Cancer Immunology

Bench to Bedside Immunotherapy of Cancers

Nima Rezaei
Editor

Second Edition

Springer

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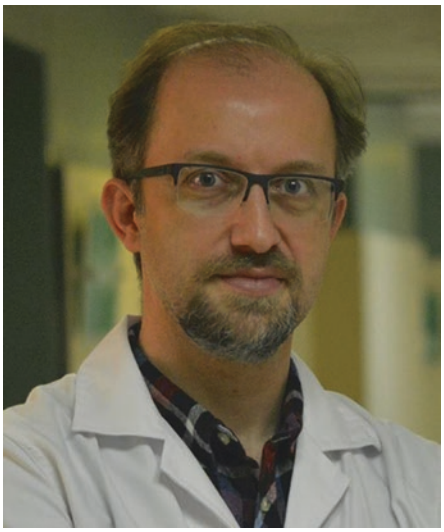
This book would not have been possible without the continuous encouragement by my parents and my wife, Maryam.
I wish to dedicate it to my daughters, Ariana and Arnika, with the hope that progress in diagnosis and treatment of these diseases may result in improved survival and quality of life for the next generations, and at the same time that international collaboration in research will happen without barriers.
Whatever I have learnt comes from my mentors. This book is therefore dedicated also to all of them, but most importantly to the patients and their families whose continuous support has guided me during the years.

Preface



The rapid flow of studies in the field of cancer immunology during the last decade has increased our understanding of the interactions between the immune system and cancerous cells. In particular, it is now well known that such interactions result in the induction of epigenetic changes in cancerous cells and the selection of less immunogenic clones as well as alterations in immune responses. Understanding the cross-talk between nascent transformed cells and cells of the immune system has led to the development of combinatorial immunotherapeutic strategies to combat cancer.

The *Cancer Immunology* series, a three-volume book series, is intended as an up-to-date, clinically relevant review of cancer immunology and immunotherapy. The first edition of the book was published 4 years ago, which was very welcomed by readers and made us to work on the second edition of the book in such a short period of time.

Volume I, *Cancer Immunology: A Translational Medicine Context*, is focused on the immunopathology of cancers. Volume II, *Cancer Immunology: Bench to Bedside Immunotherapy of Cancers,* is a translation text explaining novel approaches in the immunotherapy of cancers; and finally, volume III, *Cancer Immunology: Cancer Immunotherapy for Organ-Specific Tumors,*

thoroughly addresses the immunopathology and immunotherapy of organ-specific cancers.

In volume II, clinical applications of cancer immunotherapy are fully described. Notably, the principal focus is very much on putting the basic knowledge gained on tumor immunology in volume I into clinical perspective, with the aim to educate clinicians on the most recent approaches used in tumor immunotherapy. To meet this purpose, this volume was extended from 27 chapters in the first edition to 32 chapters in the second edition.

At the very beginning, an overview of frontiers in cancer immunotherapy is given in Chap. 1; then novel strategies in cancer immunotherapy are discussed in Chap. 2. Thereafter, personalized prevention strategies to defeat cancer, as well as tumor antigens valuable in the treatment and clinical evaluation of tumors, and strategies to target tumor immunosuppression are outlined in Chaps. 3, 4, and 5, respectively.

Due to the importance of overcoming tumor immunosuppression and cancer tolerance when treating tumors, Chap. 6 aims to tackle these crucial and challenging issues. From this point, more precise focus is given to introducing novel immunotherapeutic approaches by allocating Chaps. 7–9 to gene therapy, virus-based vaccines, hematopoietic stem cell transplantation, and lymphodepletion. Chapter 10 provides the reader with the most important detail on the combination of chemotherapy and cytokine therapy in tumor management. Thereafter, various aspects of the role of type I interferons and T lymphocytes in cancer immunotherapy are explained in Chaps. 12–14, with special attention to their synthetic biology, clinical application, role in immunosurveillance and immunotherapy, as well as optimizing chemokine receptor-mediated homing of T cells in cancer immunotherapy.

A general discussion on the multitude of monoclonal antibodies used in the clinical and preclinical setting is brought up in Chap. 15. Chapter 16 aims to familiarize readers with the role of pattern recognition receptors and Toll-like receptor pathway, while Chap. 17 discusses the role of NK cells in cancer immunotherapy. Novel vaccines produced by dendritic cells for cancer therapy are elucidated in Chap. 18. Thereafter, Chap. 19 explicates the role of tumor-associated macrophages in tumor development, while exosomes are the subject of discussion in Chap. 20.

The implication of photodynamic therapy and polarization of the tumor milieu are brought up in the two following chapters, Chaps. 21 and 22, followed by Chap. 23 which discusses targeting 5T4 oncofetal glycoprotein as an immunotherapeutic approach. Aging and cancer prognosis is discussed in Chap. 24. Novel biomarkers discovered during immune checkpoint inhibitor therapy are described in Chap. 25, while cancer nanomedicine is explained in Chap. 26. Oncolytic viruses as immunotherapeutical agents and immune targeting of oncogenic HPV are the subjects that are discussed in Chaps. 27 and 28, respectively.

Chapters 29 and 30 are focused on radioimmunotherapy. Finally, after discussing difficulties of cancer immunotherapy in Chap. 31, the book ends by pointing to the ethical considerations crucial during cancer immunotherapy in Chap. 32.

The *Cancer Immunology* Series is the result of valuable contribution of more than 300 scientists from more than 100 well-known universities/institutes worldwide. I would like to hereby acknowledge the expertise of all contributors for generously devoting their time and considerable effort in preparing their respective chapters. I would also like to express my gratitude to Springer Nature publication for providing me the opportunity to publish the book.

Finally, I hope that this translational book will be comprehensible, cogent, and of special value for researchers and clinicians who wish to extend their knowledge on cancer immunology.

Tehran, Iran Nima Rezaei, MD, PhD

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I would like to express my gratitude to the Editorial Assistants of this book, Dr. Mahsa Keshavarz-Fathi and Dr. Farnaz Delavari. With no doubt, the book would not have been completed without their contribution.

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Abbreviations

3'-UTR	3'-untranslated region
3D	Three-dimensional
3-MA	3-Methyladenine
4-OHT	4-Hydroxytamoxifen
5AC	5-Azacytidine
Ab	Antibody
ABC	Adenosine triphosphate-binding cassette
Abs	Antibodies
AC	Adenocarcinoma
ACC	Acinar cell carcinoma
ACC	Adenoid cystic carcinoma
Ad5	Adenovirus serotype 5
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADP	Anti-adipophilin
Ag	Antigen
AHR	Aryl hydrocarbon receptor
AIA	Ag-induced arthritis
AICD	Activation-induced cell death
AIDS	Acquired immune deficiency syndrome
AIF	Aapoptosis-inducing factor
AILT	Angioimmunoblastic T-cell lymphoma
AIRC	Italian Association for Cancer Research
AIRE	Autoimmune regulator
ALK	Anaplastic large cell lymphoma kinase
ALL	Acute lymphoblastic leukemia
ALP	Alkaline phosphatase
alphaGalCer	Alpha-galactosylceramide
ALPS	Autoimmune lymphoproliferative syndrome
AML	Acute myeloid leukemia
ANCs	Absolute neutrophil counts
ANN	Artificial neural network
ANT	Adenine nucleotide translocase
APC	Antigen-presenting cells
APCP	Adenosine 5'-(α, β-methylene) diphosphate
APCs	Antigen-presenting cells

APECED	Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy
APL	Acute promyelocytic leukemia
APM	Antigen presentation machinery
APS-1	Autoimmune polyendocrine syndrome type I
ARB	Average relative binding
ARDS	Acute respiratory distress syndrome
ASCs	Adult stem cells
ASM	Acid sphingomyelinase
ASPS	Alveolar soft part sarcoma
ATCL	Anaplastic large cell lymphoma
ATLL	Adult T-cell lymphoma/leukemia
ATM	Ataxia telangiectasia mutated
ATO	Arsenic trioxide
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia/Rad3-related kinase
ATRA	All-trans retinoic acid
B SLL/CLL	B-cell small lymphocytic lymphoma/chronic lymphocytic lymphoma
BAFF	B-cell activating factor
BALs	Bronchoalveolar lavage
BCA	Basal cell adenocarcinoma
BCC	Basal cell carcinoma
BCG	Bacillus Calmette-Guérin
BCR	B-cell antigen receptor
BER	Base excision repair
bFGF	Basic fibroblast growth factor
BLI	Bioluminescence imaging
Bregs	Regulatory B cells
BSO	Buthionine sulfoximine
BTK	Bruton's tyrosine kinase
BTLA	B- and T-lymphocyte attenuator
C/EBPb	CCAT/enhancer-binding protein b
CAFs	Cancer-associated fibroblasts
CaP	Prostate cancer
CARD	Caspase-recruitment domain
CBA	Cytometric bead array
CBR	Clinical benefit response
CC	Choriocarcinoma
CC	Chromophobe carcinoma
CCS	Clear cell sarcoma
CD	Clusters of differentiation
CD40-B	CD40-activated B
CD40L	CD40 ligand
CDC	Complement-dependent cytotoxicity
c-FLIP	Cellular FLICE-inhibitory protein
CFSE	Carboxyfluorescein diacetate succinimidyl ester

CGN	Chromogranin
CHL	Classic Hodgkin lymphoma
CHS	Contact hypersensitivity
CIA	Collagen-induced arthritis
CIC/CRI	Cancer Immunotherapy Consortium of the Cancer Research Institute in the USA
CIHR	Canadian Institutes of Health Research
CIMT	Cancer Immunotherapy
CIP	CIMT Immunoguiding Program
CK	Cytokeratin
CLA	Cutaneous lymphocyte-associated antigen
CLEC9A	C-type lectin domain family 9A
CLL	Chronic lymphocytic leukemia
CLRs	C-type lectin receptors
CMA	Chaperone-mediated autophagy
CMC	Chronic mucocutaneous candidiasis
CML	Chronic myeloid leukemia
CNS	Central nervous system
Con	Concanavalin
CP	Core particle
CpG-A ODN	CpG-A oligodeoxynucleotide
CpG-ODN	CpG oligodeoxynucleotide
CPS	Cancer Prevention Study
CQ	Chloroquine
CR	Complete remission
CRC	Colorectal cancer
CRCC	Clear RCC
CRDs	Cysteine-rich domains
CrmA	Cytokine response modifier A
CRP	C-reactive protein
CRT	Calreticulin
CS	Classic seminoma
CS&T	Cytometer setup and tracking
CSC	Cancer stem cell
CSF-1	Colony-stimulating factor
CSF-1R	CSF-1 receptor
CSF3R	Colony-stimulating factor 3 receptor
CSR	Class switch recombination
c-state	Cytosolic state
CTC	Circulating tumor cells
CTL	Cytotoxic T lymphocyte
CTS	Cathepsins
CTVT	Canine transmissible venereal tumor
CVID	Common variable immunodeficiency
Cyt	Cytochrome
DAMP	Damage-associated molecular pattern
DC	Dendritic cells

DCC	Deleted in colorectal cancer
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DD	Death domain
DDP	Diamindichloridoplatin
DED	Death effector domain
DES	Desmin
DFTD	Devil facial tumor disease
DHh	Desert hedgehog homolog
DISC	Death-inducing signaling complex
DKO	Double knockout
DLBCL	Diffuse large B-cell lymphoma
DNAM	DNAX-accessory molecule
DNMTs	DNA methyltransferases
DNR	Dominant-negative TGF-ß type II receptor
DNT	Double-negative T
DR	Death receptor
DRMs	Detergent-resistant microdomains
DSB	Double-strand break
DSRCT	Desmoplastic small round cell tumor
DSS	Dextran sulfate sodium
DT	Diphtheria toxin
DTE	Desmoplastic trichoepithelioma
DTH	Delayed-type hypersensitivity
DTR	Diphtheria toxin receptor
DUBs	Deubiquitinases
EAE	Experimental autoimmune encephalomyelitis
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EC	Embryonal carcinoma
ECL	Electrochemiluminescent
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EGF	Epidermal growth factor
EGFR	EGF receptor
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
EMC	Epithelial-myoepithelial carcinoma
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial–mesenchymal transition
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ER	Estrogen receptor protein
ER+	Estrogen receptor-positive
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem
ES/PNET	Ewing sarcoma/peripheral neuroectodemal tumor
EV	Epidermodysplasia verruciformis

FADD	Fas-associating protein with a death domain
FAK	Focal adhesion kinase
FasL	Fas ligand
FcγRII	Fc receptor II
FDA	Food and Drug Administration
FL	Follicular lymphoma
FLIP	FLICE-inhibitory protein
Flt3L	FMS like tyrosine kinase 3 ligand
Fluc	Firefly luciferase
FRB	FKBP12-rapamycin-binding domain
FSC	Forward scatter light
FZD	Frizzled
GAP	GTPase-activating protein
GBM	Glioblastoma multiforme
GC	Germinal center
GCLP	Good clinical laboratory practice
GEFs	Guanine nucleotide exchange factors
GEM	Genetically engineered mouse
GEMM	Genetically engineered mouse models
GFI1	Growth factor-independent 1
GFP	Green fluorescent protein
GI	Gastrointestinal
GITR	Glucocorticoid-induced tumor necrosis factor receptor-related protein
Gld	Generalized lymphoproliferative disease
Gli	Gli transcription factors
Gln	Glutamine
Glu	Glutamate
GLUD1	Glutamate dehydrogenase 1
GLUL	Glutamate-ammonia ligase
GM-CSF	Granulocyte macrophage colony-stimulating factor
G-MDSC	Granulocytic MDSC
GMP	Good manufacturing practice
GPU	Graphical processing units
GRAFT	Genetically transplantable tumor model systems
GrB	Granzyme B
GSIs	Gamma secretase inhibitors
GSK-3β	Glycogen synthase kinase-3β
GVDH	Graft-versus-host-disease
GWAS	Genome-wide association studies
HAX1	HS-1-associated protein X
HBE	Human bronchial epithelial
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCL	Hairy cell leukemia
HCV	Hepatitis C virus
HD	Healthy donors

HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HDACs	Histone deacetylases
HEV	High endothelial venules
HGF	Hepatocyte growth factor
HGPIN	High-grade prostate intraepithelial neoplasia
HGS	Human Genome Sciences
Hh	Hedgehog
HIES	Hyper-IgE syndrome
HIF2α	Hypoxia-inducible factor 2-α
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis
HNC	Head and neck cancer
HP	Human papilloma
HPC	Hematopoietic progenitor cells
HPV	Human papilloma virus
HRG	Histidine-rich glycoprotein
HRP	Horseradish peroxidase
HRR	Homologous recombination repair
HS	Herpes simplex
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem-cell transplantation
HSP	Heat shock proteins
HVEM	Herpesvirus entry mediator
IAP	Inhibitor of apoptosis protein
IB	Immunoblotting
IBCC	Infiltrating basal cell carcinoma
ICAD	Inhibitor of caspase-activated DNase
ICAM	Intercellular adhesion molecule
ICAM-3	Intercellular adhesion molecule 3
ICC	Immunocytochemistry
ICOS	Inducible costimulator
ICOS-L	Inducible costimulator ligand
ICS	Intracellular cytokine staining
IDC	Invasive ductal carcinoma
IDO	Indoleamine 2, 3-dioxygenase
IELs	Intraepithelial lymphocytes
IFN	Interferon
IFNγ	Interferon gamma
IFN-γ	Interferon γ
Ig	Immunoglobulin
IgAD	IgA deficiency
IgE	Immunoglobulin E
IHC	Immunohistochemistry
IHC/ICC	Immunohistochemistry and immunocytochemistry

IHh	Indian hedgehog
IkB	Inhibitor of kB
IKK	IκB kinases
IL	Interleukin
IL-10	Interleukin-10
IL-1Ra	Interleukin-1Ra
IL-1β	Interleukin-1β
IL-2Rα	Interleukin-2 receptor-α
ILC	Invasive lobular carcinoma
IM	Inner mitochondrial membrane
IMPT	Intensity-modulated proton therapy
IMRT	Intensity-modulated radiotherapy
IMS	Intermembrane space
INF	Interferons
iNOS	inducible nitric oxide synthase
IP	Immunoprecipitation
iPS	Induced pluripotent stem
IRF	Transcription factor
ISPC	In silico planning comparative
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITK	T-cell kinase
IVD	In vitro diagnostic
JAK	Janus kinase
JNK	Jun N-terminal kinase
KARs	Killer activation receptors
KGF	Keratinocyte growth factor
KIRs	Killer cell immunoglobulin-like receptors
KSHV	Kaposi sarcoma-associated herpesvirus
LAT	Linker of activation in T-cell
LC	Luminal cells
LCA	Leukocyte common antigen
LCMV	Lymphocytic choriomeningitis virus
LCs	Langerhans cells
LCT	Leydig cell tumor
LD	Linkage disequilibrium
LIR	LC3 interacting region
LMP-1	Latent membrane protein-1
LNA	Locked nucleic acid
LNs	Lymph nodes
LOH	Loss of heterozygosity
LOX	Lysyl oxidase
LPL	Lymphoplasmacytic lymphoma
Lpr	Lymphoproliferation
LPS	Lipopolysaccharide
LTA	Lymphotoxin-α
LUBAC	Linear ubiquitin chain assembly complex

mAb	Monoclonal antibody
Mac	Macrophages
MAC	Microcystic adnexal carcinoma
MALT	Mucosa-associated lymphoid tissue
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MC	Molluscum contagiosum
MC	Myoepithelial carcinoma
MCA	Methylcholanthrene
MCC	Merkel cell carcinoma
MCMV	Mouse cytomegalovirus
M-CSF	Macrophage colony-stimulating factor
mDCs	Myeloid-derived dendritic cells
MDS	Myelodysplasia
MDSC	Myeloid-derived suppressor cells
MEC	Mucoepidermoid carcinoma
MEXT	Ministry of Education, Culture, Sports, Science and Technology
MF	Mycosis fungoides
MFI	Mean fluorescence intensity
MGMT	Methylguanine methyltransferase
MGUS	Gammopathy of unknown significance
MHC	Major histocompatibility complex
MIACA	Minimal information on reported results including reporting information on cellular assays
MIAME	Minimal information about microarray experiments
MIATA	Minimal information about T-cell assays
MIBBI	Minimal information on biological and biomedical investigations
MIC-A	MHC class I chain-related A
MIF	Macrophage inhibitory factor
MIG	Monokine induced by interferon-γ
miRNAs	MicroRNAs
MISC	Motility-inducing signaling complex
MKPs	MAP kinase phosphatases
ML-IAP	Melanoma inhibitor of apoptosis protein
MM	Multiple myeloma
M-MDSC	Monocytic MDSC
MMP	Metalloproteases
MMR	Mismatch repair
MnO	Manganese oxide
MOMP	Membrane permeabilization
MPSC	Metastatic pulmonary small cell carcinoma
MSA	Muscle-specific antigen
MSCs	Mesenchymal stem cells
MSF	Migration-stimulating factor
MSI	Microsatellite instability

m-state	Matrix state
mTOR	Mammalian target of rapamycin
MVD	Microvascular density
MYG	Myogenin
MZL	Marginal zone lymphoma
NADPH	Nicotinamide adenine dinucleotide phosphate oxidases
NAIP	Neuronal apoptosis inhibitory protein
NCCD	Nomenclature Committee on Cell Death
NCR	Natural cytotoxicity receptor
ncRNAs	noncoding RNAs
NEC	Neuroendocrine carcinoma
NER	Nucleotide excision repair
NF	Nuclear factor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
NHANES	National Health and Nutrition Examination Survey
NHEJ	Nonhomologous end-joining
NHL	Non-Hodgkin lymphoma
Ni	Nickel
NiS	Nickel sulfide
NK	Natural killer
NKG2D	Natural killer group two member D
NKT	Natural killer T
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NLRs	The nucleotide-binding oligomerization domain-like receptors
NMC	NUT midline carcinoma
NOD	Nucleotide-binding oligomerization domain
NP	Normal prostate
NPC	Nasopharyngeal carcinoma
NPY	Neuropeptide Y
NSCLC	Non-small cell lung carcinoma
Nt	Nucleotides
NTKs	Neurothekeoma
NUT	Nuclear protein in testis
OARs	Organs at risk
OC	Oncocytoma
ODEs	Ordinary differential equations
ONB	Olfactory neuroblastoma
OPN	Osteopontin
OPRCC	Oncocytic papillary RCC
PAC	Pulmonary adenocarcinoma
PAGE	Polyacrylamide gel, and separated by electrophoresis
PAK	p21-activated kinase
PAMPs	Pathogen-associated molecular patterns
PARP	Poly ADP-ribose polymerase
PAX	Paired box

PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBMCs	Blood mononuclear cells
PC	Prostate adenocarcinoma
PCD	Programmed cell death
PCG	Protein coding gene
PD	Paget disease
PDAC	Pancreatic ductal adenocarcinoma
pDCs	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death-1 ligand
PE	Phosphatidylethanolamine
PE	Pleural effusion
PEMCs	Pleural effusion mononuclear cells
PET	Positron emission tomography
PFS	Progression-free survival
PH	Pleckstrin homology
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PIDs	Primary immunodeficiencies
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLAD	Pre-ligand binding assembly domain
PLGC	Polymorphous low-grade adenocarcinoma
PlGF	Placental growth factor
PMA	Phorbol myristate acetate
PMNs	Polymorphonuclear leukocytes
PMT	Photomultiplier tube
PNET/ES	Peripheral neuroectodermal tumor/extraskeletal Ewing sarcoma
PNP	Purine nucleoside phosphorylase
PR	Progesterone receptor
PRC	Polycomb Repressive Complex
PRCC	Papillary RCC
pre-pDCs	Precursor of pDCs
PROTOR	Protein observed with Rictor
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
PSSM	Position-specific scoring matrix
Ptc	Patched dependence receptor
PTCH1	Patched receptor
PTM	Posttranslational modification
PTPC	Permeability transition pore complex
PVDF	Polyvinylidene fluoride
PYGL	Glycogen phosphorylase
QDs	Quantum dots

QoL	Quality of life
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
Raptor	Regulatory-associated protein of mTOR
Rb	Retinoblastoma protein
RCC	Renal cell carcinoma
RFK	Riboflavin kinase
RFLPs	Restriction fragment length polymorphisms
RHIM	RIP homotypic interaction motif
RHOH	Ras homolog family member H
RIA	Radioimmunoassay
RICD	Reactivation-induced cell death
Rictor	Rapamycin-insensitive companion of mTOR
RIG-1	Retinoic acid-inducible gene I
RIP	Receptor interacting protein
RISC	RNA-induced silencing complex
RLHs	RIG-I-like helicases
RMS	Rhabdomyosarcoma
ROS	Reactive oxygen species
RS	Reference samples
SA	Sebaceous adenoma
SAP	Signaling associated protein
SBDS	Shwachman–Bodian–Diamond syndrome
SC	Sebaceous carcinoma
SCC	Squamous cell carcinoma
SCCHN	Squamous cell carcinoma of the head and neck
SCF	Stem cell factor
SCID	Severe combined immune-deficient
SCLCL	Small cell lung cancer
SCM	Small cell melanoma
SCN	Severe congenital neutropenia
SCNP	Single-cell network profiling
SCs	Stem cells
SCT	Sertoli cell tumor
SDC	Salivary duct carcinoma
SDS	Shwachman–Diamond syndrome
SDS	Sodium dodecyl sulfate
SEC	Small cell eccrine carcinoma
SED	Subepithelial cell dome
SFB	Segmented filamentous bacteria
Shh	Sonic hedgehog
SHh	Sonic hedgehog homolog
SHM	Somatic hypermutation
siRNA	Small interfering RNA
SIRP-α	Signal-regulatory protein-α
SLAM	Signaling lymphocytic activation molecule
SLE	Systemic lupus erythematosus

SMC	Skeletal muscle cells
SMM	Stabilized matrix method
Smo	Smoothened
SNEC	Small cell neuroendocrine carcinoma
SNP	Single nucleotide polymorphisms
SNUC	Sinonasal undifferentiated carcinoma
SOBP	Spreadout Bragg peak
SOCE	Store-operated Ca^{2+} entry
SOPs	Standard operating procedures
SP	Side population
SP-A	Surfactant protein A
SPECT	Single-photon emission computed tomography
SPIO	Superparamagnetic iron oxide
SPN	Solid pseudopapillary neoplasm
SS	Sjögren syndrome
SS	Spermatocytic seminoma
SSC	Side-scattered light
SSCC	Small cell squamous carcinoma
SSO	Sequence-specific probes
SSP	Sequence-specific primers
SSPCs	Salivary gland stem/progenitor cells
STAT	Signal transducer activator of transcription
STAT1	Signal transducer and activator of transcription-1
STIM	Stromal interaction molecule
SVZ	Subventricular zone
SYN	Synaptophysin
T1D	Type 1 diabetes
T2	Transitional 2 immature
TAA	Tumor-associated antigens
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TADC	Tumor-associated dendritic cells
TAM	Tumor-associated macrophages
TAMC	Tumor-associated myeloid cells
TAN	Tumor-associated neutrophils
TAP	Transporter associated with antigen processing
TApDCs	Tumor-associated pDCs
TAPs	Peptide transporters
TAS	Trait-associated SNP
TAs	Tumor antigens
TB	Tuberculosis
TBI	Total body irradiation
tBID	Truncated BID
TC/HRBCL	T-cell/histiocyte-rich B-cell lymphoma
TCF-4	T cell factor
TCL	T-cell lymphoma
TCR	T cell receptor

TDLN	Tumor-draining lymph node
TEM	Tie2-expressing monocytes
TEM	Transmission electron microscopy
TEMRA	Terminally differentiated effector memory
TFBSs	Transcription factor binding sites
TFH	T follicular helper
TGB	Thyroglobulin
TGF-β	Transforming growth factor β
Th	T helper
TIL	Tumor-infiltrating lymphocytes
TIL-Bs	Tumor-infiltrating B cells
TLR	Toll-like receptor
TLT	Tertiary lymphoid tissue
TME	Tumor microenvironment
TNC	Tenascin C
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
TNFα	Tumor necrosis factor alpha
TNF-α	Tumor necrosis factor-α
TNM	Tumor-node-metastasis
TRADD	TNF-receptor-associated death domain
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tregs	Regulatory T cells
TSC	Tuberous sclerosis complex
TSGs	Tumor suppressor genes
TSH	Thyroid-stimulating hormone
TSLP	Thymic stromal lymphopoietin
TTP	Time to progression
U1snRNP	U1 small nuclear ribonucleoprotein
UADT	Upper aerodigestive tract
UC	Urothelial carcinoma
UCH	Ubiquitin C-terminal hydrolases
ULBPs	Unique long 16 binding proteins
Unfrac	Unfractionated
UNPC	Undifferentiated nasopharyngeal carcinoma
uPA	Urokinase plasminogen activator
UPP	Ubiquitin-proteasome pathway
UPS	Ubiquitin-proteasome system
USP	Ubiquitin-specific proteases
USPIO	Ultrasmall superparamagnetic iron oxide nanoparticles
UV	Ultraviolet
UVRAG	Ultraviolet radiation resistance-associated gene
VEGF-A	Vascular endothelial growth factor-A
VIM	Vimentin
VINIII	Vulvar intraepithelial neoplasia grade III
VNTR	Variable number tandem repeat
VZ	Varicella zoster

WAS	Wiskott–Aldrich syndrome
WASp	WAS protein
WASP	Wiskott–Aldrich syndrome protein
WGS	Whole genome sequencing
WHIM	Warts, hypogammaglobulinemia, infections, and myelokathexis
WM	Waldenstrom macroglobulinemia
WT	Wild-type
X-IAP	X-linked inhibitor of apoptosis protein
XLA	X-linked agammaglobulinemia
XLN	X-linked neutropenia
XLP	X-linked lymphoproliferative disease
XLT	X-linked thrombocytopenia
YST	Yolk sac tumor

Frontiers in Cancer Immunotherapy

1

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1.1 Introduction

Our immune system is characterized by remarkable specificity, potency, and memory—the ability of a single vaccine treatment to provide lifelong protection. No pharmacologic treatment for any indication can provide the same level of safety, efficacy, and long-lasting effect that a vaccine can. Thus, researchers and clinicians alike have sought to apply these characteristics to the treatment of cancer [1]. Advances in cellular and molecular immunology over the past three decades have provided enormous insights into the nature and consequences of interactions between tumors and immune cells. This knowledge continues to lead to strategies by which the immune system might be harnessed for therapy of established malignancies [2].

Cells of the innate immune system respond to "danger" signals provided by growing tumors as a consequence of the genotoxic stress of cell transformation and disruption of the surrounding microenvironment. Under ideal conditions, these signals induce inflammation, activate innate effector cells with antitumor activity, and stimulate professional antigen-presenting cells (APCs), particularly dendritic cells (DCs), to engulf tumor-derived antigens and migrate to draining lymph nodes to trigger an adaptive response by T- and B-lymphocytes. Despite this well-orchestrated surveillance operation, the presence of a tumor indicates that the developing cancer was able to avoid detection or to escape or overwhelm the immune response. Progressing tumors often exhibit strategies that promote evasion from immune recognition [3]. This includes physical exclusion of immune cells from tumor sites, poor immunogenicity due to reduced expression of major histocompatibility complex (MHC) or co-stimulatory proteins, and disruption of natural killer (NK) and natural killer T (NKT)-cell recognition [4]. Additionally, some tumors prevent triggering of an inflammatory response by secreting proteins, such as interleukin (IL-10) or vascular endothelial growth factor (VEGF), that interfere with DC activation and differentiation [5] or by blocking the production of pro-inflammatory molecules by increasing expression of the STAT3 protein [6]. Even if a response is induced, tumor cells may escape elimination by losing targeted antigens, rendering tumor-reactive T-cells anergic, inducing regulatory T-cells, or specifically deleting responding T-cells [7, 8]. Thus, there is often a cat and mouse game with the immune system exerting pressure to eliminate the tumor and the tumor cells evading the immune response; the eventual tumor that develops reflects "immunoediting" with the selection of poorly immunogenic and/or immune-resistant malignant cells [9]. Despite these obstacles, modern immune-based therapies continue to show increased potential for treating malignant diseases. Here, we will review some of the most promising cancer immunotherapeutic approaches in development today, as recent clinical successes signal the beginning of cancer immunotherapy's transition from experimental to established therapy.

1.2 Innate Cells as Initiators of the Adaptive Immune Response

One of the first strategies to enhance immune response to cancer was the direct administration of adjuvants into solid tumors to stimulate inflammation and recruit immune effector cells. This approach is still commonly used for treating superficial bladder carcinomas and has been used to treat melanoma and neurological tumors. It is now known that many of these adjuvants contain bacterial products, such as lipopolysaccharide (LPS) or CpG-containing oligo-deoxynucleotides recognized by toll-like receptors (TLRs) on innate immune cells. This leads to the production of pro-inflammatory cytokines and facilitates productive interactions between the innate and adaptive immune responses [10]. However, many tumors render this strategy ineffective by producing proteins, such as transforming growth factor beta (TGF-ß), to prevent activation of the immune response [11].

1.3 Cellular Immunotherapy

T-cells express clonally distributed antigen receptors that in the context of MHC proteins can recognize either unique tumor antigens evolving from mutations or viral oncogenesis or self-antigens derived from overexpression of proteins or aberrant expression of antigens that are normally developmental or tissue-restricted. To mediate antitumor activity, T-cells must first be activated by bone marrow-derived APCs that present tumor antigens and provide essential costimulatory signals [12], migrate and gain access to the tumor microenvironment, and overcome obstacles to effective triggering posed by the tumor. Activation results in the production of cytokines, such as interferon (IFN) and tumor necrosis factor (TNF), that can arrest proliferation of malignant cells and prevent the angiogenesis necessary for tumor growth and also lysis of tumor cells mediated by perforin and/or Fas. Consequently, efforts have focused on identifying tumor antigens, providing the antigens in immunogenic formats to induce responses, manipulating T-cell responses to increase the number of reactive cells, and augmenting effector functions.

1.4 Active and Passive Immunotherapy

A number of immunologic interventions, which can be divided into both passive and active, can be directed against tumor cells [13]. In passive cellular immunotherapy, specific effector cells are directly infused and are not induced or expanded within the patient. Lymphokine-activated killer (LAK) cells are produced from the patient's endogenous T-cells, which are extracted and grown in a cell culture system by exposing them to interlukin-2 (IL-2). The proliferated LAK cells are then returned to the patient's bloodstream. Clinical trials of LAK cells in humans are ongoing. Tumor-infiltrating lymphocytes (TILs) may have greater tumoricidal activity than LAK cells. These cells are grown in culture in a manner similar to LAK cells. However, the progenitor cells consist of T-cells that are isolated from resected tumor tissue. This process theoretically provides a line of T-cells that has greater tumor specificity than those obtained from the bloodstream. Moreover, concomitant use of interferon enhances the expression of major histocompatibility complex (MHC) antigens and tumor-associated antigens (TAAs) on tumor cells, thereby augmenting the killing of tumor cells by the infused effector cells.

1.4.1 Active Immunotherapy

Inducing cellular immunity (involving cytotoxic T-cells) in a host that failed to spontaneously develop an effective response generally involves methods to enhance presentation of tumor antigens to host effector cells. Cellular immunity can be induced to specific, very well-defined antigens. Several techniques can be used to stimulate a host response; these may involve presenting peptides, DNA, or tumor cells (from the host or another patient). T-cells as the ultimate effectors of adaptive immune response are currently used to treat patients affected by infectious diseases and certain tumors. Recently, T-cells have been manipulated ex vivo with viral vectors coding for chimeric antigen receptors, exogenous T-cell receptors, or "suicide" genes to potentiate their efficacy and minimize possible side effects. However, the introduction of exogenous genes into T lymphocytes, particularly bacterial or viral transgene products, has occasionally produced immune-mediated elimination of transduced lymphocytes. This immune effect has recently been exploited in a trial of active immunotherapy in melanoma patients [14]. Peptides and DNA are often presented using antigen-presenting cells (dendritic cells). These dendritic cells (DCs) can also be genetically modified to secrete additional immune-response stimulants (e.g., granulocyte-macrophage colony-stimulating factor (GM-CSF). These will be discussed in more detail later.

Peptide-based vaccines use peptides from defined TAAs. An increasing number of TAAs have been identified as the target of T-cells in cancer

Table 1.1 Monoclonal antibodies, cytokines, and short peptides used in cancer immunotherapy

Type	Application	Target
Alemtuzumab	Chronic lymphocytic leukemia	CD52
Bevacizumab	Anti-angiogenic therapy	Vascular endothelial growth factor (VEGF)
Cetuximab	Colorectal, head, and neck cancer	Epidermal growth factor receptor (EGFR)
Gemtuzumab	Acute myeloid leukemia	Myeloid cell-surface antigen CD33 on leukemia cells
Ibritumomab	Non-Hodgkin lymphoma	CD20
Nimotuzumab	Squamous cell carcinoma, glioma	EGFR inhibitor
Panitumumab	Colorectal cancer	EFGR
Rituximab	Non-Hodgkin lymphoma	CD20 on B-lymphocytes
Tositumomab	Non-Hodgkin lymphoma	CD20
Trastuzumab	Breast cancer	HER2/neu receptor
Cytokines		
Interferon-gamma	Melanoma, renal and kidney cancer, follicular lymphoma, hairy cell leukemia	IFN-stimulated gene factor 3 (ISGF3)
Interlukin-2	Melanoma, renal and kidney carcinoma, hematological malignancies	Suppressors of cytokine signaling (SOCS) 1, SOCS2, dual-specificity phosphatase (DUSP) 5, DUSP6
Short peptides		
MART-1, gp100, tyrosine, MAGE-3	Melanoma	
PAP/GM-CSF	Prostate carcinoma	
MAGE-3.A24	Bladder cancer	
Follicular B-lymphoma	Idiotype/KLH conjugate	

patients and are being tested in clinical trials. Recent data indicate that responses are most potent if TAAs are delivered using dendritic cells. These cells are obtained from the patient, loaded with the desired TAA, and then reintroduced intradermally; they stimulate endogenous T-cells to respond to the TAA. Peptides can also be delivered by co-administration with immunogenic adjuvants (see Table 1.1 for representative list of monoclonal antibodies (mAbs), cytokines, and short peptides used in cancer immunotherapy).

DNA vaccines use recombinant DNA that encodes a specific (defined) antigenic protein. The DNA is incorporated into viruses that are injected directly into patients or, more often, introduced into Dcs obtained from the patients, which are then injected back into them. The DNA expresses the target antigen, which triggers or enhances patients' immune response.

Autochthonous tumor cells (cells taken from the host) have been reintroduced to the host after use of ex vivo techniques (e.g., irradiation, neuraminidase treatment, hapten conjugation, hybridization with other cell lines) to reduce their malignant potential and increase their antigenic activity. Allogeneic tumor cells (cells taken from other patients) have also been used in patients with acute lymphocytic leukemia and acute myeloblastic leukemia.

1.4.2 Nonspecific Immunotherapy

Interferons (IFN-α, IFN-β, IFN-γ) are glycoproteins that have antitumor and antiviral activity. Depending on dose, interferons may either enhance or decrease cellular and humoral immune functions. Interferons also inhibit division and certain synthetic processes in a variety of cells. Clinical trials have indicated that interferons have antitumor activity in various cancers, including hairy cell leukemia, chronic myelocytic leukemia, AIDS-associated Kaposi's sarcoma, non-Hodgkin lymphoma (NHL), multiple myeloma, and ovarian carcinoma. However, interferons may have significant adverse effects, such as fever, malaise, leukopenia, alopecia, and myalgias.

Certain bacterial adjuvants (BCG and derivatives, killed suspensions of *Corynebacterium parvum*) have tumoricidal properties. They have been used with or without added tumor antigen to treat a variety of cancers, usually along with intensive chemotherapy or radiation therapy. For example, direct injection of BCG into cancerous tissues has resulted in regression of melanoma and prolongation of disease-free intervals in superficial bladder carcinomas and may help prolong drug-induced remission in acute myeloblastic leukemia, ovarian carcinoma, and NHL.

1.5 Stimulation of Responses In Vivo

The poor immunogenicity of most tumor antigens largely reflects the nonconductive context in which these antigens are naturally presented, as well as tolerance resulting from most tumor antigens being normal proteins aberrantly expressed by the tumor. Therapeutic vaccines have attempted to circumvent these problems by presenting tumor antigens in a more enticing fashion, generally through activated DCs. This has been achieved either by the following:

- Isolating DCs and introducing the antigen ex vivo before returning the DCs to the host.
- Inoculating dead tumor cells modified to secrete factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) which promote local accumulation of DCs.
- Injecting activators of DCs, such as TLR ligands or mAb to CD40 with the antigen.
- Injecting recombinant vectors that provide both the antigen and a stimulus to the innate immune system [15].

The last category includes plasmid DNA containing the antigen and immunostimulatory CpG sequences as well as recombinant attenuated pathogens, such as adenoviruses or *Listeria monocytogenes*, that express the antigen and provide TLR ligands to trigger innate responses. However, most vaccinated patients exhibit only weak or undetectable T-cell responses to the tumor antigen and experience no clinical benefit. Therefore, methods to maintain APC activation and sustain immunogenic antigen presentation normally occurring during an encounter with a replicating foreign pathogen will likely be required before vaccines become more predictably beneficial.

An alternative to improving antigen presentation has been to mitigate negative checkpoint signals that limit the T-cell response. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a potent-negative regulator of T-cell activation. Administration of blocking antibodies to CTLA-4 has had marked effects in murine models and recent clinical trials, with lymphocytic infiltration into tumors and significant antitumor responses, including complete regressions of advanced disease in a fraction of patients [16–18]. However, global in vivo CTLA-4 blockade predictably had effects beyond the antitumor response, causing significant autoimmunity. These studies again demonstrate the potent antitumor activity of T-cells and suggest that learning how to safely and effectively disrupt checkpoint signals should yield substantial therapeutic benefit.

1.6 Adoptive Immunotherapy

There is now an emerging sense that cancer immunotherapy has the potential to effectively cure patients suffering from certain types of cancer. This hope and some of the data that supports one kind of immunotherapy (adoptive cell transfer or ACT) were recently summarized in a review article (adoptive immunotherapy for cancer: harnessing the T-cell response) [19]. Furthermore, high-dose chemoradiotherapy followed by rescue from the resulting ablation of normal bone marrow with an allogeneic hematopoietic stem cell transplant (HSCT) has also become standard therapy for many hematologic malignancies. One problem with this treatment is graft-versus-host disease (GVHD), due to allogeneic donor-derived T-cells injuring the "foreign" normal tissues of the host. However,

malignant cells that survive chemoradiotherapy are also of host origin, and patients who develop GVHD have lower relapse rates from an associated graft-versus-tumor (GVT) effect. T-cells mediate this antitumor activity, as affirmed by the complete responses sometimes observed in patients who receive infusions of donor T-cells to treat relapse after HSCT and in recipients of a newly developed non-myeloablative allogeneic HSCT regimen in whom, because of the absence of high-dose chemoradiotherapy, all antitumor effects must result from GVT effects [20]. However, the GVT activity with these regimens is often associated with severe and life-threatening GVHD. Ongoing efforts to define antigenic targets with limited tissue distribution, permitting donor lymphocytes to preferentially target malignant cells and not critical normal tissues, coupled with methods to generate and/or select T-cells with such specificities, should provide a much-needed refinement to this approach [21].

An alternative to using allogeneic T-cells to mediate antitumor responses has been to isolate autologous tumor-reactive T-cells, expand the cells in vitro, and then reinfuse the cells back into the patient. This approach circumvents many of the obstacles to generating an adequate response in vivo, as the nature of the APCs and components of the microenvironment can be more precisely controlled in vitro. However, this strategy has required the recent development of methods to extensively manipulate T-cells in vitro with retention of specificity and function, such that after infusion the cells will survive and migrate to and eliminate tumor cells.

Initial therapies used tumor-infiltrating lymphocytes as an enriched source of tumor-reactive cells, but such cells can also usually be obtained from circulating blood lymphocytes. Although optimal methods for stimulating and expanding antigen-specific T-cells in vitro are still being defined, in general, DCs presenting the antigen are used to initially trigger reactive T-cells, which can then be selected and stimulated with antibodies to CD3. Supplemental cytokines are provided during cell culture to support lymphocyte proliferation, survival, and differentiation. With this approach, it has been possible to expand tumor-reactive T-cells to enormous numbers in vitro, infuse billions of specific cells without overt toxicity to achieve in vivo frequencies beyond that attainable with current vaccine regimens, and mediate regression and occasionally complete elimination of large disseminated tumor masses. However, despite the high in vivo frequencies of tumor-reactive effector cells achieved, only a fraction of patients respond, indicating the existence of additional hurdles. One essential requirement is that infused cells must persist to mediate an effective response. Analogous adoptive therapy trials for cytomegalovirus and Epstein-Barr virus infection in immunosuppressed hosts have demonstrated increased in vivo proliferation and persistence of $CD8^+$ effector T-cells in the presence of specific $CD4^+$ helper T-cells [22]. Such $CD4^+$ T-cells likely provide many beneficial functions, including cytokine production and APC activation, which can improve the quality and quantity of the $CD8^+$ cell responses, as well as direct effector activities against infected or tumor targets. However, unlike viral responses that induce robust $CD4^+$ and $CD8^+$ responses, identifying and characterizing the specificity of tumor-reactive $CD4^+$ T-cells has proven considerably more difficult than with CD8 responses. Additionally, obstacles to safely maintaining a $CD4^+$ response reactive with a potentially normal protein remain to be elucidated. Consequently, CD4 help is largely provided to transfer tumor-reactive CD8 cells in the form of surrogate exogenous cytokines. The largest experience is with IL-2, which prolongs persistence and enhances the antitumor activity of transferred $CD8^+$ cells [23]. Alternative cytokines such as IL-15, IL-7, and IL-21, as well as activation of APCs with antibodies to CD40, are currently being evaluated in preclinical studies.

The infusion of T-cell clones, rather than polyclonal T-cell lines, represents an appealing refinement of adoptive therapy, because the specificity, avidity, and effector functions of infused cells can be precisely defined (Fig. 1.1). This facilitates subsequent analysis of requirements for efficacy, basis for toxicity, and rational design of improved therapies. The transfer of

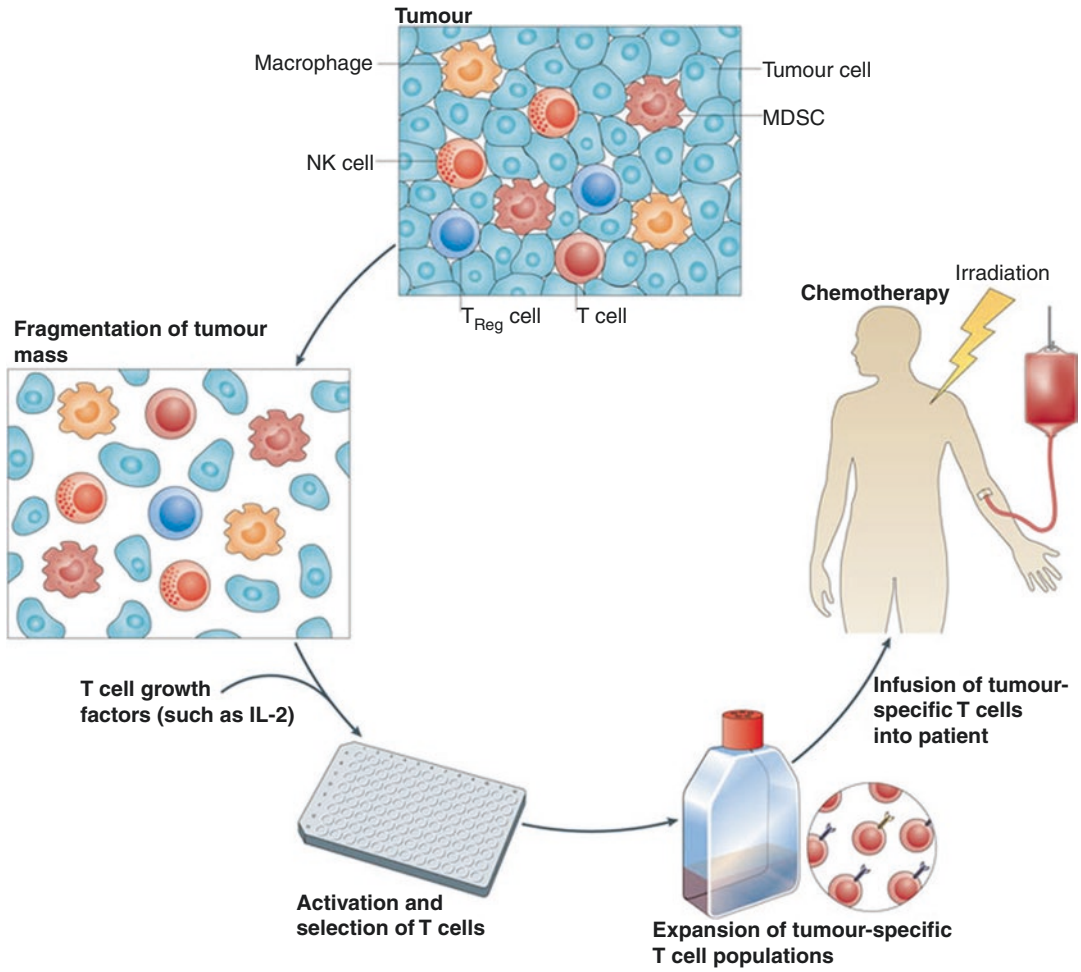

Fig. 1.1 Tumors are often complex masses containing diverse cell types. These masses can be surgically resected and fragmented, and the cells can be placed in wells into which a T-cell growth factor, such as interleukin-2 (IL-2), is added. T-cell populations that have the desired T-cell receptor (TCR) specificity can be selected and expanded and then adoptively transferred into patients with cancer. Prior to this adoptive transfer, hosts can be immunodepleted by either chemotherapy alone or chemotherapy in combination with total-body irradiation. The combination of a lymphodepleting preparative regimen, adoptive cell transfer, and a T-cell growth factor (such as IL-2) can lead to prolonged tumor eradication in patients with metastatic melanoma. *MDSC* myeloid-derived suppressor cell, *NK* natural killer, *Treg* regulatory T (Reprinted by permission from Nature Publishing Group: Restifo et al. [19])

antigen-specific $CD8^+$ T-cell clones has been shown to be effective for prevention of viral infections and treatment of malignant disease. Such studies have also formally demonstrated that low, nontoxic doses of IL-2 are sufficient to promote the in vivo persistence and antitumor activity of $CD8^+$ T-cells.

1.7 Cancer Vaccines

Therapeutic cancer vaccines target the cellular arm of the immune system to initiate a cytotoxic T-lymphocyte response against tumor-associated antigens [24]. The development of human therapeutic cancer vaccines has come a long way since

the discovery of MHC-restricted tumor antigens in the 1980s. The simplest model of immune cell-mediated antigen-specific tumor rejection consists of three elements: appropriate antigen, specific for the tumor, efficient antigen presentation, and the generation of potent effector cells. Moreover, the critical time when immune responses against the tumor are most important should also be determined. While eliminating some early transformed cells may be ongoing in an asymptomatic way as part of the immunosurveillance, if early elimination failed, equilibrium between small tumors and the immune system may be established. If the immune system is unable to maintain this equilibrium, tumors may escape, and it is this last phase when they become symptomatic. Therapeutic cancer vaccines are applied in this last phase in order to reverse the lack of tumor control by the immune system. In addition to the increasing knowledge about how to optimize the elements of antitumor immunity in order to generate clinically relevant responses, there is an ever-increasing list of immune evasion mechanisms impeding the efforts of cancer vaccines. This indicates that the elements necessary for immune-mediated tumor rejection need to be optimized [25].

Potential tumor-associated antigens (TAAs) can be identified by the elution of peptides from MHC molecules on tumor cells [26] or with proteomic approaches such as two-dimensional gel electrophoresis, MALDI-MS, and SELDI-MS (matrix-assisted or surface-enhanced laser-desorption ionization mass spectrometry) [27]. Serological analysis of recombinant—cDNA expression—libraries (SEREX) is another widely used method; it utilizes sera of cancer patients to detect overexpressed antigens from tumor cDNA libraries [28]. Furthermore, several RNA-based methods have also gained importance: transcriptome analysis that includes DNA microarrays [29], serial analysis of gene expression (SAGE) [30], comparative genomic hybridization (CGH) [31], and massively parallel signature sequencing (MPSS) [32]. These methods provide an enormous amount of information and require complex computer-aided analysis and interpretation of the data, referred to as gene expression profiling. This is necessary in order to find gene expression patterns and to distinguish them from noise [33].

Following promising in vitro immunogenicity studies [34], multicenter vaccine trials have been organized with the sponsorship of the Cancer Vaccine Collaborative (NCI and Ludwig Institute for Cancer Research). These trials have provided some information about the optimum route of administration, type of vaccine, type of adjuvant, endpoints, etc. [35]. When testing the immunogenicity of candidate antigens and defining epitopes, it should be remembered that T-cells with high avidity for self-antigen undergo negative selection during T-cell development; thus, the new TAAs may only generate T-cell responses of intermediate or low affinity. Furthermore, the wide range of restriction elements in the human population means that due to the combination of tolerance and immunodominance, potentially ideal TAAs will not be equally immunogenic in all patients. Antigen loss may also occur during tumor progression, as TAAs, which are not necessary for the maintenance of the transformed phenotype, may be deleted and tumor cells in advanced disease may express antigens different from those in early stages [36]. Another promising approach to break this immune tolerance consists of the application of anti-idiotypic (anti-Id) mAbs, so-called Ab2, as antigen surrogates. This vaccination strategy also allows immunization against non-protein antigens (such as carbohydrates). In some clinical studies, anti-Id cancer vaccines induced efficient humoral and/or cellular immune responses associated with clinical benefit (see review by Ladjemi 2012) [37].

1.7.1 Dendritic Cells

DCs are the main antigen-presenting cells in the body [38], and their generation for antitumor immunity has been the focus of a vast array of scientific and clinical studies [39]. They are the main antigen-presenting cells (APCs) in the body. Immature DC (iDC) patrols the peripheral tissues, sampling antigen from the environment. Following their activation, DCs undergo a matu-

ration process that involves the upregulation of T-cell co-stimulatory molecules (e.g., CD80, CD86) and increased cytokine secretion, a transient increase in phagocytosis followed by reduced antigen uptake, and expression of migratory molecules such as CCR7. These changes equip mature DC (mDC) to prime naive T-cells in the lymph nodes, in contrast to iDC that induces T-cell tolerance to antigen [40].

The ability of DCs to present protein tumor antigens (T-Ags) to $CD4^+$ and $CD8^+$ T-cells is pivotal to the success of therapeutic cancer vaccines. DC's specialized capacity to cross-present exogenous Ags onto MHC class I molecules for generating T-Ag-specific cytotoxic T lymphocytes (CTLs) has made these cells the focal point of vaccine-based immunotherapy of cancer (Fig. 1.2).

Dendritic cells can be loaded exogenously with TAA using whole cell populations or short peptides corresponding to epitopes from specific TAA. While the use of DC pulsed with short peptides can yield information on immune activation following therapy, they are not ideal therapeutic agents for a number of reasons. The most obvious reason is that the use of specific TAA depends on the identification of relevant TAA and not all cancers have well-defined TAA. Moreover, TAA expression within a tumor can be very heterogeneous [42]; thus, priming CTL specific for defined TAA peptides may encourage the outgrowth of non-expressing clones, leading to immune evasion. Furthermore, both MHC-1 and MHC-II epitopes are required for efficient T-cell priming. While a number of MHC-1-restricted peptides have been identified, fewer MHC-II epitopes are known. Synthetic long peptides, comprising both MHC-I and MHC-II epitopes, which require processing by DC before presentation, can overcome some of the limitations of small peptides, as they lead to extended epitope presentation.

An alternative to pulsing with peptide epitopes is to load DC with whole tumor cell preparations in the form of lysates or whole dead cells or by fusing DC with tumor cells [43]. Both allogeneic and autologous tumor material have been used to load DC with clinical trials carried out using preparations using both types [44].

Genetic modification of DC, using recombinant DNA viruses encoding TAA, has been demonstrated by several groups and can enhance T-cell priming potential via antigen presentation. DCs transduced to express the model tumor antigen β-galactosidase, using a recombinant adenoviral vector, were able to generate antigen-specific CTL responses [45]. A phase I/II trial using genetically modified DC showed that autologous DC could be transduced with high efficiency using a replication-defective adenovirus expressing full length melanoma-associated antigen recognized by T-cells (MART-1) and that the DC processed and presented the antigen for at least 10 days. Evidence of MART-1-specific $CD4^+$ and $CD8^+$ responses was found in around 50% of patients following vaccination [46].

In addition to loading DC with antigen, genetic approaches have been used to further optimize the maturation state of DC, for example, DC transfected with GM-CSF demonstrated increased antigen presentation and better migratory capacity, which translated into enhanced immune priming in vivo [47]. Other approaches include genetically modifying DC using adenoviral or retroviral vectors to directly express TH1 cytokine IL-12 [48], an adenovirus encoding *CD40L* [49], and modifying DC to express co-stimulatory molecules CD40L, CD70, and TLR4 called "TriMix" [50] and heat shock protein [51]. Furthermore, vaccines coupled to TLR ligands lead to efficient CTl activation by endogenous DC [52], and the use of oncolytic viruses also looks particularly promising [53].

Despite the use of mature DCs in vaccination trials, results from multiple clinical trials with DC-based vaccines have been contradictory, and only fractions of enrolled patients show potent antitumor or antiviral immune responses with moderate clinical response rates (approximately 10–15%) (see reviews [54, 55]). Several studies suggested that this is because of inefficient activation of Th1-polarized responses due to incomplete DC maturation. As a result, different strategies are currently being pursued in order to improve the efficacy and outcome of DC-based cancer vaccines. Considering the aforementioned powerful immune-stimulatory

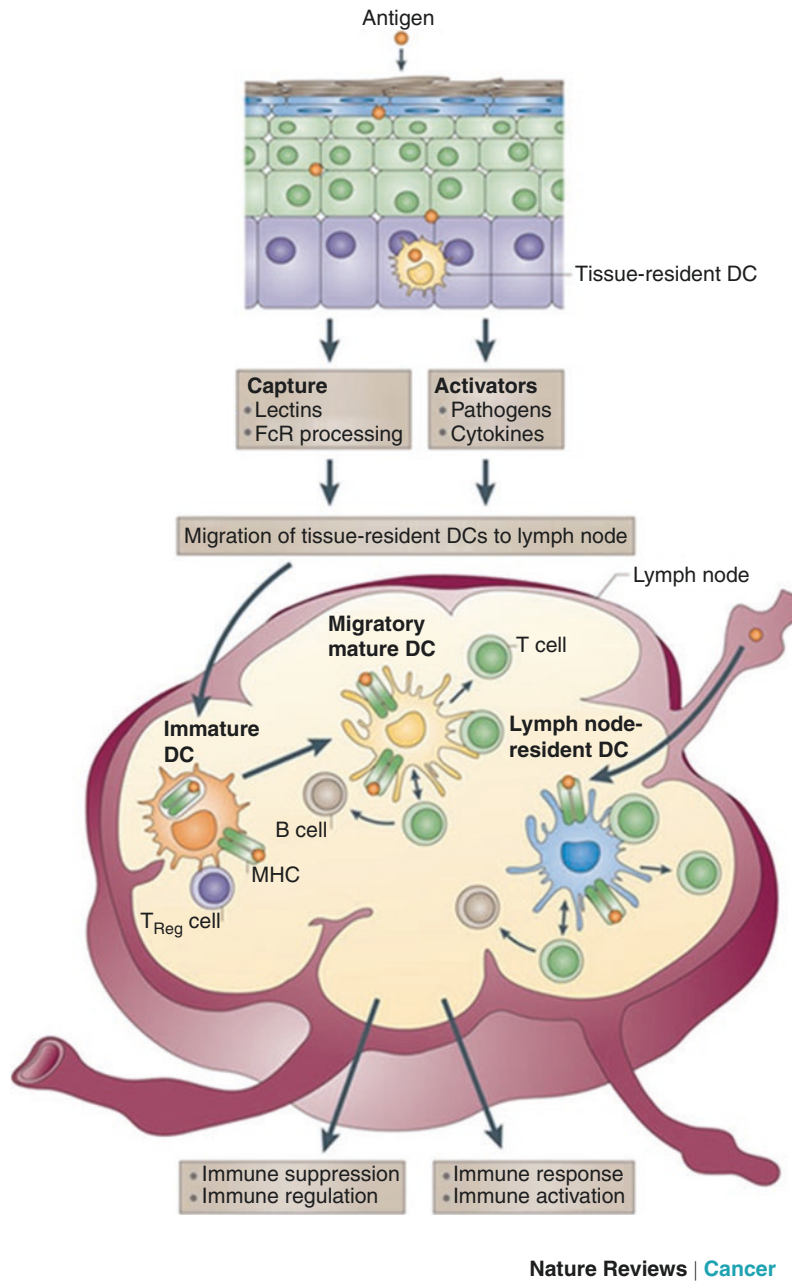

Fig. 1.2 Antigens can reach lymph nodes through two pathways: via lymphatics, where the antigen is captured by lymph node-resident dendritic cells (DCs), or via tissue-resident DCs. These immature DCs capture antigens, and DC activation triggers their migration toward secondary lymphoid organs and their maturation. DCs display antigens in the context of classical major histocompatibility (*MHC*) class I and MHC class II molecules or in the context of nonclassical CD1 molecules, which allow the selection of rare antigen-specific T-lymphocytes. Activated T-cells drive DCs toward their terminal maturation, which induces further expansion and differentiation of T lymphocytes into effector T-cells. If DCs do not receive maturation signals, they will remain immature, and antigen presentation will lead to immune regulation and/or suppression. *Treg cell*, regulatory T-cell (Reprinted by permission from Nature Publishing Group: Palucka and Banchereau [41])

properties possessed by IL-12p70, DC-based vaccination strategies may consistently benefit from incorporation or endogenous induction of this cytokine. In a first phase I clinical trial by the group of Czerniecki [56], 13 breast cancer subjects were injected intranodally with short-term DCs activated with a cytokine cocktail consisting of IFN-γ and LPS in order to induce IL-12p70-secreting DCs. The authors reported induction of robust detectable immunity as evidenced by in vitro monitoring of circulating vaccine-induced antigen-specific $CD4^+$ and $CD8^+$ T-cells, as well as both T- and B-cell infiltrates into tumor region and dramatic reductions

in tumor volume. Moreover, it has been demonstrated by others that DCs electroporated with mRNA encoding CD40 ligand, CD70, and constitutively active toll-like receptor 4, so-called TriMix DCs, display increased potential for the induction and amplification of tumor-specific responses in patients with advanced melanoma [57, 58].

One of the major obstacles against successful DC vaccination is the immunosuppressive mechanisms triggered by the tumor cells. Under the influence of the tumorigenic microenvironment, the host DCs may acquire a tolerogenic phenotype. These tumor-conditioned DCs could, in return, produce a variety of immunosuppressive molecules, thus further supporting tumor immune escape [59]. With respect to tackling different arms of the immune system, many different approaches are currently being pursued. In particular, considering the distinct ability of different DC subsets in inducing both innate and adaptive immunity, the exploitation of specific subsets of DCs to elicit the desired immune response is anticipated. Although pDCs primarily contribute to innate antiviral immune responses by producing IFN-α/β, this ability has also been reported to activate other DCs, including those involved in cross-priming and consequently greater activation of adaptive immune responses. In so doing, pDCs may play a critical role in provoking cancer immunity. Therefore, combination therapies aiming at interaction of pDCs and cDCs to stimulate T-cell priming and hence effective antitumor or antiviral immunity are needed in cancer patients and chronically infected patients.

1.7.2 Physical Barriers, Tumor Stroma, and Vessels

The tumor environment represents another challenge for cancer vaccines. Established epithelial tumors can be surrounded by basal membrane-like structures, which prevent infiltration by lymphocytes and the expansion of tumor-specific T-cells at the tumor site and in lymphoid tissues [60]. Solid tumors larger than about 1–2 mm in diameter require the presence and support of stromal cells for blood supply, growth factors, and structural support. The stroma consists of cancer-associated fibroblasts (CAF), tumor endothelial cells (TEC), and tumor-associated macrophages (TAM) and can represent more than 50% of the tumor tissue depending on the type tumor [61]. Stromal cells do not only represent a physical barrier but also release soluble mediators (TGF-β, IL-10, prostaglandin) which inhibit immune responses and promote angiogenesis and tumor progression [62, 63]. Conventional cancer treatments, such as debulking surgery, chemotherapy, or radiotherapy, not only destroy tumor cells but also destroy or damage stromal cells that may contribute to breaking immunological resistance and immunosuppression [64]. The intricate interplay between tumor and stroma attracts their simultaneous immune destruction: when highly expressed TAAs on tumor cells are cross-presented by stromal cells to T-cells, the stromal component also becomes a target of cytotoxic T-cell killing [65].

TGFβ-1 regulates the production of cytokines and growth factors by stromal and tumor cells, such as fibroblast growth factor (FGF), connective tissue growth factor (CTGF), and vascular endothelial growth factor (VEGF), which promote angiogenesis and tumor progression. The new tumor vasculature is generally both structurally and functionally abnormal, which makes trafficking/recirculation of the tumor tissue by lymphocytes and treatments including cancer vaccines extremely difficult. Anti-angiogenic treatments, including immunological targeting of antigens overexpressed on endothelial cells during angiogenesis or antibody blockade of VEGF-receptors, "normalize" the tumor vasculature [66, 67]. This treatment also reverts epithelial tumors to noninvasive type and may also aid the penetration of vaccines and other treatments in the tumor tissue. Moreover, IL-12 inhibits angiogenesis via an IFN-γ-mediated pathway [68], while adoptively transferred tumor-specific $CD8^+$ T-cells destroy the vasculature of established tumors via an antigen-independent, IFN-γ-dependent mechanism [69].

1.8 Mechanisms of Tumor-Induced Tolerance/Escape from the Immune System

Despite the evidence that immune effectors play a significant role in controlling role in tumor growth under natural conditions or in response to therapeutic manipulation, it is well known that malignant cells can evade immunosurveillance [70]. This is in part due to the fact that peptides with sufficient immunogenic potential are not presented by malignant cells to antigen-presenting cells under molecular/cellular conditions conducive to an effective immune response. From a Darwinian perspective, the neoplastic tissue can be envisaged as a microenvironment that selects for better growth and resistance to the immune attack. Cancer cells are genetically unstable and can lose their antigens by mutation. This instability, combined with an immunological pressure, could allow for selective growth of antigen-loss mutants [71]. Mechanistically, this could operate at several levels including loss of the whole protein or changes in immunodominant T-cell epitopes that alter T-cell recognition, antigen processing, or binding to the MHC. Antigen loss has been demonstrated in patients with melanoma and B-cell lymphoproliferative disease [72, 73]. Moreover, many cancer vaccines aim to induce a therapeutic $CD8^+$ cytotoxic T-cell response against TAAs. This in turn is dependent on correct processing and presentation of TAAs by MHC class I molecules on tumor cells. This pathway is complex and involves multiple intracellular components. Defects in the components of the MHC class I antigen processing pathway are frequently found in human cancers and can occur in concert with the loss of tumor antigens [74, 75].

Other cancer-related mechanisms underlying tumor immune escape include loss of TAA expression [3], lack of co-stimulatory molecules expression [76], inactivating mutations of antigen presentation-related molecules [77], and production of soluble immunosuppressive factors, e.g., transforming growth factor-beta (TGF-β), IL-10, reactive oxygen species (ROS), and nitric oxide (NO), produced by tumor cells. Furthermore, tumor-infiltrating immune cells such as suppressor immune cells, e.g., T regulatory (Treg) cells, macrophages, and myeloid-derived suppressor cells (MDSC), also influence this phenomenon and are now discussed in more detail.

1.8.1 Treg Cells

Since their discovery in the 1960s as suppressive T-cells, Tregs have been extensively studied in a wide range of both physiological and pathological conditions in human [78]. Treg suppresses T-cell responses and provides another mechanism compromising the development of effective tumor immunity [79]. These cells are usually $CD4^+$ and are distinguishable phenotypically by expression of CD25 (the chain of the IL-2 receptor required for high affinity binding), high levels of CTLA-4, the glucocorticoid-induced TNF-related receptor (GITR), and the forkhead transcription factor Foxp3. Treg cells can arise in response to persistent antigen stimulation in the absence of inflammatory signals, particularly in the presence of TGF-ß, and have been detected in increased frequency in some cancer patients. Furthermore, tumor-induced expansion of regulatory T cells by conversion of $CD4^+$ $CD25^+$ lymphocytes is thymus- and proliferation-independent [80]. Thus, depleting Treg cells in vivo may facilitate the elaboration of effective antitumor T-cell responses.

Inhibiting Treg cell function in patients with cancer is an essential step if new therapies, especially immunotherapies, are to be clinically successful. Initial studies have indicated that depleting Treg cells from cancer patients might be a valid approach; more recent preliminary data has raised the hypothesis that functionally inactivating Treg cells might be a better alternative. Studies in murine tumor models targeting all $CD25^+$ T-cells for depletion have appeared promising [81]. However, activated effector $CD8^+$ and $CD4^+$ T-cells also express CD25, and depletion of these cells during the acute phase of the antitumor T-cell response may severely limit the application of this approach. The availability of the

anti-CD25 mAb, PC61, has enabled the effects of Treg-cell depletion to be tested in murine models [82]. Despite some efficacy, intrinsic limitations apply when PC61 is used to treat established tumors as time course experiments have reported that its efficacy is lost as tumors progress [83]. Other mAbs to human CD25 that are available for clinical use, such as daclizumab, block IL-2, and receptor interactions are used to treat hematologic malignancies [84]. However, to date, most studies in humans have used the immunotoxin denileukin difitox (Ontak), a fusion protein between the IL-2 and diphtheria toxin, to selectively kill lymphocytes expressing the IL-2 receptor. The in vivo antitumor efficacy is still under preclinical and clinical investigation, and discrepant results have been reported so far.

Another approach is to inhibit tumor-specific Treg-cell expansion which could be achieved by inhibiting the indoleamine 2, 3-dioxygenase (IDO) pathway. Preclinical data confirm that the administration of an IDO inhibitor significantly decreases the rate of peripheral conversion and dramatically impairs tumor growth [85]. Another possible target is transformed growth factor (TGF), involved in both proliferation and conversion of Treg cells in tumor bearers. Genetically engineered mice that express a dominant negative form of the TGF receptor on lymphocytes show reduced, if not absent, growth of several transplanted tumors [86]. Moreover, CTLA-4 blockade or GITR triggering has been shown to reverse immune suppression as a result of Treg function both in vitro and in vivo [87].

Ultimately, by inducing Treg expansion, the tumor takes advantage of the inhibitory function that these cells exert on all the immune components. Avoiding the physical elimination of Treg cells would be potentially useful as it would prevent the induction of a new wave of peripherally converted Treg cells that are endowed with a wide TCR repertoire. Conversion would also redirect potential effector T-cells toward the Treg-cell phenotype. Alternatively, Treg-cell inactivation is a suitable strategy, which would functionally impair Treg-cell suppression without changing the TCR repertoire of the expanded Treg-cell population. Triggering of TLR8 or OX40, and potentially blocking adenosine, might improve the chances of neutralizing Treg-cell immunosuppression in cancer immunotherapy.

1.8.2 Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that expand during cancer, inflammation, and infection and have a remarkable ability to suppress T-cell responses [88]. Although suppressive myeloid cells were described more than 20 years ago in patients with cancer [89], their functional importance in the immune system has only recently been appreciated.

Accumulating evidence has now shown that that this population of cells contributes to the negative regulation of immune responses during cancer and other diseases. Common features to all MDSCs are their myeloid origin, their immature state, and a remarkable ability to suppress T-cell responses. In addition to their suppressive effects on adaptive immune responses, MDSCs have also been reported to regulate innate immune responses by modulating the cytokine production of macrophages [90]. Studies have shown that the expansion and activation of MDSCs are influenced by several different factors, which can be divided into two main groups. The first includes factors that are produced primarily by tumor cells, which promote the expansion of MDSCs through the stimulation of myelopoiesis and inhibit the differentiation of mature myeloid cells. The second group of factors is produced mainly by activated T-cells and tumor cells and is involved in directly activating MDSCs. It has also become clear that the suppressive activity of MDSCs requires not only factors that promote their expansion but also factors that induce activation. The expression of these factors, which are produced mainly by activated T-cells and tumor stromal cells, is induced by different bacterial and viral products or as a result of tumor cell death [91].

The immunosuppressive activities of MSDCs require direct cell-cell contact, suggesting that

they function either through cell-surface receptors and/or through short-lived soluble mediator. Such mediators include arginase and nitric oxide synthase (iNOS) [92], reactive oxygen species (ROS) [93], and peroxynitrite [94]. Moreover, it has been reported that MDSCs promote de novo development of the FOXP3$^+$ Treg cells in vivo [95]. As they are one of the main immunosuppressive factors in cancer and other pathological conditions, several different therapeutic strategies that target these cells are currently being explored. These include promoting myeloid-cell proliferation [96], inhibition of MDSC expansion [97], inhibition of MDSC function [98], and elimination of MDSC [99]. Ultimately, the roles of specific MDSC subsets in mediating T-cell suppression, and the molecular mechanisms responsible for the inhibition of myeloid differentiation, need to be elucidated. The issue of whether T-cell suppression occurs in an antigen-specific manner remains to be clarified, as do the mechanisms that induce MDSC migration to peripheral lymphoid organs. Some of the main priorities in this field should include a better characterization of human MDSCs and a clear understanding of whether targeting these cells in patients with various pathological conditions will be of clinical importance.

1.8.3 Macrophages

Macrophages undergo activation in response to environmental signals, including microbial products and cytokines [100]. In response to some bacterial moieties, e.g., lipopolysaccharide (LPS) and IFN-γ, macrophages undergo classic (M1) activation. Alternative (M2)-activated macrophages come in different varieties depending on the eliciting signals mediated through receptors that include IL-4, IL-13, immune complexes plus signals mediated through receptors that involve downstream signaling through MyD88, glucocorticoid hormones, and IL-10. The various forms of M2 activation are oriented to the promotion of tissue remodeling and angiogenesis, parasite encapsulation, regulation of immune responses, as well as promotion of tumor growth. Recent results have highlighted the integration of M2-polarized macrophages with immunostimulatory pathways. They have been shown to induce differentiation of Treg cells [101], and conversely, Tregs have been reported to induce alternative activation of human mononuclear phagocytes [102]. Cancer has thus served as a paradigm of in vivo M2 polarization [103].

In spite of the many pro-tumor activities described for TAM, some studies have reported that high numbers of infiltrating TAM are associated with pronounced tumor cell apoptosis and improved disease-free survival [104]. Moreover, in experimental murine tumor models, the presence of macrophages has been shown to be essential for spontaneous tumor regression. The mechanisms behind the antitumor effects of TAM have not been fully elucidated and could potentially be ascribed to the presence of significant numbers of classically activated M1 macrophages. Macrophage-mediated cytotoxicity involves diverse mechanisms including reactive nitrogen intermediates and members of the TNF receptor family. By damaging vascular cells and activating coagulation, M1 macrophages can elicit tissue- and tumor-destructive reactions that manifest as hemorrhagic necrosis. Recent evidence suggesting that TAM infiltration is positively correlated with response to anti-CD20 therapy in follicular lymphoma is likely the clinical counterpart of these properties [105]. Furthermore, it has been reported that dying tumor cells were able to cross-present antigen to DC in a toll-like receptor (TLR4) and MyD88-dependent manner and also trigger protective immune responses via the "danger signal" HMGB1, again signaling via TLR4 [106]. Thus, the challenge is to dissect pro- and antitumor activities of cancer-related inflammation and tipping the macrophage balance to "reeducate" TAM to exert protective antitumor responses.

1.9 Candidates for Immunotherapy in Oncology

Malignant melanoma, renal cancer, and prostate cancer are potentially immunogenic, making them good candidates for immunotherapeutic approaches [107, 108]. Melanoma has been the most popular target for T-cell-based immunotherapy in part as it is much easier to grow tumor-reactive T-cells from melanoma patients than any other type of human cancer [109]. However, many promising immune-based therapies have been ineffective in human clinical trials [110]. For example, although IL-2, licensed for use in malignant melanoma in the USA, can induce long-term regression of metastatic tumors, it has been associated with high levels of toxicity [111]. As yet, no approved therapy for advanced melanoma has improved overall survival to date. Other immunotherapies for melanoma have not been used outside the setting of clinical trials.

Immunotherapeutic approaches currently under investigation for renal cancer include vaccines, which have been used with limited success. In a phase I trial, a granulocyte-macrophage colony-stimulating factor (GMCSF)-secreting vaccine administered to patients with metastatic renal cancer induced significant tumor regression in one patient. Additionally, infusion with lymphocytes that secrete antitumor cytokines, such as tumor necrosis factor, has also been used in clinical trials [112].

IL-2 is approved in the USA for the adjuvant therapy of stage III renal cancer [113]. In some cases, IL-2 has been demonstrated to induce long-term regression of metastatic tumors and durable complete responses of metastatic tumors, probably by inducing T-cell activation. Interferon-α has been used in clinical trials and has demonstrated a response rate of 15–20% in patients with metastatic disease. Combination therapy with IL-2 has demonstrated improved response rates versus IFN-α alone, although this has not been shown consistently [63].

1.10 Combination Immunotherapy

A deeper understanding of the mechanisms underlying the generation of tumor immunity has provided a framework for developing more potent immunotherapies. A major insight is that combinatorial approaches that address the multiplicity of defects in the host response are likely to be required for clinical efficacy [114]. In addition to surgery, nanotechnology [115] and molecular imaging [116] are methods employed with cancer immunotherapy. The following summarizes some of the combinations that have been tested in laboratory and clinical settings.

1.10.1 Chemotherapy and mAb

Immunostimulatory mAbs directed to immune receptors have emerged as a new and promising strategy to fight cancer. In general, mAbs can be designed to bind molecules on the surface of lymphocytes or antigen-presenting cells to provide activating signals, e.g., CD28, CD137, CD40, and OX40 [117]. MAbs can also be used to block the action of surface receptors that normally downregulate immune responses, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), and PD-1/B7-H1. In combined regimes of immunotherapy, these mAbs are expected to improve therapeutic immunizations against tumors as observed in preclinical studies. Anti-4-1BB (agonistic anti-CD137) mAb has been successfully tested as an anticancer molecule in preclinical studies [118]. Clinical trials of chemotherapy and mAb have resulted in some efficacy against cancer in patients [119]. For example, tremelimumab induced durable objective responses with low-grade toxicities when used as second-line monotherapy in a phase I study with melanoma patients treated with single, escalating doses [120]. Moreover, phase I studies of ipilimumab were performed in patients with prostate, melanoma, and ovarian cancer. In these studies, patients after a single administration of ipilimumab achieved

some clinical efficacy as demonstrated by incomplete reduction of tumor size with extensive tumor necrosis with leukocyte infiltration. In phase II studies, repeated administrations with ipilimumab allowed more patients to achieve objective responses [121]. The combination of ipilimumab with chemotherapeutics (dacarbazine) [122] or docetaxel [123] and with IL-2 [124] or melanoma-associated peptide vaccines [125] improved the rate of complete responses in patients compared with the monotherapy arms.

1.10.2 Chemotherapy and Active Specific Immunotherapy

The combination of active immunization with standard treatments is provocative because of the immunosuppressive effects of most standard treatments. Clinical trials utilizing both chemotherapy and vaccine therapy have been performed in patients with different cancer types, including glioblastoma multiforme (GBM) [126], colon cancer [127], pancreatic cancer [128], prostate cancer [129], and small-cell lung cancer [130]. For example, Wheeler et al. [126] investigated the clinical responsiveness of GBM to chemotherapy after vaccination. Three groups of patients were treated with chemotherapy alone, vaccination alone, or chemotherapy after vaccination. All patients subsequently underwent a craniotomy and received radiation. The vaccination consisted of autologous dendritic cells loaded with either peptides from cultured tumor cells or autologous tumor lysate. Results demonstrated a significantly longer postchemotherapy survival in the vaccine/chemotherapy group when compared with the vaccine and chemotherapy groups in isolation. Overall, data suggests that vaccination against cancer-specific antigens can sensitize the tumor against subsequent chemotherapeutic treatment. Although the mechanisms that underlie such a synergistic effect have not yet been elucidated, it is speculated that the vaccination-induced increase in the frequency of primed T-cells constitutes a major advantage by the time the tumor microenvironment is modified by cytotoxic drugs.

1.10.3 Chemotherapy and Adoptive Lymphocyte Immunotherapy

Lymphodepletion by chemotherapy followed by the adoptive transfer of lymphocytes has been evaluated in small-scale studies in melanoma patients [131]. In a study by Dudley et al. [132], 35 patients were adoptively transferred with autologous cytotoxic lymphocytes with the administration of IL-2 1 day after cyclophosphamide and fludarabine administration. They observed a complete response in only 3 patients, partial response in 15 patients, and no response in 17 patients. Larger-scale studies are needed to assess the efficacy of this treatment modality in cancer patients.

1.10.4 Immunotherapy with Radiation Therapy

Preclinical work in murine models suggests that local radiotherapy plus intratumoral syngeneic dendritic cell injection can mediate immunologic tumor eradication. Radiotherapy affects the immune response to cancer, besides the direct impact on the tumor cells, and other ways to coordinate immune modulation with radiotherapy have been explored. In a recent review, the potential for immune-mediated anticancer activity of radiation on tumors was reported [133]. This can be mediated by differential antigen acquisition and presentation by DC, through changes of lymphocytes' activation and changes of tumor susceptibility to immune clearance. The review alluded to recent work that has implemented the combination of external beam radiation therapy (EBRT) with intratumoral injection of DC. This included a pilot study of coordinated intraprostatic, autologous DC injection together with radiation therapy with five HLA-$A2^{(+)}$ subjects with high-risk, localized prostate cancer; the protocol used androgen suppression; EBRT (25 fractions, 45 Gy); DC injections after fractions 5, 15, and 25; and then interstitial radioactive implant. Another was a phase II trial using neoadjuvant apoptosis-inducing EBRT plus intratumoral DC in soft tissue sarcoma to test if this

would increase immune activity toward soft tissue sarcoma-associated antigens. In future, radiation therapy approaches designed to optimize immune stimulation at the level of DC, lymphocytes, tumor, and stroma effects could be evaluated specifically in clinical trials.

1.11 Humoral Immunotherapy

B-cell activation results in the production of antibodies that can bind to immunogenic cell-surface proteins on tumor cells. These initiate complement-mediated cell lysis, bridge NK cells, or macrophages to the tumor for antibody-dependent T-cell-mediated cytotoxicity (ADCC). They in turn interfere with tumor cell growth by blocking survival or inducing apoptotic signals or increase immunogenicity by facilitating the uptake and presentation of tumor antigens by APCs. Thus, enhancing B-cell responses in vivo or providing a large amount of in vitro-generated antibodies has the potential to promote antitumor activity.

The widely used rituximab binds CD20 and, if given alone or with chemotherapy, can induce high rates of remission in patients with B-cell lymphomas [134], as does cetuximab, which completely inhibits the binding of epidermal growth factor (EGF) [135]. Some mAbs can mediate antitumor activity independent of effector cells, such as by blocking essential survival signals or inducing apoptotic signals. For example, two mAbs approved for clinical use, reactive with the Her-2/Neu receptor on breast cancer cells and the epidermal growth factor receptor on epithelial tumors, provide therapeutic benefits in part by blocking growth signals. The antitumor activity of mAbs can also be enhanced by attaching radioisotopes or drugs or by engineering recombinant bi-specific antibodies that simultaneously bind tumor cells and activate receptors on immune effector cells such as CD3 or FcR [136].

The efficacy of stimulating a patient's own tumor-reactive B-cells may be limited by the magnitude of the antibody response that can be achieved in vivo. Nevertheless, this approach remains appealing because of demonstrations with tumor cell expression libraries that sera from a large fraction of patients already contain tumor-reactive antibodies. The simplest means to stimulate such B-cells in vivo is to provide tumor antigens in immunogenic vaccine formulations, such as mixed with adjuvants or conjugated to antigens that can elicit helper T-cell responses. Marked clinical results have been observed after priming patients with autologous dendritic cells (discussed previously). These cells were pulsed with the unique idiotypic immunoglobulin derived from the B-cell receptor of a patient's own B-cell lymphoma followed by boosting with the immunoglobulin conjugated to the helper protein keyhole limpet hemocyanin (KLH).

Alternative approaches for activating and expanding existing B-cell responses in vivo by ligation of co-stimulatory molecules, such as CD40 or by administration of the B-cell proliferative cytokine IL-4, have not met with much success in preclinical models and could potentially induce hazardous autoreactive antibodies. Thus, humoral therapy will likely continue to be dominated by passive administration of mAbs specific for selected tumor antigens.

1.12 Concluding Remarks

Immunotherapy of cancer has long been considered an attractive therapeutic approach. While mAbs, cytokines, and vaccines have individually shown some promise, it is likely that the best strategy to combat cancer is to attack on all fronts. Different strategies demonstrate benefit in different patient populations. To improve early encouraging clinical results, biomarkers to better select patients that may benefit from immunotherapy are actively sought. Furthermore, immunosuppression associated with cancer has to be overcome to allow better immunostimulation. It may be that the best results are obtained with vaccines in combination with a variety of antigens or vaccine and antibody combinations. Finally, combination of immunotherapy with conventional treatments (chemotherapy, anti-angiogenic, etc.) should further improve this approach, both in its effectiveness and in its clinical indications.

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Novel Strategy of Cancer Immunotherapy: Spiraling Up

2

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Contents

2.1 Introduction

The early internationally accepted ideas of basic immune mechanisms date back to 1908 when the two outstanding scientists—Russian physiologist Ilya Mechnikov and German researcher Paul Ehrlich—shared the Nobel Prize for the discovery of cell immunity (phagocytosis, I. Mechnikov) and humoral immunity (antibody development, P. Ehrlich). These major immune mechanisms determine individual resistance to infections, and the later studies led to a scientific discussion on antitumor immunosurveillance and, more recently, immunoediting. Different evidence may prove active function of antitumor immunity:

- Phenomenon of spontaneous regression of a primary tumor or metastases.
- Although occasional, it is a registered fact. The regression of primary skin melanoma or lung metastases from renal cell carcinoma

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occurs in one third of the cases as partial spontaneous regression. Complete melanoma regression was observed in 1–2% of tumors. In case of palliative resection of kidney, spontaneous regression of some lung metastases was also registered.
- Detection of the cellular stromal reaction to tumor progression.
- Morphological studies reveal tumor infiltration by immune cells such as lymphocytes, macrophages, granulocytes, MDSC, etc.
- AIDS-associated tumors.
- Mechanism of tumor escape from the immune attack is primarily based on the lack of specific antigens on tumor cell surface and loss or downregulation of the expression of molecules of major histocompatibility complex (MHC), which are necessary factors for initiation of adaptive immune response and generation of antigen-specific T-lymphocytes. These findings can partly explain the poor results of most clinical trials studying the effectiveness of dendritic cell-based vaccines and some other immunization types relying on specific immunity.

Recent data have given more evidence in favor of innate immunity being the main arm of immunosurveillance in the fight against tumor development. Moreover, natural killer cells (NKs) play a crucial role as they can recognize and lyse transformed cells in an MHC and antigen-independent manner. In addition, an important part in implementation of antitumor defense is assigned to other effectors of innate immunity such as natural killer T cells (NKT). Along with the mentioned functions, innate immunity effectors can have a negative regulatory effect on antitumor immunobiological surveillance by secreting T-helper cell type 2 (Th2) cytokines. Antitumor immunity has been the subject of most thorough interest and detailed investigation over the last decades. Contemporary standpoints in understanding mechanisms of innate and adaptive immunity are the basis for development and improvement of immunotherapy approaches. Even though numerous research data on cell-based technologies offer extensive information, no comprehensive concept of the most effective implication of antitumor immunotherapy is available so far. This chapter presents an overview of the most extensively studied approaches that make the ground for an immunotherapeutic strategy at the next step of the research ladder.

2.2 Natural Killer Cells: The Key Effectors of Innate Immunity

Natural killer (NK) cells are effector cells that play a critical role in the early innate immune response to pathogens and cancer [1].

NK cells were identified in humans and mice in 1975 as a result of their specific function of lysing certain tumor cells with no prior stimulation. NK cells were qualified as lymphocytes on the basis of their morphology, expression of lymphocyte markers, and their origin from the common lymphoid progenitor cell in the bone marrow. NKs, however, are regarded as part of innate immune defense as they lack antigen-specific cell surface receptors. Unlike T- or B-lymphocytes of the adaptive or antigen-specific immunity, NK cells do not rearrange T-cell receptor or immunoglobulin genes from their germline configuration. The NK morphologic type of large granular lymphocytes shows (due to a large number of secreting granules) their high functional activity, and they have characteristic immunophenotype $CD3^{-}/CD16^{+}/CD56^{+}$. NKs account for 5–20% of total lymphocyte number in humans. NK cells can detect and lyse cells with deficient expression of MHC class I (MHC-I) molecules, which help better understanding of the function and role of NK cells in the immune response. These cells also bear receptors to IL-2, and evidently, they can be activated by this endogenous cytokine or its exogenous analogues. Being effectors of the innate immunity, NKs need no cascade of antigen presentation reactions to perform their function (Fig. 2.1). Along with neutrophils, NKs may be considered “the first line of defense” of the immunosurveillance as they can cause lysis of a transformed cell after contacting it with no additional stimuli. However, NK cell triggering function relies on a complex balance

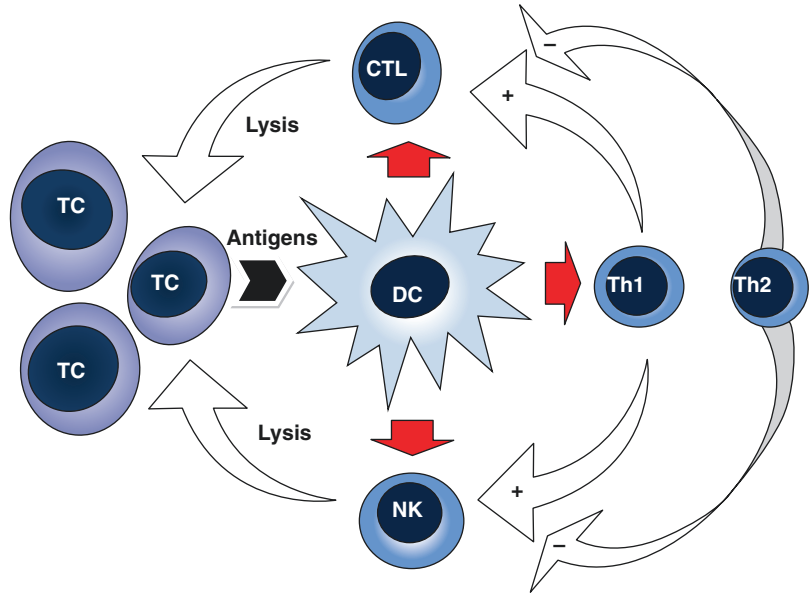

Fig. 2.1 Schematic interaction of the effectors of innate and adaptive immunity. *Abbreviations*: *TC* tumor cell, *DC* dendritic cell, *Th1* and *Th2* T helper cells of types 1 and 2, *NK* natural killer cell, *CTL* cytotoxic T-lymphocyte

between inhibitory and activating signals and requires not only a deficient MHC-I expression on target cells but also the expression of inducible ligands of activating NK cell receptors. Both points are crucial for antitumor immunity performance since transformed tumor cells may shed off MHC molecules, lose tissue-specific antigens, or acquire features of embryonic cells (low-differentiated embryocarcinomas) and thereby "escape" from specific immunity. Such particularly malignant cells may become the target for NKs. These effector cells have the ability to recognize and destroy a wide range of abnormal cells (including tumor cells, virus-infected cells, cells bound to an antibody, allogeneic cells), as well as stressed cells, without damaging the healthy and normal "self" cells. Tumors developed mechanisms to escape NK cell control such as the shedding off soluble NKG2D ligands that function as decoys for the activating NKG2D receptor on NK cells, a phenomenon correlating with poor prognosis in human melanoma and prostate cancer [2].

NK cells can regulate immune responses by activating DCs and promoting their differentiation into mature, high IL-12-producing type 1 polarized DCs (DC1) with enhanced capacity to induce Th1 and CTL responses, the response most desirable against cancer [3]. Conversely, the innate and effector functions of NK cells require close interactions with activated DCs. Cell membrane-associated molecules and soluble mediators, including cytokines and prostaglandins (PGs), contribute to the bidirectional cross talk between DCs and NK cells [4, 5].

NK cells use an array of innate receptors to sense their environment and respond to alterations caused by infections, cellular stress, and transformation. The activity of NK cells is controlled by balancing inputs from activating and inhibitory receptors. The most important ligands for inhibitory receptors are MHC-I molecules. Since normal cells express high levels of MHC-I, they are most often protected from NK cell killing. In contrast, target cells expressing downregulated levels of MHC-I are seen as "missing self" and killed [6, 7].

Three predominant superfamilies of NK cell receptors (NKRs) have been identified that can either inhibit or activate NK cell function: killer immunoglobulin (Ig)-like receptors (KIRs) that bind to classical class I MHC molecules, C-type lectin receptors that bind to nonclassical class I MHC molecules or "class I-like" molecules, and natural cytotoxicity receptors for which ligands are currently not well defined [8]. The different NK cell subsets show important differences in their cytotoxic potential, capacity for cytokine

production, and responses to cytokine activation. The $CD56^{bright}$ NK cells are the major population of NK cells that produce immunoregulatory cytokines, including interferon-γ (IFN-γ), tumor necrosis factors (TNF-α and TNF-β), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukins (IL-10 and IL-13) after monokine stimulation. On the other hand, immunoregulatory cytokine production by $CD56^{dim}$ NK cells is negligible even following specific stimulation [9].

The above-described characteristics and functions show that NKs are obviously a valuable source for adoptive antitumor immunotherapy, and they can not only recognize and lyse transformed cells with no or low expression of MHC and tumor-associated antigens but also play an important role in regulation of immune reactions, which makes a rationale for combination of antitumor vaccines and NKs in immunotherapy approaches.

2.3 Adoptive IL-2/LAK (or CIK) Therapy of Cancer

IL-2 stimulation of lymphocytes results in generation of the so-called LAK cells. LAKs are a heterogeneous population of lymphocytes that include primarily NK, NKT, and T cells, which are generated in vitro from peripheral blood mononuclear cells (PBMC) in the presence of IL-2. The major effector subset in the LAK population is NK cells, which are mechanistically regarded as peripheral blood NK cells but are more cytotoxic against tumor cells, including NK-resistant targets [10].

The first real clinical progress in immunotherapy was seen after the introduction of recombinant DNA technology used for production of immune-stimulating cytokines. Since 1985, several studies on combined IL-2 and LAK cell treatment have been performed, and the results were published [11–15].

Such clinical trials have shown that high-dose IL-2 alone or in combination with LAK cells mediates objective tumor regression in 17–28% of patients with metastatic renal cancer or metastatic melanoma, while prolonged remission was observed even in some patients with metastatic cancers [16]. Some authors have reported on clinical trials of the systemic treatment with high-dose IL-2 and tumor-infiltrating lymphocytes (autologous lymphocytes can be isolated from tumor-infiltrating cells, which presumably express tumor-specific TCRs) of patients with advanced cancer. Such treatment resulted in a 34% objective response rate of patients with metastatic melanoma [17]. Although there was considerable clinical interest in LAKs for antitumor therapy by the end of the last century, LAK therapy has failed to obtain public support as a standard therapy for cancer patients. This was largely the result of limited response to immunotherapy when compared with that to chemotherapy or radiation therapy, and there were concerns about toxicity associated with the IL-2 infused simultaneously in order to maintain LAK activation. Another confounding factor was that most studies on immunotherapy used terminal-stage patients with virtually no remaining immune response functions, as they had failed to respond to previous conventional treatments [18].

More recently, a new cell-based immunotherapy utilizing activated lymphocytes has been suggested as an adjuvant regimen to radical surgery of cancer patients. Kimura and coauthors conducted a randomized trial of 174 patients with non-small-cell lung carcinoma comparing IL-2/LAK therapy in combination with chemotherapy versus chemotherapy alone [19]. Patients had undergone curative resection of their lung carcinoma and received six to eight courses of IL-2/LAK therapy over 2 years. The authors reported an improvement in the 5- and 9-year survival rates of 21% and 28%, respectively. Other studies involved cytokine-induced killers (CIKs) (inducers: IFN-γ, Ab-anti CD3 and IL-2) for adjuvant treatment of solid tumors. CIK cells are a heterogeneous subset of ex vivo expanded T lymphocytes presenting a mixed T-NK phenotype and have unrestricted MHC antitumor activity [20]. In the setting of hepatocellular carcinoma and gastric cancers, adjuvant infusions of autologous CIK cells after surgical resection resulted in a significant increase of disease-free survival [21–23].

To improve IL-2/LAK immunotherapy effectiveness, local and locoregional infusions were performed, which increased the effective concentration of activated killers at the site of the lesion. The most significant clinical effects were achieved with intra-cavity infusions of IL-2 and LAKs in patients with malignant effusions (pleuritis, ascites, and pericarditis). Malignant effusion regression was seen in 70–95% of cases, showing good tolerance and effectiveness in chemotherapy-resistant cancer types [24]. One of the advantages of adjuvant locoregional immunotherapy is that these low IL-2 immune-stimulating doses cause no marked side effects, neither immune nor myelosuppression, which are characteristic of high-dose cytokine therapy.

These LAK- and CIK-cell immunotherapy methods aim to stimulate the innate chain of antitumor immunity, which is a reasonable approach because most tumors express either little or no MHC or tumor antigens. It is also necessary to consider the fact that T killers constitute an essential part of lymphoid cell populations and are responsible for a more specific mechanism of action—in these conditions, they are obviously not involved in the antitumor defense function.

2.4 Tumor-Infiltrating Lymphocytes (TILs) in Cancer Immunotherapy

The basic stage of antitumor immunotherapy is the generation of lymphocytes that specifically recognize tumor cells. T cells recognize short peptides derived from proteins lysed in nucleated cells and presented in the context of MHC molecules on the cell surface. Adoptive cell transfer is a treatment strategy that allows activation and expansion of tumor-reactive T cells ex vivo for subsequent reinfusion to the autologous host. Hundreds of peptides restricted to presentation on different subclasses of MHC molecules and derived from tumors of different histological types have been identified over the last decades [25]. Tumor-associated antigens fall into several major categories: (1) overexpressed normal proteins (e.g., carcinoembryonic antigen (CEA) or non-mutated p53); (2) non-mutated differentiation antigens (e.g., MART-1, overexpressed in melanoma and found in normal melanocytes); and (3) cancer-testis antigens (CTA), consisting of non-mutated genes expressed during fetal development and then silent in normal adults. The description of TILs derived from a variety of histological cancer types demonstrated that cellular immune reactions against established malignancies exist in humans. TILs are heterogeneous populations of mononuclear leukocytes, which include not only $CD4^+$ and $CD8^+$ T lymphocytes (as previously reported) but also a small and, in some cases, significant fraction of γδ T cells, with a prevalence of the Vδ1 subset [26] as well as macrophages. TILs that infiltrate melanoma can specifically recognize tumor-associated antigens [27] (e.g., MAGE and NY-ESO); (4) mutated antigens, unique to a single tumor or shared by a group of tumors (e.g., BRAF with the V600E mutation in melanoma and other solid tumors, or EGFRvIII in glioblastoma) [28].

Some authors presented early results in patients with metastatic melanoma treated with the adoptive transfer of autologous TILs selected for antitumor activity—expanded in vitro and then reinfused into patients along with IL-2, following a lymphodepleting preparative regimen [29–32].

In clinical trials with increasing lymphodepletion prior to infusion of autologous TILs, objective response rates between 49% and 72% were seen for patients with metastatic melanoma [33]. Limitations of TIL therapy, including the requirement for surgery to isolate the tumor and the need to consistently generate T cells with antitumor activity, have led to novel strategies for redirecting normal T cells to recognize tumor-associated antigens (e.g., NY-ESO-1, CEA (carcinoembryonic antigen), anti-CD20) using genetically engineered tumor antigen-specific TCRs or chimeric antigen receptor genes. As an alternative to TIL therapy, highly avid TCRs can be cloned from naturally occurring T cells, and then gene transfer vectors can be used to introduce these into the patient's lymphocytes. In this manner, large numbers of antigen-specific T cells can be rapidly generated, in comparison with the long-term

expansion required for TILs. These highly reactive T-cell clones are able to recognize and effectively lyse target tumor cells [34–36].

Recently, several clinical trials have reported clinical efficacy and benefit of gene-modified T cells for treatment of different cancers, including melanoma, colorectal and synovial cell cancers, neuroblastoma, and lymphoma. In patients with synovial cell cancer, the measurable response rate was 66%, compared to 45% in melanoma patients [37–39]. However, though a number of studies showed effective TIL therapy, the complicated methodology of lymphocyte isolation from tumors and generating a purified appropriate TIL culture still remains a strong limitation. This laborious method is mainly applied in melanoma treatment because this tumor type provides a sufficient number of lymphocytes. Besides, to achieve TIL's effect, lymphodepletion by means of chemotherapy or radiotherapy is needed, which is considered to extend the TIL's active period. Therefore, TIL therapy has a number of essential limitations resulting from the necessity to obtain an appropriate tumor sample and then isolate lymphocytes, as well as the necessity of chemotherapy or radiation therapy for lymphodepletion.

On the other hand, a promising area of TIL implication is the treatment of malignant effusions (pleuritis, ascites, and pericarditis). TILs from such metastatic material are available in large numbers and may be easily expanded ex vivo in the presence of IL-2 or INFs.

We performed a clinical trial on evaluation of the effectiveness of intrapleural IL-2/LAK immunotherapy in 85 patients (pts) with malignant effusions—primary tumor types included lung cancer, breast cancer, mesothelioma of pleura, and other cancer localizations. Autologous LAKs were generated from TILs—lymphocytes of the patient's pleural effusions. Prior to IL-2/LAK therapy, most patients (56%) with malignant effusions received radiation and chemotherapy including intrapleural infusion of cytostatics, which had no clinical effect.

Before the beginning of the immunotherapy, 500–2800 ml of serous or serous hemorrhagic liquid was evacuated from pleural cavity. Cytological examination of pleural effusion was performed in all cases.

In most cases, one-sided pleuritis developed with equal frequency from the right or left side. In 7.7% of cases, two-sided accumulation of pleural effusion was registered; such patients had drainage firstly in one pleural cavity, then if clinical effect was achieved, the other one was drained.

Intrapleural infusion of IL-2 and LAKs (generated from autologous TILs) achieved clinical effect in 88% (75 pts). 60 pts. had complete remission and 10 pts. experienced partial reduction of effusion (Fig. 2.2a, b). Recurrence of effu-

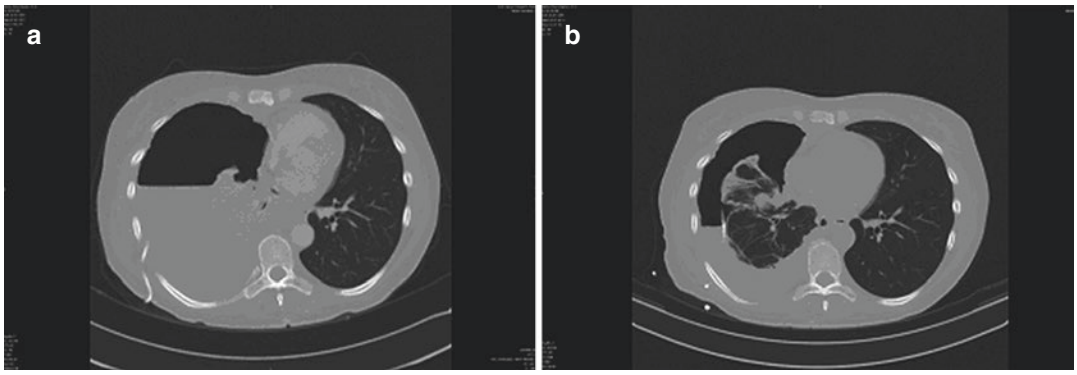

Fig. 2.2 CT of the chest during the course of IL-2/LAK immunotherapy of malignant pleural effusion. Patient Sh. Lung cancer (the right lung), right-sided pleuritis. (**a**) Prior to IL-2/LAK intrapleural immunotherapy; (**b**) 2 months after the immunotherapy. Partial effect

sion occurred in 10 (11.8%) patients 1.2–2.5 months after completed treatment. However, one or two repeated courses of IL-2/LAK therapy resulted in the regression of malignant effusion. It is important to emphasize that delay or cessation of effusion was achieved only in those cases where pleural liquid contained essential number of activated lymphoid cells including immunoblasts.

Eight patients had repeatedly several immunotherapy courses due to encapsulated pleuritis. The second course was performed after 1 month interval, and IL-2 intrapleural infusion was accurately administered into small (up to 150 ml) residual cavities; clinical effect was registered in all these cases.

Plasmic part of effusion after elimination of tumor cells if necessary may be reinfused intravenously to maintain homeostasis of cancer patients. Indications to such reinfusions are determined by the severity of the patient's performance status, edemas due to lack of proteins, or hypoalbuminemia. Reinfusion of plasmic effusion part to ten patients was totally satisfactory, and no side effect was noted. For reinfusion purposes, plasmic part was additionally centrifuged at 6000 rpm during 30 min in order to eliminate cellular fractions, and after that it was carefully examined in cytological, bacterial, and biochemical tests and then reinfused intravenously to the patients.

In some cases along with immunologic pleurodesis, there were registered decreased indexes of tumor markers and reduced size and density of metastatically modified supraclavicular lymph nodes. Elimination of effusion accumulation opens a new opportunity to treatment that was started before effusion onset: 1 patient had a successful radiation therapy, and 15 patients underwent chemotherapy due to non-small-cell lung cancer. Other patients had a dynamic follow-up during 2 months to 2 years. Course of disease within this period demonstrated other symptoms of cancer process, including disease progression but free from malignant effusion.

Analysis of autologous LAK immunophenotype showed that after cultivation of lymphocytes derived from effusion during 3–5 days in the presence of IL-2, the number of $CD4^+/CD25^+$ cells may increase, which may occur due to lymphocyte transformation into activated cells triggered by IL-2. Infusion of high doses of IL-2 can also stimulate functions of natural subpopulation of regulatory $CD4^+/CD25^+/Foxp3^+$ T cells (T-reg), which play their role in immunologic tolerance and suppress antitumor activity of NK and T cells [40, 41].

Our data showed no increase of $CD4^+/CD25^+/Foxp3^+$ T-reg in LAK population even during long-term incubation of peripheral blood lymphocytes of healthy donors or cancer patients in the presence of IL-2. If only generating LAKs from lymphocytes of the pleural effusion with enhanced initial T-reg subset, the number of suppressive T-reg subpopulation might increase [42].

2.5 Autologous Vaccines on the Base of Dendritic Cells (DC Vaccines)

Dendritic cells (DCs) are the antigen-presenting cells (APC) with a unique ability to induce primary immune response. DCs both prime naive cytotoxic T cells and activate memory cells play an important role in adaptive immunity.

Mature DCs for antitumor vaccines are typically generated from $CD14^+$ monocytes according to a well-known two-stage methodology. The initial stage is cultivation for 6–7 days in the presence of granulocyte-macrophage colony-stimulating factor and IL-4 in macrophage-conditioned medium [43].

The second stage — DC maturation — may proceed in the presence of various factors, such as bacteria (live or dead), bacterial products, lipopolysaccharide, viruses, two-strand RNA or its analog poly-I:C, proinflammatory factors and their combinations (IL-1β, tumor necrosis factor-α, IL-6, prostaglandin E2 [PGE_2]), and CD40 ligand (CD40L). During maturation, DCs lose their ability for endocytosis and antigen processing [43, 44]. Early studies on the use of DCs involved only small groups of patients, but reported potentially promising results [45, 46].

To date, over 200 clinical trials have assessed DC-based vaccines, yet their clinical effectiveness and expedience for the use in cancer patients become more and more doubtful. Rosenberg et al. argued that early optimism for DC vaccines relied rather on dubious surrogate end points, which lacked robustness, than on evidence-based proof of antitumor effects. One trial, conducted at the Surgery Branch of the National Cancer Institute on 440 patients, yielded an overall objective response rate of only 2.6%. This was comparable to the 4.0% response rate reported in 40 other smaller studies involving a total of 756 patients [47]. More recent studies showed partial or complete regression rates of 4.0–12% in patients with advanced cancer [48].

2.6 Advantages of Combined Implication of DC Vaccines and Activated Lymphocytes

Experimental studies in vitro showed that co-incubation of DCs and activated lymphocytes results in enhanced antigen-presenting function of DCs and increased cytotoxic lymphocyte activity [49, 50]. When DCs pulsed by tumor lysate (TL) are cultured with activated lymphocytes, they can induce a specific and strong immune response against renal carcinoma cells (RCC) and prostate cancer cells [51]. On the basis of their initial in vitro experiments, other authors planned and conducted a randomized controlled trial to evaluate the efficacy of adjuvant immunotherapy with autologous TL-pulsed DCs co-cultured with CIK cells for treating cancer patients. The described cell culture was used for immunotherapy against localized and locally advanced RCC. The authors mentioned that nearly 20–40% of patients with clinically localized RCC develop metastases after nephrectomy or nephron-sparing surgery [52]; therefore, such patients need effective adjuvant therapy. A recent randomized controlled trial of adjuvant combined immunotherapy by TL-DC-CIK cells showed that all patients tolerated the TL-pulsed DC-CIK cell immunotherapy very well, and side effects in the DC-CIK group were less than in the IFN-α group. The metastasis and recurrence rates were significantly decreased after TL-pulsed DC-CIK cells or IFN-α immunotherapy compared with the control group [53]. Effectiveness of TL-DC-CIK cell immunotherapy was shown in combination with chemotherapy in patients with breast cancer, advanced non-small-cell lung cancer, and multiple myeloma [54, 55]. There are ongoing clinical studies on evaluation of the effectiveness of TL-DC-CIK cell immunotherapy in patients with hepatocellular and pancreatic carcinomas [56, 57]. The authors consider combined DC-CIK cell immunotherapy as a novel strategy for treatment of cancer patients which improves effectiveness of antitumor vaccines and activated lymphocytes.

2.7 Combination of Immune Checkpoint Blockade and Adoptive Immunotherapy

The insufficient effectiveness of adoptive immunotherapy is often related to the weak antitumor immune response or to the inhibition of the immune reactions by the tumor.

Immune checkpoint blockade can probably increase effectiveness of different immunotherapy methods since blocking these inhibitory receptors triggers excessive immune reaction. Currently, a number of studies have been set off to investigate this approach. So far, various in vivo experiments and some pilot clinical studies have been performed that showed encouraging results of treatment by a combination of mAb to CTLA-4 and PD-1 with adoptive immunotherapies on the base of DCs or ex vivo activated lymphocytes.

As a rule, DCs stimulate antigen-specific T lymphocytes by interaction of MHC molecules with T-cell receptor (TCR). However, what is most important in the induction of the immune response is the co-stimulating signal that T cells receive from B-7 surface DC molecules via co-receptor CD28-stimulating molecule. At this stage, negative regulation may involve inhibiting receptor CTLA-4, which interacts with B-7

molecules with greater affinity than CD28 and can either directly compete with CD28 or decrease co-stimulating DC potential by trans-endocytosis of B-7 molecules [58]. CTLA-4 blockade (by target mAb) disrupts this interaction and disables the potential of inhibiting immune reactions at this point. Besides that, DCs have other lymphocyte-inhibiting receptor surface ligands PD-L1 and PD-L2. Interaction of PD-1 and its ligands can also decrease the immune response [59]. Blocking antibodies against PD-1 (nivolumab (Opdivo®), pembrolizumab (Keytruda®)) or against PD-L1 (atezolizumab (Tecentriq®)) can play their role at this stage. Moreover, PD-1 can regulate the immune response during the ongoing process of immunologic reaction in tissues with PD-L1.

It should be noted that other inhibiting receptors (such as lymphocyte activation gene-3 (LAG-3) and T-cell immunoglobulin and mucin-domain-containing-3 (TIM-3)) are less investigated than PD-1 and CTLA-4 [60]. Blocking antibodies to these receptors have not been approved yet.

Interestingly, inhibiting receptors PD-1 and CTLA-4 were found in NKs as well, where they also function as immune inhibitors [61]. It is well established that these effectors of innate immunity can act as antitumor factors and play an essential role in antitumor therapy on the base of ex vivo activated lymphocytes. Therefore, PD-1 and PD-L1 and PD-L2 blocking antibodies are potential therapeutic agents in such kind of treatment.

Effective combination of antitumor DC-based vaccine and immune checkpoint inhibitors was achieved in preclinical studies on mice [62, 63].

Similar results were shown in some limited clinical studies [64–66]. Blocking antibodies to CTLA-4 MDX-010 (Ipilimumab) were added along with IL-4 and GM-CSF into the cell culture of PBMC (peripheral blood mononuclear cells) of patients with acute myeloid leukemia (AML). As a result, the generated DCs induced a much stronger cytotoxic T-cell response to the malignant AML cells than those generated in standard conditions with no ipilimumab [64]. In relation to these data, it is interesting to notice that CTLA-4 was detected on the DC surface and may reduce DC antigen presentation [65]. Ribas et al. showed in a clinical trial with 16 patients with advanced melanoma a great effectiveness of combination of anti-CTLA-4 antibodies (tremelimumab) and DC pulsed by melanoma peptide MART-126-35 as compared with both monotherapies [66]. However, the authors registered significant side effects of autoimmune origin (hypophysitis, diarrhea of grade 3) in 2 out of 3 patients who received monthly tremelimumab simultaneously with DC-vaccine in the highest dose of 10 μg/kg.

In a recent phase II clinical trial, Wilgenhof et al. performed systemic administration of Ipilimumab in combination with the antitumor DC-vaccine loaded with synthetic RNA TriMixDC-MEL by electroporation in patients with advanced melanoma [67]. The study achieved a long-term significant clinical effect (objective response—38%). However, marked unfavorable immune effects were noticed, such as local redness at the site of DC injection (100%), chills (38%), a flu-like condition (84%), dermatitis (64%), hepatitis (13%), hypophysitis (15%), and diarrhea/colitis (15%). Unfavorable side effects of the immune origin of grade 3 and 4 were registered in 36% of patients.

Sioud et al. studied the effect of DC-vaccine in a patient who had received pretreatment by Ipilimumab [68]. The therapy achieved reduction of metastases and improvement of patient's general status. Therefore, it may be stated that administration of DCs pulsed with tumor antigen and simultaneous CTLA-4 blockade stimulates immune response to antigens that previously was not activated.

Antonios et al. demonstrated that PD-1 blockade improves efficacy of DC-vaccine in mice with glioma [69]. Moreover, they showed that blocking PD-1 receptor ex vivo on human tumor infiltrating lymphocytes dramatically increased lysis of the autologous tumor.

Another study showed that autologous CIK (cytokine-induced killer cells) activity against AML cells increases when blocking inhibitor receptors such as killer cell immunoglobulin-like receptors (KIR), LAG-3, PD-1, and TIM-3, but not CTLA-4 [70]. However, other diseases –

acute lymphoblastic leukemia (ALL) and multiple myeloma (MM) — were refractory to CIK treatment, and immune checkpoint blockers could not alter tumor cell resistance.

Combination of CIK and PD-1/PD-L1 blockers was found effective in the experimental model of gastric cancer therapy in mice where it demonstrated significant inhibition of tumor growth and increase of experimental animals' survival [71].

Immune checkpoint blockade may lead to enhancement of TIL function, which can be another approach in adoptive antitumor therapy [72, 73].

2.8 CART Cells

CART cells are immunocytes that are genetically modified and express surface chimeric antigen receptors along with various costimulatory molecules. The chimeric antigen receptor T (CART) cells target tumor antigens, and they are able to maintain survival and proliferation of their cell population via cytokine production. The unique points of this technology include an HLA-independent manner of cancer cell recognition, specific antigen targeting, and single-course infusion of CART cells. Such advantages make adoptive immunotherapy with CAR technologies a highly perspective approach.

Kochenderfer et al. reported high efficacy of CART therapy in treatment of CD19+ B-cell acute lymphocytic leukemia. The study was performed at National Cancer Institute and involved anti-CD19 CAR T cells containing CD3z/CD28 signaling domains in combination with low cyclophosphamide doses in patients with relapsed/refractory B-cell lymphomas. The results demonstrated an overall response rate of 73% and a CR rate of 55% [74]. Another multicenter study included seven patients with refractory diffuse large B-cell lymphoma (DLBCL). The patients received CD3z/CD28-based CAR T-cell therapy during 30 days, which involved a dose of 2×10^6 CAR T cells/kg in combination with low-dose conditioning chemotherapy of concurrent cyclophosphamide and fludarabine. Five patients achieved an objective response which lasted for 1 month, four of them had a complete effect. However, all patients developed marked unfavorable events with a maximum grade of 3, 4, and 5 reported in three (43%), three (43%), and one (14%) patient(s), respectively. The most frequent of which was neutropenia (febrile neutropenia) and encephalopathy of grades 3–4, as well as cytokine release syndrome with fever and hypotension manifestation [75]. However, no similar effect has been seen in solid tumors yet [76]. A few clinical trials enrolling a limited number of subjects demonstrated complete effect of 27% in patients with neuroblastoma, partial effect, and disease stabilization in patients with non-small cell lung cancer and prostate cancer [77, 78]. It is important that special attention is drawn to study toxicity problems, such as cytokine release syndrome, neurotoxicity, and non-tumor cytotoxicity. The grade and number of these unfavorable events of solid tumor treatment might be reduced by optimal combination of chemotherapy, surgery, radiation therapy, and immunotherapy. Another approach to achieve decrease of unfavorable events is local (intracavity) infusion of therapeutic agent. Currently, clinical trials are going on to study intrapleural and intraperitoneal infusion of CAR T cells in patients with mesothelioma and ovarian cancer [79, 80]. Recently, some reports have suggested a new method of generating CAR-transduced NK cells. They have a number of advantages compared with T cells such as an established safety in clinical trials and a specific mechanism of targeting cancer cells. Human NK cells and NK-92 cell line were successfully transduced to express chimeric antigen receptor against hematological cancers as well as solid tumors. In addition, NK cells express various activation receptors (NKR), such as CD16, NKG2D, CD226, and NKp30, which may specifically target ligands expressed on the tumor cells. However, it is necessary to note that NK transduction reaches rather low effectiveness that requires more developmental studies to improve safety and therapeutic efficacy of CAR treatment [81].

2.9 Spiral Up

Despite the theoretical rationale and experimental basis of antitumor cytotoxicity of induced lymphocytes, adoptive immunotherapy with lymphokine-activated lymphocytes, designed by Rosenberg and coauthors at the beginning of the 1980s of the last century, seems not to achieve the expected results. The initial enthusiasm about immunotherapy of cancer patients gave place to grave pessimism lasting for almost two decades, while only some research groups continued the search for effective use of activated lymphocytes. It was during that period of ruined expectations for clinical efficacy of LAK immunotherapy that a fundamentally new principle of the use of activated effectors of antitumor immunity was suggested.

Immunotherapy is not regarded as a method of standard conservative antitumor treatment anymore, when effective therapy uses maximal tolerated doses of drugs (cytokines in immunotherapy) and includes patients with advanced cancer. Finally, we reached understanding that special functions of antitumor immunity effectors are limited to certain conditions and it is important to create an effective ratio of cell targets/effectors in order to achieve good clinical results. Such effective cell ratio can be created by local and/or locoregional infusion or in adjuvant treatment after radical surgery with the aim to extend relapse-free period. Besides, immunotherapy now uses low immune stimulating cytokine doses, which do not cause significant side effects. Immunotherapy in this manner limits the area of its implication but gives a real opportunity to achieve essential clinical effect in target patients.

The next step for antitumor cell-based immunotherapy was made by designing antitumor DC vaccines, which unlike LAK (or CIK) can stimulate adaptive (specific) immune response to target antigens. However, extensive clinical trials performed over the last years showed that the real effectiveness of DC vaccines, if not counting on surrogate criteria, seemed to be even lower than that of LAK therapy. Even though at present the search for approaches to improve DC-vaccine effectiveness is still continuing, the probability of reaching the expected results is doubtful because malignantly transformed cells have no unique specific antigens and may lose or have low expression of MHC antigens. In addition, the heterogeneity of tumor cell population, where tumor cells have different expression of target tumor-associated antigens, should always be kept in mind.

Combination of cell-based antitumor vaccines and immune checkpoint blockers may be efficient in achieving optimal results. An interesting approach is presented by those studies which employ inhibitors of immune checkpoints at the stage of ex vivo generation of DCs or CIKs, but not as systemic patient's treatment. This methodology suggests much lower risk of autoimmune reactions induced in response to immune checkpoint blockade while it simultaneously enables generation of highly activated effector cells.

Over the last years, CART technologies have evoked much hope. This technology may help to overcome one of the mechanisms of tumor evasion from immune surveillance, namely, the one that takes advantage of the lack or low expression of MHC molecules. However, this method does not resolve the major problem of the lack of tumor specific antigens. That may explain why CART cells show effective results in leukemia only, where the target is a leukocyte differentiation antigen, in particular, CD19. Besides, marked side effects — such as pancytopenia — obviously reflect the fact that CART cells produce a cytotoxic effect not only on the cells expressing the target antigen but also on other hematopoietic elements. Including CAR NK cells in immunotherapy may increase the efficacy only due to their function of transformed cell recognition in an MHC and antigen-independent manner. Therefore, it is unlikely that CAR NK can significantly surpass the effects shown before by conventional adoptive immunotherapy on the base of activated NKs (LAK and CIK technologies). So far, limited clinical experience of local (intra-cavity) CART cell infusion also has not shown any advantages over LAKs or TILs. Hence, this sophisticated and expensive method of antitumor therapy will hardly have a wide clinical application in near future, and probably its

effectiveness will be restricted to several leukemia types resistant to conventional treatment. To date, the efforts of making this method more available employing allogenic CART technologies have not achieved a big success yet; clinical trials have been halted by the FDA because of significant toxicity [82].

Thus, at the new step of spiral development, cell-based immunotherapy once again returns to exploiting activated lymphocytes and NK, LAKs, CIKs, and TILs, but novel strategy uses them in adjuvant regiment or in local/locoregional treatment with simultaneous low immune-stimulating doses of cytokines. Since NKs and DCs have reciprocal activating relations, a novel strategy for improved immunotherapy suggests combined use of activated lymphocytes and tumor antigen-pulsed DCs. Such approach may not only increase activity of effectors of antitumor immunity but also stimulate both innate and adaptive immunity and thus target a wider range of tumor cells regardless their expression of MHC or tumor-associated antigens.

2.10 Concluding Remarks

Despite tremendous progress in basic immunological research, effective immunotherapies for most cancer types have been hardly set into clinical practice. However, the results of recent studies suggest that we are at the edge of a breakthrough in cancer immunotherapy. The most promising therapeutic approach for activating antitumor immunity in cancer patients may be simultaneous stimulation of the innate and adaptive antitumor immunity by the well-studied techniques. A more rational approach is to create an effective ratio of activated effector cells against tumor cells in the patient's body. Therefore, immunotherapy that aims to prevent relapses can achieve better effects in cancer patients after radical treatment as well as locoregional immunotherapy with local infusion of activated effector cells in the tumor site. Optimized methods of cancer immunotherapy based on tumor biology may be used for personalized treatment of cancer patients.

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3 Personalized Prevention Strategies to Defeat Cancer

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3.1 Introduction

Personalized treatment is, surely, one of the most urgent needs in the clinical strategies of prevention and cure of tumors.

New possibilities have been opened by the latest results [1] of the research on the aging changes specific for gender in the regulation of the redox-immune system homeostasis.

It has been demonstrated that Trx1/CD30 redox immune system (Trx1/sCD30) is a double target biomarker; it is both aging-related and specific for gender and can be used to establish the very early risk for cancer development or its progression.

Trx1/soluble CD30 (Trx1/sCD30) has been proposed as a new double pharmacological target for treatment to restore the redox-immune system homeostasis during aging and the normal levels of Trx1, RTrx1, sCD30, and cytokines T regulatory (Treg), T helper1, (Th1), Th9, and Th17. These are functional biomarkers of extracellular and intracellular pathways of Trx1/sCD30. Furthermore, the polymorphisms of killer immunoglobulin-like receptors (KIRs) and receptors for the Fc domain of IgG (FcγR) FcγRIIa-131H/R and FcγRIIIa-158V/F have been proposed as clinical stratification parameters to personalize the prognostic biomarkers in non/low/high disease risk indices.

3.2 The Thioredoxin1 System

The redox control of the cell physiology is one of the most important regulatory mechanisms in all the living organisms. The Trx1/RTrx1 system is a relevant regulator of the redox-mediated cell reactions of the whole organism.

Mammal cells contain two Trx systems. The first being Trx1/RTrx1 is normally localized in cytoplasm, but in stress conditions, it could migrate in the nucleus (inducing the transcription and transduction of target genes) or it could be secrete in the extracellular environment [2] and take part, in this way, to the network of the immune system. The second one, Trx2/RTrx2, localized in mitochondria and in the endoplasmathic reticulus, regulates the cell apoptosis [3]. In addition, literature reported other Trx systems: the Testis/sperm-specific, localized on the spermatids (Sptrx-1, Sptrx-2, and Sptrx-3), and the Trxl-2, located in the lungs and in other ciliate tissues [4].

Trx1 is a thermostable protein (constituted of 108 amino acids) that is largely distributed in all the living organism, from bacteria to mammals. It contains an S-S bridge, it does not contain metal, and it has a catalytic domain that is a donor of hydrogen for redox reactions [5, 6] (Fig. 3.1). The Trx1-reduced form is able to reduce protein disulfides by using their two active cysteine site.

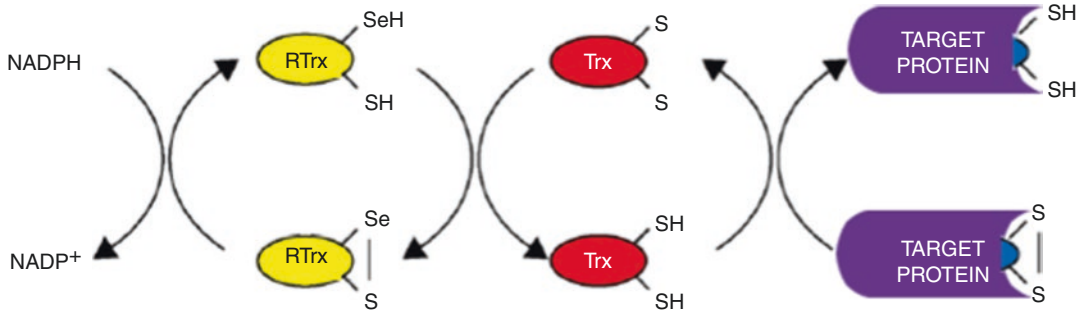

Fig. 3.1 Thioredoxin 1 (Trx1) system. Trx1 reduces protein disulfides using their two active site cysteines, and upon reduction of target proteins, it is itself oxidized in its active site. The oxidized Trx1 form is converted in the reduced form by the Thioredoxin1 reductase flavoprotein (RTrx), with the involvement of NADPH. These molecules constitute the thioredoxin redox-system1 (Trx1)

Upon reduction of target proteins, it is itself oxidized in its active site. The oxidized Trx1 form is converted in the reduced form by the Thioredoxin1 reductase flavoprotein (RTrx), with the involvement of NADPH. These molecules constitute the thioredoxin 1 (Trx1) system. Trx1 is very important for the defense of the state of health, also protecting from the tumoral pathology. Trx1 regulates the enzymatic activity, for example, of the "apoptosis signal-regulating kinase 1" [7], the caspase-3 protease that promotes apoptosis [8], and the "protein kinase C" [9]. It increases the binding and activating function on DNA [10] of different transcription factors as activator protein 1 (AP1) [11, 12], the "nuclear factor kB (NFkB) [13], the "glucocorticoid receptor" [14], and p53 [6]. Human T cells, transformed by viruses, produce a factor that is identical to the human Trx1 and that was previously called actin-depolymerizing factor (ADF) [15]. Trx1 is also secreted by activated B lymphocytes, the B lymphocytes of the type B chronic leukemia, fibroblast, and T lymphocytes [16, 17]. Trx1 is a powerful growth and survival factor [9, 12]. Its expression is increased in different types of tumor, especially in the most aggressive ones [15, 16] such as in lung cancer. In fact, increased levels of Trx1 are associated with the decrease of lung cancer patient survival. Trx1 increase has been also correlated with the inhibition of the immune system [18, 19]. Its increased expression has been identified as an independent prognostic factor of disease progression, and the expression of vascular endothelial growth factor (VEGF) and redox effector factor 1 (Ref-1) are correlated to it [20]: these are important assumptions for new therapies with monoclonal-specific antibodies for these cellular receptors.

3.3 The CD30 System

At the beginning, CD30 receptor (CD30), a member of the TNFR/NGFR family, has been identified on primary cultural cells of Hodgkin and Sternberg [21]. CD30 is also expressed on lots of other T- and B-cell lines after viral transformation; normally, peripheral blood mononuclear cells (PBMCs) express CD30 only after activation [22].

The physiological function of CD30 has not been yet clarified, but there are evidences that it could behave as a signal transducing molecule. The interaction between CD30 and its ligand (CD30L) on activated T cells, monocytes, natural killer (NK), neutrophils, eosinophils, and B cells induces the rapid activation of genic transcription factors, as JunN-kinase (JNKs) and nuclear factor NF-κB (NFkB) [23–25]. In addiction, CD30 signals induce and regulate the lymphocyte expression of cytotoxic molecules, lymphonodal traffic, proliferation, and apoptosis [22].

Advances in research have shown that CD30 is a molecule that mediates regulatory signals. These results [24–28] clarified the significance of its physiopathologic function. They showed that the interaction between CD30 and its soluble form (sCD30), released in the cell environment when CD30 interacts with CD30L, controls the physiologic homeostasis in the immune and in the neurologic systems. This is because the CD30/sCD30 interaction regulates the functions of NK, monocytes, and mature (DC) and immature (IDC) dendritic cells in order to direct the Th-cell differentiation in the respective subtypes (Treg, Th1, Th9, Th17) [24–30].

NK cells provide the first-line defense against viral infections and malignant cells. NK cells perform this important role in the immune response for their ability to kill tumor cells, for cytokine production, and for the cross-talking with the adaptive system. The cooperation with the adaptive response is mediated by the interaction between CD30 on the NK cells and CD30L on the IDC cells. This binding induces the secretion of cytokines by IDC via the mitogen-activated protein kinase pathways and promotes the differentiation of mature DC cells and the release of TNFα/IFNγ by NK cells.

At this point, it is important to highlight that from the regular development of these interactions depends the generation of DC- and Th-specific cells, a normal immune response and the protection of the health state [25].

3.4 The Functional Link Between Trx1 and CD30 Systems

Therefore, research clarified that the functional link between Trx1 and CD30 is very important for the physiologic homeostasis. Furthermore, it underlines the big potentiality of these elements as target and biomarkers in clinical treatments.

Trx1/CD30 is of key importance for Treg/Th1/Th9/Th17 cell network balance and the immune response homeostasis. In fact, the Trx1 redox system maintains balance between reduced Trx1 and oxized Trx1 which regulate, respectively, the activation/inactivation of the CD30 receptor with CD30L, modifying the stoichiometric structure of CD30 receptor (Figs. 3.2 and 3.3) [1, 31].

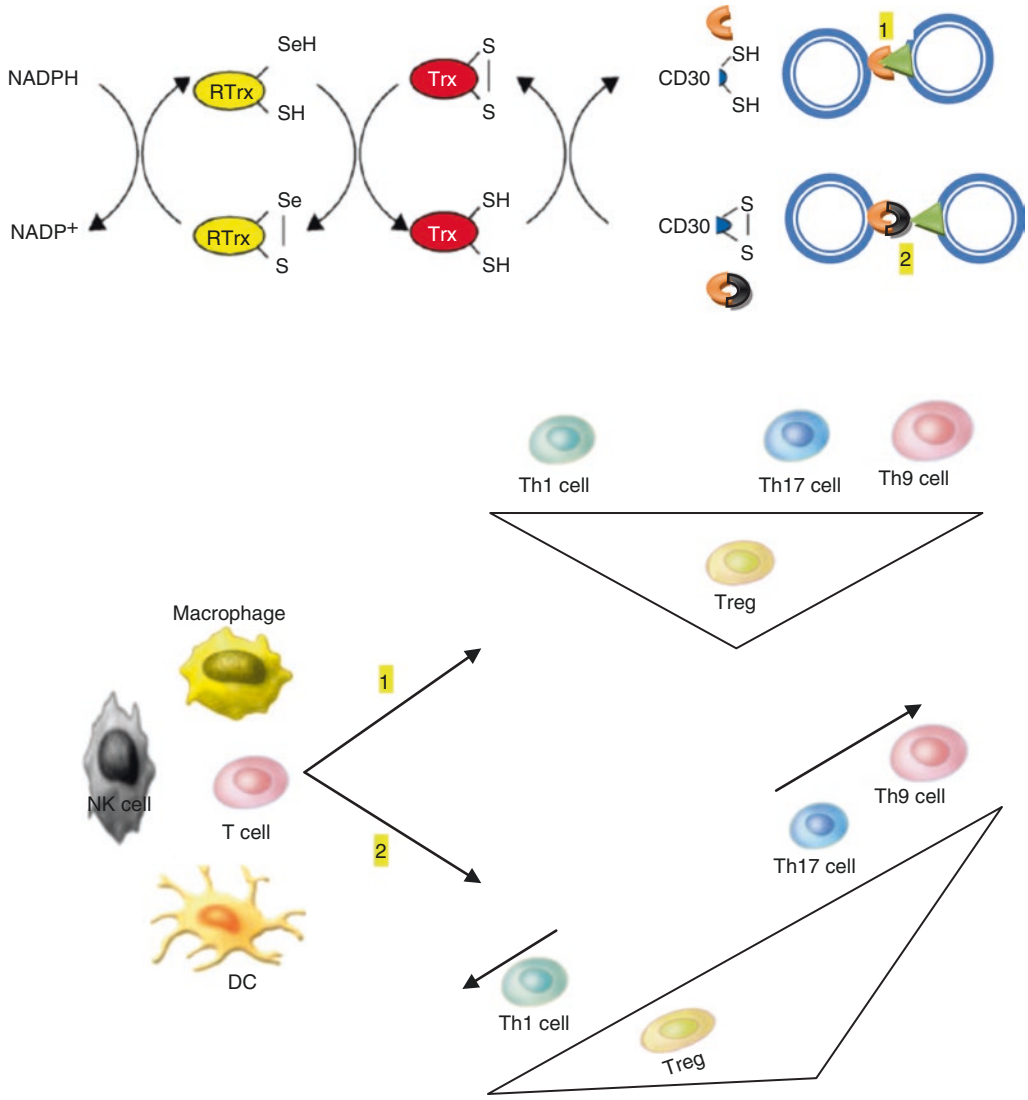

Fig. 3.2 Functional link between Trx1 and CD30 systems. Trx1 and CD30 systems regulate the Treg/Th1/Th9/Th17 network homeostasis of the immune response. The Trx1 redox-system1 maintains balance between oxidant and antioxidant Trx1, regulating the activation (1)/inactivation (2) balance of the CD30 receptor (CD30) with its ligand (CD30L). The reduced Trx1 form (Trx1-SH) is able to interact with the oxidized CD30 (CD30 S-S) and reduce it (CD30 S-H). CD30 receptor can only interact in this latter form with CD30L on activated NK, DC, monocytes, and T cells (1). On the contrary, unbalance could be the cause of non-homeostasis of the immune response and cancer development (2)

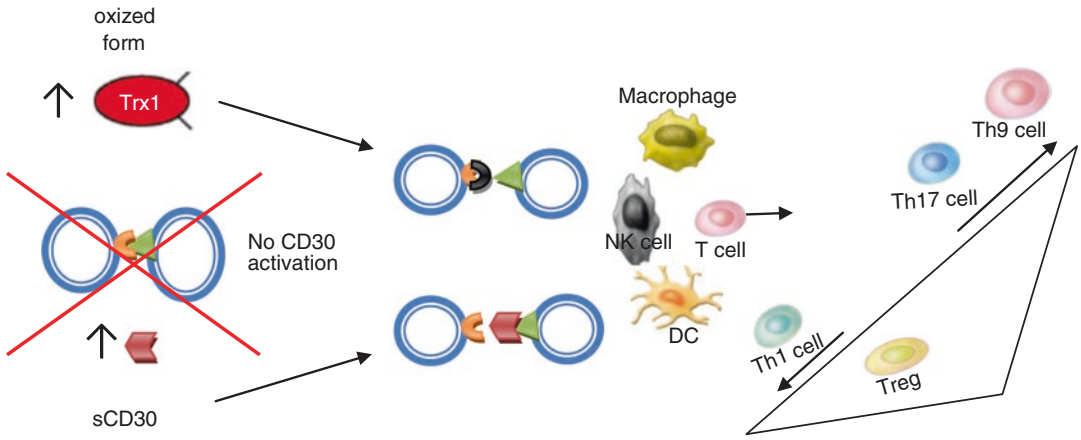

Fig. 3.3 sCD30 and Trx1 both regulate CD30R functional activation and Treg/Th1/Th9/Th17 network balance. sCD30 and Trx1 are both able to influence the CD30 capacity of mediating the activation of intracellular signals. sCD30 makes this function by binding and blocking the binding site of CD30L (◀), with which it has a strong affinity. Trx1 makes this function catalytically, modifying the stoichiometric structure of CD30. Abnormal increases in the levels of both sCD30 and Trx1oxized form result in non-activation of CD30 receptor. This causes Th9 and Th17 cell expansion and Treg and Th1 cell functional deficit, which have been noted in cancer

Furthermore, research explained that sCD30, in addition to Trx1, influences the CD30 capacity of mediating the activation of intracellular signals by CD30L. sCD30 makes this function by binding and blocking the binding site of CD30L, with which it has a strong affinity [1, 28] (Figs. 3.2 and 3.3).

The results have, also, underlined that during the inflammatory response, CD30 is largely expressed on the immune cells, and as a consequence, there is an increase of sCD30 that is released in the extracellular environment [28] (Fig. 3.3). Furthermore, it has been shown that the sCD30 level variations in the cellular or tumoral microenvironment could be used as biomarkers of the correct functioning of the immune system and the therapeutic response [1, 24–28, 32]: the sCD30 level, within the normal physiological ranges, is a positive index of the immune system homeostasis and of the therapeutic benefit. On the contrary, a significant increase of the sCD30 level is a negative index because it denotes an immunological deficit and the lack of a therapeutic response. For these reasons, both Trx1 and sCD30 have to be considered as therapeutic target.

Therefore, changes of the Trx1 and sCD30 levels are functional extracellular biomarkers of Trx1/CD30, while the Treg/Th1/Th9/Th17 cytokine levels are functional biomarkers of the intracellular pathways [1, 33–35].

These results indicate, then, that Trx1/CD30 have great potentialities to be a new double pharmacological target on which it is possible to intervene to restore the balance and the normal health state.

3.5 The Polymorphisms of KIRs, FcγRIIa-131H/R, and FcγRIIIa-158V/F Could Be Clinical Stratification Parameters to Personalize the Prognostic Trx1/CD30 Biomarkers of the Early Risk in Tumor Disease or Progression

These polymorphisms could influence the interaction between innate and adaptive immune response. In fact, as we reported above, this cooperation is mediated by the interaction between CD30/CD30L/sCD30 on NK, monocytes, DC, and IDC in order to direct the Th-cell differentiation in the respective subtypes.

It was found that only those NK cell clones expressing at least one inhibitory-specific KIR

for self-HLA class I molecule were "licensed" or functionally active. This mechanism shapes the NK repertoire and prevents NK-mediated self-damage. Thus, in tumors the downregulation of HLA class I antigen expression makes tumor cells susceptible to NK cell attack. However, often, solid tumor cells even with partial or complete loss of HLA class I expression are able to spread.

The NK cell activity is regulated by a balance of transduction signals performed by activating and inhibiting receptors [36]. The independent segregation of HLA and KIR genes, along with KIR specificity for particular HLA allotypes, makes it possible that any given individual may express KIR molecules for which there is no ligand. While gene polymorphisms encoding inhibitory KIR2DL1, KIR2DL3, and KIR2DL4 are detected in almost all individuals, those codifying for activating KIR, like KIR2DS2, are found only in a part of population. Furthermore, KIR polymorphism and its interaction with HLA alleles may influence susceptibility to inflammatory diseases, including systemic sclerosis and vascular events in systemic lupus erythematosus [37, 38], viral infections, malignancies, and pregnancy outcome [39].

Antibody-dependent cell-mediated cytotoxicity (ADCC) is, additionally, an immune defense system in mediating tumor cell killing. The FcγRs seems the only molecule on human myeloid cells capable of mediating ADCC of tumors and may be important in antibody therapy of cancer.

There are two types of FcγRs: activation receptors (CD16A and CD32A) and inhibition receptors (CD16B and CD32B) [40–42]. CD16A and CD32A activate NK lymphocytes and myeloid cells, connecting innate and the adaptive immune responses.

CD16A is expressed in NK lymphocytes and macrophages, while CD32A is widely expressed in myeloid cells [43–45]. Genes encoding for these receptors are located in the low-affinity "FCGR" locus on chromosome 1q23 [46]. FcγRIIIa gene for CD16A and FcγRIIa gene for CD32A.

Some polymorphisms of FcγR have been identified which could prove to have significant clinical relevance [43]. Two functional polymorphisms of human FcγRIIa and FcγRIIIa have been identified in the extracellular regions of these receptors: valine/phenylanine-158 of CD16A (FcγRIIIa-158V/F) and histidine/arginine-131 of CD32A (FcγRIIa-131H/R) which modulate their affinity for certain human IgG subclasses [47, 48]. Clinical studies reported that the presence of FcγRIIa-131H/H and FcγRIIIa-158V/V genotypes is associated to a more efficient ADCC antitumor response.

For these reasons, the polymorphism of KIRs, FcγRIIa-131H/R, and FcγRIIIa-158V/F has been studied as stratification parameters for the loss of the physiological homeostasis, disease risk, and its progression.

3.6 The Trx1/CD30 Double Target Is a Real Weapon to Defeat Cancer

The advances of the research have confirmed the importance of the Trx1/CD30 as double target in tumor defense. The results showed that Trx1/CD30 control the redox immunological homeostasis of the immune response both in men and women, but through different redox-immune pathways. In this control, the normal levels of Trx1/RTrx1 and sCD30 are fundamental for the preservation of IL10, TGFβ, IL4, IL6, and IL2 pathway homeostasis of immune response in the healthy subjects, also during aging. Studies in the patient groups supported this scientific rational by showing as the unbalance of the Trx1/RTrx1 and sCD30 levels generates cancer and makes it progress, through different redox-immune pathways between men and women. Then, research confirmed this role showing that the unbalance of the Trx1/RTrx1 and sCD30 levels is a biomarker of the loss of the IL10, TGFβ, IL4, IL6, and IL2 pathway homeostasis in the network of the immune response and is a risk biomarker of cancer development and progression.

Data showed also that the above redox immune unbalance is prognostic in both gender of the specific type of disease [49–59]. In men, the disease is of degenerative-destroying kind because it is

correlated to an increase of TGFβ and IL4 cytokine combination, which is a biomarker for a Th9 cell expansion [49, 50, 58, 59]. While in women, the redox-immune unbalance produces autoimmune diseases since it is correlated to an increase of the TGFβ and IL6 cytokine combination, which is a biomarker for a Th17 cell expansion [60–62]. Therefore, these and previous results [1, 52–56] showed that the susceptibility and clinical course in disease, dissimilar for genders, are caused by a different Treg, Th17 and Th9 cell polarization. This is due to the IL10, TGFβ, IL4, IL6, and IL2 cytokine pathway interactions, which vary between men and women.

The results specify, in fact, that our body produces immunological responses through physiological pathways different between men and women. However, these differences related to sex do not have consequences for the final result: the responses are activated; they perform their function and return to the initial rest phase. All this happens, normally, regardless of differences in the path between the two sexes, until there are pathological changes in these specific gender-specific pathways. In fact, if alterations occur in the pathways of IFNγ and IL6 cytokines, the effects for men and women, in terms of development of the disease, are different. This happens because in the physiological network the activity of the immune response is the result of the interactions of the activities of the entire cytokine network which is present in the microenvironment. As stated above, the cytokine pathways of IFNγ and IL6 are the main regulators of the network of the immune response of men and women, respectively. Consequently, the male gender will suffer the consequences that follow a lack of network regulation by IFNγ pathways; instead, the female sex will suffer from a lack of network regulation by the IL6 pathways.

Furthermore, it was also clarified that in these events a determining role is to be attributed to the ability of environment cytokines to activate the genic transcription factors for the differentiation of the specific Th subsets. Th1 requires the expression of Tbet transcription factor, whereas Th2 cells are controlled by expression of GATA-3 [63–65]. Treg cells differ through Forkhead boxP3 (Foxp3) transcription factor [66, 67]; instead, Th17 cells need retinoic acid-related orphan receptor gt (RORgt) [68–70], and Th9 cells need the PU.1 bet transcription factor [71–74]. There is also a mutual development relationship between Treg, Th17, and Th9 cells. TGFβ triggers the expression of Foxp3 transcription factor in naive T cells, generating Treg cells. Nevertheless, IL6 can inhibit the Foxp3 expression driven by TGFβ, and the combination of TGFβ and IL-6 cytokines is able to induce ROR-gt transcription factor, triggering the Th17 cells: nevertheless, IL2 can inhibit this induction [75]. Additionally, also IL4 inhibits induction of Foxp3 from TGFβ. The combination of TGFβ and IL4 induces the expression of PU.1 transcription factor generating Th9 cells. The co-expression of IL-9 and IL-17 was identified as a Th17 function in mediating autoimmune tissue destruction: IFNγ inhibits this generation [76].

Consequently, research has shown that Trx1/CD30 in NK, DC, monocyte, and T cells regulate the redox immunological homeostasis of the TGFβ, IL4, IL6, IL10, and IL2 gender-specific pathways. The loss of this control produces a pathological gender-specific polarization of T-cell subsets, which causes the disease development.

3.7 KIR and FcγRIIa and FcγRIIIa Polymorphisms Are Biomarkers of Low/Moderate/High Risk of Cancer Disease or Progression

The results showed that the KIR polymorphisms are stratification parameters for disease risk in healthy subjects and for its progression in patients.

The individual number of inhibitory KIR (iKIR) showed no relevance in this correlation. Instead, the number of KIR-activating receptors (aKIR) showed meaning: aKIR>2 and aKIR<3 are, respectively, biomarkers of no risk and of risk of disease and of its progression.

The increase of age is related to the increase of the disease risk, and the female gender is the

most impressed, linked to 2DS4del polymorphism. In men, the increase of risk of disease during aging is caused, primary, by the Trx1 enhance and linked to the 2DL3, 2DS4ins, and 3DL1 polymorphisms.

Furthermore, it was found that in men 3DL1 is the highest risk biomarker: it is negatively correlated with the IL2 increase and positively with the IL4 increase (prognostic for Th9 cell generation). Instead, 2DL5B is the male highest no-risk biomarker: in fact, it is positively correlated with both IL2 and IFNγ increase (prognostic for immunological response homeostasis).

As in men and also in women, 2DL5B is the highest no-risk biomarker because it is positively correlated with IL2 increase. Additionally, 2DS2/2DL2 pair is also a female no-risk biomarker: it is negatively correlated with TGFβ increase.

Results also showed that the $2DL2^+/2DS2^+$ pair is protective for tumor [77] and this is because $2DL2^+/2DS2^+$ pair is biomarker of positive interaction between innate and adaptive immunity and of immunological redox homeostasis.

Another goal of these studies is the validation of FcγRIIa and FcγRIIIa polymorphisms as gender-specific disease risk biomarkers. During aging, the FcγRIIa-131H/H combination with FcγRIIIa-158V/V is the biomarker of the lowest disease risk in both, men and women, because it is the most efficient combination for the control of redox-immune homeostasis when IL10 level is increased. The increase of IL10 level is high-risk biomarker for chronic-degenerative diseases (as tumor) and of its progression. The combinations of FcγRIIa-131H/R and FcγRIIIa-158F/F genotypes in men and of FcγRIIa-131H/R and FcγRIIIa-158V/F in women are, furthermore, biomarkers for an intermediate risk. This is because it is the most efficient combination for the control of redox-immune homeostasis when IL6 level is increased. In fact, IL6 is a pre-risk condition for the disease onset and/or its progression. The combined genotypes of FcγRIIa-131R/R with FcγRIIIa-158V/F in men and of FcγRIIa-131R/R with FcγRIIIa-158F/F in women are biomarkers for the highest risk of disease or of its progression, because they are protective only if the levels of IFNγ, IL4, and IL2 cytokines increase together. In this condition, in fact, there is no risk for the redox-immune balance.

These results showed also that in patients the combinations of H/H-F/F e R/R-V/V in men and of the H/H-V/V, H/R-V/V, and R/R-F/F in women are biomarkers of no risk of disease progression; the pair H/R-F/F is a biomarker of moderate risk only in men, while the H/H-V/F and R/R-V/F are high-risk biomarkers both in men and women; the combination H/R-V/F is a high-risk biomarker only in men.

3.8 Concluding Remarks

Therefore, research showed that the Trx1/CD30 is a gender-specific double target and biomarker of the homeostasis/non-homeostasis of the redox immune system during aging.

Homeostasis protects the state of health because it preserves our physiological ability to defend ourselves against diseases, such as cancer. On the other hand, non-homeostasis causes incapacity to defend oneself from inflammation which makes irreversible the mechanisms that generate the disease.

Consequently, the Trx1/CD30 and the selected biomarkers are a real tool for new personalized clinical strategies to defeat cancer.

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4 Tumor Antigen Identification for Cancer Immunotherapy

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4.1 Introduction

Distinguishing between the foreign and self-antigen is a key principle in proper immune system function, resulting in immune tolerance for self-antigens, while non-self-antigens are immunogenic [1]. Talking about cancer, this discrimination is hard due to its origin from normal host cells [2]. Considering that in mind, the tumor microenvironment consisting of cells, molecules, and extracellular matrix facilitates the interaction between tumor and immune system. While possessing tumor-suppressing potentials, changing the immune profile of the tumor microenvironment may result in tumor escape [3]. The immunoediting hypothesis propounds that the interaction between tumor and immune system, via three processes of elimination, equilibrium, and escape, despite initial destroying of the nascent cells, eventually leads to tumor expansion with uncontrolled manner because of selection and generating of those variants of cancer cells with increased capacity toward the immune system [4]. Altogether, tumor antigen identification remains an important issue in cancer immunotherapy, since challenging with the immune escape of the tumor on one hand and the serious side effects and toxicities of designed therapeutics due to targeting of normal cells' antigen on the other hand has made many complexities [5]. Thus, finding the target antigens via different approaches is fundamental, making it necessary to be equipped with novel various technologies in the field.

In this chapter, we will briefly review various types of tumor antigens. Further, we will discuss the approaches in identifying tumor antigens and finally will mention the clinical utility of tumor antigen identification.

4.2 Tumor Antigens

Antigen is defined as any substance capable of inducing immune system response [6]. From the point of origin, tumor antigens could be divided into two major groups: (1) native tumor-associated antigens which are also presented in normal cells but are upregulated in malignant cells and (2) tumor-specific antigens [7, 8]. Tumor-specific antigens are classified in turn into three main groups: (a) those related to tumor-specific somatic mutations which are known as neoantigens [6], (b) cancer/testis antigens that are normally expressed in male germ cells in the testis and sometimes in the female ovary and in trophoblast which can also be expressed in different tumors due to gene dysregulation in malignancies [9], and (c) antigens generated from malignant transformation via viral open reading frames [8], such as HPV16 E6 and E7 [10] and EBV [11]. These carcinogenic viruses also contribute to the generation of neoantigens in a subset of tumors like cervical or head and neck cancers [12], but as they constitute a small proportion of cancers, the majority of neoantigens are derived from tumor-specific mutations [8].

Furthermore, tumors may express antigens in a heterogeneous manner in which some antigens are presented in all malignant cells, called clonal antigens, whereas some others will present in a subset of cells instead of the whole tumor which are known as subclonal antigens [6].

Another used classification is as follows:

(a) Unique tumor-specific antigens which are raised from unique mutations in a tumor of a patient.
(b) Shared lineage-specific antigens presenting in the tumor and its matched normal tissue, prostate-specific antigen (PSA) belongs to this group.
(c) Shared tumor-specific antigens which are not seen in healthy tissues but are commonly shared between different types of tumors.
(d) Shared antigens which derive from both tumor and normal tissue, but are upregulated in tumors [13].

Based on different characteristics of these various antigen types, they rank differently as ideal candidates for immunotherapy, which is briefly discussed later.

4.3 Approaches to Identify Tumor Antigens

Namely, the main two antigen identification approaches are algorithm-based prediction, also known as indirect or reverse immunology [2], and the forward/direct immunology or HLA peptidomics, in which the HLA-peptide complexes are

isolated from samples and followed by identification of peptide sequences [14]. Although rendering many neoantigen identification, the reverse immunology approach may eventually result in a small fraction of predicted peptides to be confirmed, yielding high false-positive peptides and thus requiring validation via laborious and time-consuming techniques. Furthermore, since the validation is based on the previous immunogenicity of the peptide, they may not present by the tumor anymore in contrast to the HLA peptidomics strategy in which the antigens are actually presented even though they are not immunogene [15].

4.3.1 Prediction-Based Identification

The indirect or reverse immunology approach relies on the algorithm-based prediction of the proper antigen candidate. The steps and main implemented methods are summarized here. The main steps are illustrated in Fig. 4.1.

4.3.1.1 Antigen Identification

The initial step is the antigen identification. This could be implemented with or without sample acquisition. In the method without obtaining any sample, the candidate frequent mutations are selected from common well-characterized mutations on the basis of existing literature and databases [8]. This classic approach was one of the early methods in identifying tumor antigens. The cDNA library has shown to be very efficient in identifying many unique neoantigens such as PTPRK in melanoma [16], ACTN4 in lung cancer [17], and KIAA1440 in renal cancer [18]. However, it is laborious and low throughput and hard to clone some large, GC-rich or low-expression transcripts [2]. Sharkey MS. et al. reported the V599E mutation of BRAF codon 599, to be recognized by T cells. They provided melanoma culture by enzymatic lysis of metastatic lesions. DNA sequencing was done on genomic DNA isolated from melanoma cells and peripheral blood mononuclear cells. PCR was used for the amplification of BRAF exon 15. Due to the interference of melanin, reverse transcribed cDNA was utilized as the template for PCR [19].

In sample acquisition method, tumor and matched normal cells are obtained, followed by the DNA sequencing [8] or protein overexpression analysis including different methods such as western blotting and immunofluorescence,

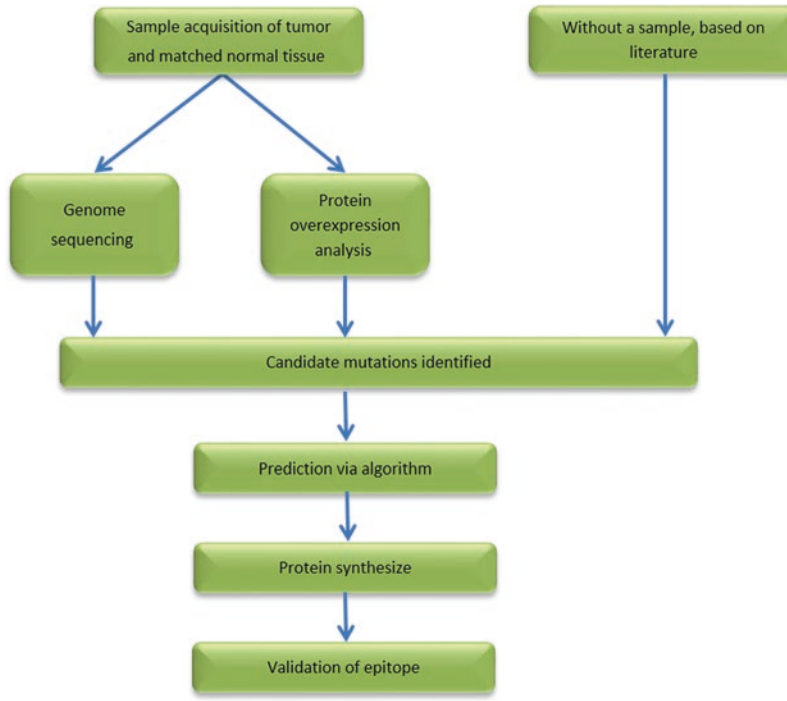

Fig. 4.1 Identification of tumor antigen by indirect immunology approach

immunohistochemistry, etc. Yang Li et al. reported glutathione S-transferase omega 1 protein as a tumor-associated antigen which could be utilized as a biomarker in early detection of esophageal squamous cell carcinoma. They used immunohistochemistry analysis to compare the GSTO1 expression between esophageal squamous cell carcinoma and the normal tissue. They also used western blotting and immunofluorescence to confirm the mentioned discovery [20].

Whole-exome sequencing is one of the most frequently used techniques. While being very efficient in identifying antigens previously missed by cDNA library screening, its efficiency could be restricted by the accuracy of HLA-peptide binding prediction algorithms, especially for HLA II and rare HLA alleles, and the failed expression of some epitopes on the cell surface. The latter could be somewhat resolved by pulsing the antigen-presenting cells with long synthetic peptides [2]. Along with DNA sequencing, RNA sequence is also determined to validate the expression levels of detected mutations [8].

Another approach has been developed by the application of tandem minigene (TMG). One minigene is designed for each mutation, which is synthesized in tandem to generate the TMG construct that encodes polypeptides comprising mutated amino acids. They are used as templates for the generation of in vitro transcribed RNA, and then each transfects the autologous antigen-presenting cell or cell lines co-expressing autologous HLA molecules [2, 8, 21].

4.3.1.2 In Silico Peptide Prediction

After identifying the mutations, in silico analysis is utilized to predict the binding affinity of peptides to autologous HLA. Moreover, the peptides predicted to be poorly processed by the proteasome, and thus poorly presented could be removed. Using the prediction algorithms, the mutations are then ranked, and the candidate peptides are synthesized [2, 8]. There are different databases and tools for prediction. The IEDB (immune epitope database and analysis resource) is an online database rendering tools such as SMM, SMMPMBEC, ARB, and Pickpocket [8]. As an example of these bioinformatics, NetMHCpan is a large database of HLA-I and peptide interactions capable of generating quantitative predictions of HLA-peptide binding affinity which acquires the data from IEDB and the data published by Sette and coworkers [22].

4.3.1.3 Validation of Antigen Presentation and Immunogenicity

To determine whether or not the synthesized neopeptides can induce the T-cell activation, their expression and immunogenicity must be validated using T-cell reactivity analysis. Thus, antigen-loaded autologous antigen-presenting cells are generated and utilized to stimulate T cells from patients or healthy donors. The expanded T cells are then studied for their activation in vitro and detected by markers such as cytokine secretion (IFN-γ), CD170a, OX-40, and 4-1BB upregulation [2, 8].

4.3.2 Forward Immunology in Tumor Antigen Identification

In the early 1990s, the first successful cloning of the human gene MAGE-1 encoding a tumor antigen of melanoma MZ2-MEL was investigated by Traversari et al. along with demonstrating the autologous cytotoxic T-lymphocyte response [23, 24]. However, different from HLA peptidomics used in recent years in forward immunology, it is often revered to as direct immunology approach as the first human tumor antigen identification.

4.3.2.1 Genome Sequencing

The initial step is determining the DNA sequence of the tumor and matching normal sample to identify the somatic mutations in malignant cells. It could be done by means of whole exome or genome sequencing [15]. Robbins et al. investigated the ability of tumor-infiltrating lymphocytes in recognizing potent antigens. They developed a screening method via mining whole-exome sequence data to identify mutated antigens. They introduced whole-exome sequencing, that is, a relatively simple and rapid genomic approach capable of providing an opportunity for the development

of different therapeutic modalities such as adoptive transfer protocols and cancer vaccines in various tumors [25].

4.3.2.2 Isolation of HLA-Peptide Complex

In this step, the tumor cells or tissues are lysed to extract the HLA-peptide complex. Due to the hydrophobic nature of the bi-lipid plasma membrane structure and poor solubility of the membrane proteins, the isolation process requires enrichment techniques [26]. They are categorized into three main groups:

(a) Isolation based on physical properties such as gradient centrifugation as the oldest method; ultracentrifugation in which the different fragments are split into groups with similar shape, density, and size; and also coating cells with cationic colloidal silica particles.
(b) Isolation with limited short-duration proteolysis via enzyme for cell surface shaving, which in turn solves the low solubility problem of the membrane. Cell integrity should be taken into consideration during the digestion process.
(c) Chemical enrichment methods with different materials, which is one of the favored strategies in recent years. Namely, some of the substantial ones are cell-surface capture techniques, glycocapture, biotinylation, etc. [14].

Along with the enrichment process, solubilization should be done in order to extract the proteins from the embedded lipid membrane. Ionic liquids, solvents, detergents, organic acids, and chaotropes are of various methods used [26]. Organic solvents lessen the performance of the enzymatic digestion; thus it is required to constantly use the fresh protease during the process or to dilute the solvent before proteolysis. The disadvantage of detergents is their incompatibility with liquid chromatography or mass spectrometry [14].

4.3.2.3 Sequencing of Neopeptide

In proteome study, label-based and label-free techniques are the main methods for protein quantification. The first includes isobaric, enzymatic, and metabolic labeling which are capable of parallel quantification of several samples resulting in time-saving and increased performance, although they will miss the identification of antigens in minority. Label-free techniques such a mass spectrometry could be applied with fewer expenses and steps while implicating more precise control of protocol employment to elude experimental errors rendering sample-to-sample variation [14]. Finally, the neoantigens are identified by comparing the data of the complete human proteome and the detected mutated proteins of the tumor [15]. MaxQuant software is one of the commonly used modules for the analysis of peptides based on genomic variations [27].

4.4 Clinical Utility of Tumor Antigen Identification

Endogenous T cells have shown promising results in cancer immunotherapy. This fact implies the ability of T cells in recognizing and thus acting against some antigens presenting on malignant cells [12]. Many other therapeutic modalities have also underlined the importance of targeting specific structures of tumors. As a result, the selection of appropriate antigens based on their various properties plays a pivotal role in designing novel treatments.

While owing low likelihood of central thymic immunological tolerance and thus being highly immunogenic, neoantigens also face challenges in immunotherapy since they are unique to each patient, resulting in expensive and laborious technical issues [7, 8]. In contrast to neoantigens, nonmutated self-antigens have been broadly applicable, due to the ability to be generally utilized among patients. Nevertheless, they result in substantial side effects due to being presented in normal cells, in addition to higher rates of acquired immune tolerance [7] that could be one reason why vaccines designed on the basis of these native antigens did not show acceptable clinical results [7], whereas studies based on neoantigens such as an individualized vaccine targeting more than 20 personal neoantigens in patients with melanoma [28] or tumor-infiltrating lymphocytes against mutant KRAS G12D in the

metastatic colorectal cancer [29] have demonstrated promising results [30].

In addition, durable clinical benefits have been reported in tumors with low subclonal in comparison to clonal mutations [31]. Altogether, the selection of ideal antigens is still under question. Nevertheless, some key facts should be taken into consideration. Antigens with these properties might be favorable:

1. The target antigens widely presented in various malignancies.
2. Antigens playing an important role in tumor progression or survival.
3. Highly immunogenic antigens.

Furthermore, personalized medicine targeting unique antigens of the individual tumor is of novel therapeutic options [13]. Identified antigens could be targeted via immune vaccines. However, there are some issues in developing neoantigen vaccines, including the variation in the mutation rate of numerous malignancies. Tumors such as melanoma with higher mutation rates are better candidates for vaccine therapy because of being more immunogene than those tumors with fewer antigenic burdens. Another challenge is that tumors utilize different mechanisms for immune escape by means of reducing antigen processing and presenting and downregulation of HLA-1 molecules. They also make changes to the tumor microenvironment by inducing suppressive cells such as regulatory T cells, macrophages, and myeloid-derived suppressor cells. Apart from antigen-induced signals of the T-cell receptor, the co-stimulatory signal is required for the activation of T cells, and tumors are capable of inducing T-cell anergy by interfering with these co-stimulatory and co-inhibitory signals. To solve the mentioned issues, some solutions have been recommended. These include the application of multi-epitope vaccines for generating a robust and durable response, which has been investigated in clinical trials. Another suggestion is the use of adjuvants such as toll-like receptor agonists and monoclonal antibodies. The delivery system of vaccines could also play a role. By acting like pathogen-associated molecular patterns, the nanoparticles are the favorable delivery system [8].

4.5 Concluding Remarks

Anti-cancer immunotherapy is becoming a milestone in the treatment of malignancies. Heterogeneity of tumors, immunoediting, and inhibition of immunosurveillance are faced challenges in the field. Based on current knowledge, identification of ideal tumor antigens will empower the diagnostic and therapeutic modalities, and recent advances in antigen identification have generated new opportunities such as antitumor vaccines and adoptive cell transfer. Combination therapy of different immunologic approaches or with conventional anti-cancer therapies may render promising results. An increasing pattern in the development and clinical application of targeted therapies is anticipated. By means of next-generation sequencing, more sensitive and precise mass spectrometry, high-throughput methods, etc., ideal identification of antigens will become more feasible.

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Strategies to Target Tumor Immunosuppression

5

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5.1 Introduction: The Balance of Immune Surveillance in the Tumor

In the beginning of the twentieth century, Paul Erlich was the first to introduce the concept of a vigilant immune system that can be manipulated to counteract tumor development [1]. However, due to lack of experimental evidence, it was not until the 1970s that Frank Macfarlane Burnet postulated the "immune surveillance theory." This theory brings to light a complex immunological mechanism capable of eliminating potentially malignant cells, mainly through recognition of tumor-specific antigens expressed on tumor cells [2]. In later years, several studies describing interactions between the immune system and the developing tumor have further refined this theory [3, 4].

Indeed, strong evidence supporting the key role of immune effector cell populations that are either tumor-specific, including B and T cells able to recognize tumor-associated antigens (TAAs) [5, 6], or non-specific, such as macrophages and natural killer (NK) cells, led to the sophisticated concept of cancer "immune editing," which spans cancer development from tumor immune surveillance to tumor immune escape [7, 8]. According to this concept, cancer development is comprised of three distinct phases [9, 10]: (1) the elimination, (2) the equilibrium, and (3) the escape, which are more extensively reviewed and discussed in separate chapters of this book. Particularly, the phenomenon of tumor immune escape according to which tumors are capable of side-tracking or completely blocking host antitumor immunity through interference with various components of the immune system is of major importance for the development of cancer immunotherapies [11]. Recently, several immune escape mechanisms have been described to hamper antitumor immune responses, either by reducing the homing of immune effector cells to the tumor site or by suppressing antitumor immune functions [12–15]. Therefore, cancer immunotherapies should attempt to stimulate homing and activation of immune effector cells and/or deplete or target pro-tumoral immunosuppressive cell populations and pathways.

Immunotherapy of cancer was selected as the breakthrough of the year 2013, according to Science [16]. Indeed, several groundbreaking clinical trials demonstrated the potency of such therapeutic approaches in patients. Yet, trials have also demonstrated that the responses vary greatly between patients. While in a selected group of patients immunotherapy leads to a full eradication of the tumor, in other patients the same treatment does not evoke a response at all. Currently, tumor immunologists are searching for biomarkers that can be used to describe the "immune signature" of the tumor [17, 18]. Defining the intratumor immunologic profile unique for every tumor type or patient may enable personalized immunotherapeutic strategies for the effective control of tumor progression [19].

This chapter gives an overview of novel strategies for reversing/reducing immunosuppression in the tumor microenvironment, illustrating their targets and the underlying mechanisms responsible for their therapeutic antitumor activity. Prior to this, the immunosuppressive mechanisms most widely encountered in human tumors are briefly addressed.

5.2 The Balance Is Tilted: Mechanisms of Tumor Immune Escape

Tumor immune escape is a consequence of the so-called "immune editing" process driven by the host immune system, through which malignant cells sensitive to immune interventions are eliminated, but in some cases allowing immune-resistant variants to survive and further develop [20, 21]. The mechanisms of tumor immune escape can be functionally divided in two categories: immune tolerance and immunosuppression.

5.2.1 Tolerance Mechanisms

Tumors frequently induce a state of T-cell unresponsiveness toward tumor-associated antigens (TAAs), attributed partly to T-cell ignorance, since tumor cells express mainly self-antigens. Additionally, tumor cells often alter their antigen processing/presentation machinery, mostly toward a defective T-cell priming in the tumor microenvironment [12, 22], but also in adoptive strategies to directly block active immune surveil-

lance, usually with the use of tumor-derived soluble factors [23]. Thus, the main targets of tumor-induced tolerance mechanisms are CD4+ T cells, cytotoxic CD8+ T lymphocytes (CTLs), dendritic cells (DCs), and the antigen presentation machinery. Both the relevance of these immune populations and the tolerance mechanisms they are the targets of are shortly addressed below.

5.2.1.1 CD4+ Helper T Cells and CD8+ Cytotoxic T Lymphocytes: Negative Polarization and Apoptosis

After proper cytokine stimulation, CD4+ mature T helper cells play a crucial role in the initiation and activation of antitumor immune responses. IL-12 polarized, type 1 CD4+ T cells (Th1) provide help to cytotoxic CD8+ T cells by stimulating their proliferation and inducing IFN-γ secretion once antigen-specific immunity has developed [24]. In contrast, IL-4 polarized, type 2 CD4+ T cells (Th2) secrete cytokines which induce neutralizing antibody production by B cells [25], thus directing immunity toward a tumor-promoting Th2 response, prevalent in the context of tumor immunology.

A major mechanism of tumor-induced apoptosis of CTLs is via cross-linking between the overexpressed death receptor FasR (CD95) on the surface of activated effector T cells and its correspondent ligand FasL on the surface of human tumor cells [26, 27]. Direct tolerization of antitumor T cells by tumor cell-induced TGF-β signaling is another highly effective mechanism, leading to a significantly decreased function and frequency of CTLs [23, 28].

5.2.1.2 Defects in the Antigen Presentation Process

The main components of the antigen processing and presentation machinery are the antigen-presenting cells (APCs), TAAs, and major histocompatibility complex (MHC) (or human leukocyte antigen (HLA) in humans) class I antigens. Tumor-induced alterations can affect the functionality of any of these factors via several mechanisms [29].

DCs are the dominant APCs capable in activating T cells but also in tolerizing them, depending on the local microenvironment [30]. Key determinants of DC competence for antigen processing and presentation are their activation and maturation status [31]. In several studies, decreased numbers of mature DCs were detected in the secondary lymphoid organs of tumor-bearing mice [32–34]. This observation is consistent with studies in patients with rapidly growing solid or non-solid tumors which exhibit significantly lower numbers of myeloid mature DCs [35–40]. In addition, isolated DC subsets have phenotypes similar to immature DCs and reduced expression of co-stimulatory molecules [41]. Downregulation of these molecules on the surface of DCs leads to inappropriate provision of co-stimulatory signals required for T-cell activation and interferes with the process of cross-presentation and thus results in death or anergy of antigen-specific CTLs [41, 42]. Moreover, DCs exposed to indoleamine-2,3-dioxygenase (IDO), transforming growth factor-beta (TGF-β) or prostaglandins [29, 43], have been shown to induce tolerance and anergy leading to failure of recognizing tumor cells.

Another means of tumor-mediated immunosuppression, as a result of genetic instability of tumors over time, is the change of their antigenic profile and selective development of "epitope loss" [44–46], by which tumors fail to be recognized and eliminated by the immune system. An additional effect of this genetic instability is a diminished or abolished expression of HLA class I antigens and antigen presentation-associated proteins [25, 47–54], with a frequency of antigenic loss or downregulation ranging from around 15% in melanoma lesions up to more than 50% in primary prostate carcinoma [53, 54].

5.2.2 Immunosuppression Mechanisms

The machinery of tumor-induced immunosuppression is highly versatile, as it has developed to target a large variety of antitumor processes. Within the tumor microenvironment, many cell populations contribute to the generation of an immunosuppressive profile. These include cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs). Furthermore, various tumor-derived factors with immunosuppressive activities also

contribute to tumor progression. The mechanisms by which these cell populations and factors give rise to tumor-immune escape are addressed below.

5.2.2.1 Cancer-Associated Fibroblasts (CAFs)

CAFs are cells that reside mostly within the tumor mass, or are often found within the tumor stroma. CAFs facilitate the malignant transformation process and promote tumor growth, angiogenesis, inflammation, and metastasis [55]. Similar to normal fibroblasts, CAFs are very heterogeneous [56, 57] and therefore difficult to classify based on expression of specific markers. However, the most widely used markers for CAF classification are α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) [58]. Notably, the latter is being studied as a potential biomarker associated with poor prognosis in colorectal cancer [59]. Unlike normal fibroblasts present in healthy tissues, CAFs are more proliferative [60] and secrete various factors that promote tumor growth (such as CXCL12 [61], TGF-β [62]) and modulate the expression of matrix metalloproteinases (MMPs) [63]. Several studies in diverse tumors suggest that CAFs are not only promoting tumor growth and metastasis but can also enhance drug resistance through various mechanisms [64]. In pancreatic cancer, CAFs decrease the sensitivity of cancer cells to chemotherapy and radiotherapy by secretion of soluble factors [65], while in head and neck squamous cell carcinoma, CAFs protect cancer cells through secretion of MMPs [66].

5.2.2.2 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs ($CD11b^+CD14^-CD33^+$) [67] represent a heterogenic, bone-marrow-derived cell population [68, 69] with an increased frequency in the peripheral circulation and tumors of patients with different malignancies [70–72]. Migration of bone marrow precursors (which are further differentiated to MDSCs) to the tumor zone has been shown to be mainly induced by CCL2 secretd by tumor cells [73]. Once MDSCs arrive, signals derived from the tumor promote their activation [69]. MDSCs are characterized by poor phagocytic activity, continuous production of reactive oxygen species (ROS), nitric oxide (NO), and several anti-inflammatory cytokines [74]. As immune suppressive cells, they have the capacity to inactivate both $CD4^+$ and $CD8^+$ T cells through various mechanisms, including depletion of L-arginine [14], decreased tryptophan levels [75], and production of ROS [76], iNOS [77], and immunosuppressive cytokines, such as IL-10 and TGF-β [78]. Although MDSC-mediated suppression mainly affects T-cell function, it has also been described that MDSCs impair T-cell activation, by inhibiting MHC class II expression [79] and thus leading to decreased antigen presentation.

5.2.2.3 Regulatory T Cells (Tregs)

Similar to MDSCs, Tregs have also been shown to accumulate in tumors of patients with cancer [80]. Intratumoral accumulation of Tregs leads to poor prognosis for patients with gastric [81] and ovarian [80] carcinomas. $CD4^+$ Tregs, characterized by the expression of FoxP3 [82], are a highly immunosuppressive subset of $CD4^+$ T cells. Two major populations of $FoxP3^+$ Tregs have been described to date: one "natural" subset, which differentiates in the thymus, and one "induced," developed in the periphery from conventional $CD4^+$ T cells [83]. Both subsets promote tumor immune escape via the following mechanisms: (1) by secretion of immunosuppressive mediators, including cytokines like IL-10, TGF-β, and IL-35 [84, 85]; (2) by induction of effector T-cell apoptosis [86], as they promote a status of metabolic disruption secondary to IL-2 [87] deprivation; (3) by engagement of contact-dependent mechanisms of immunosuppression (e.g., inhibition of DC maturation, via CTLA-4 interaction with CD80/CD86 on DCs [88]); or by (4) by expression of suppressor molecules, such as LAG-3, CD39, neuropilin 1, or galectin 1 [89].

5.2.2.4 Tumor-Associated Macrophages (TAMs)

TAMs are immune cells that modulate and promote several immunosuppressive factors in the tumor microenvironment [90]. TAMs derive from monocytes that are recruited to the tumor [91] and, in the presence of Th2 cytokines such as IL-4 or IL-13, are polarized toward an M2 ("alternatively activated") non-cytotoxic phenotype [92]. Several studies have underlined their capacity to cause tumor growth both directly, by production

of cytokines that stimulate proliferation of tumor cells [93], and indirectly, by stimulating proliferation of endothelial cells [94]. TAMs are frequently found in solid tumors, where they promote remodeling of the extracellular matrix and secrete growth factors inducing tumor-specific neoangiogenesis [95]. Moreover, TAMs are enriched in hypoxic areas in most of the solid tumors [96], where they support tumor cell proliferation by secreting cytokines and growth factors. Indeed, accumulation of macrophages within the hypoxic tumor areas of patients is correlated with poor prognosis [97]. On the other hand, increasing accumulation of TAMs in the normoxic tumor area supports M1-like macrophages, leading to an antitumor immune response [98], while blocking colony-stimulating factor-1 (CSF-1) signal decreases M2-like polarization and impedes malignant progression resulting in regression of established gliomas [99]. These processes thus underscore the therapeutic relevance of TAM polarization.

Recently, metabolic changes in the tumor microenvironment have gained attention suggesting that, during tumor progression, gradients of extracellular metabolites (like lactate) act as tumor morphogens that promote M2-like polarization [100, 101]. Moreover, it has been suggested that treating TAMs with the glycolysis inhibitor 2-deoxyglucose blocks the development of TAMs with a pro-metastatic phenotype [102]. In the same line, increasing glucose uptake specifically in TAMs outcompetes endothelial cells for glucose usage, thus reducing vascular hyperactivation and decreasing tumor angiogenesis [103], supporting the link between metabolism of TAMs and tumor angiogenesis.

TAM-mediated immunosuppression also affects T-cell function. Under IL-6 and IL-10 stimulation, expression of programmed death-ligand 1 (PD-L1) is induced in TAMs [104], thus impairing T-cell effector activity. Moreover, programmed death 1 (PD-1) expression on the surface of TAMs correlates with decreased phagocytosis [105]. PD-1/PD-L1 blockade increases both effector T-cell activity and PD-1$^+$ TAM phagocytosis, supporting the use of checkpoint inhibitors in cancer treatment. In addition, TAM-derived PGE2, IL-10, and IDO play important roles in the induction of Tregs. Furthermore, TAM-derived CCL17, CCL18, and CCL22 are chemotactic factors for Tregs [87], resulting in the suppression of T cells in the tumor microenvironment. For example, in the HPV16 E6- and E7-expressing TC-1 tumor mouse model, TAMs were shown to cause suppression of the antitumor T-cell response [106], while their secreted IL-10 subsequently induced a Treg phenotype [107].

5.2.2.5 Tumor-Derived Immunosuppressive Factors

Within the tumor microenvironment, signals that stimulate T-cell cytolytic functions can be replaced by inhibitory signals secreted by the tumor itself as a mechanism of immune escape.

Cytokines

The immunosuppressive cytokines TGF-β and IL-10 are produced by Tregs as a means to disbalance T-lymphocyte surveillance of tumor development [108, 109], by inhibiting proliferation of antitumor effector T cells. Granulocyte-monocyte colony-stimulating factor (GM-CSF) is another cytokine with immunosuppressive properties. Due to these properties, GM-CSF facilitates recruitment and expansion of MDSCs in several cancer models [110, 111] and promotes generation and expansion of TAMs [112], despite being described as immunostimulatory in other settings [113]. The GM-CSF receptor (GM-CSF-R) signals through signal transducer and activator of transcription factor 3 (STAT3) [114], which has been linked to elevated PD-L1 expression on myeloid cells [115] and regulation of IDO expression in breast cancer MDSCs [116].

Enzymes

Together with arginase and iNOS, which are central for two of the mechanisms of immunosuppression exerted by MDSCs, IDO and cyclooxygenase 2 (COX2) also present immunosuppressive properties. IDO inhibits T-cell activation by depleting tryptophan [117], one of the essential amino acids necessary for T-cell development, whereas COX2 stimulates PGE2 production, a prostaglandin involved in conversion of human DCs into immunosuppressive MDSCs [118].

Negative Regulatory Factors

Antitumor immune responses are hampered by tumor-induced activation of negative regulatory

pathways (also called checkpoints), either associated with immune homeostasis or actively facilitating tumor immune escape [119–121]. Frequently, antitumor immunity shares characteristics with chronic immune responses, such as T-cell exhaustion [122], mediated by the expression of multiple inhibitory receptors including PD-1 (also known as CD279), cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152), lymphocyte-activation gene (Lag-3), T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), CD244/2B4, CD160, TIGIT, BTLA, and others [12, 123–128]. Among them, PD-1 and CTLA-4 have been extensively studied and garnered attention due to the clinical success of antibody therapies [129–131]. PD-1 is a member of the CD28 superfamily of T-cell regulators, expressed on activated $CD8^+$ T cells during priming or expansion, and functions mainly in peripheral tissues, where T cells encounter its two corresponding ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), members of the B7 family [132]. PD-L1 is expressed in various cell types, including stromal and tumor cells, but also in immune cells after exposure to effector cytokines such as IFN-γ, while PD-L2 is mainly expressed on DCs in normal tissues [133]. In physiological situations, the PD-L1/PD-1 axis is an important negative feedback loop ensuring immune homeostasis through suppression of excessive immune activation [134] and facilitation of immune tolerance to self-antigens [132, 135, 136]. However, in the tumor, the PD-1/PDL-1 axis restricts tumor immunity [129]. Tumor-specific $CD8^+$ T cells that express lower levels of PD-1 showed less exhausted phenotypes [137], as compared with tumor-specific $CD8^+$ T cells with higher PD-1 expression. Similarly high levels of PD-1 have been found on activated $CD8^+$ T cells during chronic infections [138]. Co-inhibitory signaling via PD-L1 (but not PD-L2) is necessary for conversion of naïve $CD4^+$ T cells to adaptive $CD4^+FoxP3^+$ Tregs. In addition, PD-L1 expression in various tumors, including breast, ovarian, colorectal, pancreatic cancer, and hematologic malignancies, has been considered a predictor of poor prognosis [139–143].

Although not as disputed as the PD-1/PD-L1 axis, LAG-3 is also a member of the immunoglobulin superfamily and is expressed on the surface of activated Tregs, $CD8^+$ T cells, B cells, and NKT cells, contributing to tumor immune suppression. Interestingly, Tregs from LAG-$3^{(-/-)}$ mice present reduced regulatory activity [144]. Lastly, CTLA-4 is a receptor expressed on the surface of Tregs and upregulated on activated conventional T cells [145, 146]. CTLA-4 transmits an inhibitory signal for T-cell activation by competing with the co-stimulatory molecule CD28 for binding to their shared ligands CD80 (B7.1) and CD86 (B7.2), with opposing effects [147, 148].

Endothelin Receptors

Aberrant activation of the small bioreactive peptide endothelin 1 (ET1) and its receptors endothelin receptor type A (ETAR) and type B (ETBR), by a large array of stimuli, in a paracrine and autocrine loop [149], has multiple implications in the progression of various solid tumors, including prostate, colon, ovarian, breast, and lung cancer [150–154]. Upon binding of its ligand ET1, ETAR promotes vasoconstriction, tumor cell proliferation, and cell migration [155–158] through phospholipase Cβ and downstream activation of mitogen-activated protein kinase family members, including ERK signaling [150]. ETAR may also play a role in chemoresistance [159]. On the other hand, ETBR was shown to inhibit T-cell homing and adhesion to the tumor by inducing the suppression of intracellular adhesion molecule 1 (ICAM-1) on the endothelial cells [150]. High expression of ETAR has been reported in patients with prostate cancer and bone metastasis [160], HPV-induced neoplasia [156, 161], and renal cell carcinoma [162]. ETBR expression was associated with the absence of tumor-infiltrating lymphocytes and decreased survival of patients with ovarian cancer [163]. Additionally, ETBR overexpression is associated with an aggressive tumor phenotype in melanoma [164, 165] and correlates with tumor progression and metastasis of vulvar squamous cell carcinoma [166].

The above-described spectrum of strategies developed by tumors to evade the cytolytic activity of the immune system illustrates the complexity of the tumor immune escape phenomenon and its capacity to adapt and particularly target distinct mechanisms of the antitumor immune response. Developing tumors are able to use different functions of the immune system to sustain their own growth and to simultaneously build up mechanisms which enable them to hide from an immune-based attack. Different types of tumors develop diverse immune escape mechanisms, translating into various degrees of tumor aggressiveness. Thus, the complexity of the tumor immune escape phenomenon resides in the ability of human tumors to develop unique signatures, which pose a real challenge for development of effective antitumor therapies.

5.3 Shifting the Balance: Strategies to Target Tumor Immunosuppression

Therapeutic approaches against cancer have mainly been oriented on the activation of the immune system to directly eliminate tumor cells, thus decreasing the tumor load. More recently, the importance of cancer-induced immune suppression is being taken into consideration with apparent clinical success of antibodies against immune checkpoints [129]. Despite the therapeutic potency of those immunotherapies, still only a subset of patients exhibit durable responses, suggesting that the main challenge of these strategies is the unique immune signature of tumors, which further translates into a large variability of tumor-induced immunosuppression mechanisms. Hence, the starting point of these strategies consists of mapping this immune signature, followed by a documented selection of uni- or multimodal therapies targeting the predominant immunosuppressive mechanisms developed within each tumor type. Based on their overall target aim, these therapies can be categorized as those which attempt to increase homing of effector T cells to tumors and those that, directly or indirectly, increase antitumor activity of intratumor effector T cells, either by overcoming tumor-induced tolerance or by overriding the immunosuppression mechanisms imposed during tumor development (see Table 5.1).

5.3.1 Strategies Targeting Homing of Effector T Cells

Some of the tumor immune escape mechanisms described above interfere with the proper trafficking of effector T cells from the peripheral circulation or secondary lymphoid organs to the tumor site. A reduced homing of these effector cells to the tumor will give rise to negative regulatory processes leading to tumor progression. Several strategies to block these processes and enhance intratumor homing of effector cells have been proven effective. These include local tumor irradiation, blockade of endothelin receptors, taxane-based chemotherapy, and antibody-mediated targeting of effector CTLs.

5.3.1.1 Local Tumor Irradiation

Local tumor irradiation has long been used as a curative treatment for localized cancer and isolated metastasis, but also as a palliative treatment in patients with widespread disease. Overall, more than 50% of cancer patients receive radiotherapy, often as adjuvant therapy, in association with other therapies such as surgery, hormonal therapy [167], chemotherapy, or bone marrow transplantation. Radiotherapy has been highly effective for certain malignancies, including prostate, endometrial, and cervical cancer. Recently, irradiation has come to the attention of tumor immunologists due to its immunogenic properties and potentially antimetastatic effects [168–174].

A major immunological effect of local tumor irradiation is the induction of cell death [175] that results in release of TAAs and danger signals, which attract immune cells to the tumor site, thus favoring antigen cross-presentation, improved DC function, and therefore enhanced antigen-specific T-cell priming [170, 176, 177]. Furthermore, it has recently been demonstrated that, after irradiation, the remaining cancer cells

Table 5.1 Types of immunotherapy aimed at targeting various mechanisms of tumor-induced immune suppression

Type of therapy	Targeted pathway	Achieved effect
Local tumor irradiation	Antigen presentation and processing Release of tumor-associated antigens Production of proinflammatory cytokines and chemoattractants	Enhanced intratumor homing of effector CTLs[a]
Endothelin receptor blockade	Restoration of ICAM-1[b] expression	
Chemotherapy Taxanes	Inhibition of angiogenesis Induction of programmed cell death Antigen presentation and processing TAMs[c] cytotoxicity	
Ab-mediated targeting of CTLs[a]	Tumor and T-cell concomitant antigen binding	
Depletion/inactivation therapy MDSCs[d] Tregs[e] TAMs[c]	Inhibition of DNA replication Inhibition of tyrosine kinase signaling Enzyme inhibition Inhibition of angiogenesis	Enhanced activity of intratumor effector CTLs[a]
Cytokine therapy IL-15 IL-7 IL-12	T-cell growth factors DCs[f] activation Vaccine adjuvants	
Blockade of negative factors Anti-CTLA-4[g] (Ipilimumab) Anti-PD-1[h]/anti-LAG3[i] Anti-TGFβ[j] Anti CD40/CD40L	Blockade of T-cell checkpoints Inhibition of receptor signaling Induction of T-cell activation Antigen-presenting cell activation	

[a]Cytotoxic T lymphocytes
[b]Intercellular adhesion molecule 1
[c]Tumor-associated macrophages
[d]Myeloid-derived suppressor cells
[e]Regulatory T cells
[f]Dendritic cells
[g]Cytotoxic T lymphocyte-associated protein 4
[h]Programmed cell death protein 1
[i]Lymphocyte-activation gene 3
[j]Transforming growth factor beta

present high levels of co-stimulatory and MHC class I molecules that render them more immunostimulatory and susceptible to T-cell-mediated killing [178]. Other beneficial effects of local tumor irradiation involve the induction of proinflammatory cytokines, such as TNF-α, IL-1β, and TGFβ [168, 179, 180]; expression of chemokines, like CXC-motif chemokines such as CXCL9, CXCL10, CXCL11, and CXCL16 that result in chemotaxis of T cells; and induction of adhesion molecules and death receptors that enhance CTL responses [181, 182]. These changes within the tumor microenvironment facilitate recruitment of effector T cells to tumors via two distinct mechanisms: first, by promoting vasculature normalization [183] and, second, by stimulating overexpression of endothelial adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) [169].

In the last decade, preclinical and human studies brought forward substantial clinical evidence that local tumor irradiation has the capacity to activate the immune system. Notably, combination of immunotherapies and radiation has been shown to enhance antitumor responses. Preclinical studies in tumor-bearing mice displayed that irradiation combined with PD-1 blockade increased overall survival and decreased Treg infiltration [184], when compared with anti-PD-1 treatment alone. Consistent to that combination of anti-PD-L1 antibody and irradiation resulted in substantial tumor regression, together with significant reduction of MDSCs within the tumors and increased $CD8^+$ T-cell infiltration

[185]. Currently, multiple clinical trials are evaluating anti-PD-1 and anti-PD-L1 antibodies in combination with radiation for cancer treatment, but results are not yet published [186]. Additionally, after combination therapy of irradiation and CTLA-4 blockade [187], lung metastasis was inhibited in a mouse 4T1 primary mammary carcinoma. Recently, Vanpouille-Box et al. suggested that, in patients who did not respond to treatment with immune-checkpoint inhibitors, local tumor irradiation may induce tumor-specific CTLs [188]. Clinical studies of combination therapies with anti-CTLA-4 antibodies, such as ipilimumab, demonstrated tumor regression and improved overall survival, primarily in patients with melanoma but also with lymphoma, prostate, or renal cancer [189–194].

Taken together, these preclinical and clinical data illustrate that radiotherapy, alone or in combination with other therapies, effectively stimulates the immune system to fight tumor development. This occurs by facilitating antigen presentation and processing, causing the release of TAAs; increasing production of inflammatory cytokines, chemokines, and receptors involved in recruitment of effector CTLs; and thus enhancing migration of these active effector CTLs to the tumor site.

5.3.1.2 Blockade of Endothelin Receptors

Various studies demonstrated that endothelial cells from a variety of human cancers overexpress the ET1 receptors. Blocking these receptors seems a promising strategy to delay tumor development or stop tumor cell proliferation. In a mouse HPV-induced cervical carcinoma model, blockade of ETAR caused inhibition of tumor growth [165], mediated by an increase in T-cell homing to the tumor site. Moreover, ICAM-1 downregulation, as an effect of ETBR interaction with ET1 [163], is rescued by administration of BQ-788, an ETBR small molecule inhibitor [149]. Neutralization of ETBR by administration of BQ-788, suppressed intercellular communication and growth of melanoma cells in nude mice [165] and significantly increased T cell homing to tumors [149, 163]. In fact, selective ETAR blockade by atrasentan showed delayed progression of hormone-refractory prostate adenocarcinoma [195], enhanced the effect of paclitaxel/docetaxel treatment in prostate cancer [196], and increased the overall survival of patients with chronic lymphocytic leukemia B [197].

5.3.1.3 Taxane-Based Chemotherapy

Conventional chemotherapy is considered to act through direct killing of tumor cells or by irreversible tumor growth arrest. Most chemotherapeutics interfere with cellular processes, such as DNA synthesis and replication, or lead to specific cell cycle arrest through microtubule disruption and apoptosis induction [198]. Originally, taxanes (e.g., paclitaxel, docetaxel) have been categorized as a class of chemotherapeutic drugs which block tumor development upon induction of mitotic inhibition through disruption of microtubule functionality. Other studies suggested additional antitumor mechanisms, such as binding to and blocking the functions of the antiapoptotic molecule Bcl-2 expressed on the surface of tumor cells, thus inducing programmed cell death [199]. More recently, the idea of chemotherapeutic agents, including taxanes, as enhancers of effector CTL homing into the tumor site came into place. The immunomodulatory effects of chemotherapy span both the innate and the adaptive immune systems, highlighting the enhanced potential of chemotherapy in combination with immunotherapy [198]. For example, treatment with the angiogenesis inhibitor paclitaxel resulted in an increased infiltration of circulating effector T cells into the tumor site, in a human xenograft mouse model [200]. Additionally, paclitaxel therapy is associated with tumor regression through direct stimulation of TAM cytotoxicity [201] or indirect activation of DCs, NK, and tumor-specific $CD8^+$ T cells via IL-12, TNF-α, and iNOS secretion by TAMs [202]. Taxanes also promote antigen presentation in murine bone marrow (BM)–DCs and human monocyte-derived DCs (moDCS) in vitro via upregulation of costimulatory molecules and IL-12p70 [203, 204]. Additionally, paclitaxel specifically impairs the viability and the cytokine production of $FOXP3^+$ Tregs [205]. On the other hand,

docetaxel induces maturation of DCs in vitro [206] and selective killing of MDSCs in vitro and in vivo [207, 208].

5.3.1.4 Antibody-Mediated Targeting of Effector CTLs

Monoclonal antibody therapy is a method commonly used to functionally inactivate or deplete suppressive immune populations such as MDSCs or Tregs, as discussed below. However, various studies using bispecific monoclonal antibodies suggest that they can also exhibit antitumor therapeutic potential. These antibodies are artificial proteins composed of fragments of two distinct monoclonal antibodies that can bind to two different types of antigens. In cancer immunotherapies, they are engineered to simultaneously bind to a CTL and a tumor cell. Several examples include engagement of CD3, CD28, or CD137 receptors [209] on the T cells and various tumor cell markers, such as epithelial adhesion molecule, and human epidermal growth factor receptor expressed on the tumor cell [210]. Different studies have shown the therapeutic potency of these strategies in vitro [211] and in vivo [209, 210, 212–214].

5.3.2 Strategies Targeting the Activity of Effector T Cells

Enhancing intratumor homing of immune effector cells will most likely not be sufficient for an effective tumor control, as cells that migrate to the tumor site are often anergic or dysfunctional. As addressed above, multiple mechanisms within the tumor microenvironment, involving a diversity of immunosuppressive cell populations (e.g., MDSCs, TAMs or Tregs), negative regulatory factors (e.g., CTLA-4, PD-1, PDL-1), as well as cytokines and enzymes (e.g., TGF-β and IDO), have been implicated in generating this immune suppressive tumor microenvironment.

To increase the efficacy of immunotherapies and rationally develop novel strategies which enhance the activity of intratumor effector T cells, both inhibition of tolerance mechanisms and restriction of tumor-induced immune suppression should be targeted. To effectively target the above-described negative regulatory mechanisms, several strategies have been studied. An overview of the immunotherapeutic interventions that are most widely studied preclinically as well as in clinical trials will be addressed.

5.3.2.1 Circumventing Activity of Suppressive Immune Populations: Depletion or Inactivation Therapy

One commonly used mechanism to target innate as well as adaptive antitumor immunity is manipulation of the immune suppressive functions of MDSCs, Tregs, or TAMs. A more intrusive alternative, however extremely efficient, is depletion of suppressive immune populations. Different depletion methods, with specificity for the targeted immune population at hand, have been developed.

There are several ways to specifically target and deplete intratumoral MDSCs [215]. Studies using an engineered RNA aptamer that targets IL4 receptor alpha (IL4Rα), upregulated on MDSCs of tumor-bearing mice, showed delayed tumor growth, enhanced T-cell infiltration, and MDSC apoptosis [216, 217]. This strategy may have promising results, since ILRα expression is also elevated in MDSCs in human tumors [218]. Another way to deplete MDSCs is with broad-spectrum tyrosine kinase inhibitors, such as sunitinib [219]. In the TC-1 cervical cancer mouse model, combinations of sunitinib with a cancer vaccine targeting tumor cells expressing the E6,7 oncoproteins of HPV, resulted in MDSC depletion and led to enhanced E7-specific CTL frequencies and subsequent tumor eradication [220]. Consistent to this, sunitinib also induced reversal of Treg elevation, significant reduction of IL4 production, and increased frequencies of IFN-γ-producing T cells [219, 221]. Sunitinib is capable of inducing selective MDSC apoptosis, up to 50%, in patients with metastatic renal cell carcinoma, thus representing one of the most promising drugs for reducing tumor-induced immune suppression [219, 222]. Treatment with chemotherapeutic agents and cytostatic drugs such as 5-fluorouracil [223, 224] or gemcitabine [225,

226], as well as novel strategies, like peptibodies [227], have also been described to deplete MDSCs.

Another immune suppressive population that has been intensively targeted for improving antitumor responses is Tregs. To date, several methods to deplete Tregs have been developed. Depletion of $CD4^+CD25^+$ Tregs by monoclonal antibody therapy has been achieved in both tumor-bearing mice as well as in clinical trials [228, 229]. Selective depletion of $FoxP3^+$ Tregs in transgenic DEREG (depletion of regulatory T cells) mice, in combination with therapeutic immunization against melanoma, greatly enhanced the antitumor effect [230]. However, the potency of a combination of immunization and Treg depletion depends not only on the involvement of Tregs in the tumor model studied but also on the level of Treg induction or activation in the immunization strategy. For example, depletion of Tregs by treatment with an anti-folate receptor 4 antibody did not enhance the immune response induced by immunization with the recombinant viral vector vaccine Semliki Forest virus encoding for the early HPV viral proteins E6 and E7 (SFVeE6,7) in a mouse model of cervical carcinoma [231]. In the clinical setting, a potent method to deplete Tregs by targeting their high CD25 expression is by employing the immunotoxin denileukin diftitox (Ontak™ Ligand Pharmaceuticals), which is approved for clinical use in the treatment of cutaneous T-cell lymphoma [232]. In combination with immunization, it has also been used for treatment of other types of tumors [233]. Daclizumab (Hoffman-La Roche) is another anti-CD25 agent, previously used in patients with T-cell leukemia [234] and, more recently, in combination with a peptide vaccine for treatment of metastatic breast cancer [235] and ovarian cancer [236]. However, anti-CD25 antibodies can also target activated $CD25^+$ effector T cells. Alternatives that circumvent this disadvantage are the use of novel antibodies with human specificity such as anti-glucocorticoid-induced TNF receptor antibodies, or low doses of Treg-depleting cyclophosphamide [237].

Regarding TAMs, selective depletion can be achieved by different approaches, such as blockade of TAM chemoattractant chemokines (e.g., blockade of CCL-2 with the inhibitor molecule bindarit [238] or immunization with a legumain-based minigene DNA vaccine [239]). Notably, the most efficient depletion method in animal models involves the usage of clodronate liposomes. Clodronate liposomes are artificial spheres formed by dispersion of phospholipid molecules into an aqueous solution of clodronate bisphosphonate. Intraperitoneal or subcutaneous administration of clodronate liposomes induced efficient depletion (75–92%) of TAMs in different murine tumor models [240–244]. Furthermore, selective depletion of TAMs is promoted by IL-15 and or TGF-α in human primary colorectal adenocarcinomas [245]. In other studies, IL-15 has been shown to reverse T-cell anergy and to rescue the tolerant phenotype of CD8+ T cells [246]. Several other pharmacological drugs, such as zoledronic acid and sorafenib, may also deplete TAMs and enhance the antitumor responses [247]. Yet it should be noted that nonselective depletion of TAMs also results in the depletion of tumoricidal macrophages, whereby any beneficial effect can be counteracted. Novel strategies that repolarize the protumoral M2-like TAMs to cytotoxic M1-like macrophages should be considered.

5.3.2.2 Immunostimulatory Cytokines: Cytokine Therapy

In addition to the above-discussed IL-15, various other cytokines are viewed as promising immune-restorative drugs. IL-7, a survival cytokine crucial for T-cell development in the thymus and survival of naïve and memory T-cell homeostasis in the peripheral tissues [248], increases the numbers of peripheral $CD4^+$ and $CD8^+$ T cells in patients [249, 250]. IL-12, a cytokine naturally produced by DCs, is a potent immune adjuvant promoting IFN-γ release from immune cells and thus inducing Th1 polarization and proliferation of antitumor effector T cells [251], with encouraging results in preclinical studies on diverse mouse tumor models, including thyroid cancer, bladder cancer, metastatic breast carcinoma, and glioma [252–254].

5.3.2.3 Blockade of Negative Regulatory Factors: Antibody Therapy

Antibody therapy against developing tumors has been employed in the clinics for many years and belongs to the category of "molecular targeted therapy" of cancer. Despite the emergence of a large palette of anticancer monoclonal humanized or chimeric antibodies (MABs), only a small number are approved for patient use by the Food and Drug Administration (FDA). Among them, trastuzumab (Herceptin) is a humanized MAB targeting ERGR activity, specific for HER-2/neu-positive breast cancer and metastatic gastrointestinal cancers [255–257]. Another successful example of MABs is Rituximab (Rituxan), a human/murine MAB targeting CD20 for B-cell lymphoma, lymphocytic leukemia, but also autoimmune diseases [258, 259]. Due to their low toxicity profile and capacity to activate several distinct host effector mechanisms [260], these monoclonal antibodies are seen as very promising anticancer drugs. The mechanisms mainly employed by these antibodies are direct interference with tumor cell progression and cell-mediated cytotoxicity by ligation of Fc receptors expressed on the surface of different immune cells [261].

The blockade of PD-1/PD-L1 interaction by several immune checkpoint inhibitors is currently being used for a wide range of solid and non-solid cancers [262] and has so far exhibited durable responses without serious toxicity in the majority of treated patients. The magnitude of clinical responses achieved with checkpoint inhibitor therapy implies that patients can have preexisting tumor-specific T cells that can be reactivated by blocking the PD-1/PD-L1 interaction. Another antibody that has been approved for treatment of late stage melanoma is ipilimumab (Yervoy), a human monoclonal antibody directed against the CTLA-4 expressed on activated T cells, as discussed above. Due to its capacity to inhibit this negative signaling pathway and contribute to restoration of the antitumor antigen-specific immune response, anti-CTLA4 is nowadays used as a novel therapy for solid tumors [15]. Recently, PD-1 blockade has been shown to increase the induction of effector T cells in the spleen, prolong T-cell proliferation, and enhance recruitment of effector T cells to tumor sites. In multimodality therapy regimens, PD-1 blockade increased therapeutic efficacy of total body irradiation and DC transfer therapy [263]. Also, antibody blockade of LAG-3 in two murine models of self and tumor-tolerance increased the accumulation and effector function of antigen-specific $CD8^+$ T cells [264]. Thus, combination of MAB therapy against PD-1 or LAG-3 with immunization strategies has been recently demonstrated to restore the functions of tolerized antigen-specific $CD8^+$ T cells [265]. Several clinical trials are currently ongoing to evaluate responses in patients with cancer following anti-PD-L1 treatment [266–269]. Several approaches have been employed to induce high avidity effector T cells in an attempt to target the inhibition of tumor-induced tolerance. One such approach involves blockade of TGF-β-induced signaling that has pleiotropic functions in tumor initiation, development, and metastasis. Since cancer cells display dysregulated TGF-β signaling, TGF-β inhibitors act on TGF-β-responsive cells (e.g., fibroblastic, endothelial, and immune cells) in the tumor microenvironment. In a xenograft mouse model of prostate cancer, transfer of tumor-reactive, TGF-β-insensitive $CD8^+$ T cells led to a 50% decrease in average tumor weight, when compared with tumors of mice which underwent transfer of naïve $CD8^+$ T cells [270]. Also, monoclonal antibodies against TGF-β, which are nowadays evaluated in clinical trials, seem to be very promising antitumor candidates as they present little systemic toxicity [271]. Clinical results of TGF-β inhibition in a phase II study performed in hepatocellular carcinoma patients are promising [272]. Additionally, radiotherapy and chemotherapy can induce TGF-β activity, and combined TGF-β inhibition enhances tumor sensitivity to chemotherapy and radiotherapy [273]. Another approach aimed at manipulating TGF-β to improve antitumor immune responses involves generation of TGF-β-insensitive DC vaccines. Transduced DCs, which have been rendered insensitive to TGF-β, maintain their normal phenotype, present

upregulated expression of surface co-stimulatory molecules (CD80/CD86), and induce potent tumor-specific cytotoxic T-lymphocyte responses in vivo [274].

Another target for antibody therapy is the costimulatory molecule CD40 expressed on various APCs and tumor cells. CD40 binds to CD40L expressed on T helper cells, resulting in APC activation as indicated by HLA classs II upregulation and IL-2 production [275, 276]. Agonistic antibodies against CD40 and/or CD40L tested in clinical trials seem to have a promising therapeutic potential [277].

5.4 Concluding Remarks

In the last few decades, major progress has been achieved within the field of cancer immunotherapy, highlighting the underlying therapeutic potential. However, despite the clinical success of antibody therapies against immune checkpoints, especially in the context of CTLA-4 and PD-1/PD-L1 axis blockade, still only a subset of patients shows sustained responses. This illustrates the complexity of tumor immunity and the interplay between antitumor responses, immune tolerance, and immune suppression within the tumor microenvironment. For cancer immunotherapy to be effective, sufficient homing and activation of antigen-specific immune effector cells in the tumor and suppression of immune-suppressive mechanisms is pivotal. This calls for multimodality treatment regimens to achieve long-term tumor regression. A desirable, highly effective immunization strategy should therefore accomplish two purposes. On the one hand, it should aim at increasing both the recruitment of antigen-specific effector T cells to the tumor site and their intratumor arrest for the time necessary to exert their antitumor activity. For this purpose, combinations of immunization regimens with ways to enhance homing of immune effector cells to the tumor site, such as local tumor irradiation, endothelin B receptor blockade, antibody-mediated targeting of effector CTLs, or taxane-based chemotherapy, could be promising strategies. On the other hand, only targeting the homing of vaccine-induced effector T cells to the tumor site might not be enough. We may speculate that once these cells have reached the tumor, they can be anergized or tolerized by diverse immune-suppressive mechanisms developed by the tumor itself or by secondary immune-suppressive populations. To counteract this effect, strategies that aim at maintaining or potentiating the activity of these intratumor antigen-specific effector T cells, such as depletion or functional inhibition of immune-suppressive populations, or blockade of negative regulatory factors are necessary.

Concluding, the development of new multimodality strategies in which immunization therapies are combined with effective antitumor immunological or conventional approaches aimed at increasing homing of immune effector cells to tumors and their intratumor activity is of crucial importance and represents the next step forward in cancer immunotherapy.

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6 Overcoming Cancer Tolerance with Immune Checkpoint Blockade

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6.1 Introduction

In 1957, Thomas and Burnet proposed the immunosurveillance theory, contending that the immune system is continuously patrolling, recognizing, and eliminating individual or groups of transformed cells [1]. This theory together with the identification of tumor-associated antigens (TAAs) led to much of the work in cancer vaccines to date. Based on this theory, it stands to reason that if the immune system has failed to recognize or mount a sufficient immune response to cancer, thus allowing a cancer to grow until it is clinically evident, stimulating the immune system sufficiently against the cancer could correct the immune system's failings and destroy the cancer. While there is considerable data in support of this theory, a number of discrepancies have also been noted. Most notably, athymic nude mice, which are T-cell deficient, and immunosuppressed individuals (transplant patients) do not develop neoplasms that are not virally linked at rates much drastically higher than their immunocompetent counterparts [2, 3]. While better models have since confirmed the role of the immune system in protecting against cancer development, it is clear that the immunosurveillance theory alone is not sufficient to explain the role of immune systems in cancer development.

Active immunotherapy for cancer based on the immunosurveillance understanding of cancer has, for the most part, been characterized by promising preclinical and early phase trials with, ultimately, disappointing clinical results in later phase trials [4]. Vaccination techniques have focused on stimulating the immune system by exposure to single or multiple tumor-associated antigens with immunoadjuvants such as cytokines (GM-CSF, IL-2) or toxins. While a variety of different techniques have been tried, with the exception of sipuleucel-T, a cancer vaccine approved for treatment of metastatic prostate cancer, these techniques have largely proven insufficient to overcome the local and systemic immunosuppression of advanced cancer in order to achieve a clinically significant improvement [5]. Historically, various types of active immunotherapy have shown excellent results in eradicating or preventing tumors in relevant murine models. In early phase clinical trials, active immunotherapies have generally had minor, well-tolerated toxicity profiles and shown promising immunologic results; however, these have not translated to clinically meaningful endpoints

when tested in larger-scale controlled trials. As noted above, an exception to this is the sipuleucel-T vaccine, which demonstrated significant benefit in overall survival in castrate-resistant prostate cancer (CRPC) in two phase III trials and has been FDA approved based on these results [5, 6].

The immune system-cancer interaction is now recognized to be more complex than once imagined. The cumulated results of experimental evidence have led to the "immunoediting theory," a modification of the previous immunosurveillance theory that explains how immunocompetent individuals develop cancer and how the immune system can help shape the biologic activity of the cancers themselves. The theory proposes that cancer proceeds though three phases: elimination, equilibrium, and escape. The elimination phase describes the recognition and elimination of nascent cancer cells as in the immunosurveillance theory. The equilibrium phase is a period where the cancer cells that avoid immune destruction are held at bay by the immune system and which, through selective pressure (immunoselection), can change the cancer's phenotype into a less immunogenic and more tolerance-inducing tumor. The escape phase describes the setting in which cancer cells have evolved to evade immune pressure and can replicate to become a clinically apparent neoplasm [7].

Cancer avoids immune destruction in the equilibrium phase and then is able to enter the escape phase through multiple mechanisms that have become increasingly well characterized. Cancer cells can escape immune detection by downregulating production of TAAs or the major histocompatability (MHC) complexes that the antigens are presented on [8, 9]. Tumor tissue can promote lymphocyte anergy, or unresponsiveness, by downregulating necessary co-stimulatory signals, which are necessary for functional lymphocyte activation, or upregulating coinhibitory signals, which are necessary for preventing autoimmunity. Tumors, through contact-mediated and soluble signals, recruit and cause proliferation of inhibitory cell populations such as regulatory T lymphocytes (Tregs), tolerogenic dendritic cells, and myeloid-derived suppressor cells. Additionally, tumors alter the cellular microenvironment through secretion of inhibitory cytokines and metabolic byproducts, all of which hamper effective immune response [10].

Given our increased understanding of how tumor cells actively inhibit and escape host immunity and the disappointing results of most cancer vaccine therapies, it has become increasingly clear that these failures do not stem from lack of ability to stimulate an appropriate immune response but rather from the inability of the immune response to overcome immunosuppressive mechanisms. In other words, regardless of how many stimulated, cancer-specific effector cells are created with a given vaccine, if the cells are rendered ineffective in the "immunoedited" tumor microenvironment, ultimately the therapy will fail [11]. A large amount of research effort is underway to identify, characterize, and target cancer escape mechanisms in hope of delivering more effective immunotherapeutic treatments.

As mentioned earlier, one major mechanism of immune resistance is through multiple costimulatory and inhibitory receptor-ligand combinations (immune checkpoints) that create a context for the effector and target cell (or antigen-presenting cell) interaction. Multiple immune checkpoints have now been identified and have been found to play an integral role in cancer escape (Fig. 6.1). Blockade of two of these checkpoint pathways, CLTA-4 and PD-1/PD-L1, has led to commercially available therapeutic drugs in patients with multiple different types of malignancy. Many other immunomodulatory checkpoints are being actively investigated and will, in all likelihood, lead to further therapeutic options for patients with cancer. In addition, the potential for combination therapy with multiple checkpoints targeted (such as CTLA-4, PD-1, PD-L1) or together with standard therapies or cancer vaccines remains great. This chapter will review the role of therapeutic checkpoint targets to overcome tumor-mediated immune suppression through targeted checkpoint modulation.

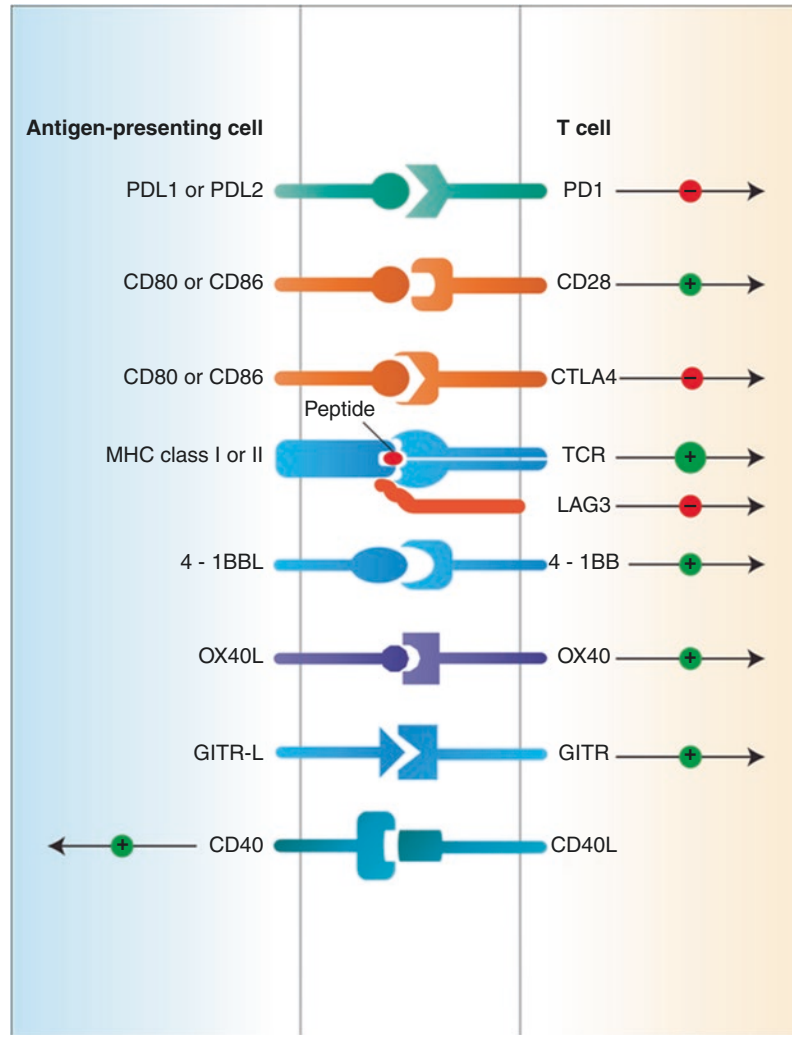

Fig. 6.1 Multiple immunomodulatory coinhibitory and costimulatory receptor-ligand pairs have been identified (although not all are depicted here). These pathways set the immunologic context when an antigen is presented on a T-cell receptor (TCR) to a major histocompatibility (MHC) complex

6.2 Neoantigens: Targets for the Immune System

With the development of multiple commercially available checkpoint blockade drugs, considerable research has been devoted to determining in which tumor types and in which clinical setting the drugs are beneficial. With this new focus, factors that make certain tumors more immunogenic are becoming clearer. All malignancies that become clinically apparent are able to evade immune destruction, but this is often due to immunosuppressive factors (rather than lack of immunogenicity of the tumor itself) that can be countered with checkpoint inhibitors and, potentially, other immunostimulatory drugs in development. Neoantigens are unique antigens generated from gene mutations during neoplastic transformation. Each neoantigen produced represents a potential target for the host immune system to differentiate the tumor from normal tissue. However, not all neoantigens are inherently immunogenic. It is presumably a matter of chance whether the mutations a tumor acquires produce neoantigens immune system is capable of recognizing and targeting. As a consequence, in general, tumors with a higher mutational load, such as melanoma, NSCLC, and microsatellite unstable tumors, are more likely to respond to checkpoint inhibitors [12–17]. However, this is not entirely predictive as tumors with relatively lower somatic mutations (HCC, clear cell carcinoma)

have shown benefit, albeit with lower response rates, to checkpoint inhibitor therapy [18]. Checkpoint inhibitors allow the ineffective immune responses to be more effective (but there has to be an immune response to begin with), illuminating why checkpoint inhibitors are not effective in all patients.

At this time, there are five checkpoint inhibitors approved by the US Food and Drug Administration for a variety of cancers, including ipilimumab (melanoma), pembrolizumab (melanoma, non-small cell lung cancer [NSCLC], head and neck squamous cell cancer, classical Hodgkin's lymphoma [cHL], urothelial carcinoma, microsatellite instability [MSI]-high colon cancer, gastric cancer), nivolumab (melanoma, NSCLC, renal cell carcinoma [RCC], cHL, MSI-high colon cancer, hepatocellular carcinoma [HCC]), atezolizumab (urothelial carcinoma, NSCLC), avelumab (Merkel cell carcinoma [MCC], urothelial carcinoma), and durvalumab (urothelial carcinoma) [19].

6.3 Cytotoxic T-Lymphocyte-Associated Antigen-4 (CTLA-4): The First Checkpoint Pathway to Demonstrate Clinical Benefit

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, CD152) was the first recognized inhibitory immune checkpoint molecule [20, 21]. CTLA-4 is the target of the first FDA-approved checkpoint-targeting drug, ipilimumab. During the development of CTLA-4 blocking monoclonal antibodies (mAb), much has been learned about dosing, toxicity, combination therapy, and tumor response that are now and will continue to be useful as other immune checkpoint therapies are developed.

6.3.1 CTLA-4 Function

When CTLA-4 (CD152) was first reported in 1987, it was presumed to play a role in controlling T-cell activation given its close sequence homology with CD28, its proximity to CD28 on chromosome 1, and its expression on cytotoxic T lymphocytes (CTLs) coinciding with T-cell activation [20]. The first CTLA-4$^{-/-}$ knockout mice, created in the mid-1990s, confirmed that CTLA-4 played a key role in T-cell homeostasis as the mice quickly succumbed to polyclonal lymphoproliferative disease characterized by massive expansion of activated T cells [22]. Since then, it has become clear that CTLA-4 functions as a negative counterpart to CD28, the required costimulatory signal for the activation and expansion of T cells.

For T lymphocytes to be activated, an antigen-specific T-cell receptor (TCR) must bind to an MHC complex containing the appropriate peptide in its binding grove. While this is necessary, it is not sufficient to complete activation. A number of additional regulatory pathways have since been elucidated that closely control T-cell activation to ensure appropriate, directed immune responses under normal circumstances. Among these pathways, co-stimulation with CD28 (on the T cell) binding to B7-1 (CD80) or B7-2 (CD86) on the antigen-presenting cell (APC) is perhaps the most important and best known. B7-1 and B7-2 are expressed on APCs and are typically upregulated after activation [23, 24].

As a competitively binding counterpart to CD28, CTLA-4 is an inhibitory checkpoint molecule expressed on activated T cells and constitutively expressed on regulatory T cells (Treg) [21]. After TCR-antigen-mediated activation of T lymphocytes, expression of CTLA-4 on the cell membrane increases dramatically. CLTA-4 suppresses immune activation through multiple pathways, and the relative importance of each in overall immune homeostasis and in disease-related autoimmunity and immune suppression is not clear [25].

The CTLA-4 receptor controls effector T-lymphocyte activation by competitive binding with CD28 as well as through internal and external signaling. CTLA-4 binds the same ligands as CD28 (B7-1 and B7-2) but with 20 to 100 times greater avidity and can accommodate two ligands, whereas CD28 can only bind one [26–28]. CTLA-4 appears to blunt T-cell responses by not only competitively binding the CD28 ligands, B7-1 and B7-2, but also by recep-

tor-mediated induction of cell cycle arrest, decreasing production of IL-2, limiting T-cell dwell time, and enhancing Treg function, among other mechanisms [29]. There is evidence that competitive binding of B7-1 and B7-2 by CTLA-4 remains the most important function in counteracting CD28-mediated T-cell stimulation, as treatment of CLTA-4-deficient mouse models with CTLA-4-immunoglobulin fusion protein (CLTA-4Ig) can abrogate the lymphoproliferative autoimmunity which would otherwise be fatal [30]. Additionally, the singular importance of B7-1 and B7-2 in these pathways is demonstrated by the fact that mice deficient in CTLA-4 as well as B7-1 and B7-2 do not demonstrate lymphoproliferative autoimmunity [31]. Unlike CD28, which has some level of constitutive expression on most T cells, CTLA-4 is only expressed in significant quantity on effector T cells after activation. CTLA-4 reaches a maximal expression level as long as 48 h after the T cell is activated serving as a negative feedback loop to turn off or prevent an overly robust immune response as well as to prevent autoimmunity (Fig. 6.2) [27, 32].

In addition to directly and indirectly inhibiting effector T-lymphocyte activation and proliferation, CTLA-4 interacts with Tregs in a manner important to its overall function. As previously stated, CTLA-4 is expressed at some constitutive level on Treg cells, and higher levels of expression may be rapidly mobilized from an intracellular source [25]. The exact role that Treg-mediated immune suppression plays in the overall context of CTLA-mediated immune control is not entirely clear. There is evidence from

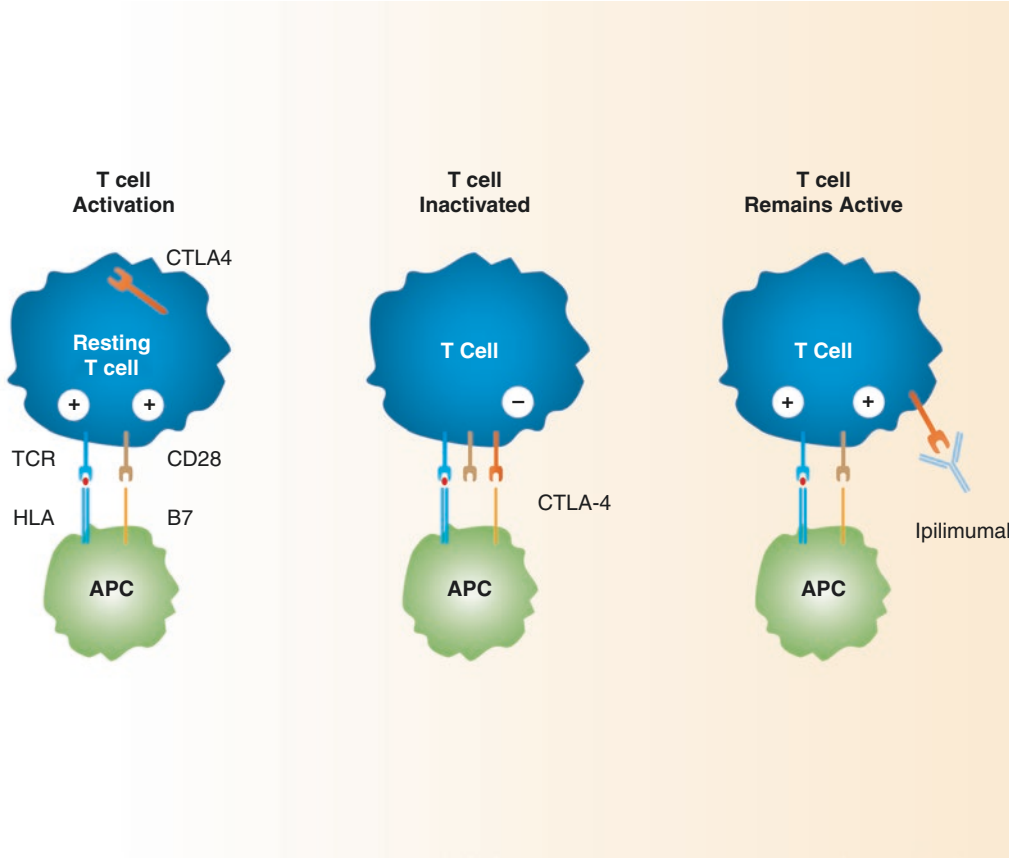

Fig. 6.2 Mechanism of action of CTLA-4 in suppressing activated T cells and proposed mechanism of action for ipilimumab

lymphocytes treated with anti-CTLA-4 monoclonal antibodies (mAbs) in vitro, which suggests that CTLA-4 blockade mediates the immune system by both direct activation of effector T lymphocytes and Treg depletion, dependent on the mAb subtype and its ability to stimulate antibody-dependent cytotoxicity (ADCC) [33, 34].

The important role of CTLA-4 in Treg homeostasis and immune control has become clear in multiple experiments. Treg-mediated CLTA-4 inhibits B7-1 and B7-2 expression on dendritic cells [35]. Murine models with CTLA-4-deficient $CD4^+$ $FOXP3^+$ (Treg) lymphocytes developed lymphoproliferative disease [35]. Additionally, CTLA-4 plays an active role in Treg homeostasis as blocking the receptor with anti-CTLA-4 mAbs leads to a rapid proliferation in peripheral Treg cells [36–38]. This action is thought to be due to CTLA-4 counteraction against CD28-stimulated proliferation of Tregs as blocking both CTLA-4 and CD28 leads to a contraction in the peripheral Treg population [24, 36]. However, expansion of Tregs with CTLA-4 blockade does not appear to lead to increased Treg function [39]. Similarly, in murine organ transplant models, deficiency of CD28 or both B7-1 and B7-2 leads to a significant decrease in the Treg population; however, the mice get paradoxical acceleration of graft rejection inversely proportional to the Treg level [39].

As work progresses in deciphering the mechanisms of the CTLA-4 receptor's complex interplay within broader immune homeostasis, the CTLA-4 receptor remains an active target of investigation for modulating the immune system for therapeutic purposes. The identified roles that CTLA-4 plays in human disease are substantial and ever-growing. There is evidence that CTLA-4 polymorphisms plays a role in autoimmune conditions such as type 1 diabetes, thyroiditis autoimmune hypothyroidism, and Graves' disease [40–43].

6.3.2 Tremelimumab

Tremelimumab (formerly CP-675, 206, ticilimumab, previously licensed to Pfizer, New York, NY, now licensed to AstraZeneca, London, UK) is another humanized anti-CTLA-4 mAb that has been evaluated in human clinical trials [29, 44]. Tremelimumab is an IgG2 antibody that, similar to ipilimumab, blocks the binding site of CLTA-4. It has a longer half-life of approximately 22 days compared to 12–14 days for ipilimumab [44]. In vitro testing of tremelimumab revealed enhanced T-cell activation, demonstrated by increased cytokine production. Based on this, as well as initial experience with ipilimumab, the drug proceeded with human trials.

The first dose escalation phase I trial of tremelimumab enrolled metastatic melanoma ($n = 34$), renal cell carcinoma ($n = 4$), and colon cancer patients ($n = 1$). The trial did note dose-limiting autoimmune toxicity, but determined that the drug was tolerated up to 15 mg/kg in a single dose. The trial also noted complete or partial response in 4 of the 29 patients with measurable melanoma [45]. Ongoing evaluation of tremelimumab is occurring in a phase II hepatocellular carcinoma study in combination with durvalumab (NCT02519348).

A phase I/II trial further evaluated dosing in metastatic melanoma patients and recommended dosing at 15 mg/kg every 3 months for further study given equivalent efficacy and better safety to more frequent dosing [46]. A subsequent single-arm, phase II trial of tremelimumab was conducted in 251 patients with relapsed or refractory metastatic melanoma. Patients were treated with tremelimumab at 15 mg/kg every 90 days (as recommended in the previous trial) for 4 doses and allowed up to 4 additional doses in patients with a tumor response or stable disease. The trial revealed an objective response rate of 6.6%. The trial reported an overall OS of 10.0 months, which is comparable with what was found in the previously described phase III trial of ipilimumab in similar patients. Serious adverse events (≥grade 3) were seen in 21% of patients [47].

The phase III trial of tremelimumab monotherapy in treatment-naïve unresectable stage III or stage IV melanoma began enrolling in March 2006. Patients were randomized to receive tremelimumab at 15 mg/kg every 90 days until symptomatic disease progression or standard-of-care

chemotherapy (temozolomide or dacarbazine) for 12 weeks or until disease progression. The primary end-point was OS. The trial was terminated by the data safety monitoring board at the second interim analysis (after two-thirds of planned events had occurred) because the test statistic crossed the prespecified futility boundary [48]. Survival follow-up continued after the trial was stopped. At final analysis, the median overall survival was 12.6 months in the tremelimumab arm compared to 10.7 months in the chemotherapy arm ($p = 0.127$). Objective response rates were similar in both arms (10.7% vs. 9.8%, respectively). Grade 3 or 4 adverse events occurred in 52% of tremelimumab patients compared to 37% of chemotherapy patients [49]. More recent work has suggested that the lack of tremelimumab efficacy may stem from the fact that it is an IgG2 isotype mAb, thus less able to produce reduction in intratumoral Tregs than ipilimumab, an IgG1 mAb [34]. Despite its lack of proven effect in this trial, tremelimumab remains under active investigation in other patient populations (discussed further below).

6.3.3 Toxicity

As previously described, CTLA-4 blocking antibodies can lead to unique, immunologic toxicities termed "immune-related adverse events" (irAEs) through nonspecific activation of the immune system. While the majority of these are minor and manageable, they occur relatively frequently, particularly at higher doses and can be severe. In the first phase III trial of ipilimumab, with treatment at 3 mg/kg, 14 patients (2.1%) receiving ipilimumab died from causes deemed treatment-related, with 7 of the deaths were from irAEs [50]. In a pooled analysis of 325 patients treated with ipilimumab at 10 mg/kg every 3 weeks for 4 doses, 72.3% experienced irAEs and 25.2% were ≥grade 3 [51]. In the phase III trial combining ipilimumab with dacarbazine for treatment naïve melanoma, 56.3% of patients in the combination arm experienced grade 3 or 4 adverse events. The most frequent irAEs are of the skin, gastrointestinal tract, liver, and endocrine system. These adverse events tend to occur at predictable times after receiving CTLA-4 blocking antibodies [51].

Skin toxicity is the most frequent irAE in some series, with roughly half of the patients receiving ipilimumab experiencing some form of rash. The rashes can typically be managed with symptom control and topical medication until they become more severe when systemic steroids and/or withholding or discontinuing treatment may be necessary. There are rare reported cases of toxic epidermal necrolysis that have been fatal [52].

Diarrhea is another frequent adverse event seen in CTLA-4 blockade treatment, occurring in between 32.8% and 51% of patients in phase III trials of ipilimumab and tremelimumab [49, 50, 53]. Severe diarrhea, colitis, and perforation are less common but can occur. Like skin toxicity, initial management is symptomatic. A high degree of suspicion for colitis with a low threshold for endoscopic evaluation is necessary for more severe (≥grade 2) diarrhea. The diagnosis of colitis or grade 3 or higher diarrhea necessitates more aggressive treatment with fluid replacement, systemic steroids, and treatment cessation. Infliximab treatment has been effective for severe colitis. A high index of suspicion for perforation with involvement of gastroenterology and surgery is also warranted in these cases [52].

Hepatotoxicity is seen less frequently (3–9%) with CTLA-4 blocking antibodies but can be severe. In general, liver function tests should be followed during treatment, and ≥grade 3 hepatotoxicity requires systemic treatment with systemic steroids and occasionally mycophenolate mofetil along with drug cessation [51].

Endocrine toxicities consist of hypophysitis and, less frequently, autoimmune thyroid dysfunction and adrenal insufficiency. Hypophysitis appears to occur in less than 5% of cases but typically has permanent sequelae and can lead to life-threatening adrenal insufficiency if not properly recognized and managed. Suspicion for hypophysitis should lead to pituitary MRI and laboratory testing. Treatment consists of systemic steroids and withholding CTLA-4 blocking treatment. Monitoring of

serum chemistries and thyroid function panels is recommended with ipilimumab treatment [54].

Other less frequent irAEs seen with CTLA-4 blocking therapies include episcleritis, uveitis, pancreatitis, neuropathies, and lymphadenopathy. Screening for a history of autoimmune disease and consideration of risk factors and expected benefits are recommended given the potential for serious toxicity with CTLA-4 blocking antibodies. National Comprehensive Cancer Network (NCCN) guidelines recommend participation in a risk evaluation and mitigation strategy (REMS) program when using ipilimumab [55].

Interestingly, multiple phase I and II trials of ipilimumab have noted a higher rate of clinical response in patients with irAEs and, in particular, grade 3 and 4 irAEs [52, 56–62]. A similar correlation was not addressed in the phase III trials of CLTA-4 blockade antibodies, and further evaluation may help clarify this as well as the underlying mechanisms.

6.4 Programmed Death 1 (PD-1) Pathway

6.4.1 Function

Programmed death 1 (PD-1) is a more recently discovered immune checkpoint receptor that has generated considerable excitement based on favorable preclinical profiling and initial clinical results. PD-1 was first discovered in 1992 by subtractive mRNA hybridization in an attempt to identify genes involved in programmed cell death [63]. Its protein structure was deduced based on the mRNA sequence obtained; however, its function remained unclear until PD1$^{-/-}$ knockout mice were noted to develop lupus-like autoimmune disease [64]. At that time, it was correctly suspected that PD-1 played a role in inducing peripheral tolerance.

Since its discovery, the function and significance of PD-1 has become more clear [65]. Like CTLA-4, PD-1 is a transmembrane protein expressed on effector immune cells [66]. Also like CTLA-4, expression of PD-1 is inducibly expressed with lymphocyte activation, although it is expressed more broadly than CTLA-4 as it is also found on activated B lymphocytes and NK cells [67–69]. PD-1 is bound principally by programmed death ligand 1 (PD-L1, B7-H1) but also, to a lesser degree, by programmed death ligand 2 (PD-L2, B7-DC) [70]. PD-L1 is constitutively expressed in certain tissues such as lung and placental macrophages [71]. Its high level of expression in the placenta has been implicated in mediating maternofetal tolerance [72, 73]. PD-L1 expression can also be induced on a broad range of hematopoietic, endothelial, and epithelial tissues in response to proinflammatory cytokines, such as interferon, GM-CSF, IL-4, and IL-19 [67, 74–77]. PD-L2 expression is more limited as it is inducibly expressed on dendritic cells, macrophages, and mast cells [71].

The PD-1 receptor pathway is an important negative regulator of the immune system. PD-1 appears to play a role primarily in dampening immune response in the setting of peripheral inflammation as opposed to CTLA-4, which plays a greater role in regulating T-cell activation [71]. As mentioned before, PD-1 knockout mice helped initially reveal the function of PD-1. The initial B6-PD-1$^{-/-}$congenic mice developed varying degrees of autoimmune arthritis and glomerulonephritis by 6 months of age and exaggerated inflammatory response to infection, in contrast to CTLA-4 knockout mice who die of diffuse lymphoproliferative disease shortly after birth [22, 64, 78]. Remarkably, later PD-1$^{-/-}$ knockout mouse models (BALB/c-PD-1$^{-/-}$ and MLR-PD-1$^{-/-}$) developed fatal autoimmune dilated cardiomyopathy early in life due to production of autoantibodies [79, 80]. In contrast, mice deficient in PD-L1 do not manifest autoimmunity, but can have increased accumulation of CD8^{+} lymphocytes in the liver and increased tissue destruction with experimental autoimmune hepatitis [81].

Ligation of PD-1, which again is found primarily on immunologic cells, counters CD28-mediated signaling through multiple mechanisms. PD-1 is phosphorylated upon ligand engagement, initiating a cascade of intracellular events [82, 83]. PD-1 signaling decreases the production of several proinflammatory cytokines such as IFN-

γ, TNF-α, and IL-2 [71]. It may also serve to retard cell activation mediated via CD28 and IL-2. PD-1 ligation has also been implicated in inhibiting transcription factors and initiation of several cell death pathways [84–86]. Importantly, PD-1 and its ligands also appear to play a role in shifting lymphocyte response from activation to tolerance when exposed to antigens, an attribute that is particularly significant for cancer immunotherapy [87]. Interestingly, PD-L1 was discovered to function not only as a ligand for PD-1 but also as a receptor bound by B7-1 (CD80) capable of delivering an inhibitory signal [88]. This finding not only demonstrates the complexity of lymphocyte regulation but suggests that blockade of these molecules could result in functionally different outcomes [78].

The PD-1 and PD-L pathways have been implicated in a variety of human diseases. Higher than normal expression levels of PD-1 and single nucleotide polymorphisms of PD-1 have been implicated in multiple autoimmune diseases such as systemic lupus erythematosus, Sjogren's disease, type 1 diabetes, and rheumatoid arthritis. As such, this pathway remains an active therapeutic target in these conditions [65]. In infectious diseases, the PD-1 and PD-L pathways play an important role in preventing unnecessary immune-mediated tissue destruction and have also been implicated in preventing the clearance of chronic viral, bacterial, and parasitic infections [71, 89].

6.4.2 PD-1 Pathway in Cancer

Just as the PD-1 pathway plays a central role in tolerance of chronic infections, it also appears to have a primary role in cancer tolerance and immune escape. PD-1 ligand expression, particularly of PD-L1 expression, has been demonstrated at various levels on a large variety of human cancer tissues. Higher expression of PD-L1 on tumor cells is associated with worse prognosis, more aggressive features, and/or resistance to immunotherapy in the large majority of cancers in which it has been characterized [90–101]. However, in some cases higher expression appears to have little influence on prognosis, as was found in NSCLC, and has even been associated with a more favorable prognosis, as found in colorectal cancer without mismatch repair (MMR) deficiency [102, 103]. $CD8^+$ tumor-infiltrating lymphocytes ($CD8^+$ TILs) have been noted to have high levels of PD-1 expression in many cases; nonetheless, correlation between PD-L expression and prognosis is mixed [97, 102, 104, 105]. Circulating NK cells in cancer patients have been noted to express PD-1, while healthy control NK cells do not [106]. Furthermore, preclinical data demonstrates that increasing tumor expression of PD-L1 makes it less susceptible to immunotherapy, while blocking it increases its vulnerability to immune-mediated destruction [107–110].

Some of the differences observed in tumor PD-L1 expression and correlation with cancer prognosis may be due to tumor-host interaction. Two recent studies examining human melanocytic lesions and colorectal cancer found a strong positive correlation between tumor PD-L1 expression and patient survival, in contrast to the majority of tissue types previously examined. However, in addition to this, higher PD-L1 expression was associated with both increased tumor infiltrating lymphocytes and interferon gamma (INF-γ) levels or gene expression in the tumor microenvironment [103, 111]. In these cases, the higher levels of PD-L1 expression may be in response to INF-γ signaling, as observed in normal human tissue [112, 113]. Thus, upregulation of PD-L1 expression may represent an adaptive tumor response to tumor-specific immunity, termed "adaptive resistance." [111, 114] The effective host immune response may explain the more favorable outcomes observed in these patients. Other evidence implicates different transcriptionally related oncogenic pathways in the upregulation of PD-1, which may or may not be related to external inflammatory signaling [92]. The adaptive resistance hypothesis may help further explain how tumors are able to escape immune stimulation from active immunotherapy and lead to blockade of the PD-1 pathway of particular therapeutic interest.

6.4.3 PD-1 Blockade

In preclinical studies with murine cancer models, anti-PD-1 and anti-PD-L1 blockade demonstrated antitumor effect as monotherapy and augmented the effects when given comitant with cancer vaccination [115–120]. Similarly, ex vivo blockade of PD-1 or PD-L1 improved the ability of human lymphocytic function against tumor tissue in multiple studies [107, 121–123]. Based on the functional importance of PD-1 in cancer as well as promising preclinical therapeutic results, several blocking mAbs have proceeded to human clinical trials.

6.4.4 Nivolumab

Nivolumab (MDX-1106, BMS-936558, Bristol-Myers Squibb, New York, NY) is a fully humanized IgG4 mAb that binds to PD-1, blocking its binding site. It was initially tested in a phase I, dose escalation trial on 296 patients with heavily pretreated advanced melanoma (n = 104), colorectal cancer (n = 19), CRPC (n = 17), NSCLC (n = 122), and renal cell carcinoma (n = 34). Nivolumab was given at 0.3, 1, 3, or 10 mg/kg in six patient cohorts followed by expansion cohorts at 10 mg/kg. Patients were initially given a single dose and allowed additional doses if they demonstrated clinical benefit; however, the trial transitioned into a phase Ib where patients were dosed every 2 weeks and reassessed every 8 weeks. Treatment was continued for up to 96 weeks or until disease progression or complete response. Overall, treatment with nivolumab was better tolerated than treatment with CTLA-4 blocking antibodies with no maximum tolerated dose achieved. Only 14% experienced serious (≥grade 3) drug toxicity, leading to the discontinuation of therapy in only 5%. There were drug-related adverse events in 41% and serious drug-related adverse events in 6% of patients that were likely irAEs, including pneumonitis, diarrhea, colitis, hepatitis, hypophysitis, and vitiligo. Pneumonitis, which occurred in 3% of patients, is of special interest, since it was not typically seen with CTLA-4 blocking mAbs and led to only three treatment-related deaths [124]. This toxicity may be secondary to constitutive expression of PD-L1 in alveolar macrophages.

Nivolumab treatment demonstrated substantial antitumor effect, with partial or complete responses (by RECIST criteria) observed in patients with melanoma, NSCLC, and renal cell carcinoma but not colorectal cancer or CRPC. Responses were observed across various doses at rates of 19–41% in melanoma, 6–32% in NSCLC, and 24–31% in renal cell carcinoma. One patient with melanoma and one with renal cell carcinoma had complete response to treatment. Responses tended to be durable with over half of melanoma and renal cell responses lasting for greater than 1 year. In addition, disease stability and mixed response (as described in irRC) were observed in a substantial portion of patients. Further analysis of PD-L1 expression from 61 patients who had pretreatment specimens available demonstrated an objective response in 36% of tumors expressing PD-L1 and none in PD-L1-negative tumors [124].

This data raises the possibility that PD-L1 could serve as a biomarker for response to therapy, an idea that is being actively investigated. PD-L1 has been shown to be a prognostic biomarker in the tumor cells of head and neck squamous cell cancer [125]; however, a recent review indicates that PD-L1 expression alone is insufficient for patient selection for most malignancies, both as monotherapy and combination therapy [126]. Another group showed the association between the mutational load of >100 nonsynonymous somatic mutations or neoantigens and ipilimumab or tremelimumab therapy with long-term clinical benefit in patients with advanced melanoma [127]. Another study in melanoma patients showed an association between that same mutational load and clinical benefit (complete or partial response or stable disease with overall survival longer than 1 year). Interestingly, only 0.04% of the identified antigens were present in more than one patient who showed clinical benefit, suggesting that most neoantigens associated with immunotherapy success are patient specific. Most recently, however, a systematic review and meta-analysis of 6664

patients found that PD-L1 expression was predictive of favorable response across tumor types including non-small cell lung cancer, melanoma, bladder cancer, renal cell carcinoma, gastroesophageal cancer, head and neck cancer, merkel cell carcinoma, and small cell lung cancer (OR 2.26, 95% CI, 1.85–2.75, $p < 0.001$), with the greatest effect observed in non-small cell lung cancer, where quantitative PD-L1 testing is now recommended prior to treatment (OR 2.51, 95% CI 1.99–3.17, $p < 0.001$) [12, 127].

Nivolumab has now been approved by the US Food and Drug Administration for use in humans in multiple cancer types. It was first approved in 2014 for patients with unresectable or metastatic melanoma and disease progression following ipilimumab and a BRAF inhibitor if applicable. Approximately 1 year later, nivolumab was approved for metastatic squamous and nonsquamous NSCLC with progression on or after platinum-based chemotherapy, unresectable or metastatic melanoma in combination with ipilimumab in BRAF V600 wild-type patients, and renal cell carcinoma in patients who received prior antiangiogenic therapy. In 2016, approval was granted for classical Hodgkin lymphoma (cHL) that progressed after hematopoietic stem cell transplantation and recurrent or metastatic head and neck squamous cell carcinoma that progressed on or after platinum-based chemotherapy. To date, additional approvals have been granted in locally advanced or metastatic urothelial carcinoma on or following platinum-based chemotherapy, adult and pediatric microsatellite high (MSI-H) or mismatch repair-deficient metastatic colon cancer that has progressed following chemotherapy, and HCC in patients previously treated with sorafenib [17, 19, 128–134].

6.4.5 Pembrolizumab

Pembrolizumab (Keytruda, Merck, Whitehouse Station, NJ) is a humanized monoclonal antibody that binds to PD-1 and blocks interaction with PD-L1 and PD-L2. At this time, it is FDA approved in patients with unresectable or metastatic melanoma, select NSCLC, recurrent head and neck squamous cancer, refractory cHL, locally advanced or metastatic urothelial carcinoma, and select gastric cancers. Most notably, pembrolizumab has received a broad indication for all adults and pediatric MSI-H or mismatch repair deficient solid tumors who have progressed following prior treatment, and colorectal cancer that has progressed following chemotherapy.

Deserving special mention is the first-of-its-kind MSI-H, and mismatch repair deficient (dMMR) indication was obtained in five uncontrolled, open-label, multi-cohort, multicenter, single-arm trials[45], known respectively as KEYNOTE-016, −164, −012, −028, −158. A total of 149 MSI-H or dMMR patients met inclusion criteria, and 98% had metastatic disease. Most had received two or more prior therapies. Patients received either 200 mg every 3 weeks or 10 mg/kg every 2 weeks. The majority (60%) of patients had colorectal cancer, and the remainder consisted of multiple solid tumors most commonly endometrial, biliary, and gastric/GE junction tumors. The overall response rate was 39.6% (95% CI 31.7–47.9), with 78% of patients demonstrating a durable response at 6 months [19, 135–140].

6.4.6 PD-L1 Blockade

Initial results of the PD-1 pathway blockade are very encouraging. The findings of objective clinical responses of up to 41% of subgroups of patients with nivolumab and relatively high response rates in NSCLC, a disease historically resistant to immunotherapy, are unprecedented in cancer immunotherapy. Additionally, lower rates of toxicity, in particular, serious irAEs, compared to CTLA-4 blockade have given hope that this pathway will yield more widely applicable and better-tolerated therapies. Much work remains and is currently in progress to bring these therapies into general clinical use. Determination of optimal dosing, duration of treatment, and the subsets of patients who benefit from treatment are all underway. As with CLTA-4 blockade, preclinical data supports a possible synergistic effect when PD-1 pathway blockade is combined with other cancer

treatments such as chemotherapy, radiation, and immunotherapy; this deserves and is receiving further investigation [107, 119, 121, 141]. As these investigations move forward, one area of particular interest will be whether PD-L1 expression on tumors continues to serve as a reliable biomarker for predicted therapeutic benefit, thus increasing the ever-growing trend of more personalized, tailored treatment for individual tumors.

6.4.7 Atezolizumab

Atezolizumab is an Fc-engineered, humanized, monoclonal antibody that binds to PD-L1, blocking its interaction with PD-1 and B7-1 receptors. It is now FDA approved in patients with unresectable or metastatic urothelial carcinoma who are not eligible for platinum-based chemotherapy or who progressed on such therapy and metastatic NSCLC with progression on or after platinum-based chemotherapy. The urothelial carcinoma indication was granted accelerated approval in 2015 based on early-phase results in 310 patients who had disease progression after platinum-based therapy. Compared to historical controls with a 10% overall response rate, an objective response rate of 15% with a median follow-up of 11.7 months was achieved. In addition, increased levels of PD-L1 expression on immune cells were associated with increased response [142–145].

NSCLC approval was based on two randomized, open-label clinical trials (POPLAR and OAK) where atezolizumab 1200 mg IV every 3 weeks was compared with docetaxel and an overall survival benefit of 2.9 months in POPLAR at a median survival of 12.6 months and 4.2 months in OAK at a median survival of 13.8 months [144, 146].

6.4.8 Durvalumab

Durvalumab (MEDI-4736) was recently approved for locally advanced or metastatic urothelial carcinoma who progressed after platinum-based chemotherapy. It was approved under accelerated approval based on a phase I/II open-label study in 182 patients who had disease progression on or after platinum-based chemotherapy and received durvalumab 10 mg/kg IV every 2 weeks for 12 weeks. 31 patients (17%) demonstrated clinical responses, with 5 complete responses at a median follow-up of 5.6 months [147].

Additional approval has been granted for patients with unresectable stage III NSCLC without disease progression following platinum-based chemotherapy and radiation. This approval was granted based on the PACIFIC study, a multicenter, randomized, double-blind, placebo-controlled study enrolling 713 patients who had completed at least two cycles of platinum-based chemotherapy and definitive radiation. Patients who received durvalumab demonstrated a statistically significant overall response rate of 28.4% compared to 16% in the placebo group ($p < 0.001$), with a longer median duration of response in the durvalumab group (72.8% vs. 46.8% had an ongoing response at 18 months post-randomization). Median progression-free survival was 16.8 months for durvalumab versus 5.6 months for placebo (95% CI 4.7–7.8) [148].

6.4.9 Avelumab

Avelumab is another PD-L1 blocking antibody that received accelerated FDA approval in 2017 for metastatic Merkel cell carcinoma in adults and children age 12 and older. This approval was granted based on a prospective, open-label, phase II trial in patients with stage IV, chemotherapy-refractive Merkel cell carcinoma who were given avelumab 10 mg/kg every 2 weeks. 88 patients received at least one dose, and 28 (32%) patients achieved an objective response (20 partial, 8 complete) at a median follow-up of 10.4 months [149, 150].

6.5 Immune-Related Response Criteria

Initial WHO response criteria and later RECIST criteria, which have undergone many revisions over the years, were developed to identify and

Table 6.1 Comparison of World Health Organization (WHO) and immune-related response criteria (irRC) for tumor response

	Word Health Organization (WHO)	Immune-related response criteria (irRC)
CR	Disappearance of all lesions in two observations at least 4 weeks apart	Disappearance of all lesions in two observations at least 4 weeks apart
PR	≥50% decrease in SPD of all index lesions in the absence of progression of nonindex lesions or new lesions in two observations at least 2 weeks apart	≥50% decrease in total tumor burden in two observations at least 4 weeks apart
SD	<50% decrease compared to baseline and <25% increase compared to nadir measurements of the SPD of index lesions, in the absence of progression of nonindex lesions or new lesions	<50 decrease compared to baseline and <25% increase compared to nadir
PD	≥25% increase in SPD compared with nadir or progressions of nonindex lesions or appearance of new lesions	≥25% increase in tumor burden compared to nadir in two observations at least 4 weeks apart

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *SPD* sum of the products of the largest dimensions of lesions

standardize definitions of tumors responsive to cytotoxic therapy and not as a surrogate for survival [151]. They have been used in early phase clinical trials as a surrogate for response to therapy. The use of these criteria assumes that tumors will shrink or stabilize at the outset of therapy. Tumor growth or the appearance of new metastases constitutes progressive disease and, therefore, lack of response. In immunotherapy trials, including those evaluating ipilimumab, it has been shown that tumors often progress or remain stable before responding, therefore making RECIST criteria less helpful in predicting treatment response. Based on these observations, new immune-related response criteria (irRC) were proposed (Table 6.1). The new criteria do not necessarily consider the appearance of new lesions or growth of isolated lesions as progressive disease but, instead, consider overall tumor burden. Based on retrospective observations of 487 metastatic melanoma patients in three phase II trials of ipilimumab at 10 mg/kg dosing, 9.7% of treated patients initially classified as progressive disease under WHO criteria later had evidence of response to therapy. In retrospective reclassification by irRC, response to therapy appears to correlate better with overall survival than WHO criteria [152]. Immune-related response criteria have been used alongside WHO criteria in multiple ipilimumab trials since it was first introduced [153, 154]. Further prospective validation will be needed to determine to what degree it correlates with overall survival.

6.6 CTLA-4 Blockade Monotherapy

Two mAbs, ipilimumab and tremelimumab, were developed in parallel. The therapies underwent phase III trials that ultimately led to approval for ipilimumab for treating metastatic melanoma and showed disappointing results for tremelimumab.

6.6.1 Ipilimumab

Based on the work in murine models, fully humanized IgG1 CTLA-4 mAbs were created by Medarex Inc. (Princeton, NJ; purchased by Bristol-Myers Squibb, New York, NY, in 2009) using a transgenic hybridoma HuMAb mouse model. The proprietary mouse model has multiple genetic modifications designed to facilitate production of high-avidity human IgG mAbs [155]. The mAb used for initial in vivo testing was selected based on affinity and specificity for CTLA-4 as well as ability to block the binding site. The antibody, called 10D1 (later designated MDX-010 and ipilimumab), also had cross-reactivity with macaques monkey CTLA-4. It was initially tested in this setting where it was shown to increase antibody response to hepatitis

surface antigen as well as a human melanoma cell vaccine. Additionally, the macaques did not demonstrate polycolonal T-cell activation or autoimmunity [156]. Based on this work, ipilimumab proceeded with human trials.

6.6.1.1 Ipilimumab in Uveal Melanoma

Uveal melanoma is a rare cancer that, like cutaneous melanoma, shares melanocyes as the cell of origin but has different pathogenesis and clinical behavior. Similar to melanoma, it has a very poor prognosis when it has metastasized (typically to the liver) and is resistant to systemic chemotherapy [156, 157]. Three open-label, multicenter, single arm phase II trials have been conducted using ipilimumab in uveal melanoma. The GEM-1 trial enrolled 32 patients treated with 10 mg/kg ipilimumab. At a median follow-up of 5.5 months, 13 patients had evaluable responses, with 1 having a partial response (7.7%) and 6 having stable disease (46.2%) [158].

The DeCOG treated 53 pretreated and treatment-naïve patients with metastatic uveal melanoma with ipilimumab at a dose of 3 mg/kg. Overall, they reported a relatively disappointing median progression-free survival (2.8 months) and overall survival (6.8 months) [159].(NCT01585194). The GEM-1402 trial is a phase I/II trial looking at ipilimumab in combination with nivolumab in the adjuvant setting for high-risk uveal melanoma after completion of standard treatment. In an interim analysis, it showed progression-free survival of 4.99 months at a median follow-up of 4.6 months (NCT02626962).

6.6.2 Phase III Trials of Checkpoint Inhibitors in Melanoma

The first phase III study of ipilimumab, sponsored by Bristol-Meyers Squibb, began enrolling patients in September 2004. The trial enrolled 676 HLA-A*0201$^+$ patients with pretreated, unresectable stage III or IV melanoma. The patients were randomized 3:1:1 to receive either ipilimumab with gp100 peptide vaccine, ipilimumab alone, or gp100 alone. The gp100 peptide had demonstrated effectiveness in previous phase II trials in melanoma, particularly when combined with ipilimumab [56–58, 160]. Ipilimumab was dosed at 3 mg/kg every 3 weeks for four doses. Patients were not routinely offered maintenance therapy; however, those who progressed after responding to therapy or who had stable disease after 12 weeks were allowed "reinduction" therapy. The primary endpoint of the trial was OS. The trial demonstrated an OS benefit in all patients who received ipilimumab (median OS: 10.0 months for ipilimumab with gp100, 10.0 months for ipilimumab alone, and 6.4 months for gp100 alone; $p < 0.003$). There was no difference in survival in patients who received ipilimumab with gp100 and those who received ipilimumab alone. There were four cases of complete responses and multiple cases of long-term disease control in patients who received ipilimumab. Approximately, 60% of patients treated with ipilimumab experienced some irAE, with the rates of serious irAEs (≥grade 3) of 10–15% in the ipilimumab groups [50]. Of the 31 patients who met criteria for and received "reinduction" therapy (progression after complete or partial response or stable disease), 19% achieved a complete or partial response and 68% achieved disease control with similar toxicity to the original induction therapy [161]. Based on this study, ipilimumab achieved FDA approval at a dose of 3.0 mg/kg to treat unresectable stage III and stage IV melanoma.

When ipilimumab was approved for therapy, it generated considerable interest because it represented a therapeutic success for nonspecific immunostimulation, a new modality in cancer treatment. In addition to this, it raised hope for future successes for cancer immunotherapy, particularly coming on the heels of the FDA approval of another cancer immunotherapy, sipuleucel T (Provenge; Dendreon, Seattle, WA), the first therapeutic cellular immunotherapy to prove effective in phase III trials [5, 6]. It gave hope to clinicians treating and patients with metastatic melanoma, as this was the first therapy to show an overall survival benefit in a randomized,

phase III trial for metastatic melanoma [162]. Significant questions remain and are currently under evaluation regarding the treatment of melanoma with ipilimumab. As discussed previously, a randomized, double-blind phase II trial comparing the dosing of ipilimumab demonstrated the superiority of 10 mg/kg dosing over 3 mg/kg dosing (used in the phase III trial and currently approved) in pretreated patients [163]. This data was not available at the initiation of the phase III trial.

The randomized, double-blind, multicenter phase III trial comparing 10 mg/kg versus 3 mg/kg ipilimumab in 727 patients with previously untreated or previously treated unresectable stage III/IV melanoma without previous treatment with BRAF inhibitors or immune checkpoint inhibitors showed a significant overall survival advantage with 10 mg/kg therapy over 3 mg/kg therapy (15.7 vs. 11.5 months, $p = 0.04$). The 10 mg/kg group did demonstrate a higher frequency of treatment-related adverse events and adverse events leading to discontinuation [164].

An additional question raised by the previous trials is the duration of treatment. Many of the previous phase II trials included maintenance dosing every 3 months after completion of the "induction" phase [52, 153, 163, 165]. The phase III trial of ipilimumab monotherapy applied a somewhat different approach, using "reinduction" therapy, in which the patients were redosed every 3 weeks for four doses if they had evidence of progression after initial response to treatment. Both long-term dosing schedules appear to be well tolerated. It remains to be seen if one is clearly superior. Ipilimumab monotherapy in metastatic melanoma has largely been replaced by combination therapy of ipilimumab with PD-1 inhibitors pembrolizumab and nivolumab. Phase III data for pembrolizumab was obtained in the KEYNOTE-006 study, in which 834 ipilimumab-naïve patients with advanced melanoma were randomized 1:1:1 to receive pembrolizumab 10 mg/kg every 2 weeks or 3 weeks or four doses of ipilimumab 3 mg/kg every 3 weeks. In the final analysis, pembrolizumab in both dosages provided a superior overall survival to ipilimumab at a median follow-up of 22.9 months. Median overall survival was not reached in either pembrolizumab group and was 16 months in the ipilimumab group. Twenty-four month overall survival was 55% in both the 2 and 3 weeks pembrolizumab dosing group and 43% in the ipilimumab group [138, 166]. In addition, patient-reported health-related quality-of-life scores were superior for patients who received pembrolizumab [167].

Nivolumab was evaluated in a phase III trial in ipilimumab-refractory melanoma patients who had unresectable or metastatic disease, comparing nivolumab to the investigator's choice of chemotherapy. In an analysis after 120 patients were enrolled in the nivolumab arm, there was an objective response rate of 31.7% (95% CI 23.5–40.8%) in the nivolumab arm versus 10.6% (95% CI 3.5–23.1%) in the chemotherapy arm. Additionally, nivolumab was associated with fewer toxic effects than chemotherapy [132].
Another study, known as CheckMate-066, examined untreated patients in a phase III study in previously untreated melanoma patients without a BRAF mutation and compared nivolumab with dacarbazine. Nivolumab was associated with improved overall survival at 1 year (72.9% vs. 42.1% respectively, $p < 0.001$) and progression-free survival (median 5.1 vs. 2.2 months, respectively, $p < 0.001$) [134].

6.6.3 Adjuvant Checkpoint Inhibitors

Ipilimumab was first approved as adjuvant therapy for melanoma due to results from a double-blind, phase III trial in patients with stage III cutaneous melanoma after resection, who received 10 mg/kg ipilimumab or placebo every 3 weeks for four doses and then every 3 months for up to 3 years.

951 patients were randomized, and median recurrence-free survival was 26.1 months (95% CI 19.3–39.3) in the ipilimumab group vs. 17.1 months (95% CI 13.4–21.6) in the placebo group. In patients who received ipilimumab, 52% discontinued therapy due to adverse events, most commonly gastrointestinal, hepatic, and endocrine [168].

Ipilimumab (10 mg/kg) was compared to nivolumab (3 mg/kg) in resected stage IIIB/IIIC/IV melanoma patients. 12-month recurrence-free survival was 70.5% (95% CI 66.1–74.5%) in the nivolumab group versus 60.8% (95% CI 56.0–65.2%) in the ipilimumab group. Grades 3 and 4 treatment-related adverse events were significantly worse in the ipilimumab group (45.9% vs. 14.4% in the nivolumab group), with two deaths in the ipilimumab group. The hazard ratio for death or recurrence favored nivolumab over ipilimumab (HR 0.65, 0.51–0.83, $P < 0.001$) [169].

Pembrolizumab was evaluated in a phase III double-blind trial in patients with completely resected stage III melanoma. Patients were randomized to receive either 200 mg pembrolizumab IV every 3 weeks for 18 doses or placebo. Pembrolizumab was associated with significantly longer recurrence-free survival at 1 year, 75.4% (95% CI 71.3–78.9) versus 61.0% (56.5–65.1) for placebo. Grades 3–5 trial-related adverse events were reported in 14.7% that received pembrolizumab compared to 3.4% in the placebo group [170].

Combination therapy involving checkpoint inhibitors is an active area of study. Recently, improved survival was observed using ipilimumab in combination with nivolumab in late-stage melanoma [129]. This will be covered in more detail in a later section.

6.7 Checkpoint Inhibitors as Combination Therapy

While CTLA-4 blockade, specifically ipilimumab, has found success as monotherapy in metastatic melanoma, and more trials are underway to test its effectiveness in a variety of malignancies and different clinical scenarios, its greatest potential may lie in combining it with other antineoplastic agents. The hope is that by combining CTLA-4 blocking therapy with other antineoplastic therapies that carry different toxicity profiles, a synergistic effect of the agents will be achieved. Recognizing these issues, researchers have been actively pursuing combination therapy with CTLA-4 blockade since its inception. The primary areas of research focus on combining CTLA-4 blockade with chemotherapy, radiation, surgery, and other immunotherapy.

6.7.1 Checkpoint Inhibitors and Chemotherapy

Given the known immunosuppressive effects of most chemotherapeutic agents, it has been thought that combining chemotherapy with immunotherapy would be unsuccessful. However, there is increasing evidence for a possible synergistic role between the two modalities. The immune system appears to play an important role in antitumor activity of chemotherapy, an effect which may be further augmented by immune checkpoint blockade [171, 172]. In murine models of mesothelioma, CTLA-4 blockade given between cycles of chemotherapy has been demonstrated to increase tumor-infiltrating lymphocytes and inflammatory cytokines and inhibit cancer cell repopulation [173]. Additionally, chemotherapy, when given appropriately, may enhance the effect of specific immunotherapy [174]. Evidence from clinical trials reveals that combining chemotherapy with cancer vaccination can be more effective than either therapy alone [175–177]. The mechanisms by which chemotherapy may increase anticancer immunity include reduction of immunosuppressive influences by decreasing tumor mass, inducing the expression of TAAs on the cell surface, exposing the immune system to TAAs through cell death, and "resetting" the immune posture through depletion of inhibitory cell populations (i.e., Tregs and myeloid-derived suppressor cells) [171]. Indeed, there is growing evidence that the success of certain chemotherapy regimens is dependent on the drug's ability to cause immunogenic cell death of tumors, where TAAs are presented in the appropriate context to elicit a broader immune response [178]. While this is a promising area for future development, clearly the timing of drug administration, chemotherapeutic regimen used, and dosing are integrally important to successful application. Highly dosed cytotoxic treatment has the

potential to quash a developing therapeutic immune response. Optimizing these factors will be necessary in future trials of combining checkpoint blockade with chemotherapy.

Clinical trials have been performed combining chemotherapy with CTLA-4 blockade. A randomized phase II trial testing the combination of chemotherapy with ipilimumab was conducted in patients with treatment-naïve metastatic melanoma. Seventy-two patients with unresectable, metastatic melanoma were randomized to receive ipilimumab at 3 mg/kg every 4 weeks for four doses with dacarbazine compared to ipilimumab monotherapy. The trial demonstrated an increased objective response rate (14.3% vs. 5.4%, by RECIST criteria) and increased median OS (14.3 vs. 11.4 months) for the combination therapy group, although neither reached statistical significance due to the smaller number of patients. Toxicity was higher in the combination group, including 17.1% ≥ grade 3 irAEs compared to 7.7% in the monotherapy arm [179].

Based on these results, the concept was tested in a randomized phase III trial evaluating ipilimumab with dacarbazine versus dacarbazine alone [163]. Additionally, based on the results of the phase II ipilimumab monotherapy trial that showed a benefit of higher dosing, 10 mg/kg of ipilimumab was used in combination with dacarbazine. Five hundred two patients were enrolled and randomized 1:1 to receive ipilimumab plus dacarbazine every 3 weeks for four doses followed by dacarbazine every 3 weeks until week 22 or placebo plus dacarbazine at the same schedule. Patients with stable disease or RECIST criteria objective responses were able to receive maintenance ipilimumab or placebo every 12 weeks. Of note, based on emerging consensus from previous work with CTLA-4 blockade and other immunotherapy, the primary endpoint was changed, with FDA approval, from progression-free survival to OS prior to unblinding of the treatment groups or data analysis [152, 180]. Ultimately, the trial showed that patients who received the combination of ipilimumab with dacarbazine survived longer (11.2 months) compared to dacarbazine alone (9.2 months, $p < 0.001$). The difference became more pronounced with time, as the combination arm had 20.8% of patients alive at 3 years compared to 12.2% in the chemotherapy only arm. Toxicities were greater in the combination arm and also greater than in many of the previous ipilimumab studies (56% ≥ grade 3), likely secondary to the higher dose (10 mg/kg) of ipilimumab used as well as the addition of chemotherapy. Interestingly, the toxicity profile was different. There were lower rates of gastrointestinal toxicities, such as diarrhea and colitis, and endocrine toxicity but a higher rate of hepatic toxicity compared with previous ipilimumab trials. No treatment-related death was reported [53]. Differences may reflect the effect of the combination therapy; however, clinician's experience managing the drug may have affected the outcome as well. Based on the results of this study, the combination of ipilimumab and dacarbazine is approved as the first-line therapy for unresectable melanoma.

However, the potential for unanticipated toxicity exists with combining CTLA-4 blockade, particularly with other targeted therapies. Initial results from a phase I study of combination therapy with both ipilimumab (dosed at 3 mg/kg) and vemurafenib, a BRAF inhibitor approved for treatment of BRAF-V600E-mutated melanoma, demonstrated an unacceptably high level of hepatotoxicity, leading to early termination of the trial [181].

Additional trials of combination chemotherapy and ipilimumab were conducted in patients with advanced non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Advanced-stage NSCLC carries a poor prognosis with a median survival of 8–12 months despite first-line chemotherapy [172, 182]. In a phase II trial, 204 patients with stage IIIB or IV NSCLC were enrolled in a randomized, double-blind trial of ipilimumab plus chemotherapy (paclitaxel and carboplatin) given concurrently, ipilimumab plus chemotherapy given phased with two doses of chemotherapy given prior to starting ipilimumab and chemotherapy given together, or placebo plus chemotherapy. Ipilimumab was dosed at 10 mg/kg every 3 weeks for up to 18 weeks with the option for

maintenance therapy (or maintenance placebo) every 12 weeks. The primary endpoint was immune-related progression-free survival (irPFS). The concept of immune-response criteria for immunotherapy in cancer (different from classic World Health Organization RECIST criteria) came from observations with ipilimumab and other immunotherapies (discussed further below) [152]. The trial showed improved irPFS with phased ipilimumab and chemotherapy (median: 5.7 months, HR: 0.72, $p = 0.05$), while concurrent ipilimumab and chemotherapy did not reach statistical significance (median: 5.5 months, HR: 081, $p = 0.13$) compared to the control regimen (median 4.6 months). Improvement was also noted in PFS by WHO criteria ($p = 0.02$), and an improvement in OS by 3.9 months ($p = 0.23$) was observed for phased ipilimumab over chemotherapy alone. Overall toxicity was similar across the treatment arms; however, there was more severe toxicity (grade ≥ 3) in the combination arms. A phase III trial was conducted using phased ipilimumab and chemotherapy in patients with squamous NSCLC, the group that derived the greatest benefit in subset analyses [154]; however, the addition of ipilimumab to first-line chemotherapy consisting of paclitaxel and carboplatin did not prolong OS [183].

A similar phase II trial was conducted in patients with extensive disease-small cell lung cancer (ED-SCLC). Chemotherapy remains the first-line and only effective therapy in this disease process with a median overall survival of 8–11 months [184]. Eligible patients ($n = 130$) were randomized to receive concurrent therapy with ipilimumab and chemotherapy (paclitaxel and carboplatin), the phased combination, or placebo with chemotherapy. In this trial, again the phased combination of ipilimumab and chemotherapy was superior with an improvement in irPFS (median: 6.4 months, $p = 0.03$), while concurrent therapy did not improve irPFS (median: 5.7 months, $p = 0.11$), compared to the control arm (median: 5.3 months). There was no significant difference in mWHO PFS or OS. The combination of ipilimumab plus etoposide and platinum chemotherapy versus etoposide and platinum alone has been evaluated in a phase III trial. 954 patients were randomized with no significant OS benefit (11.0 vs. 10.9 months), with increased rates of diarrhea, colitis, and rash in the ipilimumab group [185].

The combination of ipilimumab has been further studied in a phase II trial in prostate cancer. Forty-three patients with CRPC were randomized to receive either ipilimumab monotherapy at 3 mg/kg every 4 weeks for four doses or ipilimumab (dosed the same) with a single dose of docetaxel at the start of therapy. The number of responses to therapy were small with three patients having a decrease of >50% in each arm [186]. However, this study may be limited by underdosing of both the ipilimumab and docetaxel, concurrent (instead of phased) administration of the two drugs, as well as the small number of patients tested.

The combination of tremelimumab and sunitinib, an oral small-molecule tyrosine kinase inhibitor, was tested in a phase I dose escalation trial in patients with metastatic renal cell carcinoma. Unexpectedly, the trial demonstrated a high (4/28 patients) rate of sudden onset grade 3 renal failure in addition to other toxicity associated with CTLA-4 blockade. Further testing of this combination at doses of tremelimumab >6 mg/kg with sunitinib was not recommended by the study authors [187].

6.7.1.1 PD-1/PD-L1 Inhibitors and Chemotherapy

Pembrolizumab in combination with chemotherapy recently received FDA approved based on results of a double-blind phase III trial in which 616 patients with metastatic NSCLC without sensitizing EGFT or ALK mutations with no previous treatment were randomized to receive pemetrexed and a platinum-based drug plus either 200 mg pembrolizumab or placebo every 3 weeks for 4 cycles, followed by maintenance pemetrexed and pembrolizumab or placebo for 35 cycles. At a median follow-up of 10.5 months, estimated overall survival at 12 months was 69.2% (95% CI, 64.1–73.8) in the pembrolizumab group versus 49.4% (95% CI, 42.1–56.2)

in the placebo group, corresponding to a hazard ratio for death of 0.49 (95% CI, 0.38–0.64, $p < 0.001$). In addition, progression-free survival was significantly greater in the pembrolizumab arm: 8.8 versus 4.9 months. Adverse events of grade 3 or higher were comparable between arms (67.2% for pembrolizumab vs. 65.8% for placebo) [188].

There are no current FDA indications for nivolumab in combination with chemotherapy; however, multiple clinical trials are evaluating this (NCT02477826, NCT03101566).

6.7.2 Checkpoint Inhibitors and Radiation

Much like chemotherapy, there is evidence that the local and systemic effects of radiation therapy can increase the effectiveness of immunotherapy, in general, and CTLA-4 blockade, specifically. Radiation therapy damages tumor cells that are in the path of the focused energy, which, like chemotherapy, can result in cell death and antigen cross-presentation, leading to an effective, targeted immune response toward remaining tumor cells [189]. Radiation-induced cell damage may lead to several cellular changes that promote effective presentation of TAAs such as the release of high mobility box group 1 (HMBG1), which signals migration of immune cells to the tumor microenvironment, and upregulation of MHC I complexes, Fas, and ICAM-1, all of which increase susceptibility to T-cell-mediated death [189–192]. Additionally, localized radiation does not typically produce the same level of lymphodepletion and immunosuppression associated with high-dose chemotherapy. As with chemotherapy, reduction in the mass of a viable tumor may help decrease cancer-related immunosuppression. All of these factors make the combination of radiation with immunotherapy appealing [193]. The concept of combining radiation with immune checkpoint blockade is particularly attractive. Unlike more specific, directed immunotherapy (cancer vaccines), CTLA-4 blockade helps overcome cancer immunosuppression, but ultimately relies on the body's preexisting immunity toward a neoplasm. Radiation, by damaging cancer cells and releasing a wide array of TAAs in an inflammatory context, especially with immunosuppression checked, may allow the immune system to mount a response that is appropriate both for the individual and the tumor.

There is considerable preclinical data that supports the combination of CTLA-4 blockade and radiation. In one study, a mouse model of poorly immunogenic mammary carcinoma, 4T1, was treated with control IgG, CLTA-4 blocking IgG (9H10), radiation therapy, or a combination of 9H10 IgG and radiation. CTLA-4 blockade alone did not affect tumor growth or mouse survival. Radiation therapy slowed tumor growth but did not affect survival. The combination of CTLA-4 blockade and radiation therapy inhibited metastases and increased survival compared to the control [193]. Subsequent studies in this model revealed that treatment with the combination in mice deficient in invariant natural killer (NK) T-cell lymphocytes led to an even more effective response with some mice becoming disease-free and resistant to tumor rechallenge, highlighting the important role for this cell type in regulation of cancer immune responses [194]. Finally, an additional study in TSA mouse mammary carcinoma and MCA38 mouse colon carcinoma models again demonstrated the effectiveness of combining radiation and CTLA-4 blocking antibody; moreover, they showed that the use of a fractionated radiation schedule (but not single dose radiation) along with CTLA-4 blockade could significantly inhibit tumor foci out of the radiation field, a phenomenon known as the abscopal effect [195].

The abscopal effect refers to the regression of tumors in remote areas following localized radiation of tumors. This phenomenon has been documented in melanoma, renal cell carcinoma, and lymphoma [196–198]. Several cases of this occurrence have been documented in patients receiving ipilimumab. In one notable case, a patient with recurrent melanoma with paraspinal, right hilar lymphadenopathy, and splenic metastases was enrolled in an ipilimumab monotherapy trial in September 2009. She received treatment at 10 mg/kg dosing per protocol with

slow progression of her disease over the subsequent 15 months. In December 2010, she received directed, external beam radiation to her symptomatic paraspinal lesion followed by an additional dose of ipilimumab in February 2011. Surprisingly, follow-up imaging revealed significant regression of metastatic lesions outside the radiation field, which remained stable at minimal disease for at least 10 months after her radiation treatment. Along with this clinical effect, the patient was noted to have a marked increase in peripheral antibodies to the tumor antigen NY-ESO-1, an increase in $ICOS^{high}$ T cells, and a decrease in myeloid derived suppressor cells [199]. Similar cases of abscopal regression of metastatic melanoma in patients on ipilimumab have since been reported [200].

A phase I/II study examined the effects of ipilimumab with radiation therapy (RT) in patients with metastatic CRPC. Patients were treated with dose escalation ipilimumab monotherapy (3, 5, or 10 mg/kg) or ipilimumab (3 mg/kg or 10 mg/kg) with external beam RT, although the trials were not designed to directly compare the two arms. Ipilimumab was given every 3 weeks for a total of 4 weeks [201]. An overall of 71 patients were treated; 33 patients were treated in the dose escalation phase, and the 10 mg/kg arm was expanded to a total of 50 patients. At the 10 mg/kg dosing level, 16 were given ipilimumab monotherapy and 34 received ipilimumab with radiation. In the 10 mg/kg dosing group, there were four (25%) PSA declines >50% in the ipilimumab monotherapy arm and four (12%) PSA declines >50% in the ipilimumab with radiation group; however, a higher proportion of patients in the monotherapy group were chemotherapy naïve. A phase III trial examining radiation with ipilimumab compared to radiation alone in advanced CRPC has not shown a difference in overall survival [202].

A retrospective study was performed analyzing patients treated with pembrolizumab for NSCLC on the phase I KEYNOTE-001 study to determine the effect of previous radiotherapy on clinical outcomes. Of 98 patients that received pembrolizumab, 43% received previous radiotherapy. At a median follow-up of 32.5 months for surviving patients, progression-free survival was significantly increased in patients that received previous radiotherapy (4.4 months; 95% CI, 2.1–8.6) versus no radiotherapy (2.1 months; 95% CI, 1.6–2.3), corresponding to a hazard ratio of 0.56 (95% CI 0.34–0.91), $p = 0.019$. Median overall survival was increased in patients who received any radiotherapy (10.7 months; 95% CI, 6.5–18.9) versus no radiotherapy (5.3 months; 95% CI, 2.7–7.7), corresponding to a hazard ratio of HR 0.58 (95% CI 0.36–0.94), $p = 0.026$ [203].

There are no current FDA indications for PD-1/PD-L1 inhibitors in combination with radiation; however, multiple clinical trials are attempting to answer this question (NCT02830594 in pembrolizumab, NCT03148327 in durvalumab).

6.8 Combination Immunotherapy

Results from trials of CTLA-4 and PD-1 pathway blocking mAbs as monotherapy or in combination with conventional therapies are encouraging. Immune checkpoint blockade has delivered clinical responses in patients with limited or no therapeutic options remaining. However, in all of the immune checkpoint blockade trials covered, only a minority of patients have responded which is usually transient. It is true that the vast majority of the patients treated in these trials have advanced disease, are immunosuppressed, and have limited time and options remaining. Targeting earlier stage disease and combining immune checkpoint blockade with other therapies will undoubtedly yield more impressive results. However, it is naïve to think that targeting any one checkpoint will be a "silver bullet" therapy. Just as cancer, under immunologic pressure, learns to evade the immune system to become a clinically evident disease initially, as we modulate coinhibitory and costimulatory receptors, some cancers will adapt to escape through alternative pathways. Combining active immunization (cancer vaccines) with checkpoint blockade may ultimately prove effective; nonetheless, initial

results have not been convincing. Other techniques under investigation, targeting multiple checkpoints simultaneously or in sequence, may limit the escape routes.

6.8.1 CTLA-4 Blockade and Vaccination

Early on in the development of CTLA-4 blocking therapy, anti-CTLA-4 antibodies were combined with cancer vaccines in preclinical models [204]. In multiple cancer animal models, tumors, which were poorly responsive to CTLA-4 blocking therapy alone or active immunotherapy alone, responded significantly better to the combination of the two [37, 204–216]. These studies have helped elucidate the function and significance of the CTLA-4 receptor and have led to clinical trials in patients.

Some of the first human trials of ipilimumab used a combination of peptide vaccines from gp100, a tumor-associated antigen expressed by the majority of malignant melanomas [217]. Gp100 peptides have been shown to be immunogenic and elicit an antigen-specific T-cell response in the majority of melanoma patients [160]. One peptide, gp100:209–217(210M), when combined with IL-2 therapy, has also been shown in a randomized phase III trial to significantly increase clinical response and PFS compared to IL-2 alone in HLA*A0201$^+$ metastatic melanoma patients [218]. Three phase I and II trials were conducted using ipilimumab combined with gp100 in unresectable melanoma patients. While these trials did not directly compare the efficacy of the addition of the peptide vaccines to ipilimumab monotherapy, they did show impressive response rates and manageable toxicity [56–58]. Based on these (and other) results, ipilimumab proceeded to the phase III trial comparing ipilimumab monotherapy, ipilimumab plus two gp100 peptides (gp100:209–217 and gp100:280–288), or the gp100 peptides alone. As previously detailed, the trial demonstrated a survival advantage for ipilimumab therapy but also showed that the addition of the peptide vaccine to ipilimumab offered no improvement over ipilimumab monotherapy [50]. It is not clear why the peptide vaccine did not prove efficacious in this setting, particularly given its proven efficacy when given with IL-2 therapy in a similar patient population. There is speculation that CTLA-4 blockade may augment CD4$^+$ lymphocyte activity more, while gp100 peptides preferentially generate a CD8$^+$ lymphocyte response, a hypothesis that has mixed preclinical data to support it. Another proposed possibility is that the antitumor effect of ipilimumab may stem largely from its ability to deplete intratumoral Tregs, a mechanism which may not function synergistically with MHC class I peptide vaccination [34]. Certainly, there are other possibilities to explain the results; further studies will be necessary to clarify.

Additional trials on combining CTLA-4 blocking antibodies with cancer vaccines have been conducted in melanoma and prostate cancer. In melanoma, the combination of multiple tumor-associated antigen peptides (gp100, MART-1, tyrosinase) emulsified with immunoadjuvant (Montanide ISA 51) has been combined with ipilimumab in a dose escalation trial [62]. Additionally, in prostate cancer, ipilimumab has been given in phase I trials in combination with Tricom-PSA (PROSTVAC; Bavarian Nordic Immunotherapeutics, Mountain View, CA), a poxvirus-based vaccine that expresses transgenes for PSA and costimulatory molecules, and GVAX (Aduro Biotech; Berkeley, CA, USA), a GM-CSF-transduced allogenic prostate cancer vaccine [59, 219]. In all of these phase I trials, ipilimumab combined with cancer vaccination was found to elicit a cancer-specific immune response, a low rate of clinical response, and toxicity compared with ipilimumab monotherapy. Further trials will be necessary to prove the efficacy of these combinations and multiple other combinations, which are currently under investigation (NCT01810016, NCT01302496, NCT01838200).

6.8.2 PD-1/PD-L1 and Vaccination

Nivolumab has been tested in combination with ISA 101, a synthetic long-peptide vaccine directed against human papilloma virus (HPV)

16 in patients with incurable oropharyngeal cancer. The phase II trial accrued 22 patients who received 100mcg/peptide ISA 101 on days 1, 22, and 50, plus nivolumab 3 mg/kg IV every 2 weeks for up to 1 year. Eight patients demonstrated a clinical response, with two complete responses and eight partial responses, corresponding to an overall response rate of 36%, greater than the historical nivolumab monotherapy rate of 16% [220]. At a median follow-up of 8.6 months, median progression-free survival was 2.7 months (95% CI, 2.3–8.0). Median overall survival was not reached [221].

Nivolumab has also been tested with or without a peptide vaccine in a phase I study in 90 patients with ipilimumab-naive or refractory unresectable stage III or IV melanoma. Nivolumab was dosed at 1 mg/kg, 3 mg/kg, or 10 mg/kg and was well tolerated at all doses. The median duration of response was 8.1 months, and the overall response rate was 25% [222].

Ongoing studies include PD-1/PD-L1 and vaccination in melanoma (NCT03047928), non-squamous non-small cell lung cancer (NCT03380871), and multiple solid tumors (NCT02897765, NCT02432963).

6.8.3 CTLA-4 Blockade and Cytokine Therapy

Another area of combined immunotherapy undergoing active investigation is combining CTLA-4 blockade with cytokine therapy. IL-2 therapy has been used as adjuvant treatment for melanoma and renal cell carcinoma with benefit in a small subset of patients [223]. IL-2 stimulates T-cell activation, as does CTLA-4 blockade, but through different mechanisms. A phase I/II dose escalation/expansion trial combining ipilimumab with IL-2 was conducted in metastatic melanoma patients. The trial demonstrated a 22% (5/36) tumor response rate and toxicity similar to prior ipilimumab studies [61]. There are multiple ongoing trials examining the combination of ipilimumab and high-dose interferon alpha, the cytokine therapy used most frequently as adjuvant therapy in melanoma (NCT01274338 ongoing, NCT01708941 ongoing). GM-CSF has been used in combination with ipilimumab in a phase I dose escalation trial in CRPC demonstrating an immunologic response to treatment as well as a favorable PSA response in the highest dosing cohort (ipilimumab 3 mg/kg and GM-CSF 250 mg every 4 weeks) with expected toxicities. A recent randomized trial pairing ipilimumab with GM-CSF versus ipilimumab alone in patients with unresectable stage III/IV melanoma demonstrated longer overall survival (17.5 vs. 12.7 months), with no different in progression-free survival [47]. Additional trials of ipilimumab and GM-CSF in CRPC and melanoma are currently underway, NCT01530984).

A recent phase II trial compared talimogene laherparepvec (a genetically modified herpes-simplex virus that expresses GM-CSF) with and without ipilimumab in patients with unresectable stage IIIb and IV melanoma. One hundred ninety-eight patients were randomized, with a 39% objective response rate (ORR) in the combination arm compared to 18% ORR in the ipilimumab monotherapy arm (OR 2.9, 95% CI 1.5–55, $p = 0.002$). Additionally, more patients in the combination arm demonstrated regression of visceral lesions (52% vs. 23%), with severe toxicity comparable between arms (45% vs. 35%) [46].

6.8.4 Combination Checkpoint Blockade

There is ample preclinical data supporting dual checkpoint blockade in murine cancer models [215, 224–228]. Based on these principles, investigators have initiated trials of dual checkpoint blockade in humans.

Preliminary phase I results of combination of nivolumab (PD-1 blocking mAb) and ipilimumab (CLTA-4 blocking mAb) in patients with advanced melanoma demonstrated the potential of this combination [229]. This led to a multicenter randomized controlled phase III trial, the CheckMate 067 study. This trial enrolled patients with previously untreated stage III (unresectable) or stage IV melanoma and randomized them (1:1:1) to ipilimumab (3 mg/kg every

3 weeks for four doses) and nivolumab (1 mg/kg every 3 weeks for four doses followed by 3 mg/kg every 2 weeks), nivolumab (3 mg/kg every 2 weeks), or ipilimumab (3 mg/kg every 3 weeks for four doses). The overall survival rate at 36 months was 58% in the nivolumab-ipilimumab combination group, 52% in the nivolumab group, and 34% in the ipilimumab alone group. At 36 months follow-up, the median overall survival had not been reached in the combination group and was 37.6 months in the nivolumab group and 19.9 months in the ipilimumab group, corresponding to a hazard ratio for death with nivolumab plus ipilimumab versus ipilimumab of 0.55 ($p < 0.001$) and 0.65 ($p < 0.001$) for death with nivolumab versus ipilimumab. Treatment-related adverse effects of grades 3 and 4 occurred in 59% of the combination group, 21% receiving nivolumab, and 28% receiving ipilimumab [129].

6.9 Other Checkpoint Pathways Under Development

6.9.1 Lymphocyte Activation Gene-3 (LAG-3)

Lymphocyte activation gene-3 (LAG-3, CD223) is an additional immune coinhibitory checkpoint molecule under investigation for therapeutic purposes in cancer. LAG-3 was first discovered in the 1990s on activated T lymphocytes and NK cells [230]. LAG-3 is structurally similar to CD4, and, like CD4, binds to MHC II complexes on antigen-presenting cells (APCs), but with greater affinity. While some early functional data from experiments is mixed, it appears that LAG-3 plays a predominantly inhibitory role in T-cell activation, while promoting APC activation at the same time [114, 231–235].

LAG-3 is expressed on a subset of Treg cells that secrete immunosuppressive cytokines and are more potent than other LAG-3 negative cells of the Treg phenotype (CD4+, CD25highFoxP3+). They are preferentially expanded in patients with cancer. LAG-3 ligation on $CD8^+$ lymphocytes inhibits lymphocyte function and proliferation, independent of Tregs [18]. Notably, high expression levels of LAG-3 are seen on tumor infiltrating lymphocytes and, like PD-1, appear to represent an anergic phenotype. In contrast to its coinhibitory function on T cells, when soluble LAG-3 binds MHC II complexes on dendritic cells, it promotes activation and maturation [235–238].

Just as with CTLA-4 and PD-1 pathways, tumor cells are able to utilize the LAG-3 pathway to escape host immunity. MHC class II molecule (LAG-3 ligand) expression is sometimes upregulated to varying degrees in a variety of cancers and can be associated with a worse prognosis. Increased expression of LAG-3 on TILs, corresponding with increased $CD8^+$ T-cell anergy, has been noted in Hodgkins lymphoma, melanoma, and ovarian cancer [239, 240]. Additionally, MHC class II expressing melanoma cells (but not MHC class II negative cells) were resistant to FAS-mediated apoptosis when exposed to LAG-3 transfected cells or soluble LAG-3, indicating a bidirectional signaling in the LAG-3 pathway that effects both lymphocytes and tumor cells [114, 239–241].

Removing or blocking the LAG-3 pathway improves immune-mediated antitumor effects. Blocking LAG-3 with mAbs has been shown to increase CTL expansion and improve $CD4^+$ lymphocyte cytokine production. In melanoma, anti-LAG-3 mAb blockade improved the antitumor function of tolerized $CD8^+$ lymphocytes when coupled with a viral cancer vaccine [242]. In murine cancer models, PD-$1^{-/-}$ LAG-$3^{-/-}$ knockout mice were capable of rejecting tumors that PD-1 or LAG-3 alone knockout mice could not. It is worth noting that LAG-$3^{-/-}$ knockout mice display a very mild phenotype, similar to PD-$1^{-/-}$ knockout mice, while PD-$1^{-/-}$ LAG-$3^{-/-}$ knockout mice develop lethal autoimmunity at about 10 weeks of age, underscoring the potential toxicity of dual blockade therapy [225, 227, 243]. Similar to the knockout mice, dual mAb blockade of PD-1 and LAG-3 was able to cause complete regression in several established tumor models in mice, while blockade of the individual receptors was not [227, 243].

Since LAG-3 binding of MHC II complexes on APC promotes activation and maturation of the

APC, soluble LAG-3 protein has been tested as an immunoadjuvant in cancer. Theoretically, the unbound LAG-3 can promote APC activity while, at the same time, can prevent LAG-3-mediated T-cell inhibition through competitive binding. Supporting this, soluble LAG-3 in the serum of breast cancer patients was associated with improved survival. Based on these findings, a fusion protein of the extracellular portion of LAG-3 and the Fc portion of IgG1 were recognized as IMP321. IMP321 has been tested as a vaccine immunoadjuvant where it was well tolerated and produced encouraging immunologic results. IMP321 has also undergone testing as monotherapy in a phase I dose escalation trial in 21 patients with advanced renal cell carcinoma. The drug produced no significant adverse events and was associated with significantly more disease stability at higher dosing. More recently, IMP321 was tested at two different doses in a phase I trial together with gemcitabine in 12 patients with advanced pancreatic cancer. IMP321 again did not produce significant adverse events but also failed to show any change in immunologic markers after therapy was given [244–248].

LAG-3 has been shown to be synergistic with PD-1/PD-L1. In a murine model, dual anti-LAG-3/anti-PD-1 antibody treatment cured most mice of established tumors that were resistant to single antibody treatment [48] and demonstrated that LAG-3 is required for long-term peripheral CD8 but not CD4 immune tolerance [49]. High level dual LAG-3/PD-1 expression is largely restricted to tumor-infiltrating lymphocytes which are likely advantageous due to focused "attack" instead of nonspecific or self-antigen-specific immune responses.

Ongoing studies of LAG-3/IMP321 are being performed in glioblastoma (NCT02658981), metastatic breast cancer (NCT02614833), and hematologic neoplasms (NCT02061761).

6.9.2 4-1BB

4-1BB (CD137), unlike the inhibitory molecules CTLA-4, PD-1, and LAG-3, is a co-stimulatory molecule. It is a member of the tumor necrosis factor receptor (TNFR) superfamily that is inducibly expressed on activated $CD8^+$ and $CD4^+$ lymphocytes (including Tregs), NK cells, dendritic cells, macrophages, neutrophils, and eosinophils, as well as in some tumor tissue. The 4-1BB receptor is bound by the 4-1BB ligand (4-1BBL) expressed on antigen-presenting cells. 4-1BB functions as a costimulatory signal after a T-cell receptor is bound by an antigen-MHC ligand along with CD28 costimulation to promote $CD4^+$ and $CD8^+$ lymphocyte proliferation, activation, and protection against activation induced cell death. 4-1BB ligation is able to costimulate $CD8^+$ lymphocytes to activation even in the absence of CD28-B7-1/B7-1 signaling and prevent or reverse established anergy in lymphocytes. Additionally, 4-1BB appears to function across both the innate and adaptive immune system as it is able to increase the activity of NK cells which, once activated, are further able to stimulate lymphocyte function. 4-1BB also appears to be functionally important in inhibiting Treg function and promoting antigen priming by dendritic cells. Interestingly, 4-1BB activation via agonistic mAbs is able to prevent or treat antibody-mediated autoimmunity in mouse and primate models by increasing $CD4^+$ (but not $CD8^+$) lymphocyte anergy, a process that is not completely understood [249–258].

Preclinical data with agonistic 4-1BB mAbs has demonstrated a robust antitumor effect. In multiple mouse models, mAb treatment has led to increased tumor-specific $CD8^+$ lymphocyte response and substantial tumor regression. Additionally, melanoma cells transfected to express 4-1BB agonist single chain Fv fragments and given to mice as an autologous tumor cell vaccine led to rejection of poorly immunogenic tumors. Treatments were well tolerated in animal models, although polyclonal T lymphocyte accumulation in the liver was noted. Combination of agonist 4-1BB mAb treatment with immunotherapy appears to function synergistically with immunotherapy and chemotherapy. To further test its efficacy and safety, one 4-1BB mAb, BMS 663513, was tested in primates along with a prostate-specific antigen DNA vaccine where it demonstrated encouraging immunologic results [228, 249, 252, 254, 259–266].

Two mAbs have moved into clinical testing in humans. Urelumab (BMS-663513;Bristol Myers-Squibb, New York, NY) is a fully human agonist 4-1BB mAb that was given to advanced cancer patients in a dose escalation trial. Initial results from 83 patients with melanoma (54 patients), renal cell carcinoma (15 patients), ovarian cancer (13 patients), and prostate cancer (1 patient) who were given 0.3–15 mg/kg of the mAb with expansion cohorts at the 1, 3, or 10 mg/kg level of dosing have been reported. Results revealed that there were significant toxicities including grade 3 or 4 transaminitis in 11% and grade 3 or 4 neutropenia in 5% of patients. There were three objective partial responses in melanoma patients and several other patients with stable disease along with increased levels of peripheral activated T lymphocytes and interferon in posttreatment biopsies [267]. A phase II trial in advanced melanoma was conducted; however, as the incidence of grade IV hepatitis was higher than expected, the trial was terminated. Several other trials were terminated at that time. Phase I trials have been performed in which urelumab was given as monotherapy in advanced solid malignancies or non-Hodgkins lymphoma (NCT01775631, completed, results not reported) and in combination with rituximab in non-Hodgkins lymphoma or chronic lymphocytic leukemia (NCT0177563, study withdrawn). A second drug, PF-05082566 (Pfizer, New York, NY), is currently recruiting for a phase I trial as monotherapy in solid tumors or in combination with rituximab in non-Hodgkins lymphoma (NCT01307267).

Multiple studies are in progress evaluating combination therapy with urelumab and nivolumab including urothelial carcinoma (NCT02845323), metastatic melanoma (NCT02652455), and multiple advanced tumor types (NCT02534506). Hepatotoxicity appears to be the limiting factor with 4-1BB monotherapy, but combination therapy is promising.

6.9.3 OX-40

OX-40 (CD134, TNFRSF4) is another member of the TNFR superfamily which is a costimulatory receptor of particular interest in cancer. Like many of the previously described immune checkpoint pathways, OX-40 functions to modulate T-cell activation and proliferation in the setting of inflammation to ensure an adequate immune response, but prevent autoimmunity or unnecessary tissue damage. OX-40 is predominantly expressed on activated $CD4^+$ lymphocytes; however lesser degrees of expression is observed on other cells such as activated $CD8^+$ lymphocytes, Tregs, NK cells, and neutrophils. The only known ligand to OX-40 is the OX-40 ligand (OX-40L), which is primarily expressed on activated APCs. OX-40 stimulates $CD4^+$ lymphocyte clonal expansion, survival, and cytokine production, particularly in late phases of activation. OX-40 is also important in the generation of functional memory T-cell pools. Signaling through the OX-40 pathway does expand Treg populations, but the expanded cells are functionally impaired with an exhausted phenotype. The function of OX-40 was further shown in transgenic mice engineered to have constitutive T-cell expression of OX-40L. These mice developed expansion of $CD4^+$ T-cell (but not $CD8^+$ T cell) pools and an autoimmune phenotype. This is in contrast to OX-$40L^{-/-}$ knockout mice or mice treated with OX-40L blocking mAbs, which demonstrate impaired lymphocyte priming but normal lymphocyte localization and humoral immune responses. While OX-40 appears to function primarily through $CD4^+$ lymphocytes, there is evidence that this ultimately leads to augmented $CD8^+$ lymphocyte function as well [268–283].

In cancer, agonistic therapies to the OX-40 pathway have proved successful in overcoming cancer immune tolerance. In mouse models, agonist OX-40 mAbs have led to complete regression of established tumors and protective immunity against repeat inoculation. The antitumor effect was dependent on both $CD4^+$ and $CD8^+$ lymphocytes. Treatment with agonistic OX-40 mAbs was more effective than blocking CTLA-4 mAbs in generating antigen-specific memory T-cell pools after antigen inoculation. Finally, OX-40 mAbs have been shown to function synergistically with other cancer immunotherapies, surgery, and radiation in murine models. These findings along with observations

that OX-40 has been noted to be relatively overexpressed in tumor-infiltrating lymphocytes and lymphocytes from draining lymph nodes from human melanoma, head and neck, and breast cancers led to trials in primates and then humans [273, 284–291].

A mouse agonist OX-40 mAb was used to treat 30 patients with advanced solid tumors in a dose escalation phase I trial that completed enrollment in 2009. The mAb was given as three doses over 5 days along with tetanus toxin and keyhole limpet hemocyanin. Initial results indicate that the treatment was well tolerated with evidence of clinical response in heavily pretreated patients. A humanized agonist OX-40 mAb has been developed and is currently undergoing trials combined with stereotactic radiation therapy in metastatic breast cancer (NCT01642290 in progress), combined with low-dose cyclophosphamide and radiation in metastatic CRPC (NCT01303705, in progress) renal cell carcinoma (NCT03092856), metastatic colorectal cancer (NCT02559024), and head and neck SCC or melanoma (NCT03336606) [54].

A recent study investigating combination therapy of OX-40 agonist alone or in combination with ipilimumab, durvalumab (anti-PD-L1), and rituximab was terminated at the sponsor's discretion (NCT02205333); however, ongoing studies of combination therapy include OX-40 agonists and atezolizumab (NCT02410512) and durvalumab (NCT02221960) in solid tumors [55].

6.9.4 Glucocorticoid-Induced TNFR-Related Protein (GITR)

Glucocorticoid-induced TNFR-related protein (GITR) is a third member of the TNFR superfamily with costimulatory properties. Like OX40 and 4-1BB, it has a low basal expression level on naïve T-lymphocytes, but is significantly upregulated upon activation. It is also expressed constitutively on Tregs and to a lesser degree on NK cells and mast cells, but expression is increased with activation in all cases. Also like OX40 and 4-1BB, GITR is instrumental in modulation of T-cell responses to infection and cancer; however, it operates through non-redundant pathways. GITR is bound by GITR ligand (GITR-L), which is expressed predominantly on APCs after activation, but also at lower levels on endothelial tissue and activated T cells. GITR ligation enhances T-lymphocyte activation, proliferation, resistance to activation-induced cell death, and resistance to Treg-mediated suppression. However, the in vivo effect in immunomodulation may be subtle as $GITR^{-/-}$ knockout mice demonstrate a mild phenotype with differences in response to certain infection and severe inflammatory conditions [292–304].

In preclinical studies, agonistic GITR mAbs were shown to stimulate T lymphocytes and overcome Treg-mediated tolerance. This finding led to a series of experiments in mice that demonstrated agonist GITR mAbs enhance antitumor immunity [107, 290, 305–307]. Agonistic GITR mAbs have also shown to improve the effectiveness of cancer vaccines in animal models. Based on these results, a humanized agonist GITR mAb, TRX518, is being tested in phase I trials in metastatic melanoma and other advanced solid tumors (NCT01239134, still recruiting). Multiple other studies using GITR agonists are in progress in solid tumors (NCT02628574), in combination with checkpoint inhibitors (NCT02553499, NCT02132754, NCT02598960), and using GITRL proteins (NCT02583165).

6.9.5 CD40

CD40 is another costimulatory molecule of interest in cancer immunotherapy. Like OX-40, it is a member of the TNFR superfamily. CD40 is expressed and functionally important on APCs, but it is also found on a broad range of normal and tumor tissue. On cells such as monocytes and dendritic cells, ligation of the CD40 receptor acts to license the cells into mature, active APCs. For example, ligation of CD40 on monocytes and dendritic cells leads to increased survival, increased expression of MHC complexes and costimulatory molecules, and increased cytokine production. In other tissues, CD40 appears to primarily play a role in modulating local inflammation. It is bound

primarily by CD40 ligand (CD40L); however, binding by mycobacterial heat shock protein 70 and C4b binding protein has also been identified. CD40L is expressed primarily on active (but not resting) T lymphocytes, in particular, $CD4^+$ lymphocytes, although some level of expression has been identified on other cell types. By playing a role in APC maturation, CD40 is also integrally important to lymphocyte priming and activation. Activated $CD4^+$ lymphocytes express CD40L which bind to CD40 on APCs, allowing the APCs to mature and effectively cross prime $CD8^+$ lymphocytes. The central role of the CD40 pathway in immunity is revealed by X-linked hyper IgM syndrome, a severe immune deficiency characterized by neutropenia, susceptibility to opportunistic infection, and autoimmunity, which is due to genetic mutations in the CD40L gene [308–318].

Interest in the CD40 pathway in cancer has come from observations that CD40 ligation is necessary for immune-mediated destruction of cancer cells and that CD40 is expressed on a variety of malignant tissues and from preclinical trials with CD40 mAbs. Treatment of established tumors in mice with agonistic CD40 mAbs has resulted in impressive immune-mediated tumor regression and protective immunity, while treatment with CD40L blocking mAbs results in abrogation of the antitumor immune response. The mechanism of action for agonistic CD40 mAbs is likely twofold and dependent on tumor CD40 expression level and antibody subtype used. In CD40 expressing tumors, anti-CD-40 IgG1 mAbs are able to bind and induce antibody-dependent cytotoxicity (ADCC) of the tumor cells. There is also evidence that high level of ligation of CD40 in certain cancers, particularly multiple myeloma and high-grade B-cell lymphoma, can inhibit cancer growth. The second mechanism of tumor inhibition, which is independent of CD40 expression on tumor cells, is through the immunostimulatory effects of CD40 ligation [319–329].

Multiple strategies have been investigated to therapeutically target CD40 in human malignancy. The first human trials involved treating advanced solid tumors and non-Hodgkins lymphoma with recombinant human CD40L (Avrend; Immunex Corp, Seattle, WA). Treatment was given to 32 patients with dose-limiting toxicity of grade 3 and 4 transaminitis seen with higher dosing. There was evidence of clinical activity with partial responses seen in patients with laryngeal carcinoma and non-Hodgkins lymphoma [330]. More recent efforts have focused on targeted mAb blockade of CD40, with multiple drugs currently under investigation in clinical trials.

CP870,893 (now RO7009789, Selicrelumab) (Pfizer, New York, NY is a fully humanized anti-CD40 IgG2 mAb with strong agonistic properties that has been tested in several clinical trials. Interestingly, CP870,893 with its IgG2 Fc domain has a relatively low binding affinity to human FcgRs when compared to second generation drugs, and may function by binding to a unique epitope on human CD40. It was first given as a single dose, dose escalation phase I trial to 29 patients with advanced malignancy where partial objective responses were noted in 27% (4/15) of melanoma patients but not in other tumor types. A second phase I trial evaluated weekly dosing of CP870,893 in 27 patients with advanced malignancies. Less evidence of clinical benefit was seen with no objective responses observed. CP870,893 was tested in combination with chemotherapy in two trials; in combination with gemcitabine in pancreatic carcinoma and in combination with carboplatin and paclitaxel in a variety of advanced malignancies. In these trials partial objective responses were seen in 19% (4/21) and 20% (6/30) of patients, respectively. [327, 331–334].

In all trials, the immunomodulatory properties of the mAb were evident with transient elevation in IL-6 and TNF-α, as well as depletion and stimulation of B lymphocytes. The most common toxicities were cytokine release syndrome (typically grade 1 and 2) and transient elevation of transaminases. Ongoing studies with CP870,893 include additional trials in combination with gemcitabine in advanced pancreatic cancer, and combination trials with peptide vaccines and CTLA-4 blocking tremelimumab in metastatic melanoma (NCT01456585 completed without reported results, NCT01008527 completed without reported results, NCT01103635 ongoing). Current studies

investigating CD40 combinations include combining anti-PD-L1 in solid tumors (NCT02304393), anti-Ang2/VEGF in solid tumors (NCT02665416), anti-CSF1 R in solid tumors (NCT02760797), and gemcitabine/nab-Paclitaxel in pancreatic carcinoma (NCT02588443).

APX005M is a humanized rabbit IgG1 CD40 agonist being tested in multiple trials, in combination with anti-PD-1 (NCT02706353, NCT03123783) and CD40 alone (NCT02482168).

ADC-1013 is a fully human IgG1 CD40 agonist being studied as monotherapy in multiple studies (NCT02379741, completed without reported results, NCT02829099).

SEA-CD40: non-fucosylated humanized IgG1 agonist, CD40 alone (NCT02376699, recruiting).

Dacetuzumab is a humanized anti-CD40 IgG2 mAb that has been tested in B-cell hematologic malignancies, which have high constitutive expression of CD40. Dacetuzumab was first given as a phase I dose escalation trial in 44 multiple myeloma patients where the addition of steroid premedication was found to increase the tolerated dose; however, it demonstrated no objective clinical response. Similarly, it was tested in a phase I dose escalation trial in 12 patients with chronic lymphocytic leukemia, and again, no objective responses were seen. Based on preclinical data suggesting synergy with rituximab (anti-CD20 mAb), dacetuzumab was tested along with rituximab (and gemcitabine) in 33 patients with refractory diffuse large B-cell lymphoma (DLBCL). In this trial, the combination generated six (20%) complete responses and eight (27%) partial responses. However, a randomized phase II trial comparing this combination with chemotherapy alone in DLBCL was terminated early based on perceived futility. In these trials, dacetuzumab therapy also caused cytokine release syndrome in a minority of patients, but was generally well tolerated. There are no ongoing trials registered for dacetuzumab [326, 335–338].

A third agonistic anti-CD40 mAb being tested is Chi Lob 7/4. This chimeric IgG1 mAb has undergone phase I testing in patients with $CD40^+$ advanced solid malignancies or DLBCL. 15/29 treatments were accompanied by disease stabilization for a median of 6 months with acceptable toxicities when single-dose corticosteroids were administered [339]. No further studies are registered.

The fourth anti-CD40 mAb under investigation is lucatumumab, a fully humanized IgG1mAb, which, unlike the previously described CD40-targeted therapies, is antagonistic. As previously discussed, there is evidence that CD40 ligation can promote proliferation and cell growth in low grade B-cell malignancies as in normal B lymphocytes, although the data is mixed. Thus, the proposed mechanisms of action for lucatumumab include blocking of CD40 ligation on malignant cells and ADCC, but not immunostimulation. Lucatumumab has been tested in two dose escalation phase I trials in chronic lymphocytic leukemia and in multiple myeloma with minimal toxicity but only modest clinical responses. No further studies are currently registered [328, 329, 340–342].

There is currently one actively recruiting study evaluating CDX-1140, a fully human monoclonal anti-CD40 antibody (NCT03329950). No results have been reported.

6.9.6 TIM-3

The function of T-cell immunoglobulin and mucin domain 3 (TIM-3) is becoming better understood. TIM-3 is expressed on multiple cell types including IFN-gamma secreting $CD8^+$ T-cells, Treg cells, and cells of the innate immune system (macrophages, dendritic cells), affecting both adaptive and innate immune responses. TIM-3 is expressed on Th1 cells and generates an inhibitory signal-inducing apoptosis of Th1 cells. It is also expressed on some dendritic cells leading to apoptotic cell phagocytosis and disruption of cross-antigen presentation. TIM-3 is upregulated in tumor-specific $CD8^+$ T cells and $CD8^+$ TILs, while administration of TIM-3 increases proliferation and activity of antigen-specific T cells. In multiple cancers, TIM-3 expression has been associated with tumor progression and shorter survival. Preclinical data suggests that TIM-3 blockade may be most

effective when given in combination with PD-1 mAbs. In addition, since TIM-3 is expressed on non-T cells, a possible mechanism for penetration of the tumor microenvironment is theorized. In general, TIM-3 is seen as a negative regulator of antitumor immunity. Its selective expression on intratumoral T cells may reduce nonspecific toxicity and even offers theoretical synergy with checkpoint inhibitors [343–349].

There are two TIM-3 monoclonal antibodies in development. MBG 453 (Novartis, Basel, Switzerland) is being studied in a phase Ib/II open-label trial comparing single-agent therapy to combination therapy with PD-1 antibodies in adults with advanced malignancies (NCT02608268 recruiting, NCT03066648 recruiting).
TSR-022 (TESARO, Waltham, USA) is being evaluated in a phase 1 study (NCT02817633, recruiting) as a single agent in adults with advanced solid malignancies. Some select patients will receive combination therapy with anti-PD-1 antibodies.

6.9.7 TGN1421: A Cautionary Tale

A word of caution is warranted about trying new individual or combination immune checkpoint therapies. While some immunomodulatory therapies have been well tolerated, it is clear that they have the potential for severe, lasting, and sometimes fatal toxicities. Just as animal models have proven inadequate for reliable prediction of human cancer responses to therapy, they are also inconsistent predictors of treatment toxicity. The most notable example of this is experience with TGN1412 (TeGenero). TGN1412 is a novel agonist anti-CD28 mAb, which was under development for treatment of chronic lymphocytic leukemia. In animal models, the drugs showed encouraging immunologic results without detectable toxicities. Thus, the drug was given as a single infusion to six healthy volunteers. Within 90 min, all displayed signs of cytokine release syndrome, and within 16 h all were critically ill. All patients suffered from multisystem organ failure including acute lung injury, renal failure, and disseminated intravascular coagulation. Fortunately, all six survived and recovered [350]. This example underscores the care that is necessary when designing and conducting clinical trials in order to maximize patient safety.

6.10 Conclusion

If decades of cancer research and, in particular, cancer immunotherapy research have taught us anything, it is that cancer is a resilient and adaptable foe. For now, checkpoint inhibition has added another weapon to our arsenal in the battle against cancer. As its current indications are expanding, it serves as proof of principle that immune checkpoint blockade can overcome cancer immune tolerance and escape in a clinically meaningful way. It has also reinvigorated research in cancer immunology and spurred the search for new immune coinhibitory and costimulatory checkpoints to target. While the initial work in new targets is encouraging, many large trials, at the cost of millions of dollars, are needed before its full potential is established. As we further elucidate the mechanisms by which cancer evades immune detection and destruction and learn to counter them, more effective and better-tolerated therapies are sure to emerge. Additionally, further characterization of the interactions between cancer and host immune system and how this changes with checkpoint blockade may help us understand and discover biomarkers for predicting which patients will respond, allowing treatment to be tailored and toxicity to be minimized.

Perhaps the greatest potential for improving outcomes and achieving broader applicability lies in using immune checkpoint blockade as combination therapy, by using blocking antibodies on coinhibitory receptors and agonist antibodies on costimulatory receptors. By combining checkpoint blockade therapy with conventional therapies such as chemotherapy and radiation, the destructive power of these therapies can be parlayed into a purposeful, long-lasting, cancer-specific immune response. Similarly, checkpoint blockade may help break down the barriers that have prevented most cancer vaccines from working and thus fulfill the long sought-after promise of active immunotherapy—a

stimulated, long-lasting, cancer-specific immune response that eliminates established tumors or prevents their recurrence.

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7 Gene Therapy and Genetic Vaccines

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7.1 Introduction

Delivering genetic components into tumor cells for a therapeutic approach is the main goal of gene therapy. This appealing concept has been studied in various in vitro and preclinical researches for perturbing oncogenic or tumor suppressor mutations. However, a few of these approaches are successfully implemented in the clinic. Clinical effectiveness of these therapies depends on many factors including gene delivery to tissues, transfection efficacy, duration of expression, and more importantly finding an effective gene in diverse tumors. Therefore, progressing researches are conducted to discover targeted delivery vehicles for locally high but systemically low cytotoxic effect.

Aside from therapeutic approaches, preventing tumorigenesis is an interesting area of research. One of the suggested approaches for preventing this event is called cancer vaccination. Introducing tumor-associated antigens (TAA) to the immune system is the key step for endogenous antitumor activities. The majority of vaccine strategies involve the presentation of TAAs for activating tumor-specific T cells. Continuous in vivo presentation of antigen proteins can be maintained through a specific protein-expressing DNA cassette, which is the main concept of generating DNA vaccines. Clinically available cancer vaccines require optimization in vaccine delivery methods from simply needle injection of naked plasmid DNA to administering complex vectors.

7.2 Gene Therapies

7.2.1 Gene Delivery Methods

Clinical application of gene therapy requires an appropriate route of gene delivery. There are various vectors, which differ in the amount of the gene introduced and maintaining the long-term expression of the gene. Viruses are ideal vectors used in the delivery of therapeutic genes. Different types of viruses can be transformed into viral vectors by replacing the infection-inducing genes with the transgenes of interest. Nonviral vectors can also be administered for transferring genes of interest. These include chemical transfection using lipids, proteins, polymers, etc. [1].

7.2.1.1 Viral Vector

Gene therapy-engineered viral vectors are replication-defective or selectively replicating viruses. These vectors can be classified based on their origin, integration ability, etc. Genome-integrating viral vectors facilitate the long-term expression of genes, whereas they increase the risk of perturbing the regulatory/transcriptionally active genes. Specificity of viral vectors for cancer cells can be maintained through cell-type specific ligand or antibody [2]. However, many solid tumors lack a specific tumor ligand/antigen. Transcriptional targeting using conditional promoters such as hypoxia-inducible systems can successfully target solid tumors due to their hypoxic microenvironment. The hypoxia-specific regulatory system is constructed from the hypoxia-response element (HRE) binding to a basal promoter. Systemic administration of these constructs is not feasible due to the similar states of some tissues to the hypoxic tumor microenvironment. Designing a dual regulatory system consisting of HRE sequence and the tissue-specific promoters can reduce the off-target gene delivery and following cell toxicity [3]. A combination of survivin promoter (Sur-P) and HRE could specifically induce apoptosis in breast cancer cells [4]. Another combinatory approach is estrogen response elements (ERE) and HRE for selective breast cancer gene therapy [5].

Adenovirus

Adenoviral vectors can infect a broad spectrum of host cells based on their various subtypes. Adenoviruses are classified based on their genomic homology, agglutination capacity, and oncogenic potential. Most of the recombinant adenoviral vectors are derived from Adenovirus serotypes 2 and 5. Their infection capacity is not confined to dividing cells as they are usually expressed in the cytoplasm without the risk of gene insertion mutagenesis [7]. The adenovirus genome contains eight transcription units,

which are flanked by two ITRs: early units (E1, E2, E3, E4, and E5), units with delayed expression after viral replication (IX and IVa2), and a late unit (subdivided into L1, L2, L3, L4, and L5 genes) [6]. Advexin is an AD5 vector in which E1 and E3 genes are deleted. Deletion of these genes minimizes the toxicity of adenoviruses due to the inflammatory responses. Several tumor types have been reported to be retarded using P53 expressing Advexin including head and neck squamous cell carcinoma (HNSCC), Li-Fraumeni syndrome, colorectal cancer, hepatocellular carcinoma (HCC), non-small cell lung cancer, prostate cancer, breast cancer, ovary cancer, bladder cancer, and glioma [8, 9]. Sitimagene ceradenovec is an advexin-like vector that expresses the herpes simplex virus (HSV) thymidine kinase (TK) inserted into the omitted E1 region. This vector is applied in eradicating residual glioma cells in which vector-driven TK can convert ganciclovir (GCV) to ganciclovir monophosphate. This final product can induce apoptosis in remained tumor cells, which undergo rapid DNA synthesis. Several studies report the safety of adenoviral administration as no adverse event was observed in studies [10, 11].

Adeno-Associated Virus Vector (AAVVs)

Nonpathogenic parvoviruses similarly infect both dividing and non-dividing cells with various mechanisms of cell entry. Broad host tropisms of AAVs are the result of their different serotypes [12]. Efficacy of some serotypes of adenoviral vectors and AAVs may be diminished due to the presence of neutralizing antibodies. The genome consists of three open reading frames (ORF) with several genes. The rep ORF, which encodes for proteins, is required for replication and packaging. The cap ORF (VP1, VP2, VP3) and the third ORF placed within the cap gene encode proteins for viral capsid assembly. The fIanking ITRs are necessary for viral replication, packaging, and integration. In order to produce the gene therapy vector, the gene of interest is inserted between the ITRs, in the place of rep and cap [13]. AA2 vectors have been widely studied in preclinical animal cancer models through various approaches. For example, in antiangiogenic therapy designs, a soluble splice variant of VEGF receptor 1 (sFlt1) AAV2 was delivered in the ovarian cancer model [14]. Moreover, pigment epithelium-derived growth factor (PEDF) AAV2 inhibited angiogenesis in a mouse model of Lewis lung carcinoma (LCC) [15].

Suicide gene delivery can also be conducted with AAV2 vectors such as AAV2-HSV-TK in a mouse model of breast cancer (MCF-7). AAV2-TRAIL is studied as a potent apoptosis inducer in a mouse model of lymphoma [16]. Also, AAV2-IFN-β enhanced survival in mice lung cancer and colorectal cancer under the control of the hTERT promoter [17]. Moreover, snail and slug siRNAs have been reported to be delivered through AAV2s in pancreatic and cholangiocarcinoma cancer, respectively. AAV2 has recently attracted attention in studies including AAV2-CEA, AAV2-MUC1, and AAV2-aquaporin (AAV-hAQP1), which can enhance parotic function in patients undergone radiotherapy for head and neck cancer [18, 19].

Herpes Simplex Virus Type 1 Vectors (HSVVs)

HSVVs can infect a broad spectrum of dividing and non-dividing host cells including nerves. Endothelial and dendritic cells are also targeted host cells for HSVVs [20]. In order to produce high titers of safe HSVV, infective capacity of HSV-1 is abrogated through introducing null mutations into viral early genes. Due to the ability of replication-defective viruses to remain latent in host cells, this vector can be beneficial for long-time expression of transgenes. Its non-integrating DNA genome is divided into long and short unique segments (UL and US) and flanked by inverted repeated sequences (TRL/IRL and TRS/IRS), which can deliver large pieces of foreign DNA more than 150 kb in length [21]. Amplicon vectors of HSVVs have been used in most anticancer applications [22]. As tumors exert various characteristics, the ability of HSVVs to accommodate multiple genes makes them an appropriate vector for cancer gene therapies such as melanoma, gliosarcoma, or glioblastoma [23].

Retrovirus Vectors (RVVs)

The retrovirus genome consists of three essential genes: gag, pol, and env. Unlike previously described vectors, retroviruses integrate the host cell genome, which may increase the risk of mutagenesis and tumor progression [24]. However, integration to host DNA facilitates the longer expression of transgenes in transfected cells. The large family of retroviruses can be classified into six subgroups: alpha-, beta-, delta-, and gammaretroviruses, lentiviruses, and spumaviruses. Gammaretrovirus was among the first viruses engineered for gene therapy. Gammaretroviruses can only transfect cells while undergoing mitosis [25]. In contrast, lentiviral vectors do not require the disruption of the nucleus membrane to insert the genome, which enables them to also transfect nondividing cells [26]. In order to reduce the risk of mutagenesis in AAV and retroviral vectors, several methods have been used for achieving targeted integration. These methods include DNA double-strand break-enhanced homologous recombination and Sleeping Beauty transposon system [27].

Lentivirus Vectors (LVVs)

Complicated genome of lentiviruses is based on HIV1, and it can also transfect non-dividing cells. Lentiviral vectors have the capacity to deliver large pieces of transgenes. Although long-term transgene expression is maintained through integration into the host genome, the risk of mutagenesis is limited for lentiviral vectors. In the most recent generation of lentiviruses, all the nine genes of HIV are omitted except gag, pol, and rev [28].

Poxviruses

Poxviruses have a self-sufficient replication system as they encode all the necessary transcription machinery. DNA of poxviruses can be replicated in the cytoplasm without the risk of insertional mutagenesis. Vaccinia subgroup of poxviruses such as MVA can affect a wide range of mammalian cells. MVA is often used in designing cancer vaccines, which are evaluated in various clinical trials (discussed in Sect. 7.3.3) [29].

7.2.1.2 Nonviral Vector

Nonviral vectors can protect the naked DNA from degradation without any inflammatory response in contrast to viral vectors. Besides, their production and administration are more cost-effective than the same quantities of viral vectors. However, their transfection capacity is inefficient. Cationic polymer carriers, cationic lipids, etc. are commonly used nonviral vectors. Inorganic nanoparticles including gold, silica, iron oxide, and quantum dots can also be used for gene delivery in various stabilized sizes and shapes [30].

Cationic Polymers

Polylysine (PLL), poly(ethyleneimine) (PEI), and chitosan are well-known cationic polymers that can form polyplexes containing negatively charged DNA. PLLs are biodegradable peptides, which may lose their function in lysosomes following endocytosis. Therefore, PLLs are usually modified with histidyl/imidazole groups to enhance their transfection capacity. PEIs can transfect a broad spectrum of cells more efficiently in comparison with PLLs. However, PEI can induce membrane disruption and lead to apoptosis of transfected cells. Transfection capacity of PEIs depends mostly on their molecular weight. Chitosan is a biodegradable polysaccharide, which is an attractive carrier due to its higher transfection efficacy and nontoxicity [31]. Several in vitro studies determined the capacity of PEI and PLL for antitumor gene or specific siRNA delivery in breast cancer [32, 33].

Lipid Polymers

Lipofectin and lipofectamine have been broadly used in gene therapy clinical trials. The cationic polar head of lipids interacts with phosphate groups of nucleic acid, and the hydrophobic part forms the main structure of liposomes. Lipid-based hybrids such as stabilized plasmid-lipid particle (SPLP) and stable nucleic acid-lipid particle (SNALP) can also be administered for systemic gene delivery [34].

7.2.2 Gene Therapy Strategies

Cancer gene therapy can be conducted using different gene therapy strategies with different gene transfer vectors. These include tumor cell killing through induction of apoptosis, antiangiogenic, and suicide gene transfer for prodrug activation enhancing chemotherapy. Moreover, the correction of gene defects and abnormal upregulation of oncogenes through antisense and RNA interference (RNAi) is also an appealing strategy. However, most of these strategies are just approved in animal models, and only a few of these approaches have been evaluated in clinical trials. In the 1990s, the first attempt to genetically treat cancer was conducted in which melanoma-infiltrating lymphocytes were transduced with TNFα gene in vitro [35]. Today, nearly 1200 cancer gene therapy clinical trials have been conducted worldwide.

7.2.2.1 Tumor Cell Killing Therapies

Suicide Gene

The optimum dose of chemotherapeutic drugs is difficult to manage due to its hazardous effects on normal cells. Designing "suicide gene therapies" enables the tumor cells to exclusively convert harmless prodrugs to cytotoxic factors. Examples of this approach are the delivery of *herpes simplex virus* (HSV)-thymidine kinase (TK) and bacterial cytosine deaminase (CD) [36].

TK is an ATP-thymidine 5′-phosphotransferase naturally present in all living cells. HSV-TK can phosphorylate analogue of ganciclovir (GCV). Integration of phosphorylated GCV into newly synthesized DNA triggers the apoptotic signaling cascade. This approach becomes more effective considering the bystander effect in which toxic metabolites can be transferred to adjacent cells by gap junctions [37]. TK delivery has been used in clinical trials to treat glioma, prostate cancer, hepatocellular carcinoma, breast cancer, etc. [38–41].

CD converts the nontoxic 5-fluorocytosine (5FC) into the toxic chemotherapeutic drug, 5-fluorouracil (5FU). 5FU inhibits nucleic acid synthesis selectively in CD-delivered tumor cells [42]. Plasmid DNA containing CD has been injected into breast cancer patients, which showed specific expression in tumor cells. However, tumor growth was minimally retarded [43]. ICasp9, as a newly introduced suicide gene, can induce cell death when combined with AP20187 small molecule. Inoculating mesenchymal stromal/stem cells (MSC) showed promising outcomes in cancer gene therapies. For instance, MSC co-expressing iCasp9 and TRAIL exerted promising anticancer effects in an aggressive sarcoma [44].

Apoptosis

Resistance of tumor cells to apoptosis is also an important etiology of tumor progression. Therefore, several genes have been studied for inducing apoptosis in tumor cells. TNF-related apoptosis-inducing ligand (TRAIL) is a potent mediator of apoptosis in tumor cells. TRAIL can affect cells through four receptors among which TRAIL-R1 and TRAIL-R2 contain the cytoplasmic death domain. Complex interactions of factors downstream the activation of DD would result in caspase activations and apoptosis. Due to its specificity and high expression, gene therapies are designed using TRAIL. Adenoviral-mediated TRAIL gene therapy has been evaluated in prostate cancer [45]. Adipose mesenchymal stromal/stem cells (AD-MSC) can be designated as anticancer carriers as they can reside in the tumor environment after local injection. In a study, AD-MSC was armed to constantly release a soluble variant of TRAIL [46]. Improvement in TRAIL transfection efficacy has been made through a nonviral vector called fluorinated polydendrimer (G4-F7 35) [47].

Inhibition of the mitochondrial apoptotic pathway is one of the major causes of tumor cell resistance to chemotherapies. Targeting the intrinsic apoptosis pathway was studied through BAX adenovirus in gastric cancer. However, it exerted toxicity in healthy cells [48]. Inhibition of antiapoptotic factors is also an attractive approach for cancer therapies. Administration of miR-195, miR-24-2, and miR-365-2 showed promising BCL2 downregulation and further apoptosis induction in MCF-7 breast cancer cells

[49]. X-linked inhibitor of apoptosis (XIAP) specifically inhibits the mitochondrial apoptotic pathway, which was induced by caspases 3, 7, and 9. Direct downregulation of XIAP via antisense RNA augmented apoptosis induction in human gastric cancer in vitro [50]. E3 ubiquitin ligase can bind to multiple mRNAs and upregulate their expression such as XIAP. In a dual inhibiting approach using E3 ubiquitin ligase-specific siRNA, XIAP downregulation and consequent apoptosis induction are resulted [51]. Melanoma differentiation-associated gene-7 (MDA7) or IL-24 exerts various antitumor functions such as tumor suppression, antiangiogenesis, and apoptosis induction. MDA7 transfection demonstrated promising outcomes in HER2+ breast cancer, laryngeal carcinoma cell, and osteosarcoma [52–54].

Antiangiogenic

Tumor growth, further progression, and metastasis require an increased supply of blood flow. Angiogenesis in tumor area is dependent on several growth factors including interleukins (ILs), vascular endothelial growth factor (VEGF), proteolytic enzymes (cathepsin, urokinase-type plasminogen activator, gelatinases A/B), and basic fibroblast growth factor (bFGF) and endoglin. VEGF, survivin, and endoglin siRNAs have been designated as antiangiogenesis gene therapies [55]. NK4 is a potent antiangiogenesis factor, which indirectly inhibits VEGF. Usefulness of adenoviral-mediated NK4 gene therapies has been confirmed in syngeneic mice melanoma, lung, and digestive system cancers [56, 57]. Angiostatin, endostatin, IL-24, IL-18, etc. are other angiogenesis inhibitors for gene therapy. Administration of antiangiogenic modulators has advantages of lower systemic toxicity because of the higher sensitivity of the tumor environment to these therapies. However, due to the lower capacity of antiangiogenesis therapies alone, combinational strategies are more beneficial, for instance, co-transfection of angiostatin with p53, IL-12, FAS, etc. [55].

Tumor Suppressor Insertion

Several "tumor suppressor" genes are responsible for ending the cell cycle in order to inhibit further growth of abnormal cells. For instance, P53 is a well-known tumor suppressor, which participates in apoptosis induction during cellular stress. Abnormal function or downregulation of tumor suppressor genes is one of the most important etiologies of tumor initiation and propagation. Restoring the normally expressed tumor suppressors is considered an efficacious approach in cancer gene therapy. P53 is one of the common target genes in cancer gene therapies. Intratumoral injection of adenoviral vector encoding P53 has been evaluated in recurrent malignant gliomas [58]. Gendicine™, a trade product for P53 gene therapy, showed promising outcomes in several cancer clinical trials including laryngeal cancer and head and neck squamous cell carcinoma [59]. Oncorine™ and ONYX-015 are similar adenoviral P53 delivering products but allow replication only in tumor cells. These have been proved as safe therapies in glioma and head and neck, ovarian, and pancreatic cancer [60, 61].

Another deregulated tumor suppressor gene in cancer is phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PTEN controls the tumor cell growth and apoptosis through inhibiting the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway. Adenoviral PTEN gene therapy demonstrated amendatory effects in a mouse model of small cell lung cancer [62].

7.2.2.2 Oncogene Blocking

Proto-oncogenes promote cell division and survival in normal conditions. However, mutant proto-oncogenes (termed as oncogenes) are associated with neoplastic transformation. The biological activity of oncogenes can also be modulated at the RNA or DNA level for treating cancer. Antisense RNA, siRNA, ribozymes, and DNAzyme are reported to be promising strategies to target oncogenes. Antisense DNA targeting EGFR, a member of tyrosine kinase receptors, was directly injected into HNSCC patients' tumors [63].

C-MYC is a transcription factor that also participates in RNA metabolism and various cellular processes. C-MYC upregulation is associated with almost every characteristic of tumor life from initiation to maintenance and resistance to apoptosis. Disruption of c-MYC with antisense oligonucleotides could lower the cell growth rate in melanoma cells [64].

K-RAS mutation is considered major oncogene in human colon cancers, lung adenocarcinomas, and pancreatic cancers. Targeting K-RAS by antisense RNA suppresses tumor growth in preclinical animal models including pancreatic cancers. Retroviral K-RAS antisense RNA delivery was used in a clinical trial on NSLC [65].

Oncogene knockout through genome editing is one of the progressing research areas. Zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and, more recently, clustered regularly interspaced short palindrome repeats (CRISPR) are important gene editing tools. Finger-like structure of ZFN functions as transcription factors, which specifically binds to DNA based on amino acid modifications. Multiple endonucleases such as FOK1 can be fused with finger arrays to edit the targeted genome areas [66]. Transcription activator-like effector (TALE) has a DNA-binding domain consisting of tandem repeats. The mechanism of specific DNA recognition and editing is similar to ZNFs. CRISPR/CAS is considered a bacterial immune system for destroying the foreign genome. CRISPR consists of a guide RNA, which guides the CRISPR-associated system nuclease (CAS) to the specific site of DNA. Modifications in CAS structure and truncated guide RNA enable CRISPR/CAS to accurately target several genomic sites. In vivo genome editing has been validated for the treatment of HPV-induced cervical cancer. HPV contains two important oncoproteins termed E6 and E7 which inactivate tumor suppressor genes P53 and RB, respectively [67]. Targeting these genes through CRISPR/CAS and TALEN-mediated plasmids enhanced cancer cell apoptosis. CRISPR/CAS9 system has also been used for eliminating PD1 expression in genetically modified lymphocytes for lung cancer gene therapy [68].

7.2.2.3 Antitumor Immunity Enhancement

Due to cell cycle dysfunctions, tumor cells are generated coincidentally. Normally, these abnormal cells are recognized and eliminated by the immune system. Dysfunctions in innate and adaptive immunity, specifically T cells, result in tumor development. Besides, some tumor cells produce factors that diminish immune responses through increasing regulatory T cells, myeloid derived stem cells, etc. [69]. To enhance the cytotoxic factors in the tumor area, upregulating factors of TNF-α superfamily such as CD40 ligand demonstrated suppressant effects on malignant cells in the bladder [70].

Defective tumor infiltrating lymphocytes (TILs) can be genetically modified for enhanced antitumor responses. For example, regression in tumor size was observed when TILs of melanoma patients were transduced with an anti-MART1 TCR transgene utilizing retroviral vectors [71]. This approach was also beneficial in arming T cells with NY-ESO-1-specific TCR [72]. Newly FDA-approved hematologic cancer therapy is introducing a chimeric antigen receptor (CAR) to T cells. CAR structure is composed of an antigen-specific single-chain variable fragment (scfv) ectodomain, a transmembrane domain, and a signal perpetuating endodomain (CD3ζ). Later generations of CAR T cells also comprised of CD28/4-1BB costimulatory molecules alone or both (second and third generation, respectively) [73]. The process of manufacturing CAR T cells is briefly elucidated in Fig. 7.1. T cells are isolated from donor cell peripheral blood. They are transduced with CAR-expressing construct, and genetically modified T cells are expanded to increase the number of CAR T cells for further infusion to patients [74].

Although CD19+ CAR T-cell therapies demonstrated highly effective antitumor responses against B-cell acute lymphoblastic leukemia (B-ALL), its application in solid tumor therapies encounters obstacles. Inefficient infiltration of transfused CAR T cells to the solid tumor environment, immunosuppressive environment, and lack of specific tumor antigen in these tumors are important challenges [75].

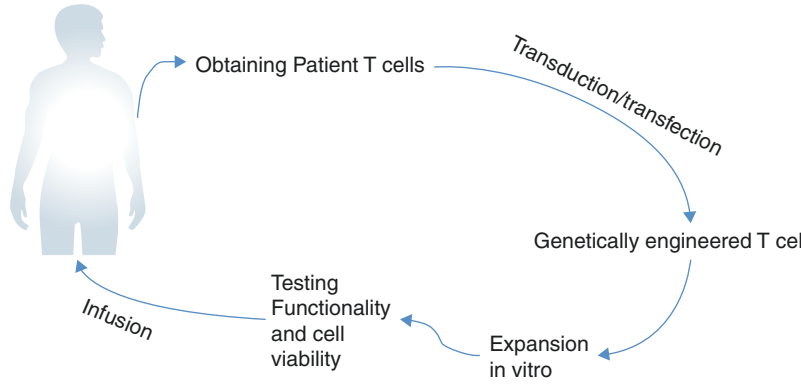

Fig. 7.1 Schematic representation of manufacturing CAR T cells

7.3 Genetic Vaccines

Genetic vaccines are synthesized from nucleic acid construct including plasmid/viral DNA or mRNA. These genetic constructs are engineered to express a specific gene using promoter elements and a transcriptional terminator. Depending on cancer type, various vaccines can be used. For example, cervical cancer is mostly caused by specific subtypes of human papillomavirus (HPV 6, 11, 16, 18). Gardasil and Cervarix contain a major particle of HPV capsid (L1) and aluminum hydroxyphosphate sulfate as an adjuvant. For example, HER2 and Mammaglobin-A (Mam-A) cDNA vaccine elicited antitumor responses against metastatic breast cancer [76].

7.3.1 DNA Vaccines

APCs play the most important role in uptaking the target antigen and representing it to T cells for antitumor responses. The DNA construct is usually composed of a bacterial plasmid vehicle, which contains the gene encoding the desired tumor antigen. A constitutive promoter is also placed near the gene such as cytomegalovirus (CMV) or SV40 promoters [77]. Application of genuine plasmid construct lacked efficiency in conventional intradermal or intramuscular injections due to its poor penetration to cells and further entry to the nucleus. Besides, interactions of tissue resident APCs with lymphocytes are less effective in the absence of strong APC stimulants such as inflammatory cytokines [78]. Therefore, various methods have been studied to introduce the optimum administration routes and DNA construct modifications to increase immunogenicity (Table 7.1). Aside from traditional adjuvants such as aluminum salts and monophosphoryl lipid A (MPL), recently investigated methods are summarized in Tables 7.2 and 7.3. Recent updates in proteomics studies revealed that human serum amyloid P (SAP) reduces the efficiency of plasmid transfection due to enhanced clearance [79]. Additionally, it has been reported that calcium/calmodulin-dependent protein kinase (CaMK) type IV expression downregulated the vector titers. These high-throughput screening technologies provide novel information for designing further effective adjuvants [80]. In various DNA vaccine delivery methods, poor transfection of APCs has resulted. Direct transfection of dendritic cells (DCs) can be achieved using in vitro engineered DC vaccines. Other strategies include using molecules such as lipophilic albumin-binding tail to target DC-specific proteins [81]. Also, nanoparticles and rabies-driven glycoprotein on protamine residues can be used for targeting APCs [77].

7.3.2 RNA Vaccines

In order to directly express the antigen of interest, genetic vaccines can contain the mRNA, which can be translated in the cytoplasm. Advantages of RNA vaccines are their easier transfection efficiency, less oncogenic potential, as they do not require entering the nucleus and incidental insertions to the genome.

Table 7.1 DNA vaccine administration routes

Administration	Mechanism	Trade product/application in cancer therapies
Magnetofection	Use of cationic magnetic nanoparticles guided by a by an external magnetic field	Liver cancer [87]
Cellular sonication	Use of ultrasound for temporary permeabilization of the cell membrane	Lymphocytic leukemia [University of California, san Diego; ID: NCT00849524]; Melanoma [88]
Gene gun	Coated DNA vaccine is directly transfused to the resident dermal APC	Melanoma [89]; Oncept™, canine melanoma [90]
Electroporation	Use of short electrical pulses for modifying the permeability of the cell membrane specially in muscles	Melanoma and prostate cancer [91]
Nanoparticles	Delivering DNA to target cells using specific cell binding sites	Gp-100 loaded chitosan nanoparticles PEI nanoparticles for further clinical administration [92]
Cationic lipids or cationic polymers (lipoplexes/polyplexes)	Described in Sect. 7.2.1.2	Allovectin™, Melanoma [93]
"Danger signal" mediated	Heat-shock proteins bind antigenic peptides	Ovarian carcinoma [94]

Table 7.2 Characteristics of viral vectors in gene delivery [6]

Virus	Type	Advantages	Concerns
Adenoviruses	dsDNA	High transfection efficacy also in non-dividing cells; no insertional mutagenesis	Transient expression; cellular inflammatory responses
AAV	ssDNA	Easily transfected; replication defective	Risk of mutagenesis due to gene insertion; low capacity for gene delivering (up to 5 kb)
Herpes simplex	dsDNA	Allowing transfection of large pieces of DNA (>50 kb)	Nonspecific cell targeting, cell toxicity, and transient expression
Retroviruses	ssRNA	Broad-spectrum tropism; high titers and allowing prolonged expression	Nonspecific cell targeting, cell division-dependent transfection
Lentiviruses HIV-1, HIV-2	ssRNA	High transfection efficacy in dividing and non-dividing cells; stable expression	Risk of mutagenesis and infection
Poxviruses	dsDNA	High transfection efficacy in dividing and non-dividing cells	High immunogenicity

Table 7.3 Adjuvants in designing DNA vaccines

Immunogenicity improvement	Mechanism	Application in previous studies related to cancer
CpG modifications	Activation of transfected DCs via stimulation through TLR-9 leading to Th1 production and proinflammatory response	Metastatic melanoma [82]
plasmids encoding inflammatory cytokines(e.g., *IL-2, IL-12, GM-CSF)*	Augments activation of APCs leading to effective vaccination	Advanced Melanoma [83]
DNA vaccine with suppressing immunoinhibitory factors	DNA encoding small interfering RNA (siRNA), for instance, targeting SOCS-1 IDO, PD-L1, or PD1	Ovarian cancer [84]
plasmids encoding costimulatory molecules (e.g., *CD80, CD86 and its ligand CD28, BAFF–E7 fusion DNA vaccine*	Enhances immune response of APCs	Colon cancer treatment(CD40 and IL2) [85], BAFF–E7 fusion DNA vaccine against TC1 tumor growth [86]
Plasmids encoding signaling molecules (e.g., *TRIF, MyD88, IRFs)*	Induce specific immune-stimulating signaling pathways for example the interaction of TRIF with TLRs and MYD88 to activate NF-kB, IRFs induce Th1 immune responses	Not yet validated in cancer models

However, in comparison with DNA, single-stranded RNA vaccines are less stable in the cytoplasm [95]. Besides, much higher immunogenicity of naked mRNAs makes them unfeasible for clinical administrations. Inserting pseudo-uridines in mRNA can diminish the immunogenicity of these vaccines while enhancing its translational capacity [96]. In order to protect the mRNAs from degradation, several complexing agents can be used such as cationic/lipid polymers, protamine, etc. [97].

7.3.3 Virus-Based Vaccines

Viral vectors containing tumor antigens are considered as an enhanced vaccination method (Table 7.4). This is partly because of the high immunogenicity of virus particles and direct transfection to APCs such as dendritic cells. High immunogenicity of viral vectors is double-edged as it could result in the immune response against the viral vector instead of the tumor antigen [98].

7.3.4 Prime-Boost Cancer Vaccines

Neutralizing antibodies against DNA vaccines lower the efficacy of cancer vaccines. To overcome this challenge, a second administration of different viral/bacterial vector (following the primary DNA) is the strategy called "prime-boost vaccine". Synergistic immune activation in prime-boost genetic platform exploits higher protection from tumor development. The sequential administration of plasmid DNA and adenovirus is well known in PROSTVAC-VF [101]. Safety of administering plasmid DNA (HER2 and GM-CSF encoding) and a booster adenoviral vector (only HER2) was evaluated in a cohort study on patients with metastatic breast cancer [102]. In another clinical trial on metastatic colorectal cancer, guanylyl cyclase C (GUCY2C) targeting DNA and Ad5 combined vaccine-enhanced antitumor efficacy through increasing T-cell receptor (TCR) avidity [103].

Table 7.4 Viral-based cancer vaccines [99, 100]

Vaccine trade name	Type of cancer	Construct	Virus vector
PROSTAVAC	Prostate cancer	PSA and a triad of T-cell costimulatory molecules (TRICOM)	Prime-boost regimen of two different recombinant poxvirus vectors
PANVAC	Colorectal cancer	CEA, MUC1, and TRICOM	Pox virus
TROVAX	Metastatic renal cell carcinoma	5T4-specific antibody with/without exogenous IFN-α and IL-2	Mammalian poxvirus: Modified virus Ankara (MVA)
TG4010	Metastatic renal cell carcinoma	Recombinant MUC-1 and IL-2 transgenes	Mammalian poxvirus: Modified virus Ankara (MVA)
ALVAC	Multiple melanoma	Multiple melanoma antigens and CD80 and CD86 costimulatory molecules	Avipox
MVA-BNO` HER2	Breast	HER2	Non-replicatingvaccinia virus Ankara (MVA)
ISF-35	Non-Hodgkin's lymphoma	CD154	Adenovirus
AD-PSA	Prostate cancer	PSA	Adenovirus
ETBX-011	Diverse tumors	CEA	Adenovirus

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8 Hematopoietic Stem Cell Transplantation and Lymphodepletion for the Treatment of Cancer

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8.1 Introduction

The first successful HSCT occurred in the late 1950s, when Dr. E.D. Thomas and colleagues successfully harvested bone marrow cells from an identical twin and infused them intravenously to the other twin [1]. Shortly thereafter, the discovery of the human leukocyte antigen (HLA) complex by Jean Dausset and the recognized existence of minor histocompatibility antigens led to the development of allogeneic HSCT. In the 1960s, Dr. Thomas demonstrated that infused marrow cells could repopulate all blood cell subsets in an allogeneic recipient, and in 1990 he was awarded the Nobel Prize for his pioneering work in the field of allogeneic HSCT [2]. HSCT has been demonstrated to be an effective treatment for hematologic malignancies [3–7], and more recently it has shown efficacy in the treatment of some solid tumors [8–11]. Since the first attempts of HSCT, intensive chemotherapy or radiation regimens have been used before transplantation of previously harvested hematopoietic progenitor cells. The preparatory therapy is intended for the elimination of cancer cells and the hematopoietic compartment. Later studies revealed that cytotoxic chemotherapy or radiation can promote cancer eradication by mechanisms beyond their direct cellular toxicities. One of these mechanisms is the development of a reorganized immune system with robust anticancer potential following lymphodepletion caused by cytotoxic therapy [12]. This chapter explores current methods of HSCT and lymphodepletion for the treatment of cancer.

8.2 Hematopoietic Stem Cell Transplantation (HSCT)

HSCT is the infusion of hematopoietic stem cells into an individual in order to reestablish all hematopoietic cell lineages. Daughter cells that retain stem cell properties do not differentiate into a specialized cell subset and instead are infinitely self-renewing and serve to provide a lifetime source of blood cells.

8.2.1 Sources of Hematopoietic Stem Cells (HSCs)

Bone marrow, peripheral blood, and umbilical cord blood can all serve as sources of hematopoietic stem cells (HSCs). Bone marrow, which contains the HSCs, can be aspirated from large bones such as the pelvis. For the harvest of HSCs from peripheral blood, the donor is treated with an agent, such as the cytokine granulocyte colony-stimulating factor (G-CSF), which "mobilizes" the hematopoietic stem cells from the bone marrow compartment to the peripheral blood. The HSCs can then be removed from the donor peripheral blood via leukapheresis, a preferred method of HSC harvest, because this technique is less invasive than a bone harvest. The HSCs may be further enriched based on CD34 expression. There is a controversy regarding the best source of HSCs for transplant (Table 8.1). Some studies suggest that peripheral blood is superior to bone marrow as the source of HSCs [13, 14], while others have demonstrated that there is no significant difference in outcomes based upon the source of stem cells [15].

Cells collected from the umbilical cord and placenta after childbirth can also be used as a source of HSCs [16–21]. Advantages of using cord blood are as follows: (1) no risks to donors, (2) immediate availability of cells, and (3) lower risk of GVHD with increased HLA incompatibility [16, 18, 22]. Although HSCs are present at higher concentrations in cord blood, there is an overall smaller quantity that limits the use of cord blood for HSCT. Investigation into methods designed to expand umbilical cord HSCs is an

Table 8.1 Characteristics of HSC source

	Bone marrow	Peripheral blood	Cord blood
Limiting factor	HLA match	HLA match	Cell quantity
Minimal HLA match	4/6	9/10	9/10
GVHD risk	Yes	Yes	No
Biggest risk	GVHD	GVHD	Delayed immune recovery

active area of research [23–25]. Compared to other sources of HSC, immune reconstitution is delayed following transplantation using umbilical cord blood as the HSC source. Slower immune reconstitution challenges umbilical cord blood HSCT due to increased risk of posttransplantation infections [26].

8.2.2 Autologous and Allogeneic HSCT

Autologous HSCT refers to the infusion of hematopoietic stem cells that were harvested from oneself. Hematologic cancers that are commonly treated with myeloablation and autologous HSCT include multiple myeloma (MM), non-Hodgkin lymphoma (NHL), Hodgkin lymphoma (HL), and acute myeloid leukemia (AML). Treatment of solid tumors such as neuroblastoma, ovarian cancer, and germ cell tumors may also include autologous HSCT [4]. Syngeneic HSCT refers to a transplant in which the donor and recipient are genetically the same. This term is used for HSCT between identical twins and for HSCT in animals when the donors and recipients are inbred and genetically identical.

Allogeneic HSCT refers to donor-derived cells that were obtained from a genetically nonidentical individual. Hematologic neoplasms that are often treated with allogeneic transplantation include AML, myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), NHL, HL, chronic lymphocytic leukemia (CLL), MM, chronic myeloid leukemia (CML), juvenile CML, and other myeloproliferative disorders [4]. Additionally, the application of allogenic HSCT has been reported in solid tumors, with most experience in the treatment of renal cell carcinoma (RCC) [27]. Allogeneic transplantation became feasible during the 1960s with the identification of the major histocompatibility complex (human leukocyte antigen or HLA) and the advent of HLA tissue typing. Matching of donors and recipients is based upon the number of shared HLA antigens. Better HLA antigen matching between the donor and the recipient is associated with higher rates of HSC engraftment and a lower risk for developing life-threatening graft-versus-host disease (GVHD).

Haploidentical HSCT refers to a more recent approach that expands the use of allogeneic HSCT to be performed with "half" HLA allele mismatching. While this approach significantly expands the donor pool, disadvantages include greater risk of graft rejection, more severe GVHD, and delayed immune reconstitution. Several strategies such as administration of post-transplant cyclophosphamide or combined α/β T-cell and B-cell depletion are being developed in order to overcome these obstacles [28–30].

8.2.3 Graft-Versus-Host Disease and the Graft-Versus-Tumor Effect

Mismatches in major histocompatibility proteins and polymorphic differences in host proteins (so-called "minor" histocompatibility antigens) both contribute to the generation of alloreactivity between the donor and host. GVHD is a complication that occurs when transplanted donor T-cells become activated to host alloantigens. GVHD is a three-step process that involves antigen-presenting cell (APC) activation, donor T-cell activation upon alloantigen recognition on host APC, and induction of pro-inflammatory cytokines [31]. As a consequence, the host-reactive donor T-cells expand and release pro-inflammatory cytokines that support the recruitment of other immune effector cells. Together, the activated immune cells can eventually destroy host tissues [32]. The pretransplant conditioning causes tissue injury that leads to the release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). These molecules stimulate APC through interaction with pattern recognition receptors (PRR), which in turn triggers immune responses against host tissues. Further release of DAMPs and PAMPs following initial tissue injury by immune cells may maintain the GVHD process [33, 34].

GVHD can present as either acute or chronic, and in either case, it is a major barrier to successful

cancer-free survival. Acute and chronic GVHD are defined by their timing of occurrence after HSCT. Acute GVHD typically occurs within the first 100 days posttransplant. During acute GVHD, newly transplanted T-cells recognize host alloantigens that are either directly presented by host APC or indirectly presented by donor APC. The major tissues that are targeted for destruction include the skin, liver, and the intestinal tract. Chronic GVHD is defined as occurring after 100 days posttransplant, and it is induced when T-cells recognize host antigens as foreign after the donor HSCs have engrafted. The pathophysiology of chronic GVHD resembles an autoimmune disease process as opposed to the acute inflammatory process occurring during acute GVHD. Both acute and chronic GVHD can be fatal. Precautions, in the form of immune-suppressive therapies, are taken with patients that receive allogeneic HSCT to reduce the incidence and severity of GVHD. It is important to note that minimal levels of GVHD can be beneficial for generating a graft-versus-tumor (GVT) effect that results in the elimination of residual tumor cells.

There is an estimated 30% lower life expectancy in cancer patients that receive an allogeneic transplant as compared to the general cancer population [35–38]. The leading causes for this increase in mortality include recurrent malignancies, infection, secondary cancers, respiratory disease, and chronic GVHD [37]. Autologous HSCT has minimal treatment-related morbidity and mortality and little risk for GVHD; however, autologous HSCT is associated with a higher incidence of tumor relapse as compared to allogeneic HSCT. Occasionally, a syndrome resembling GVHD, often referred to as autologous GVHD, can occur after an autologous HSCT. Autologous GVHD appears to occur as a result of immune dysregulation by autoreactive T-cells [39].

Despite the devastating consequences of GVHD, low levels of alloreactivity can be beneficial for generating a graft-versus-tumor (GVT) effect [40]. The GVT effect can occur after an allogeneic transplant when donor T-cells reactive to host alloantigens present on the tumor cells eliminate the residual cancer. The GVT effect was discovered when physicians attempted to avoid GVHD by extensively depleting donor T-cells from the allogeneic HSC graft. Despite a reduction in GVHD incidence and severity, T-cell depletion of the graft correlates with a decrease in leukemia-free survival [41]. It has since been demonstrated that T-cells are required for an optimal GVT effect, and removal of either $CD4^+$ or $CD8^+$ T-cells compromises GVT reactivity [42]. GVT effects have been identified in MM, NHL, HL, CLL, and acute leukemia (ALL and AML) [43]. GVHD and GVT both include three interlinked phases: (1) induced pro-inflammatory environment, (2) donor T-cell activation and proliferation, and (3) migration of immune effector cells to target tissues [44].

In addition to T-cells, natural killer (NK) cells have also been shown to induce GVT effects. NK cells quickly replicate, produce numerous cytokines, kill aberrant cells, and therefore can be useful for boosting an antitumor response [45]. NK cells eliminate tumor cells in a MHC-unrestricted manner either by direct cytotoxicity or by the production of inflammatory cytokines [45]. Clinical experiences using NK cells as part of transplant immunotherapy have observed mixed results regarding malignancy relapse after HSCT, justifying the need for further research on determinants of the antitumor effects of NK cells [45, 46].

Although the mechanisms of GVHD and GVT both involve the activation of donor T-cells against host alloantigens, it appears that these outcomes can occur independent of each other [47, 48]. Approaches that induce a GVT effect while minimizing GVHD focus on reducing pro-inflammatory processes in the recipient while increasing the reactivity of tumor-specific donor T-cells [44]. Transfer of regulatory T-cells to HSC recipients [49–52], use of CD34+ HSC selected grafts [53, 54], and selective eradication of graft naïve T-cells [55] have been shown to prevent chronic GVHD without any comparable difference in the risk of relapse.

Continued research is needed to advance the field of HSCT for the treatment of malignancy. Specifically, research is needed to (1) optimize the antitumor effect that occurs following an

autologous HSC transplant, (2) uncover mechanisms that promote alloreactive effects against tumor cells, and (3) reduce the incidence of severe GVHD following allogeneic transplantation [40, 41].

8.3 Conditioning Regimens Before Hematopoietic Stem Cell Transplantation (HSCT)

HSCT is currently preceded by administration of a preparative regimen. The purposes of the conditioning regimen are multifaceted. While it can destroy malignant cells, the regimen may also inhibit cells that play roles in suppressing anticancer immune responses. Depletion of the endogenous HSCS via the conditioning regimen is a critical prerequisite for the successful engraftment of transplanted HSCs. The conditioning regimen prevents immunologic rejection of the graft and provides sufficient space for the incoming transplanted stem cells to divide and expand [4].

Based on regimen intensity, conditioning regimens are classified into three groups: myeloablative (MA) conditioning, reduced-intensity conditioning (RIC), and non-myeloablative (NMA) conditioning [56, 57]. Bone marrow destruction that occurs from MA conditioning results in severe cytopenia which is irreversible unless new HSCs are provided. RIC and NMA conditioning regimens are associated with less profound cytopenia that may recover even without stem cell support. The main advantage of RIC/NMA conditioning regimens is that they are less toxic, making them more tolerable for older patients and patients with comorbid conditions [56–58]. However, graft failure is generally more frequent in RIC/NMA conditioning than MA conditioning [58].

8.3.1 Myeloablative Conditioning

MA conditioning is accomplished through the administration of chemotherapy drugs with or without total body irradiation (TBI). Typically, TBI between 8 Gy (800 rad) and 14.4 Gy (1440 rad) is combined with an alkylating chemotherapeutic agent such as cyclophosphamide. Cyclophosphamide is a commonly used chemotherapeutic agent and is often administered for its global lymphodepleting effects as well as for its ability to eliminate malignant cells such as those present in HL, NHL, acute and chronic leukemias, and MM, as well as solid tumors such as neuroblastoma, retinoblastoma, rhabdomyosarcoma, lung cancer, testes cancer, and ovarian cancer. Listed in Table 8.2 are chemotherapeutic drugs that are commonly used for MA conditioning.

Total body irradiation (TBI) in combination with chemotherapeutic drugs has shown benefit over chemotherapy alone for the elimination of hematologic malignancies. Several advantageous effects of TBI include the following: (1) a homogeneous effect regardless of blood supply as the myeloablative effects of TBI can more effectively reach body areas that are underperfused, (2) targeting of specific areas through the use of shields to prevent exposure to body areas where TBI is undesirable, (3) different doses of TBI can result in differential myeloablative and immunosuppressive outcomes, (4) a reduction in the requirement for drug detoxification, (5) TBI is effective against a wide variety of malignancies, and (6) TBI is effective against chemotherapy-resistant malignancies [4]. Originally, myeloablative TBI was given as a single high-dose irradiation. The advantage of this approach was the elimination of theoretically all hematologic cancerous cells in the host. However, a major disadvantage included extended cell death beyond the hematopoietic compartment, resulting in debilitating negative side effects. As a result, when TBI is now used for MA conditioning, dosing is typically fractionated. Even though each fraction consists of a lower dose of radiation, the combined myeloablative effect is equivalent to that obtained by a single high dose of radiation. The fractionated radiation is sufficient to eradicate malignant cells and destroy the patient's HSCs. The time allotted between each TBI treatment allows for some repair of normal tissue damaged by the radiation. Fractioning the

Table 8.2 Chemotherapeutic drugs used for myeloablative conditioning

Name	Type	Details	Use
Busulfan	Sulfonate	Cross-linkage of DNA strands	Leukemia
	Alkylating agent	Prevents DNA replication and transcription	Lymphoma
			Multiple myeloma
			Testicular carcinoma
			Breast cancer
			Ewing's sarcoma
Carmustine	Nitrosourea	Cross-linkage of DNA strands	Hodgkin disease
	Alkylating agent	Prevents DNA replication and transcription	Non-Hodgkin lymphoma
			Lymphoma
			Multiple myeloma
			Brain cancers
Carboplatin	Heavy metal	Cell cycle nonspecific	Ovarian cancer
	"Alkylating-like"	Causes cross-linkage of DNA strands	Lung cancer
		Inhibits DNA repair	Head/neck cancers
		Prevents DNA synthesis and cell division	
Cisplatin	Heavy metal	Cell cycle nonspecific	Sarcomas
	"Alkylating-like"	Causes cross-linkage of DNA strands	Lymphoma
		Inhibits DNA repair	Ovarian cancer
		Prevents DNA synthesis and cell division	Testicular cancer
Cyclophosphamide	Nitrogen mustard	Cell cycle nonspecific	Hodgkin disease
	Alkylating agent	Causes cross-linkage of DNA strands	Non-Hodgkin lymphoma
		Prevents DNA synthesis and cell division	Leukemia
			Multiple myeloma
			Neuroblastoma
			Retinoblastoma
			Solid cancers
Ifosfamide	Nitrogen mustard	Cell cycle nonspecific	Hodgkin disease
	Alkylating agent	Causes cross-linkage of DNA strands	Non-Hodgkin lymphoma
		Prevents DNA synthesis and cell division	Acute and chronic leukemia
			Lung, breast, and ovarian cancer
Melphalan	Nitrogen mustard	Cell cycle nonspecific	Multiple myeloma
	Alkylating agent	Causes cross-linkage of DNA strands	Ovarian cancer
		Prevents DNA synthesis and cell division	
Oxaliplatin	Heavy metal	Cell cycle nonspecific	Colorectal cancer
	"Alkylating-like"	Causes cross-linkage of DNA strands	Gastric cancer
		Prevents DNA synthesis and cell division	Ovarian cancer
Thiotepa	Organophosphorus	Cross-linkage of DNA strands	Lymphoma
	Alkylating agent	Prevents DNA replication and transcription	Melanoma
			Solid cancers
Etoposide	Topoisomerase inhibitor	Interferes with action of topoisomerase	Leukemia
		Inhibits DNA synthesis in S and G2 phases	Lymphoma
		Cells do not enter mitosis	Kaposi's sarcoma
		Poor immunosuppressive agent	Ewing's sarcoma
			Lung cancer
			Testicular cancer
			Glioblastoma

TBI has been shown to result in lower toxicity and better survival outcomes when compared to single high-dose treatment. When the toxic side effects of TBI conditioning are of particular concern to certain individuals, such as children and the elderly, radiation-free conditioning methods can be employed instead. For instance, the combination of cyclophosphamide and busulfan can induce a myeloablative outcome similar to that of TBI-containing regimens.

8.3.2 Reduced-Intensity and Non-myeloablative Conditioning

RIC/NMA conditioning results in transient depletion of lymphocytes and other leukocytes without completely ablating the host HSC compartment. Although HSCT may not be required following NMA conditioning, HSC transplant may still be given in an effort to generate a state of mixed donor-host chimerism. The goal of RIC/NMA conditioning is to eradicate hematologic malignant cells while preserving the HSC compartment and some normal mature hematopoietic cells including immune cells. The usual doses of RIC/NMA conditioning are considered to be insufficient for eliminating the underlying malignancy of patients. Cancer cell destruction, and thus disease control, is mainly provided by the GVT effect following RIC/NMA conditioning HSCT [56].

NMA conditioning consists of reduced doses of irradiation and/or chemotherapy. Irradiation of 2 Gy (200 rad) is sufficient to induce damage to quickly replicating cells such as peripheral blood cells and tumor cells. Sublethal doses of irradiation do not eliminate HSCs, allowing for relatively rapid repopulation of the depleted lymphocyte compartment.

The chemotherapeutic drugs used for NMA conditioning are often similar to those used for myeloablative conditioning (see Table 8.3); however, these drugs are administered at lower doses. Non-chemotherapeutic agents, such as alemtuzumab, can also be used for NMA conditioning. Alemtuzumab is a monoclonal antibody (mAb) that binds to CD52, a protein present on the surface of mature lymphocytes, resulting in their depletion. Since CD52 is not present on HSCs, alemtuzumab will only target mature lymphocytes for depletion allowing the HSCs to remain viable for reconstitution of the immune cell repertoire.

Table 8.3 Drugs used for non-myeloablative conditioning

Name	Type	Details
Total lymphoid Irradiation	Sublethal irradiation	2 Gy of radiation Induces damage to quickly replicating cells
Fludarabine	Chemotherapy	Inhibits DNA synthesis
	Purine analog	Interferes with ribonucleotide reductase and DNA polymerase
Cladribine	Chemotherapy Purine analog	Inhibits DNA synthesis through cell's ability to process DNA Inhibits the enzyme adenosine deaminase
Pentostatin	Chemotherapy Purine analog	Inhibits DNA synthesis through cell's ability to process DNA Inhibits the enzyme adenosine deaminase
Alemtuzumab	Chemotherapy	Binds CD52 protein on mature lymphocytes
	Purine analog	Results in depletion of lymphocytes only

Total lymphoid irradiation (TLI) is a type of NMA conditioning that induces lymphodepletion prior to HSCT or is used alone as a cancer treatment. During TLI, all lymph nodes and the thymus and spleen are irradiated using a linear accelerator, while nonlymphoid tissues are spared. Individuals do not require HSCT after TLI; however, TLI is known to establish allograft tolerance in humans and animals when allogeneic bone marrow cells are transplanted immediately following the TLI [59, 60]. The major advantage of TLI versus non-myeloablative TBI is an observed reduction in organ toxicity and decreased severity of GVHD [60, 61].

All MA conditioning, RIC, and NMA conditioning can stimulate antitumor immunity by causing tumor cell death and subsequent release of tumor antigens that can facilitate the activation of antitumor immunity. The tumor antigens released by apoptotic tumor cells can be processed and presented to T-cells by APC leading to activation of tumor-reactive cytolytic T-cells.

Other mechanisms that may promote antitumor immunity include the elimination of immune-suppressive T-cells and a decrease in cellular competition for immune stimulatory cytokines [62–65]. For these reasons, all MA conditioning, RIC, and NMA conditioning regimens have been incorporated into treatment protocols for a variety of hematologic malignancies and solid tumors.

8.4 Lymphodepletion for the Treatment of Solid Tumors

Changes in the hematopoietic compartment after myeloablative and non-myeloablative conditioning have the potential to alter antitumor immunity in several ways. Conditioning eliminates or reduces all hematopoietic cells including immune-suppressive myeloid-derived suppressor cells (MDSC) and regulatory T-cells (Tregs). During lymphodepletion, reduction of lymphocytes results in a generalized state of immune suppression. However, decrease in immune-suppressive regulatory cells, as well as the reduction in lymphocytes and innate immune cells, allows the remaining T-cells to have increased access to cytokines important for their proliferation and activation (IL-7 and IL-15) [66]. Lymphodepletion enhances cytokine release which provides a favorable environment for the expansion of adaptive immune cells [67]. Creating space in the hematopoietic cell compartment is a prerequisite for the promotion of homeostatic proliferation (HP), which allows for the skewed production of tumor-reactive memory T-cells. Moreover, lymphodepletion favors the maturation of APC necessary for efficient presentation of tumor antigens to tumor-reactive T-cells, thereby facilitating antitumor immunity [68]. Inhibition or loss of inhibitory regulatory cells, the availability of cytokines, as well as the space provided by lymphodepletion provide an environment that promotes the expansion of cytolytic T-cells capable of recognizing tumor antigens.

8.5 Reconstitution of the T-Cell Repertoire After Lymphodepletion

Reconstitution of lymphocyte cell subsets is critical for the survival of patients treated with lymphodepleting regimens. Myeloid, NK, and B-cells repopulate the hematopoietic compartment relatively quickly, while T-cell recovery is more delayed [66]. Timely reconstitution of T-cells after lymphodepletion is of great importance since these cells are the main killers of cancer cells and defend the host against opportunistic infections [69]. T-cell reconstitution after non-myeloablative conditioning results from thymopoiesis, the homeostatic proliferation of host T-cells that have survived the conditioning, and/or from the adoptive transfer of allogeneic or autologous T-cells. Early T-cell reconstitution after myeloablative conditioning results primarily from the homeostatic expansion of mature donor T-cells present in the HSC graft, while thymopoiesis may contribute to T-cell reconstitution at later times. Adoptively transferred T-cells often consist of a specific phenotype (e.g., effector cells) in an attempt to skew the T-cell repertoire toward a specific antigen-reactive subset. T-cell reconstitution by homeostatic proliferation and thymopoiesis will be further explained in the following sections.

8.5.1 Lymphodepletion-Induced T-Cell Thymopoiesis Is Important for Reconstitution of the T-Cell Repertoire

Thymopoiesis is the process whereby bone marrow-derived T-cell progenitors which have migrated to the thymus undergo maturation,

expansion, and selection, which results in a broadly diverse repertoire of mature T-cells that express unique T-cell receptors (TCRs). After non-myeloablative conditioning and thymopoiesis, the proportion of T-cells with a naïve phenotype increases [70, 71]. Thymopoiesis is influenced by cytokines, growth factors, and hormones. Interleukin-7 is important for the survival of developing thymocytes [72]. As a result, IL-7 administration after transplant enhances donor-derived thymopoiesis [73]. The importance of IL-7 in thymopoiesis was further supported by the reduced T-cell maturation observed in IL-7-deficient and IL-7a-deficient transgenic mice [72]. Keratinocyte growth factor (KGF) boosts thymic productivity by expanding thymic epithelial cell populations, and KGF-deficient mice are more susceptible to thymic damage [74]. Growth hormones, such as insulin-like growth factor-1 (IGF-1) [75], IL-22 [76], FLT3 ligand [77], and sex steroid hormones [69] are also important for the thymic output of T-cells.

Thymic activity is dependent upon age. The thymus is most productive during the first 6 months of life. Over time the thymus dramatically involutes, and the expansion of early thymocytes declines. In older lymphodepleted patients, T-cell expansion is primarily the result of homeostatic proliferation. Thymic contribution to T-cell expansion may be minimal or delayed depending on the functional status of the thymus which can be influenced by radiation, chemotherapeutic drugs, and GVHD [66]. T-cell reconstitution in children is relatively quick and results in generation of a normal CD4:CD8 T-cell ratio of 2:1 [78]. Adult T-cell reconstitution, however, typically results in a CD4:CD8 cell ratio closer to 1:1 due to decreased number of CD4 T-cells [78]. In addition, reconstituted CD4 T-cell populations in adults tend to skew toward a memory (CD45RO) phenotype because impaired thymic output increases the duration of lymphopenia, resulting in a longer period of homeostatic proliferation (HP) [78, 79].

8.5.2 Lymphodepletion-Induced Homeostatic Proliferation as Strategy to Augment Antitumor Immunity

T-cell homeostatic proliferation (HP) is the spontaneous proliferation of existing peripheral T-cells that expand to fill “empty space” in the T-cell compartment. HP is different from normal homeostatic maintenance, which occurs when dying T-cells are replaced in hematopoietic tissues. HP occurs when the T-cell compartment has been severely depleted by drugs, radiation, antibodies, or by other means. The kinetics of T-cell HP depends upon the degree and duration of T-cell lymphopenia.

T-cells undergoing HP are activated in the presence of γ-chain cytokines such as IL-7 and IL-15. These rapidly expanding T-cells have an activated memory phenotype during proliferation [66]. Cells with a memory phenotype revert back to a naive phenotype after proliferation ceases and homeostasis is restored [80]. HP in the absence of primary antigen stimulation can mediate a secondary response to antigen, suggesting that lymphopenia can promote polyclonal T-cell differentiation [81]. Memory T-cells produced during homeostatic proliferation have potent antitumor activity [82]; thus, they produce efficacious immune responses to eliminate the existing cancer cells.

The lymphodepleted environment can create ideal conditions to promote the expansion of tumor-specific cytolytic T-cells. During homeostatic proliferation, T-cells can expand to produce a repertoire which is skewed to recognize antigens abundantly processed and presented by APC. Hence, vaccination with tumor antigens during periods of lymphopenia may facilitate activation of cytolytic T-cells that specifically recognize weak tumor self-antigens. In addition to tumor antigens, the availability of cytokines during lymphodepletion can promote the expansion of specific tumor-reactive T-cell subsets. IL-7 promotes T-cell lymphopoiesis [83]. T-cells in IL-7-deficient mice do not undergo HP, dem-

onstrating that IL-7 is required for stimulating naïve T-cell HP and sustaining survival of these cells [84, 85]. Administration of IL-7 drives proliferation of naïve T-cells and restricts T-cell expansion following the recovery of T-cell numbers [84, 85]. IL-7 also restricts T-cell expansion following T-cell recovery to prevent an overabundance of naïve T-cells [66]. IL-15 and IL-21 both promote the expansion and survival of memory $CD8^+$ T-cells [86, 87]. Increased concentrations of IL-7 and IL-15 are produced during whole-body irradiation [88], and increased IL-7 and IL-15 signaling causes T-cells to undergo HP [88–90]. Naïve T-cells also require TCR activation with self-peptide/MHC complexes to undergo HP [88], and exposure of these naïve T-cells to tumor antigens may help to skew reactivity toward these antigens. HP of memory T-cells is dependent on IL-15 signaling but does not require interaction with antigen or MHC molecules [88]. T-cell repopulation is also influenced by other growth factors and hormones [66].

During HP, antitumor immune responses can be further enhanced by blocking T-cell inhibitory receptors that interfere with activation. Our laboratory reported that a combination of lymphodepletion, induced by sublethal whole-body irradiation, and administration of a programmed death receptor ligand-1 (PD-L1)-specific antibody results in increased survival of myeloma-bearing mice [91]. Therefore, during homeostatic proliferation, it may be possible to manipulate the repopulating T-cells so that they can function as more potent tumor cell killers. Other strategies designed to promote the expansion of tumor-reactive T-cells include the following: (1) adoptive transfer of mature tumor-reactive T-cells during a state of lymphopenia, (2) depletion of CD4 regulatory T-cells from the donor HSC graft to enhance an antitumor effect [92–94], (3) ex vivo manipulation of T-cells to promote expansion of tumor-reactive T-cells for adoptive transfer, (4) adoptive transfer of chimeric antigen receptor (CAR) T-cells to lymphodepleted individuals in order to specifically target cancer cells with a particular antigen [95], and (5) exogenous administration of γ-chain cytokines to promote homeostatic proliferation [68]. Studies have shown that adoptive T-cell transfer into lymphodepleted mice results in extensive T-cell proliferation and that proliferating naive T-cells will adopt a memory T-cell phenotype and function [96–98].

8.5.3 Use of Animal Models to Address Immunological Effects of Lymphodepletion

Mouse models have provided excellent systems for determining the underlying mechanisms responsible for the immunological effects of lymphodepletion. As mentioned earlier, transgenic mouse models (e.g., IL-7-deficient mice) were instrumental in dissecting the role of IL-7 for both thymopoiesis and HP expansion [66, 84, 85]. Chronically, lymphophenic strains of mice have proven crucial for investigating the immunological effects of lymphodepletion; these include RAG-deficient, SCID, Nude, and NOD mice. These strains of mice completely lack T-cells, allowing for adoptive T-cell transfer and investigation of the mechanisms involved in HP. In addition, thymectomized mice are not only useful for investigation of HP but also for studying effects of the thymus on HP. When lymphodepleted thymectomized mice receive T-cell transfer, HP is increased as compared to lymphodepleted naïve mice that have an intact thymus, demonstrating cross-regulation between thymopoiesis and HP following lymphodepletion [79]. Information gathered from these models can provide further insights to new cancer therapies that involve lymphodepletion and HSCT.

8.6 Concluding Remarks

Lymphodepletion and HSCT have now been used for more than three decades in the treatment of various cancers. Myeloablative or non-myeloablative "conditioning" serves to eliminate or reduce malignant cells present in the patient, create "space" for expansion of transplanted cells, and provide an environment that is conducive to the proliferation of tumor-reactive immune

cells. Allogeneic HSCT replenishes the T-cell repertoire with malignant-free cells, and mature T-cells in the graft can provide a beneficial GVT effect. Research advances have shown that cytokine antagonists and elimination of regulatory T-cells can drive homeostatic proliferation in the direction of effective antitumor immunity. In addition, research has demonstrated that combined therapeutic approaches appear to be the most promising strategies to improve overall survival in cancer patients. Therefore, it is critical to continue to test novel therapeutic combinations to improve treatment and ultimately translate these approaches from the bench to the bedside.

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9 Recent Advances in Haploidentical Hematopoietic Cell Transplantation for Pediatric Hematologic Malignancies

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Contents

9.1 Introduction

Allogeneic hematopoietic cell transplantation (HCT) can be curative for many patients with hematologic disorders and malignancies. The preferred donor source is a human leukocyte antigen (HLA)-matched sibling. However, less than 30% of patients will have a matched sibling donor (MSD), a probability that continues to decline in developed countries due to decreasing birth rates [1]. Notably, the likelihood of having an MSD is estimated to be only 22% for the US

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pediatric population (0–19 years) and is even lower in younger patients (1–5 years) at 17% [1].

Traditionally, seeking a matched unrelated donor (MUD) is considered the second-best alternative after an MSD. However, this option is being contested due to the resurgence of haploidentical HCT (haplo-HCT). A haploidentical donor is a relative who shares a single identical HLA gene complex (haplotype) with the recipient, inherited on chromosome 6. Generally, the unshared haplotype is mismatched but may randomly have additional HLA class I or II genes in common.

9.2 Advantages of Haploidentical Hematopoietic Cell Transplantation

The benefits of haploidentical over unrelated donor (URD)-HCT are numerous, with arguably the most notable being that haplo-HCT extends donor availability to nearly all patients. Since every patient shares one HLA haplotype with each biological parent, 50% of full or half siblings, and less frequently second-degree relatives, a haploidentical family donor is available in >95% of cases. The nearly universal donor accessibility afforded by haplo-HCT is particularly significant for ethnic and racial minorities and for patients of mixed race. While marrow registries have diversified and expanded in an attempt to increase access to unrelated donors, finding an MUD has continued to be a challenge for minority populations. The possibility of finding an 8/8 antigen (HLA-A, HLA-B, HLA-C, and DRB1) MUD is 16–19% in African Americans, 34–40% in Hispanics, 27–42% in Asians, and 36–52% in Native Americans compared to 75% for whites of European descent [2]. Furthermore, the use of haplo-HCT extends the availability of transplantation to patients in less-developed countries that do not have established donor registries. Haplo-HCT offers additional advantages over URD-HCT by avoiding the delays and costs associated with unrelated donor searches and hematopoietic stem cell procurement. The time required to acquire a stem cell product from a URD, although shortened in recent years, is significantly longer than opting for a haploidentical familial donor. Haplo-HCT, therefore, can expedite transplantation in time-sensitive circumstances potentially preventing relapses in patients with aggressive hematologic malignancies. Moreover, haploidentical familial donors, especially parents, are often eager to donate and readily available for not only the initial harvest but also potential additional collections of bone marrow, peripheral blood stem cells (PBSCs), or donor leukocyte infusions (DLI), if needed.

Younger pediatric patients who do not have an MSD may have the option of receiving umbilical cord blood (UCB) in place of an MUD transplant. As UCB units are cryopreserved and stored, they are readily available. The low numbers of T cells in UCB allows for mismatched units to be utilized, thereby expanding the donor pool for younger pediatric patients. However, disadvantages of UCB include low numbers of hematopoietic stem cells, which are associated with slow engraftment, and the high cost of the cord blood unit. The current trend in the USA and especially Europe now favors the use of haplo-HCT over UCB transplants, particularly for malignant diseases [3, 4].

9.3 Lessons from Adult Haploidentical Hematopoietic Cell Transplantation Studies

Early attempts at haplo-HCT in the 1980s proved to be challenging for a variety of reasons, including a high incidence of graft rejection and delayed immune reconstitution leading to infections and relapse [5, 6]. However, the evolution of conditioning regimens, graft manipulation, and graft-versus-host disease (GvHD) prophylaxis has resulted in reduction of acute and chronic GvHD, improved immune reconstitution, decreased non-relapse mortality (NRM), and improved overall

and disease-free survival (OS, DFS). These recent advances in haplo-HCT approaches have allowed remarkable expansion in its global use. Countless primarily adult haplo-HCT trials for acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and lymphoma (though less common) have been conducted over the last decade. These studies were heterogeneous, utilizing assorted reduced intensity conditioning (RIC) or myeloablative conditioning (MAC) regimens, bone marrow or PBSC, and T cell-replete or engineered grafts.

Results from adult haplo-HCT trials are generally comparable to concurrently reported MUD-HCT with 1- to 3-year OS averaging 60%, NRM at 15%, relapse rates around 37%, and grade III–IV acute GvHD (aGvHD) and chronic GvHD (cGvHD) at 6% and 15%, respectively [7–10]. There are no randomized trials comparing haplo- to MUD-HCT. Such trials would be difficult to conduct given the diverse disease conditions, conditioning regimens, donor characteristics, stem cell sources, and GvHD prophylaxis utilized. However, contemporaneous studies have indicated that haplo-HCT may be associated with less acute and chronic GvHD with no differences in NRM, relapse, and OS compared to MUD-HCT. Use of RIC in haplo-HCT is also associated with lower acute and chronic GvHD and decreased NRM but at the expense of increased relapse rates resulting in OS comparable to MUD transplantation. It has been noted that in approximately a third of relapses following haplo-HCT, the leukemia cells escape T cell surveillance and control through their loss of the mismatched HLA haplotype. However, these relapses do not appear to have a worse prognosis than when the mismatched haplotype is retained [11].

Similarly, there have been no randomized studies directly comparing mismatched unrelated donor (MMUD) and haplo-HCT, but most reports indicate that outcomes with MMUD-HCT are inferior to haplo-HCT with OS for MMUD in the range of 19–49% [12–17]. This has led most centers including ours to favor the selection of a haploidentical donor over a MMUD.

9.4 Evolution of T Cell Depletion Strategies in Pediatric Haploidentical Hematopoietic Cell Transplantation

The concept of graft engineering is based on decades of research that have demonstrated the roles of various immune cells in the initiation and propagation of GvHD, including T, natural killer (NK), and B cells. αβ T cells are primarily responsible for GvHD, while other lymphoid populations, such as NK cells and γδ T cells, contribute to antitumor activity. As a result, current haplo-HCT approaches seek to eliminate or retain certain cell populations in the donor graft to optimize immune reconstitution while simultaneously attempting to suppress GvHD. The resurgence of haplo-HCT has been a function of advances in graft manipulation and alterations in both conditioning and post-transplant regimens. In order to improve clinical outcomes in haplo-HCT, it is essential to understand the role that various immune cell populations play. Given the rising popularity of graft engineering, including the αβ T cell/CD19 B cell depletion approach, NK cells and γδ T cells are becoming increasingly important, prompting research focused on enhancing GvL effects (Table 9.1).

9.4.1 $CD34^+$ Megadose

Positive selection and infusion of high doses of $CD34^+$ hematopoietic stem cells and infusion (megadose CD34) was utilized as one of the earlier T cell depletion approaches in haplo-HCT in an attempt to remove the T cells that cause GvHD and the B cells that may lead to post-transplant lymphoproliferative disease (PTLD). This treatment modality evolved from murine studies demonstrating that high numbers of transplanted stem cells depleted of T cells can overcome HLA barriers with sustained engraftment without GvHD [18]. Handgretinger et al. from Germany reported on $CD34^+$ megadose haplo-HCT in 31 pediatric patients with advanced hematologic malignan-

Table 9.1 Pediatric haploidentical hematopoietic cell transplant studies for hematologic malignancies

	Year	N	Age	Graft	Disease (%)	Remission (BMT) %	Regimen (%)	Engraft (%)	aGvHD III–IV (%)	cGvHD (%)	NRM (%)	Relapse (%)	OS (%)	DFS (%)	F/U mo
Handgretinger (Germany)	2001	31 MD 8 NMD	1–18	CD34	41 ALL 13 AML 10 MDS 8 CML 8 Lymp 20 NMD	16 CR1 19 CR2 6 < CR2 58 NR	MAC	92	3	3	26	42		38 (all) 28 (MD) 39 (CR) 14 (NR)	24
Klingebiel (EBMT)	2010	102	1–16	CD34	100 ALL	17 CR1 38 CR2 25 CR3	MAC	91	9	17	37	36	29	27	60
Lang (Germany)	2014	46	1–24	CD3/ CD19 Dep	46 ALL 37AML 7 MDS	13 CR1 24 CR2 20 > CR2 43 NR 41 > 1st	MAC	87	7	21 (12e)	11	63		26	51 (M)
Diaz (Spain)	2016	70	6–19	CD3/ CD19 Dep	51 ALL 49 AML	25 CR1 40 CR2 16 > CR2 19 NR 32 > 1st	MAC	94	13	33	23	32		52	22 (M)
Lang (Germany)	2015	36 MD 5 NMD	2–18	*αβ T/ CD19 Dep*	49 ALL 22 AML 7 MDS 10 ST 12 NMD	33 CR1–2 33 > CR2 33 NR 54 > 1st	MAC	88	15	19 (9e)	7	47		51	19 (M)
Locatelli (Italy)	2017	80	1–21	*αβ T/ CD19 Dep*	70 ALL 30 AML	39 CR1 56 CR2 5 > CR2	MAC	98	0	5	5	24	72	71	46 (M)
Klein (Baltimore)	2017	40	1–25	BM PT-CY	24 ALL 24 AML 12 MDS 35 HD 7 O	CR 45 PR 55	RIC	94	13	24	13	52	56 52	43 32	12 24

Berger (Italy)	2016	33	1–21	BM PT-CY	45 ALL 21 AML 12 MDS 15 Lymp 6 O	24 CR1 30 CR2 15 > CR2 30 NR	58 RIC 42 MAC	97	3	4	9	24	72	61	12
Jaiswal (India)	2015	20	2–20	PBSC PT-CY	35 ALL 65 AML	100 NR	MAC	100	20	5	20	25	64	59	24
Katsanis (Tucson, Arizona)	2018	13	4–26	BM PT-CY PT-CY/ BEN	54 ALL 15 AML 8 AUL 8 CML 15 Lymp	23 CR1 46 CR2 8 > CR2 23 NR 15 > 1st	MAC	100	0	23 (15e)	0	0	100	100	15 (M)
Liu (China)	2013	212	3–18	BM +PBSC	63 ALL 37 AML	56 CR1 28 CR2 5 > CR2 11 NR	MAC	100	15	40 (27e)	15	29 ALL 16 AML	63 ALL 73 AML	57 ALL 73 AML	41 (M)

N number, *MD* malignant disease, *NMD* nonmalignant disease, *PT-CY* post-transplant cyclophosphamide, *PT-CY/BEN* post-transplant cyclophosphamide/bendamustine, *ST* solid tumor, *AUL* acute undifferentiated leukemia, *Lymp* lymphoma, *HD* Hodgkin's disease, *CR* complete remission, *NR* not in remission, *M* median, *MAC* myeloablative conditioning, *RIC* reduced intensity conditioning, *GvHD* graft-versus-host disease, *e* extensive, *NRM* non-relapse mortality, >1st. previous transplant, *OS* overall survival, *DFS* disease-free survival, *F/U* follow-up

cies, and the European Group for Blood and Marrow Transplantation (EBMT) later expanded this study with 127 pediatric patients [19, 20]. Although this approach resulted in 91% engraftment and a low incidence (9%) of grade III–IV aGvHD, there were substantial risks associated with the removal of both T and B cells from the graft, such as delayed immune reconstitution, leading to opportunistic infections with a NRM rate of 37% and a relapse rate of 36% with DFS of 27%. T cell reconstitution in these studies was dependent on the number of infused $CD34^+$ cells.

9.4.2 CD3/CD19 Depletion

In an attempt to decrease the incidence of infections and relapse from $CD34^+$ selection, the aforementioned group from Germany applied the Miltenyi CliniMACS device to deplete $CD3^+$/$CD19^+$ cells from granulocyte colony-stimulating factor (G-CSF)-mobilized PBSC collections [21]. In utilizing this technique, grafts retained NK and monocyte activity against pathogens and leukemia. This modality of haplo-HCT was utilized in a study involving 46 pediatric patients with acute leukemia. Forty-three percent of these patients were not in remission, and for 41% it was their second or third transplant. Conditioning was myeloablative, initially consisting of OKT3, which was replaced with ATG when the monoclonal antibody was no longer available, fludarabine or clofarabine (based on risk/active disease), thiotepa, and melphalan [21]. Primary engraftment occurred in 87% of patients, with grade III–IV aGvHD in 7% and cGvHD in 21%. NRM was 11%, with a relapse rate of 63%, which was expected in this very high-risk population, and 26% DFS.

More recently, a group from Spain reported on their experience using $CD3^+$/$CD19^+$-depleted haplo-HCT in 70 pediatric leukemia patients with 19% in a state of refractory disease and 32% receiving a second or third transplant, which was an overall lower-risk population than the German study [22]. Their myeloablative conditioning regimen consisted of fludarabine, busulfan, and thiotepa. Engraftment occurred in 94%, grade III–IV aGvHD in 13%, cGvHD in 33%, and NRM in 20%. With a median follow-up of 22 months, the probability of relapse was 32%, and DFS rate was 52%. Looking further into the grafts, they found that recipients of killer-cell Ig-like receptor (KIR) B haplotype grafts developed a rapid NK cell expansion early after haplo-HCT, resulting in a lower probability of relapse and suggesting an advantageous NK-mediated graft versus leukemia (GvL) effect.

9.4.3 αβ T Cell and CD19 B Cell Depletion

A more refined approach to T cell depletion consists of haplo-HCT with αβ T and B cell-depleted grafts. This transplant methodology allows the transfer of $CD34^+$ stem cells, without αβ T cells, which are primarily responsible for GvHD, but with γδ T and NK cells, both of which are capable of eliciting antileukemic and anti-pathogenic effects. The German group reported their preliminary results with this approach using the same MAC regimen outlined above for CD3/CD19 depletion [23]. In this study, they performed an anti-T cell receptor (TCR)-αβ microbead depletion via the Miltenyi CliniMACS device, rather than a total T cell depletion via CD3 microbeads. Forty-one patients (32 with hematologic malignancies, 4 with relapsed solid tumors, and 5 with nonmalignant conditions) underwent αβ T cell/CD19 B cell-depleted haplo-HCT. Twelve of the 36 patients with malignant disease were not in remission (33%), and for 22 of the 41 patients (54%), it was their second or subsequent transplant. Engraftment occurred in 88%, grade III–IV aGvHD in 15%, and cGvHD in 19% (9% extensive), and the relapse rate was 47%. With a median follow-up of 19 months, the disease-free survival was 51%. Of note, the ten patients who received their first transplant in complete remission (CR) showed a DFS of 100% at 1 year, illustrating the importance of disease status at the time of transplantation.

Locatelli et al. from Italy added to the investigation of αβ T cell/CD19 B cell-depleted

haplo-HCT with 80 acute leukemia pediatric patients in complete remission (39% CR1 and 56% CR2) [24]. All patients received a myeloablative preparative regimen (75% TBI based) and no post-transplant GvHD prophylaxis. Two patients experienced primary graft failure (98% engraftment), there was no grade III–IV aGvHD, and only 5% limited cGvHD was observed. NRM was only 5%, and relapse rates were 24%. With a median follow-up of 46 months, the 5-year probability of OS and DFS was 72% and 71%, respectively. As part of this study, they also compared the outcomes of the 80 haplo-HCT patients to that of 92 acute leukemia patients in CR that received MSD-HCT ($n = 41$) or MUD-HCT ($n = 51$) during the same time period. All three groups had comparable disease characteristics with the exception that 98% and 78% of MSD and MUD recipients were given bone marrow grafts (instead of PBSC) and all received post-transplant GvHD prophylaxis. Haplo-HCT was associated with a lower incidence of grade III–IV aGvHD and cGvHD and no significant difference in DFS among the three transplant groups.

In the context of αβ T cell depletion, recent studies have examined the effects of zoledronic acid (ZOL) on γδ T cell activity. A prospective analysis of 27 pediatric patients that underwent αβ T cell/CD19 B cell-depleted haplo-HCT demonstrated that γδ T cells were the predominant T cell population during the first few weeks post-transplant, with the central memory Vδ1 and Vδ2 subsets being most prevalent [25]. Vδ1 cells proliferated in response to CMV reactivation, while Vδ2 cells expanded in vitro in response to ZOL exposure, becoming cytotoxic against lymphoid and myeloid blasts. These findings suggest a potential use of ZOL as a strategy to enhance a graft's antileukemic effects. The same group proceeded to treat 43 pediatric patients that had undergone αβ T cell/CD19 B cell-depleted haplo-HCT with ZOL [26]. ZOL administration started as early as 4–5 weeks post-HCT and was given every 28 days, with most patients receiving ZOL at least twice. Increased in vitro cytotoxicity of Vδ1 and Vδ2 cells was observed against primary leukemic blasts. Cytotoxic activity was further increased in Vδ2 but not Vδ1 cells in those patients given more than one treatment. More importantly, patients who received at least three ZOL infusions were found to have significantly improved survival (86%) compared to those who did not (54%). These studies have laid the foundation for further evaluation of ZOL following HCT.

9.4.4 Donor Selection Considerations in T Cell-Depleted Haploidentical Transplants

Several factors should be taken into consideration during the pretransplantation period, including donor characteristics. Donor age has been reported to affect patient outcomes in the T cell-depleted haploidentical setting. The Spanish group expanded their analysis of the patient outcomes noted above, transplanted with CD3/CD19-depleted grafts, with an additional 25 patients receiving αβ T cell and CD19 B cell-depleted grafts [27]. Patients receiving grafts from younger donors (<40 years) had significantly faster recovery of $CD3^+$, $CD4^+$, and $CD8^+$ T cells and B cells but not NK cells. Moreover, in the cases of αβ T cell and CD19 B cell-depleted grafts, earlier γδ T cell recovery was observed when compared to grafts from donors older than 40 years. They postulated that donor age was the main risk factor for higher NRM (13% vs. 43%) due to a higher infection rate in patients with older donors. Lower grade II–IV aGvHD was observed with younger donors (32% vs. 51%). Age of donor did not significantly affect relapse rate, which was found to instead be dependent on disease status at time of HCT, receiving NK KIR genotype A rather than B (KIR genotype A 79% relapse vs. 25% with KIR genotype B) and absence of cGvHD. By univariate analysis, donor age was also found to influence DFS (35% vs. 59%) but not by multivariate analysis, with which only disease status and NK cell recovery at day +30 were significant.

KIRs are of particular importance in regulating NK cell function. The KIR gene family con-

sists of numerous genes located on chromosome 19 and are inherited as haplotypes [28]. Inhibitory KIRs recognize HLA-A, HLA-B, and HLA-C alleles as ligands, with every individual expressing a unique KIR pattern. Donor NK cells can attack patient hematopoietic cells when they lack the ligand for the corresponding inhibitory KIR, leading to an NK-mediated GvL effect. There are two human KIR haplotypes: group A haplotype, which has a fixed number of genes encoding inhibitory receptors (except for the activating receptor KIR2DS4), and group B haplotypes, which have a variable gene number of one or more KIR B-specific genes (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, KIR2DL2, and KIR2DL5). Activating forms of KIRs have been identified, with KIR2DS1 and KIR2DS4 having specificity for HLA class I molecules. Among haplotype B individuals, a KIR B content score can be determined based on the number of centromeric and telomeric KIR B haplotype motifs. Miller et al. at the University of Minnesota found that both centromeric and telomeric B motifs can protect against AML relapse, but centromeric B homozygosity had the strongest effect [29]. Oevermann et al. analyzed the effect of donor KIR gene haplotype on relapse and DFS in children with ALL who received $CD34^+$-selected T cell-depleted haplo-HCT [30]. The KIR gene haplotype was evaluated in 85 donors, and the KIR B content score was determined in the 63 KIR haplotype B donors. Patients receiving a KIR haplotype B donor had a superior DFS than those transplanted from a KIR haplotype A donor (50.6% vs. 29.5%). Moreover, a high donor KIR B content score was associated with a significantly lower risk of relapse. These data indicate that KIR genotyping should be included in the donor selection algorithm for at least T cell-depleted haplo-HCT where NK cells may play a more critical role, with the aim of enhancing GvL effects by choosing KIR haplotype B donors with high KIR B content scores.

9.5 Pediatric Haploidentical Hematopoietic Cell Transplantation with T Cell-Replete Grafts

Yet another approach of haplo-HCT in pediatric leukemia is the transplantation of T cell-replete grafts. In the setting of T cell-replete grafts, GvHD prevention becomes of utmost importance, given that the graft contains all of the immune cells necessary to attack the immunocompromised host. Furthermore, graft rejection is similarly a risk, given that the graft also contains all of the cellular components recognized as foreign. Therefore, pre- and especially post-transplant immunosuppression are essential.

9.5.1 Post-transplant Cyclophosphamide (PT-CY)

More than half a century ago, it was demonstrated that a single dose of CY was able to prolong the survival of a skin allograft from a haploidentical donor if given between the first and fourth day following implantation of the graft [31]. The use of PT-CY originated from experimental HCT in murine models performed at Johns Hopkins University. This approach has been critical in the progression of T cell-replete haplo-HCT [32]. PT-CY is effective for several reasons, including the targeting of rapidly dividing alloreactive donor T cells that are responsible for GvHD while not affecting quiescent hematopoietic stem cells due to their relatively high levels of aldehyde dehydrogenase [33, 34]. Additional benefits of PT-CY are that it is inexpensive and simple to use and thus can be applied by any center performing allogeneic HCT. T cell-replete haplo-HCT with PT-CY has therefore emerged as the most widely applied regimen, at least in the USA, as it circumvents the need to manipulate stem cell grafts. While many of the initial studies focused primarily on adult patients, T cell-replete haplo-HCT with

PT-CY has become an increasingly utilized transplant approach in pediatric patients afflicted by both malignant and nonmalignant diseases. A concern with the use of PT-CY is that donor hematopoietic stem cells may be exposed to its mutagenic effects, which is particularly problematic in children. However, a recent analysis of 790 long-term survivors of PT-CY demonstrated that this is a rare occurrence present in only 5 patients (0.6%) [35]. The reports of haplo-HCT with PT-CY summarized below were all comprised of advanced hematologic malignancies including >CR2 and, in many cases, refractory disease. Some of these studies also involved patients that had previously undergone myeloablative transplantation.

Klein et al. from Johns Hopkins demonstrated the effectiveness of PT-CY in 40 pediatric and young adult patients (1–25 years) using their haplo-HCT RIC regimen (CY 14.5 mg/kg ×2, FLU 30 mg/m^2 ×5, and 200 cGy of TBI) with PT-CY [36]. Engraftment occurred in 94% of patients, while grade III–IV aGvHD and cGvHD developed in 13% and 24%, respectively, with a NRM of 13% and relapse rate of 52%. The OS at 1 year and 2 years was 56% and 52% with DFS at 43% and 32%, respectively. When compared to adult reports from the same institution using the same regimen, GvHD and NRM were somewhat increased, while the high relapse rate was similar. This underscores the necessity of MAC regimens for disease control in pediatric haplo-HCT for refractory and advanced hematologic malignancies.

Berger et al. described a cohort of 33 pediatric patients from 5 Italian centers who received haplo-HCT for hematologic malignancies [37]. The Johns Hopkins RIC was used in 19 patients, while 12 patients received chemotherapy-based MAC regimens and 2 patients were treated with TBI-based MAC. All but one patient engrafted (97%), with rates of grade III–IV aGvHD (3%) and cGvHD (4%), NRM at 9%, and relapse at 24%. The 1-year OS was 72%, with DFS of 61%. Of interest in this study, relapse was significantly decreased in patients that received a maternal graft (0% versus 53%), and these grafts were not associated with a higher risk of GvHD, suggesting the maternal T cells had preferential GvL effects.

Jaiswal et al. reported on the use of unmanipulated PBSCs in India, following MAC with busulfan 0.8 mg/kg ×12, fludarabine 30 mg/m^2 ×5, and melphalan 140 mg/m^2 [38]. All patients had detectable leukemia prior to starting conditioning chemotherapy. Engraftment occurred in 100%, with grade III–IV aGvHD and cGvHD seen in 20% and 5%. Interestingly, all of the severe GvHD was seen in patients younger than 10 years, despite them having received equivalent number CD3$^+$ cells/kg as their older counterparts. The authors hypothesized that PT-CY did not completely eliminate all alloreactive T cells in younger patients, possibly due to variable CY metabolism in this age group. The NRM in this study was high at 20% with relapse low at 25%, 2-year OS at 64%, and DFS at 59%.

We have treated 13 pediatric and young adult patients aged 4–26 years (7 ALL, 2 AML, 1 acute undifferentiated leukemia, 1 CML, 1 non-Hodgkin's, and 1 Hodgkin's lymphoma) at the University of Arizona Cancer Center with haplo-HCT and PT-CY or PT-CY/bendamustine (BEN) [39]. We have an ongoing phase I/Ib study of de-escalating PT-CY and replacing it with BEN based on our findings that the latter may preserve GvL effects better than CY [40]. Our preparative regimens were myeloablative and consisted of TBI 1200 cGy + fludarabine 30 mg/m^2 ×4 for ALL [7]. For the other hematologic malignancies, we used a less intense MAC regimen than that in the Jaiswal study, consisting of busulfan 0.8 mg/kg ×12, fludarabine 30 mg/m^2 ×4, and melphalan 100 mg/m^2 [41]. All patients engrafted between days 12 and 26. The incidence of grade II–IV and III–IV aGvHD was 30.8% and 0%. cGvHD and extensive cGvHD were also low at 23.1% and 15.4%, respectively. With a median follow-up of 15.6 months (1.5–31.2 months), the OS and DFS stand at 100%. Taken together with the aforementioned published reports, our results strongly indicate that MAC haplo-HCT with

PT-CY is well tolerated by children and young adults and can be effectively applied in patients with advanced hematologic malignancies.

9.5.2 The Chinese Experience with GIAC Protocol

The group from Peking University has developed the GIAC protocol used to describe the four main components of their T cell-replete haplo-HCT approach. GIAC stands for *G*-CSF stimulation of the donor pre- and patient post-HCT; *i*ntensified immunosuppression via cyclosporine A, mycophenolate mofetil, and methotrexate; the addition of *a*nti-thymocyte globulin to the conditioning regimen to assist with engraftment and decrease the incidence of GvHD; and a *c*ombination of PBSC and bone marrow. G-CSF exposure, in addition to mobilizing $CD34^+$ stem cells, is believed to increase the production of IL-4 and promote polarization of T helper 1 (Th1) to Th2, enhancing immune tolerance and reducing the incidence of GvHD. In the context of pediatric leukemia, the GIAC protocol was used in a large cohort of 212 pediatric patients with AML and ALL who received haplo-HCT with an unmanipulated bone marrow and PBSC graft [42]. Study participants received an intense MAC regimen consisting of cytarabine, busulfan, cyclophosphamide, semustine, and anti-thymoglobulin in conjunction with multi-agent GvHD prophylaxis. All patients engrafted, with a NRM rate of 15%. The incidence of grade III–IV aGvHD was 14%, while cGvHD was reported to be 40% with 27% of patients having extensive cGvHD. OS and DFS were 63% and 57% for ALL and 73% and 73% for AML. While this regimen demonstrated excellent overall survival, which was identical to a contemporary cohort of pediatric patients receiving MSD transplants, the incidence of extensive cGvHD appeared high when compared to other haplo-HCT approaches.

9.6 Conclusion

In summary, haplo-HCT has quickly become an accepted transplant modality in pediatrics, comparable to MUD-HCT and MSD-HCT in treating patients with hematologic malignancies. Various options exist with respect to the choice of conditioning regimen, graft manipulation, and GvHD prophylaxis. It is clear that patients in remission and those that receive MAC regimens have lower rates of relapse. However, research is still needed to determine the optimal donor and graft characteristics, as well as to refine conditioning regimens, GvHD prophylaxis, and improve immune reconstitution for better pathogen and disease surveillance.

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Combination of Chemotherapy and Cytokine Therapy in Treatment of Cancers

10

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10.1 Introduction

The classical approaches to cancer therapy include the use of chemotherapeutic combinations and radiation, principally in advanced patients with unresectable tumors. On the other hand, emerging novel strategies such as antiangiogenic agents or immunotherapy include molecular targeted therapies. Despite the wide range of therapeutic options, for some specific tumor types the improvement of clinical response and survival of patients remain limited. Increasing

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evidence suggests that immune responses are involved in the control of cancer and that the immune system can be manipulated in different ways to recognize and attack tumors. During the last two decades, it is being observed a growing area of research focused on the combination between classical chemotherapy and novel strategies such as the use of cytokines, acting not only at the induction but also at the effector phase of the immune system regulating the innate and the adaptive immunity. The latest studies indicate that reducing the dose of conventional chemotherapy could act in synergy to generate immunity against many tumors. In this chapter we will discuss how the combination approach can be harnessed to achieve the maximal benefit to eradicate tumors.

10.2 Immune Response in the Control of Cancer

The natural history of a tumor includes subsequent phases starting with "in situ" growth, invasion, and metastasis. During these phases, crosstalk exists among all components of the tumor microenvironment and immune cells (macrophages, natural killer cells, lymphocytes, dendritic cells, and mast cells, among others) which may result in the promotion of cancer [1]. In solid tumors, e.g., colorectal carcinoma or liver cancer, immune cells could infiltrate tumors playing a key role in the control of cancer aggressiveness [2, 3].

The influence of chronic inflammation on the promotion of cancer growth has been well studied. The source of inflammatory stimuli may derive from microbial infections, as is the case of *Helicobacter pylori* infection and its association with gastric cancer or mucosal lymphoma [4]. On the other hand, chronic inflammatory diseases such as ulcerative colitis predispose to colorectal carcinoma [5]. The role of activated macrophages in chronic inflammatory processes is illustrated by the production of reactive oxygen and nitrogen species as well as by the secretion of growth factors and cytokines such as vascular endothelial growth factor (VEGF) and other pro-angiogenic molecules into avascular areas, resulting in angiogenesis stimulation [6]. Macrophages may promote tumor invasion by secreting proteases and cytokines such as IL-1 and IL-6 [7]. In addition, macrophages could suppress both arms of the immune system by blocking dendritic cell maturation and inhibiting cytotoxic T-cell responses [8]. On the contrary, experimental and clinical data support that those macrophages might exert antitumoral effects [9]. For example, liver-resident macrophages (Kupffer cells) have the ability to engulf and kill circulating tumor cells, and their depletion resulted in increased metastasis in a rat model of colorectal carcinoma [9]. Thus, plasticity is a characteristic of macrophages that could result in heterogeneity of phenotypes inside the tumor milieu. In this context, macrophages are generally categorized into two distinct subsets as either classically activated and pro-inflammatory M1 or alternatively activated and immunosuppressive M2, although this nomenclature has become over interpreted [10].

Contrarily to some pro-tumoral effects observed under chronic inflammation, the presence of NK and lymphocytes, especially $CD45^+$ and $CD8^+$ T-cells, was associated with good prognosis in many cancers [11, 12]. The density of tumor-infiltrating T lymphocytes with cytotoxic and memory phenotypes is highly predictive of favorable clinical outcome in some cancers such as melanoma, non-Hodgkin's lymphoma (NHL), breast, ovarian, head and neck, non-small cell lung, and esophageal cancer [12, 13]. These immune cell populations might induce antitumoral activity through different mechanisms such as direct tumor killing and, importantly, by the generation of memory $CD8^+$ T-cells. Then, suppressive cells and molecules such as ciclooxigenase-2 (COX-2) or as enzymes indoleamine 2,3-dioxygenase enzyme (IDO) or arginase and cytokines (IL-6, IL-10, transforming growth factor beta (TGF-β), M-CSF) might promote tumor growth, whereas other components, on the contrary, have a protective role.

10.2.1 Cancer Immunoediting Theory

In the last 30 years, we have witnessed a dramatic change in basic concepts related to tumor immunology, from the strict theory of tumor immunosurveillance postulated by Burnet and Thomas [14] to the very recent immunoediting concept

developed by Schreiber and colleagues [15]. As a result, we know that the immune system is able to recognize and eliminate cancer cells, but also part of the relationship between immune cells and cancer cells shows that inducing some selective pressure on cancer cells may facilitate their escape from the immune system's action. Therefore, the result of this tumor-immune system interaction could be anti- or pro-tumoral [15]. In summary, the cancer immunoediting hypothesis postulates three subsequent phases: (1) *elimination*, in which the immune system can recognize and eliminate nascent tumor cells (immunosurveillance); (2) *equilibrium*, between the host and cancer cells; and (3) *escape* of cancer cells from the immune attack (immunoediting) [16].

A number of mechanisms are used by cancer cells to escape from the immune recognition and tumor elimination: (1) impairment of appropriate antigen presentation mechanisms, (2) production of immunosuppressive factors, (3) inactivation of co-stimulatory signals, and (4) promotion of suppressor cells such as regulatory T-cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and immature dendritic cells (DCs) [17].

10.2.2 Tumors Escape from the Host Immune Response

Most cancer immunotherapeutic strategies are aimed at stimulating the immune system. Unfortunately, these therapies are hampered, at least in part, by complex immunosuppressive mechanisms originated mainly within the tumor microenvironment. Selective recruitment and expansion of a variety of regulatory cells such as tolerogenic DCs, natural and inducible Tregs, MDSCs, TAMs, and natural killer T (NKT) has been observed [17]. Accordingly, removal of these cells or their functional inactivation may contribute to tumor elimination. From the therapeutic point of view, these cell populations may be used as targets for immunomodulation therapy in order to generate immunity against cancer cells.

10.2.2.1 Regulatory T Lymphocytes

Regulatory T-cells were identified by Sakaguchi et al. as a subtype of $CD4^+$ T-cells that constitutively express the CD25 molecule and suppress T-cells' effector responses by $CD4^+$ and $CD8^+$ T-cells in vivo [18]. The transcription factor forkhead box P3 (Foxp3) is essential for their suppressive activity and represents a reliable intracellular marker in combination with CTLA-4 (CD152), TNF receptor-induced glucocorticoids (GITR), and lymphocyte-activation gene 3 (LAG-3) [19]. In addition, two $CD4^+$ $CD25^+$ Tregs subpopulations have been identified: "natural Tregs originated in the thymus, whose function is highly dependent on the expression of Foxp3, and 'induced" Tregs or Tr-1 cells that are characterized by their ability to inhibit the effector T-cell response by the secretion of IL-10 and TGF-β [18]. In addition to secreting immunosuppressive cytokines such as IL-10 and TGF-β, Tregs inhibit tumor-infiltrating lymphocytes (TILs) in part through the expression of CTLA-4. Also, Tregs block antitumor immunity impairing NK cell cytokine production, inducing tolerant DCs, and increasing the activity of IDO which is responsible for tryptophan degradation resulting in $CD4^+$ and $CD8^+$ T-cell apoptosis [20].

Increased number of $CD4^+$ $CD25^+$ $Foxp3^+$ cells has been reported both in circulation and within the tumors in patients with lung, pancreatic, breast, ovarian, and skin cancer [21], thus, these particular type of cells are considered therapeutic targets. In line with this, monoclonal antibodies (mAbs) directed against specific epitopes located on the cell surface of Tregs such as CD25 and CTLA-4 have been developed [20]. Nevertheless, systemic depletion of Tregs by checkpoint inhibition may induce autoimmune responses [22]. One interesting strategy aimed at inducing antitumor immunity without the induction of autoimmunity is to target effector Tregs. In addition, the high proliferation capacity of Treg cells can be directly downregulated [23]. For example, chemotherapy agents can be used to eliminate Tregs as was demonstrated by using low doses of cyclophosphamide, which selectively removes $CD4^+$ $CD25^+$ cells and induces tumor regression and antimetastatic effects in several experimental models [24, 25]. Mechanisms behind this effect are, at least in part, based on alteration of the cytokine profile from Th1 to Th2 and increased proliferation of activated T lymphocytes [26]. Importantly, Scurr et al. recently

demonstrated that cyclophosphamide reduced Tregs, B-cells, and NK cells with the subsequent activation of IFN-γ+ tumor-specific T-cells in patients with metastatic colorectal cancer [27].

10.2.2.2 Myeloid-Derived Suppressor Cells and Their Immunosuppressive Activity

MDSCs constitute a heterogeneous population of immature cells composed of certain types of macrophages, granulocytes, DCs, and other myeloid-derived cells in early stages of differentiation that exert immunosuppressive activity [28]. In mice, MDSCs are characterized by the expression of Gr-1 and CD11b molecules. MDSCs accumulate in the spleen and, in some cases, in lymph nodes in tumor-bearing mice [29]. In humans, MDSCs are $CD11b^{+}$ $CD14^{-}$ $HLA\text{-}DR^{-/low}$ $CD33^{+}$ $CD15^{+}$ and are increased in cancer patients (e.g., in renal cell carcinoma (RCC)) and associated with poor outcome [29]. MDSCs can take up antigens in vivo and process and present to T-cells resulting in anergy. In this sense, it has been widely demonstrated that the PD1/PD-L1 signaling pathway mediates immune tolerance in the tumor microenvironment. MDSCs express the inhibitory ligand, PD-L1, resulting in an exhausted phenotype of effector T-cells. MDSCs also express Galectin 9, the ligand for TIM-3 on T-cells, capable of inducing lymphocyte apoptosis [30]. Moreover, MDSCs can release NO and peroxynitrite inhibiting T-cell activation and may induce expansion of regulatory T-cells, $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ cells, in vivo [31]. More recently, Deng et al. demonstrated that MDSC-derived exosomes polarize macrophages to an M2 phenotype, showing that some of the tumor-promoting functions of MDSCs could be mediated by secreted exosomes [32]. In summary, it is possible to increase the efficacy of cancer immunotherapy for example by inhibiting MDSCs activity, and by the use of blocking antibodies against cell surface molecules [33] or drugs affecting the number and activity of these cells [34]. Recent studies have demonstrated that gemcitabine, 5-fluorouracil, or indomethacin can promote antitumor immune response by selectively removing MDSCs in mice [35, 36].

10.3 Immunotherapy of Cancer

Cancer immunotherapy aims to control the growth and dissemination of malignant tumors by the activation of a specific immune response [37]. A number of strategies destined to induce an effective immune response against cancer cells and to revert the immunosuppressive milieu have been carried out: (1) adoptive T-cell therapy, (2) indirect immunological approaches (cytokines, immune checkpoint blockade monoclonal antibodies, dendritic cells-based vaccines), and (3) indirect non-immunological strategies (antigen-encoding mRNA, metronomic chemotherapy, oncolytic viruses). Some of them are under evaluation in the clinic, but others, particularly the immune checkpoint inhibitors, have gained a place in the daily anticancer armamentarium [38].

Although several immunotherapeutic strategies have demonstrated to be potent in animal models, it was not until a few years ago with the use of immunostimulatory mAbs (e.g. ipilimumab, tremelimumab, daclizumab, nivolumab, atezolizumab) that clinical results were more satisfactory [39, 40]. A partial explanation for the frustrating clinical results is based on the presence of immunosuppressive mechanisms used by tumor cells to escape from the host immune system. This has led to the design of strategies to block factors derived from tumor microenvironments responsible for the inactivation of the immune system. As mentioned above, the use of mAbs directed against specific epitopes located on the cell surface of regulatory T-cells, such as CD25 and CTLA-4, aimed at reducing the amount and/or block its function, is under active investigation [41]. On the other hand, some drugs have been investigated to inhibit MDSC activity such as retinoic acid, vitamin D, the COX-2 inhibitor celecoxib, and others with dissimilar results [42].

In the design of a therapeutic strategy, the need to implement multiple approaches to block immunosuppressive mechanisms has to be taken into account. In this context, protocols of combined therapy consisting of a chemotherapeutic agent such as cyclophosphamide, gemcitabine, paclitaxel, or doxorubicin associated with immunostimulatory cytokines might act in synergy [43].

10.3.1 Enhancing Antitumor Immunity Using Cytokines

Cytokines are secreted by different immune cell types in response to several pathogens and antigens acting not only at the induction but also at the effector phase of the immune system, regulating innate and adaptive immunity in an autocrine or paracrine fashion. In the clinic, some cytokines (e.g., IFN-α or IL-2) have been used until very recently in patients with metastatic RCC or melanoma [44, 45].

Cytokines are classified according to their main functions as follows: (1) mediators of innate immunity, whose major cytokine sources are macrophages and NK cells, for example, TNF, IL-1 and IL-12, type I IFNs (α, y, β), IL-6, IL-15, IL-18, IL-23, IL-27. (2) Regulators of adaptive immune response that are produced mainly by T lymphocytes. Different types of antigens may stimulate naïve T $CD4^+$ lymphocytes to differentiate into Th1 profile with IFN-γ and IL-12 as predominant cytokines or Th2 type of response with IL-4, IL-10, and IL-13 as the main cytokines. Typically, IL-2, IL-4, IL-5, IFN-γ, TGF-β, IL-13, and IL-17 belong to this type of cytokines. (3) Hematopoietic cytokines: they stimulate the growth and differentiation of bone marrow hematopoietic progenitor cells. Some cytokines of this group are called colony-stimulating factors (CSFs) which are produced by leucocytes and stromal cells in bone marrow.

Several strategies are used to modulate the immune response by exogenous administration of systemic cytokines for the treatment of cancer. Strategies involving systemic administration, intra- or peritumoral injection, or the use of cancer cells engineered to secrete cytokines have been extensively investigated. The first cytokine approved by the Food Drug Administration (FDA) for the treatment of metastatic melanoma was IL-2 [46]. Unfortunately, its toxicity and low potency make it unsuccessful as a standard therapy. Its mechanisms of action involve enhanced NK cell and $CD8^+$ T-cell activity. Its low efficacy could be related, at least in part, to the expansion of Tregs resulting in the suppression of an effective antitumor response [47].

Interleukin-12 is a potent cytokine that showed antitumoral activity in a number of tumor models. Multiple mechanisms of action are known for this cytokine including the activation of NK cells, cytotoxic T lymphocytes, and the induction of a TH1 type of response as well as the ability to inhibit neoangiogenesis or to enhance the expression of adhesion molecules on endothelial cells, thus facilitating the homing of activated lymphocytes to the tumor [48, 49]. However, IL-12 was shown to eventually induce severe toxicity when administered systemically as a recombinant protein (in a phase II clinical trial) [50]. Unspecific toxic effects of systemic IL-12 administration might be solved by the use of gene therapy strategies allowing local tumoral/peritumoral expression of IL-12 with low systemic concentrations [51]. The use of GM-CSF confers some clinical advantages in melanoma, prostate cancer, and pulmonary metastases by inducing immune stimulation and enhancing tumor antigen presentation [52].

One of the most explored cytokines is interferon alpha (IFN-α). The IFN-α antitumor mechanism of action includes direct effect on tumor cells, induction of lymphocyte, and macrophage cytotoxic activities and antiangiogenesis [53]. Forni and colleagues were the first to show that the peritumoral injection of specific cytokines, particularly IL-2, could enhance tumor rejection through a coordinated host reaction composed of neutrophils, eosinophils, macrophages, NK cells, and lymphocytes [54]. On the other hand, intra-tumoral injection of viral vectors, such as an adenovirus carrying IL-12 gene (AdIL-12), proved to be safe and to generate some biological activity in patients with advanced gastrointestinal carcinomas such as an increase in tumor infiltration by both $CD4^+$ and $CD8^+$ T-cells [55]. Moreover, recently, an autologous, dendritic cell-based vaccine Sipuleucel-T [APC 8015, Provenge®] was approved by the FDA. This vaccine is produced by ex vivo exposure of DC precursors to PA 2024, a recombinant protein target (PAP) fused to GM-CSF. Studies revealed that T-cell proliferation was specific to GM-CSF and human PAP, both vaccine components [56].

10.4 Overcoming Tumor Resistance and the Use of Chemotherapeutic Agents

Based on the concept of tumor resistance, in the 1970s chemotherapy was designed in combinatorial schemes in order to improve individual drug efficacy avoiding resistance and reducing toxicity. Despite these advances, cancer remains a major cause of illness and death, and conventional cytotoxic chemotherapy schemes have proved unable to cure most human cancers [57].

10.4.1 Chemotherapy Plus Immunotherapy

Combinatorial strategies against cancer could either consist in a simultaneous application of different immunotherapeutic approaches or a combination with standard chemo- or radiotherapy. Some chemotherapeutic agents showed ability to upregulate the expression of tumor-associated antigens (such as CEA) or to reduce tumor cell resistance to specific cytotoxic T lymphocytes [58]. Although lymphopenia is frequently induced after chemotherapy with the subsequent impact on immune system [59], some of these combinations have been found to generate synergistic rather than additive effects.

10.4.2 Rationale for Drug Selection

In spite of its frequent toxicity and immunosuppression, conventional chemotherapy represents the core of cancer therapy nowadays. Chemotherapy could lead to tumor cell death by apoptotic and/or non-apoptotic mechanisms such as autophagy or necrosis, and both events may occur simultaneously [60]. DNA damage and subsequent apoptosis is the mechanism of cancer destruction by drugs such as doxorubicin, cyclophosphamide, gemcitabine, cisplatin, and others [61]. Some other drugs induce non-apoptotic cell death; for example, paclitaxel modulates the activity of small Rho GTPase family members [59]. Apoptosis has been considered as a non-immunogenic cell death; however, it is now clear that innate immunity can be triggered by apoptosis. Doxorubicin, an anthracycline drug which works by intercalating DNA, induces immunogenic apoptosis mediated by the release of the histone HMGB1, which in turn activates TLR-4 [62]. Doxorubicin and methotrexate also promote apoptosis by inducing upregulation of FAS-L in some cancer cells [63]. In normal tissue turnover, apoptotic death resulted tolerogenic, whereas necrotic death immunogenic.

Chemotherapy-induced apoptosis in vivo does not sequester tumor antigens and may induce cross presentation. One possible direct effect of chemotherapy on cross priming has been attributed to alkylating agents. Indeed, cyclophosphamide has an impact on DC homeostasis mediated by endogenous type I INFs induction leading to the preferential expansion of $CD8^+$DC, the main subset involved in the cross presentation of cell-derived antigens [63].

Toxicity induced by chemotherapy is extremely frequent in the clinic. However, experimental evidence shows that reducing the dose of conventional chemotherapy could act in synergy to generate immunity against many tumors. For example, it has been demonstrated that low-dose paclitaxel can reduce the number of tumor-infiltrating MDSCs in melanoma-bearing mice. Moreover, tumor-infiltrating MDSCs from paclitaxel-treated mice showed a reduced capability to suppress T-cell proliferation [64]. Gemcitabine and 5-FU can also selectively deplete MDSCs. In a murine model of thymoma, 5-FU-mediated MDSC depletion increased IFN-γ production by tumor-specific $CD8^+$ T-cells and also enhanced the survival of treated mice [35]. In a novel study, Blidner et al. characterized the effect of the non-steroidal anti-inflammatory drug indomethacin (IND) on MDSCs [36]. Mice with lung adenocarcinoma treated with IND inhibited the suppressive activity exerted by MDSCs on CD8 (+) T Cells.

On the other hand, besides its direct cytotoxic effect, cyclophosphamide is able to modulate the

immune system in a wide range of doses. Several researches, including the authors, have demonstrated that the use of low-dose cyclophosphamide promotes a Th2/Th1 shift in cytokine production, modulates the homeostatic equilibrium in different hematopoietic and immune compartments, induces the preferential expansion and persistence of antitumor T-cells, and selectively suppresses $CD4^+CD25^+$ naturally occurring Tregs [65–67].

The kind of immune response that would be favorable to tumor elimination should include the generation of cytotoxic T-cells with the capacity to directly lyse tumor cell targets. To this end, exogenous cytokines such as IL-2, INF, TNF, or IL-12 are good candidates to work in synergy with chemotherapy.

10.5 Combined Therapies

10.5.1 Preclinical Experience

The therapeutic use of certain cytokines in combination with systemic chemotherapy has been widely pursued in preclinical models. IL-2 was the first cytokine, which demonstrated an antitumoral effect by activating immune effector cells [68]. For example, it has been shown that combined treatment of IL-2 with low doses of doxorubicin induces an increased cytotoxic T-cell response and animal survival in mice with lymphoma (EL4 cells) [69]; $CD8^+$ T-cell depletion abolished the effect of combined therapy [69]. This therapeutic profile was confirmed in a syngeneic E0771 breast cancer model in mice; the combined therapy reduced tumor-induced immunosuppression, and its therapeutic effect involved $CD8^+$ T-cell response [70].

TNFα is a cytokine also used in combination with chemotherapy in a number of murine models. TNFα is produced by activated macrophages, $CD4^+$ T lymphocytes, and NK cells. Studies describe that the combination of TNFα and doxorubicin leads to complete tumor regression in C57BL/6 mice inoculated with EL4 lymphoma cells. Moreover, the combination showed a synergistic effect, since complete regression could not be elicited in tumor-bearing mice treated with single agents [71]. TNFα combined with doxorubicin could also induce complete regression and long-term tumor-free survival in C57BL/6 mice inoculated with EO77l mammary tumor cells [72]. In addition, Regenass et al. have demonstrated that TNFα and doxorubicin combined therapy induced complete and partial regressions in a sarcoma model developed in BALB/c mice. Importantly, the use of an intermediate dose of doxorubicin was more effective than a higher dose [73]. TNFα in combination with cyclophosphamide was also explored in this model, showing that a low dose of cyclophosphamide combined with TNFα resulted in 80% complete tumor eradication, while higher doses of cyclophosphamide were less effective [73].

In several murine models, GM-CSF has demonstrated to be a potent immunostimulatory cytokine due to its capacity to enhance tumor antigen presentation by DCs and macrophages and to stimulate $CD4^+$, $CD8^+$ T, and NKT cell activity [74]. The optimal schedule and mechanisms of action of a vaccination with irradiated tumor cells engineered to secrete GM-CSF in combination with chemotherapy have been studied in a variety of tumor models [74]. For example, the antitumor efficiency of paclitaxel in combination with the vaccine was examined in a mouse model of RM-1 prostate cancer [75]. The results showed that the GM-CSF surface-modified tumor cell vaccine was more potent at inducing the uptake of tumor antigens by DCs than irradiated tumor cells plus free GM-CSF. The administration of paclitaxel followed by the vaccination induced an increase of $CD8^+$ T-cell infiltration in tumors, suggesting a possible induction of tumor-specific immune response [75]. Immunomodulating doses of chemotherapy were also tested in combination with GM-CSF-secreting, HER-2/neu (neu)-expressing whole-cell vaccine. Studies describe that neu transgenic mice exhibit immune tolerance to the neu-expressing tumors similarly to what is observed in cancer patients. Machiels et al. have demonstrated that cyclophosphamide, paclitaxel,

and doxorubicin enhanced the capacity of this vaccine to delay tumor growth in neu transgenic mice by a mechanism that involves T helper 1 neu-specific T-cell induction [76].

As mentioned above, IL-12 is a cytokine that acts as a link between the innate and the specific immune response [77]. IL-12 has been shown to induce tumor regression and rejection in a variety of murine tumor models by activation of mechanisms that involve IFN-γ, CD4, and CD8 cells. IL-12 has the potential to be used as an immunomodulatory cytokine in the therapy of malignancies as well as in gene therapy-based protocols [78]. Brunda et al. have shown that systemic administration of murine IL-12 inhibits the growth of established subcutaneous tumors, experimental pulmonary or hepatic metastases of melanoma, sarcoma, or RCC, and local peritumoral injections of IL-12 can also result in the eradication of established tumors [48].

Importantly, it has been demonstrated that the combined administration of IL-12 with systemic chemotherapy results in potent antitumoral activity in mice. For instance, combination of a single low-dose cyclophosphamide with an adenovirus encoding interleukin-12 genes (AdIL-12) might represent a successful therapeutic strategy for experimental gastrointestinal tumors. This approach ameliorated immunosuppressive mechanisms elicited by cancer cells and showed synergistic antitumor immune response. In this sense, evidence shows that combined treatment overcomes tolerance by reducing the number of $CD4^+$ $CD25^+$ $Foxp3^+$, both in peripheral blood as in the spleen, as well as the number of MDSCs in the spleen of tumor-bearing animals [67, 79]. Synergistic effects were also observed in squamous cell spontaneous tumors in C3H mice combining cyclophosphamide with a plasmid carrying IL-12 genes [80]. More recently, bone marrow-derived DCs (BMDCs) stimulated with tumor antigens or with IL-12 were used to treat MC38 colorectal carcinoma tumor-bearing mice. Notably, after combined treatment, high cytotoxic activity of effector cells and the elimination of Treg cells from spleens and tumors were observed [81].

10.5.2 What Have We Learned from the Clinical Practice?

Immune checkpoint inhibitors (e.g. CTLA-4 and PD-1/PD-L1 inhibitors) have revolutionized the therapy of cancer, and several up to now nonresponders tumors show potent overall response rates and duration of responses. However, the suppressive tumor microenvironment is still a major obstacle for an effective antitumor response, particularly for immunotherapeutic strategies [82]. In general, immunotherapeutic protocols involve patients with advanced cancer disease that decreases the possibility of success. In addition, the immune system of the majority of treated patients is deteriorated or unable to recognize tumor antigens. Cytokines were used in combination with chemotherapy in order to improve its efficacy. The most widely used cytokines are INFα and/or IL2 in patients with metastatic melanoma or RCC. In fact, these cytokines are approved by the FDA as the standard treatment of these malignancies when used alone.

INFα is commonly used in this kind of combined strategy in the treatment of patients with advanced RCC. In a phase II clinical trial, the combination of INFα and vinblastine improved patient response rate but did not impact on overall survival [83]. Similar results in terms of survival were achieved in a phase III trial combining INFα with cis-retinoic acid [84]. In contrast, in a randomized phase III trial, which included patients with similar characteristics, the addition of cis-retinoic acid to INFα significantly increased progression-free and overall survival [85]. Another promising combination was 5-FU with IFN-α which has produced response rates of 23% [86] and 30% [87] when used together. However, even though one complete and six partial responses were observed, the combination of IFN-α and 5-FU was moderately active, since these response rates were similar to those seen in patients on IFN-α monotherapy. These results were improved with the addition of IL-2 reaching an approximate response rate of 50% [88, 89]. Nonetheless, their efficacy remains a matter of controversy [90]. IFN-α was tested in patients

with advanced hepatocellular carcinoma (HCC). A randomized, phase II trial compared INFα combined with hepatic arterial infusion of 5-FU plus cisplatin (CDDP) and 5-FU alone. The authors observed an increase in progression-free survival period in combined regimens including IFN-α [91]. Another study evaluated the efficacy of combined 5-FU and pegylated interferon (PEG-IFN)-α2b in patients with advanced HCC with similar results [92]. In contrast, a recent publication describes an open-label, multicenter, randomized phase III trial where 5-FU, cisplatin, and IFN-α2b combined with radiotherapy did not improve the survival rate compared with 5-FU monotherapy in patients with advanced pancreatic adenocarcinoma [93]. More recently, patients with advanced intrahepatic cholangiocarcinoma received subcutaneous PEG-IFN-α2b along with hepatic arterial infusion of 5-FU [94] In this study, median survival time was 14.6 months indicating that this combination may be useful for patients with advanced cholangiocarcinoma. Currently, the efficacy of gene based-therapy using an adenovirus to deliver IFN-α-2b (rAd-IFN) in combination with Celecoxib and Gemcitabine is evaluating in patients with malignant pleural mesothelioma (NCT03710876)

As described above, IL-2 is another potent cytokine used in metastatic melanoma and RCC patients in high doses and is usually poorly tolerated. When used in combination with different chemotherapeutic agents, no beneficial activity was generated [95]. However, the safety and efficacy of F16-IL-2 (a variant of IL-2 retargeted to the extracellular domain A1 of tenascin C, TNC) administered in combination with doxorubicin were evaluated in patients with advanced solid tumors, including breast cancer. As a result, toxicities were controllable, and 67% disease control rates were observed in phase I and II studies, respectively [96].

In addition, G-CSF has been evaluated in a phase I trial in order to overcome the neutropenia associated with irinotecan and high doses of amrubicin. This study showed that amrubicin can be administered at 78% of the recommended single-agent dose in combination with irinotecan [97].

Safety and efficacy of G-CSF also have been assessed in combination with 5-FU, epirubicin, cyclophosphamide, and paclitaxel in breast cancer patients (NCT02225652) [98]. Combination of G-CSF with chemotherapy was associated with severe side effects, resulting in the early closure of the study. More recently, the impact of higher or lower dose cladribine, cytarabine and mitoxantrone in combination with G-CSF has started testing in patients with acute myeloid leukemia (NCT03012672) (for details, please see Table 10.1).

Finally, different forms of immunotherapy including cytokines and immune checkpoint inhibitors should be investigated for overall clinical benefits along with conventional chemotherapy and/or radiotherapy in patients at early stages of the disease such as after surgical removal of tumors with increased likelihood of recurrence. Further research is required to optimize the combination of different immunotherapy plus chemotherapy to obtain maximal clinical benefit.

10.6 Concluding Remarks

Combined immunotherapy clinical trials in cancer patients are challenging, and several strategies have been opened for clinical applications. In general, for all solid tumors, the common scenario chosen to test immunotherapeutic protocols almost always involves patients with advanced diseases that decreases the possibility of success. Then, due to the advanced status of the cancer disease, the immune system of the majority of treated patients is deteriorated and unable to recognize tumor antigens. Thus, conventional chemotherapy (even radiotherapy) could act in synergy to generate immunity against many tumors. The different forms of immunotherapy including the use of cytokines should be tested for overall clinical benefits along with conventional treatment regimens evidencing improvements in survival.

Table 10.1 Cytokine plus chemotherapy combination in clinical trials

Cytokine	Condition	Chemotherapy	Phase	State	Reference	Outcome
IL-2	Melanoma	Dacarbazin	II	Active, not recruiting	NCT00553618	
	Melanoma	+Cy	II	Withdrawn	NCT01833767	
	Breast cancer	+Doxorubicin	II	Terminated	NCT01131364	
	Pancreatic cancer	+Gemcitabine	I	Terminated	NCT01198522	
IL-2+ IFN-α	Melanoma	Cisplatin+dacarbazin+vinblastine	III	Completed	NCT00002882	
IL-15	Metastatic melanoma	+Cy+TILs	I	Protocol was closed due to autoimmunity	NCT01369888	
	Skin Cancer	+Flu+TILs	II		NCT01369888	
IFN-α	RCC	+Vinblastine	III	Completed	72	Increased RR* similar OS*
	RCC	+Cis-retinoic acid	III	Completed	73	Similar RR* similar OS*
	RCC	+Cis-retinoic acid	II/III	Completed	74	Increased OS*
	RCC	+5-FU	II	Completed	76	No additional side effects
	HCC	+5-FU+cisplatin	II	Completed	80	No additional side effects similar OS*
	Ovarian carcinoma	+Carboplatin/paclitaxel	III	Completed	NCT00047632	
	GI, renal, and lung cancer	+5-FU	II	Completed	NCT01658813	
	Malignant pleural mesothelioma	+Gemcitabine/celocoxib	III	Recruiting	NCT03710876	
GM-CSF	Breast cancer	+FLAC	I	Completed	NCT00001269	
G-CSF	Breast cancer	+Fluorouracil, epirubicin, and cyclophosphamide followed by paclitaxel	II	Completed	NCT02225652	/97
	Acute Myeloid Leukemia	+ cladribine, cytarabine, and mitoxantrone	II	Recruiting	NCT03012672	

5-FU 5-fluorouracil, *Cy* cyclophosphamide, *FLAC* 5-fluorouracil, leucovorin, doxorubicin, cytoxan, *Flu* fludarabine, *GI* gastrointestinal carcinoma, *GM-CSF* granulocyte macrophage colony-stimulating factor, *G-CSF* granulocyte colony-stimulating factor, *HCC* hepatocellular carcinoma, *IFN-α* Interferon-alpha, *IL-2* Interleuquin-2, *IL-15* Interleuquin-15, *NCT* National Clinical Trial Code, progression-free survival, *RCC* renal cell carcinoma, *RR* response rate, immunotherapy versus chemotherapy alone, *OS* overall survival, *TILs* tumor-infiltrating lymphocytes, * combined chemoimmunotherapy versus chemotherapy alone

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11 Type I Interferons: History and Perspectives as Immunotherapeutic Agents Against Cancer

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Contents

11.1 Introduction

Six decades ago, it was observed that heat-inactivated *influenza A* virus "interfered" (i.e., prevented) with the infection of chorioallantoic membranes of chick embryos by live *influenza* [1]. Up until then, some of the most popular explanations for the interfering activity of heat-inactivated viruses included either exhaustion of food supply and resources within the experimental model or enzymatic digestion/physical blockade of cell receptors necessary for infection [2]. However, studies performed by Isaacs, Lidenmann, and other researchers including, but not limited to, Andrewes [3], Fazekas de St Groth [4], and Edney [5], led to the conclusion, in 1957, that viral interference of heat-inactivated particles was dependent on the secretion of a soluble

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macromolecule, the very first description of a substance then called interferon (IFN).

Since then, IFNs have been classified into three distinct protein families (namely, type I, II, and III IFNs), according to their general biological and genetic properties, as well as their signaling pathways [6]. Interferons α and β (IFN-α and IFN-β, respectively) are the main proteins in the type I IFN family and shall be the focus of this chapter. Type II IFN consists of IFN-γ, also known as immune IFN, produced by immunocompetent cells and whose modulatory properties are well established [7], while type III IFN comprises IFN-λ, the latest addition to the family, whose function is involved in antiviral immunity as well as in the protection of "barrier organs" such as the skin [8]. In humans, there are 14 known isoforms for IFN-α and 1 for IFN-β [9] that are secreted by a multitude of cell types following activation of pattern recognition receptors (PRRs) on the cell surface, cytosol, or endosomal compartments.

PRRs include the Toll-like receptor (TLR) family of proteins, sensitive to microbial components/products and ectopically expressed endogenous components, such as nucleic acids [10, 11] and the RNA helicase retinoic acid-inducible gene protein I (RIG-I) [12]. Activation of PRRs is necessary for the induction of large quantities of type I IFN during infections, and ultimately type I IFN signaling triggers: (a) an antiviral cellular program both in the cell of origin and in the surrounding population, (b) stimulation of the innate arm of the immune response, and finally (c) driving of the adaptive immunity to elicit a pathogen-specific response [9]. A remarkable and more recent finding is that basal levels of IFN-β are found in healthy tissues and are responsible for several physiological functions, such as maintenance of the hematopoietic cell niche, bone remodeling, and stimulation of the immune system (IS) [13].

Different cell subsets of the IS maintain a basal level of IFN-β production driven by the microbiota, which sustains the expression of signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 9 (IRF9) and tunes these cells to rapidly react to an eventual scenario of infection [13]. In a pathogen-free setting, macrophages keep their phagocytic abilities positively influenced by basal levels of type I IFNs [14], while NK (natural killer) cells heavily rely on type I IFN regulated stimulation in order to maintain proliferative and effector functions [15]. In addition, loss of basal IFN-β production impairs responsiveness to other cytokines, such as IFN-γ and interleukin-6 (IL-6) [16, 17]. There are mainly two possible explanations for this hypothesis: (1) there is cross talk between type I IFN receptors and receptors for other cytokines, generating concomitant engagement of downstream targets belonging to multiple pathways and (2) basal, extremely low amounts of IFN-β regulate intermediate components, such as STATs 1 and 2, that participate in the signaling networks of other cytokines [18, 19]. The signaling pathway for virally induced IFN-β production is dependent on transcription factors IRFs 3 and 7, whereas constitutive IFN-β production requires the participation of c-Jun and nuclear factor kappa B (NF-κB) [20, 21].

In general, type I IFNs signal through a heterodimer composed of the interferon alpha receptor (IFNAR) chains 1 and 2 (IFNAR1 and IFNAR2, respectively) or through a homodimer composed of two units of IFNAR1, which binds IFN-β more efficiently [22, 23]. Both receptors are ubiquitously expressed in almost all cell types [24]. In the canonic activation pathway, binding of the receptor leads to the phosphorylation of STATs 1 and 2 by the receptor-associated proteins Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) [9, 22, 25]. Phosphorylated STATs 1 and 2 enter the nucleus to bind IRF9 and induce the transcription of several IFN-stimulated genes. Albeit traditionally being tightly associated with antiviral immunity, type I IFNs have gained considerable space in the fields of oncology and cancer immunotherapy due to accumulating evidence of their direct action on tumor cells, as well as on the variety of cells that orchestrate and execute the innate and adaptive immune responses against tumors.

These immunocompetent cells interact with tumor cells from transformation to metastatic dissemination in a dynamic multistage process termed immunoediting. Immunoediting is generally divided into three phases: elimination (also referred to as immunosurveillance) [26], equilibrium, and escape [7, 26]. The concept of cancer immunosurveillance originally stated that transformed cells appear within our bodies rather frequently but are recognized and eliminated by the IS before leading to clinically observable diseases [27].

The regulatory role of the IS in the initial phases of carcinogenesis is evidenced by the fact that animals lacking the main components of the innate and adaptive immune response are more vulnerable to spontaneous and chemically induced tumors than those with an intact IS [26, 28]. This points to a more sophisticated notion of immune recognition that goes beyond differentiating self from phylogenetically distant pathogens but that is able to elegantly pick up on differences between self and transformed self [7]. However, even individuals with an intact IS frequently develop cancer, leading researchers to believe that immunosurveillance is only one facet through which the host's IS interacts with tumor cells. The experimental observation that tumors derived from immunocompetent hosts are less immunogenic than those obtained from immunodeficient hosts [29, 30] led to the conclusion that some phenotypical features of tumor cells are derived from the immunologic context in which they have arisen [7].

Therefore, not only can the IS recognize tumor cells, but it can also modulate tumor cell immunogenicity, in the phase of equilibrium, leading to the selection of immunologically "silent" variants that cross to the third and final phase of immunoediting termed escape, in which there is evasion from the effector mechanisms of the IS and progression of the disease [26]. Remarkably, researchers have found that type I IFNs participate in all the three immunoediting phases, a feature that can be exploited not only to better understand the cellular mechanisms underlying these processes but also for the development of novel therapeutic approaches. Several of these mechanisms and the correspondent phase of cancer immunoediting in which they happen will be discussed in the sections that follow.

11.2 Role of Type I IFNs in Malignant Transformation

The early observations that type I IFNs have a critical regulatory role over the transformation and growth of tumor cells were reported in the 1960s by Gresser [31], who injected animals with oncogenic viruses and demonstrated that those treated with IFN developed fewer tumors and lived longer [32]. At the time, it was unclear whether IFNs acted directly on tumor cells or indirectly via modulation of the host's IS. In the 1970s, Stewart et al. showed that IFN-treated murine and human fibroblasts were more likely to undergo cell death following viral infection than non-treated fibroblasts [33]. Subsequent studies showed that direct antitumor effects of type I IFNs include antiviral activity, which in turn diminishes the occurrence of virus-associated tumors, and modulatory action over growth, proliferation, cell cycle, and cell death [32, 34]. Additionally, a human leukemia cell line, resistant to IFN *in vitro*, can be inhibited in vivo [35], leading to the idea that the effects of IFN involve other defense components of the host, especially the immunocompetent cells. This notion of cross talk between the IS, type I IFNs, and transformed cells was reinforced by the early observations that murine tumor cells increase the expression of major histocompatibility complex (MHC) molecules on their surface upon IFN treatment [32].

It was observed that several hematologic tumors and some solid ones have chromosomal deletion or defects at 9p22, where the IFN genes are located [36–39], making researchers wonder if these or other related proteins work as tumor suppressors. Now, it is well established that IFN-induced cellular products that trigger an antiviral state also have antitumor activity when expressed in uninfected cells [40].

In the 1990s, it was observed that transfection of K562 cells (a human chronic myelogenous leukemia cell line) with cDNA of a subunit of the IFNAR induced cell differentiation, slowed their proliferation rate, and rendered these cells non-tumorigenic in nude mice [41]. More recently, research performed on silencing of *Ifnar* or of its downstream targets has demonstrated the relevance of type I IFN signaling in the protection from cellular transformation and tumorigenesis [42, 43]. Transformed and non-transformed cells treated with IFN-α and IFN-β, but not IFN-γ, show increased levels of p53 and this effect is abrogated in the absence of type IFN signaling, further suggesting the participation of the type I IFN system in the transcription of the p53 gene and the protection from malignant transformation [44]. Samples from late-stage lung cancer

patients present lower expression of IFN-α/β genes than samples obtained from patients at earlier stages of the disease [45]. Similarly, in metastatic tissue, there is downregulation of IFN-α/β genes in comparison to non-metastatic. These results suggest that cells lose expression of IFN--α/β-related genes during lung tumorigenesis as well as during progression of the disease.

11.3 Role of Type I IFNs in Cancer Immunoediting

The nature of immune components infiltrating the tumor microenvironment can either hinder or benefit the clinical outcome of several human malignancies, while providing a valid prognostic tool [46]. In the past, several groups reported experimental findings that supported the participation of the IFN family on the interface between tumor and IS. Mice treated with different IFN-rich preparations have an increased survival time following intraperitoneal inoculation of tumor cells [47]. Conversely, treatment of mice with anti-IFN antibodies resulted in larger and biologically more aggressive tumors, as well as defective natural killer (NK) cell expansion [48]. Antibody-mediated neutralization of IFN-α/β in immunocompetent mice increases their susceptibility to intraperitoneal transplanted tumor cells [49].

Mice treated with blocking antibodies against IFNAR1 do not reject highly immunogenic chemically induced tumors, which are readily rejected by control animals [24]. IFNAR1 signaling is necessary both in early stages of tumor recognition by the IS and in the effector phases of rejection. In a murine model of 3′-methylcholanthrene (MCA) induced sarcomas, tumors generated in *Ifnar1*$^{-/-}$ animals display an immunogenic phenotype similar to that observed in *Rag*$^{-/-}$ derived tumors. This means that lack of type I IFN signaling is extremely relevant to the editing role of the IS. Moreover, responsiveness of the hematopoietic compartment to type I IFN is necessary and sufficient for eliciting the rejection of tumors [50]. Priming of CD8^{+} T-cells with adequate stimuli is crucial for an antitumor immune response and it has been shown that CD8^{+} T-Cell infiltration in tumors correlates with the expression of a range of IFN-stimulated genes [51], such as lymphocyte-recruiting chemokines in melanoma and non-small cell lung cancer (NSCLC) [52, 53].

11.3.1 Type I IFNs and Natural Killer (NK) Cells

Perhaps the very first indication of the immunomodulatory property of type I IFNs comes from the observation that IFN-α/β participates in the induction of cytotoxic activity, proliferation, and survival of NK cells in the context of viral infection [54, 55]. Now, type I IFNs and IL-12 are considered the main stimulatory cytokines for NK cells, which are originated in the bone marrow and comprise approximately 5–10% of circulating human lymphocytes [56]. Phenotypically, human NK cells are characterized by the expression of the marker CD56 and absence of CD3. The majority of human NK cells display the phenotype CD56^{dim} CD16^{bright}, generally associated with cytotoxic activity [57, 58]. NK cells kill virus-infected and tumor target cells by perforin-induced osmotic lysis, apoptosis induced by perforin/granzymes, or by ligand-dependent cell death [59].

In cancer, experimental models of NK cell depletion show that type I IFNs play important roles in the maturation, activation, and maintenance of this cell population [60]. NK cells lacking IFNAR display impaired early maturation in the spleen [61, 62] and decreased surveillance *in vitro* [15, 50, 61]. It must be considered that in vivo this defect could be compensated by other cytokine signaling networks such as those involving IL-12 and IL-15 [60]. An example is that stimulation of dendritic cells (DCs) with type I IFNs induces production of IL-15 that is required to sustain the proliferation and activity of NK cells through a contact-dependent mechanism known as trans-presentation [63]. Tumor cells themselves can also signal through type I IFNs to enhance NK cell activity since upregu-

lated expression of NKG2D in their surface (e.g., induced by DNA-damaged cells) facilitates their elimination by NK cells [64].

Type I IFNs also modulate metastatic dissemination and NK cell-mediated elimination of circulating tumor cells. In a murine model of peritoneal metastasis, it was observed that treatment with IFN-β inhibits ascites accumulation via modulation of vascular hyperpermeability, although this effect seems to be unrelated to the already ascribed antitumor effect of IFN-β [65]. In a murine model of breast cancer that spontaneously metastasizes to the bone, metastatic tumor cells display downregulation of a large number of genes involved both in induction of type I IFN production and in signaling after type I IFN stimulation [66]. Conversely, forced expression of IRF7—an inducer of type I IFN production—in tumor cells resulted in enhanced immunomediated recognition in the bloodstream, dependent on the circulating population of $CD8^+$ T-Cells and NK cells [66]. Depletion of the aforementioned immune populations significantly accelerates metastatic spreading and decreases survival time. Similar patterns of *Irf7* expression were found in human primary breast tumors and matched bone metastasis. In accordance with these results, it was observed, in two models of spontaneous and orthotopic transplantable breast cancers, that *Ifnar1*$^{-/-}$ mice developed bone metastasis more rapidly than their WT counterparts [67]. In addition, NK cells isolated from *Ifnar1*$^{-/-}$ mice are not able to kill tumor cells *in vitro* or reduce metastatic burden in the bone in vivo after adoptive cell transfer therapy.

In a human disease setting, Hockland and colleagues have shown a short-term increase in the cytotoxic activity of ex vivo cultured NK cells isolated from IFN-treated lung cancer patients [68]. More recently, it was shown that circulating NK cells from pancreatic cancer patients treated with low-dose IFN therapy present an increase in NKG2D expression immediately after administration [69]. Similar results were reported by Edwards et al. in a study involving patients with multiple types of cancers [70].

11.3.2 Type I IFNs and Dendritic Cells (DCs)

Successful elimination of malignant cells by IS depends on the proper stimulation of tumor-specific T lymphocytes by antigen presenting cells (APCs). The most powerful and competent APCs are DCs, a rare cell type originated in the bone marrow, which act as sentinels of the environment through a wide variety of molecular sensors. These cells capture and process antigens, transmitting the message to lymphocytes in order to generate both effector and memory cells, working as the "bridge" between the innate and adaptive immunity [71]. DCs present processed antigen peptides complexed with major histocompatibility complex (MHC molecules) to naïve T-Cells in lymphoid organs [72]. When mature/activated DCs present antigens to naïve T lymphocytes, it triggers the generation of a clone of cells displaying effector functions such as cytokine production and cytotoxicity in order to eliminate tumor cells [71].

Different subsets of human DCs have been characterized. Conventional DCs (cDCs) develop through expression of the Id2 transcription factor and mainly express the phenotypical marker CD141. On the contrary, plasmacytoid DCs (pDCs) differentially express the transcription factor E2-2 and are characterized by the CD303 marker [71, 73–75]. Among the variety of functions attributed to pDCs, the most notable is their ability to secrete high quantities of type I IFNs during viral infection [76]. Even though the role for cDCs in antitumor immunity is much more prominent, there have been reports of the participation of pDCs in the elimination of tumors in mice [77, 78]. Human $CD141^+$ cDCs are the "equivalent" of the murine $CD8^+$ population of cDCs, sharing the unique and critical ability to cross-present antigens, that is, presenting exogenous peptides in the context of MHC class I molecules to $CD8^+$ T-Cells [71]. This cross-presentation is required for the launching of an antitumor immune response with the generation of specific cytotoxic tumor lymphocytes (CTLs). Due to their pivotal role

in tumor rejection, several DC-based immunotherapies are still under investigation since the approval of Sipuleucel-T by the Food and Drug Administration in 2010 [79], as discussed in Chap. 18 (Romagnoli and Kaneno).

Diamond and others observed that deletion of IFNAR1 on murine DCs fully prevents rejection of immunogenic sarcomas, while adoptive transfer of IFN-competent DCs restores rejection of tumors [80]. These findings not only emphasize the importance of endogenous type I IFNs but also highlight the fact that DCs are primary targets for endogenous type I IFN production in vivo. Thus, strategies aiming to boost DC-based cancer vaccines via exogenous addition of type I IFN could be beneficial to this modality of immunotherapy. In fact, addition of IFN-α to human blood-derived DCs stimulated with an anti-CD40-MART-1 fusion protein enhances the frequency of MART-1 specific $CD8^+$ IFN-γ^+ CTLs [81]. The same authors also reported that human blood DCs stimulated with the fusion protein anti-DEC205-IFN-α have their phenotype shifted to a more activated profile, with increased expression of CD80, CD86, CD40, and MHC class I [81].

Treatment of two lymphoma cell lines with a mixture of retinoic acid and IFN-α induced a particular form of apoptosis known as immunogenic cell death (ICD) that renders these cells more attractive targets for phagocytosis by DCs [82]. Moreover, IFN-α conditioned DCs pulsed with lysate of IFN-α treated tumor cells display a more activated phenotype and stimulate more efficient CTLs than controls. Treatment of animals with a mutated form of IFN-α (with low affinity to its receptors) coupled with an anti-Clec9A antibody to target cross-priming DCs increases the number of effector and memory $CD8^+$ T-Cells in the draining lymph node in comparison to controls. This new form of DC-targeted therapy synergizes well with checkpoint blockade therapy with anti-PD-1 antibody, low-dose tumor necrosis factor (TNF) treatment, and immunogenic chemotherapy with doxorubicin to eliminate B16 tumors in mice [83].

11.4 Immunotherapeutic Approaches

Nowadays, type I IFNs are approved for the treatment of a number of cancers, such as chronic myeloid leukemia, myeloproliferative neoplasms, melanoma, renal cell carcinoma, and Kaposi's sarcoma [83–85]. However, dose-limiting toxicity and the pleiotropic nature of these cytokines oftentimes compromise the success of treatment and their applicability on the clinic. So, in order to avoid systemic toxicity and safely deliver the cytokines to their targets, immunotherapeutic approaches are highly demanded. The activation of the innate compartment through PRR signaling and the consequent induction of type I IFNs has gained much attention within the field of tumor immunotherapy, with satisfactory tolerability and, in general, no need for specific tumor markers [86]. Properly activated innate mechanisms in the tumor microenvironment could determine the success or failure of some forms of immunotherapy through blocking of immune evasion and activation of adaptive immunity [86]. TLRs, RIG-I-like receptors (RLRs) and the stimulator of interferon gene (STING) are prominent candidates and are currently under investigation, and so the following sections are a roundup of some of the most recent publications reporting preclinical data and available clinical trials on the subject.

The issue of patient tolerability to treatment with IFN-α/β is still not quite solved [85]. The biggest challenge today in the therapeutic use of type I IFNs, as well as other cytokines, is the toxic side effects, including fatigue, fever, nausea, depression, leukopenia, and others, compromising the efficacy of the treatment and reducing patient's quality of life [85]. As early as the 1970s, there have been reports on IFN-mediated toxicity, initially attributed to the low purity of IFN preparations [87]. However, even more purified preparations still induced the same symptoms, proving to be the main dose-limiting factor [88]. The route of administration is also a focal point and intravenous infusion was shown to allow the administration of higher doses in comparison to intramuscular route [89].

Hematological malignancies were the first group to benefit from type I IFN treatment. In the early 1980s, pioneer clinical studies were conducted to verify the feasibility of clinical use of type I IFNs in the clinic. Gutterman and colleagues reported a favorable response in two myeloma patients, one who had been resistant to cytotoxic treatment and the other who had relapsed after cytotoxic treatment [90]. Solid tumors, such as renal cell and breast carcinomas, melanoma, and lung cancers, were also evaluated as targets for IFN treatment; however, both for hematological and solid tumors, results were timid and not encouraging, with no response in late-stage patients and moderate responses for tumors of viral origin [32]. The low rate of success of antitumor IFN as a monotherapy drove researchers to seek other strategies to apply this cytokine in the clinic, such as in combination with cytotoxic drugs, or as an adjuvant to radiotherapy or surgery in earlier stages of disease [32].

Recombinant DNA technology eventually led to large-scale production of pure preparations of IFNs, which were subsequently the first cytokines to be approved as an anticancer treatment [40]. However, the issue of toxicity still remains and several strategies are still under investigation to circumvent this problem and benefit from IFN signaling in a more "physiological" fashion. An important finding was that the conjugation of polyethylene glycol (PEG) with IFNs reduce both their clearance rate and their immunogenicity, leading to less frequent administration and consequently less adverse side effects [91]. Pegylated IFN-α2b (PEG-IFN-α2b) is currently the main choice for the long-term treatment of viral hepatitis, presenting with less toxicity than the non-pegylated form [92].

11.4.1 Toll-Like Receptors (TLRs) Agonists

There are ten different TLRs characterized in humans, each one specialized in the recognition of different pathogen-associated molecular patterns (PAMPs). TLRs 1, 2, 4, 5, and 6 are found on the cell surface, while TLRs 3, 7, 8, and 9 are expressed in the cytosol on endosomal membranes [86]. Bacillus Calmette-Guérin (BCG), monophosphoryl lipid A (MPL), and imiquimod, which signal through TLRs 2/4, 4, and 7, respectively, have been approved for the treatment of bladder, breast, and other types of cancer [93–95].

Phase I/II clinical trials evaluating the intratumoral administration of oligodeoxynucleotides containing unmethylated cytosine-guanosine motifs (CpG-ODN), a TLR9 agonist, for the treatment of neurological malignancies have been conducted, with reasonable tolerability but modest results [96, 97]. Intrathecal and subcutaneous injections of this compound were well tolerated by patients with neoplastic meningitis, with lymphopenia and inflammatory reactions being the most significant symptoms [98]. Association of oligodeoxynucleotides with bevacizumab (an anti-vascular endothelial growth factor monoclonal antibody) improves median survival, highlighting the advantages of combining different immunotherapeutic approaches. Both intradermal and intramuscular injections of a 9-polypeptide vaccine derived from breast carcinoma, along with a helper tetanus peptide and TLR3 ligand poly-ICLC, have minimal toxicity to patients but very low immune responses to two out of nine vaccinated peptides [99].

Sato-Kaneko et al. demonstrated, in a preclinical model of cutaneous squamous cell carcinoma, that a combination of checkpoint inhibition with anti-PD-1 antibody and TLR7 and 9 agonists enhanced the antitumor properties of either agent alone, both at injection and distant sites. This effect correlates with differentiation of M1 macrophages and infiltration of IFN-γ producing $CD8^+$ T-Cells in the tumor and spleen [100]. Biweekly injections of a TLR7 agonist called 852A in heavily pretreated, high risk chronic lymphocytic leukemia patients were well tolerated and induced the production of inflammatory cytokines and IgM [101]. Interestingly, in a single patient, exposure to the TLR7 agonist seemed to render drug-resistant tumor cells more sensitive to a vincristine-based chemotherapeutic regimen. Indeed, there have

been numerous reports of synergy between IFNs and cytotoxic drugs (addressed in this book by Malvicini et al.), as well as of a chemosensitizing property of type I IFNs [102–105].

The emerging field of nanotechnology/nanomedicine is investing in TLR-based immunotherapies in order to precisely deliver agonists to their cellular targets. For instance, encapsulation of resiquimod, a TLR7 ligand, into pegylated polymer-based nanoparticles is successfully uptaken by APCs, including DCs, which migrate to draining lymph nodes [106]. Such an approach could be a tool to enhance specific antitumor T-Cell responses, especially in combination with other immunotherapies like antigen vaccination. However, TLRs signaling in immune cells and also in cancer cells is a complex network that has not been fully elucidated. In fact, activation of TLRs in malignant cells can lead to tumor-promoting effects, such as immune evasion, chronic inflammation, and metastatic dissemination [107, 108].

11.4.2 RIG-Like Receptors (RLRs) Agonists

RIG-like receptors are a family of PRR specialized in the sensing of cytoplasmic viral RNA. They are members of DExD/H box RNA helicases and divided into three subgroups: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation-associated factor 5), and LGP2 (laboratory of genetics and physiology 2) [109–111]. Currently, growing experimental evidence suggests that the use of RLR ligands in the treatment of cancer can trigger beneficial effects, such as the preferential induction of cell death in malignant cells via IFN-dependent and independent mechanisms and immunostimulatory effects on APCs and NK cells [86, 112, 113].

As reviewed by Wu et al., RIG-I and MDA5 activation is able to induce tumor cell apoptosis in melanoma, prostatic, breast, neurological, gastric, and hepatic cancers [109]. Many silencing RNA molecules have been investigated in order to simultaneously achieve silencing of various genes as well as RIG-I signaling through binding of RNA and the consequent type I IFN production. For example, murine models show that the treatment of the pancreatic cell line Panc02 with different RLR agonists induces increased expression of IFN-β mRNA, IL-6, and IP-10 (an IFN regulated chemokine that attracts $CD8^+$ T-Cell via binding of CXCR3) as well as cell death with immunogenic features [114]. This last effect can be abrogated after RIG-I or MDA5 silencing, highlighting the role of this signaling network to the effects observed. Moreover, the culture of $CD8^+$ DC with treated Panc02 cells improves their maturation, making them more efficient in the cross-presentation of tumor antigens to $CD8^+$ T-Cells. Finally, in vivo vaccination with 5′-ppp RNA-treated Panc02 cells renders mice immune to a subsequent challenge, and therapeutic administration of poly(I:C) (an MDA5 ligand) decreases tumor burden in tumor-bearing mice.

Very similar results were obtained with transfection of different human pancreatic cancer cell lines with poly(I:C) complexed with lipofectamine (to deliver it to the cytosol and bind to RLRs) or with systemic administration of PEI-conjugated poly(I:C) to mice bearing Panc02 tumors [115]. The use of a glutaminase silencing 5′-ppp RNA both inhibits this essential enzyme and induces type I IFN production [116]. This silencing RNA acts through intrinsic apoptotic mechanisms in tumor cells only, with no cytotoxic effect in non-transformed cells. Additionally, silenced cells produce IFN-β and IP-10 and express more MHC class I and Fas molecules, facilitating CTL-mediated killing. RIG-I signaling also induces production of reactive oxygen species and impairs autophagic degradation of damaged mitochondria, leading to tumor cell death.

11.4.3 Stimulators of Interferon Genes (STING) Agonists

The STING receptors are located in the membranes of the endoplasmic reticulum and their signaling pathway is triggered via sensing of DNA in the cytosol by cyclic GMP-AMP synthase (cGAS). STING activation can lead to type

I IFN production via IRF3 or to secretion of pro-inflammatory cytokines via NF-κB [117]. Thus, STING is thought to be involved in the genesis of DNA-mediated inflammatory disorders such as systemic lupus erythematosus or Aicardi-Goutières syndrome [118, 119]. In cancer, its importance comes mainly from the fact that in the tumor microenvironment, DNA released by dying tumor cells or DNA containing vesicles can gain access to the cytosol of infiltrating DCs, which in turn initiate an antitumor immune response [120].

Use of *Ifnar*$^{-/-}$ mice and administration of IFN-blocking antibodies show that radiation-mediated antitumor responses are dependent on type I IFN signaling, through sensitization of the host's hematopoietic compartment and posterior infiltration in the tumor microenvironment [121, 122]. Moreover, given that MyD88 (myeloid differentiation primary response gene 88), a downstream effector of TLR signaling, is essential for the efficacy of chemotherapy, researchers have investigated whether that is the case for radiation therapy as well. However, Woo and colleagues found that antitumor responses following radiation of *MyD88*$^{-/-}$ and WT animals are very comparable [122]. Conversely, the authors also observed that tumor-bearing, irradiated mice knocked out for STING signaling have impaired radiation-mediated antitumor effects due to the abrogation of IFN-β production by tumor-infiltrating DCs.

Knocking out IRF3, a downstream target of STING activation, has similar effects. Other authors reported that deletion of STING or IRF3 renders mice unable to reject transplanted immunogenic tumors due to inefficient priming of CD8$^+$ T-Cells by DCs in the tumor tissue, while no such effect was observed through deletion of TLR, MyD88, IRF7, or mitochondrial antiviral signaling protein (MAVS), these last two being downstream targets of RLR signaling [122, 123]. Taken together, these results highlight the role of STING as the predominant innate immune pathway of tumor detection and rejection in vivo.

Intratumoral injection of the flavonoid 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a STING agonist compound, promotes potent tumor rejection of B16, TRAMP-C2, and 4T-1 tumors, induces long-lasting immunologic memory, and increases the frequency of IFN-γ producing tumor-specific CD8$^+$ T-Cells in the spleen. Conversely, STING-deficient mice are refractory to this agent [124]. Tumor-infiltrating macrophages and DCs respond to treatment with STING agonists by producing high concentrations of type I IFNs, and the main effector cells were found to be CD8$^+$ DCs. In addition, a synthetic modified cyclic dinucleotide molecule induces IFN-β production in human peripheral blood mononuclear cells, indicating that this pathway could be an effective target for novel immunotherapies.

The phenomenon of cell senescence has been described as an antitumor mechanism, given its ability to promote cell cycle arrest, preventing damaged/mutated cells from proliferating. Gluck and colleagues demonstrated that cGAS (cyclic GMP-AMP (cGAMP) synthase, a molecule directly involved in the activation of STING) knockout murine embryonic fibroblasts (MEFs) do not undergo senescence in the same fashion as their WT counterparts. Gene expression profiling revealed that shutting down the cGAS-STING pathway prevented MEFs from expressing crucial senescence-regulating genes [125]. Moreover, cGAS or STING deletion in MEFs or human cell lines prevented them from entering into senescence under conditions of oxidative stress, but treatment with IFN-β *in vitro* reversed this condition. In an in vivo model, cGAS knockout and WT mice were injected with transposons encoding Nras^{G12V} and markers of senescence were analyzed 6 days later. The livers of cGAS knockout mice displayed decreased levels of the molecules p21 and senescence-associated β-galactosidase and these animals had limited capacity to produce the cytokines and chemokines of the *senescence*-associated secretory phenotype (SASP) with immunostimulatory properties. As a result, clearance of Nras^{G12V+} cells was impaired in these animals.

Recently, a dual role has been ascribed to STING signaling. Liang et al. observed that following irradiation of MC38 tumor-bearing mice, there is an accumulation of myeloid-derived suppressor cells (MDSCs) in the tumor microenvi-

ronment that relies heavily upon CCR2 induction by STING activation and type I IFN signaling [126]. MDSCs impair T-Cell-mediated anti-tumor responses, promoting radio resistance. So, in order to benefit from the immunostimulatory potential of STING while restraining its regulatory mechanisms, they report that administration of anti-CCR2 antibody combined with radiation and cGAMP (cyclic guanosine monophosphate–adenosine monophosphate, a secondary messenger of the STING pathway) depleted $CCR2^{+}Ly6c^{hi}$ population in tumors, enhanced the $CD8^{+}$/MDSC as well as $CD8^{+}$/T reg ratios, and promoted tumor rejection in 60% of the animals.

11.5 Concluding Remarks

Type I IFNs have distinct characteristics that render them important tools in the development of new therapeutic strategies. They have been linked to direct anti-proliferative properties over tumor cells, enhancement of immunogenicity by upregulation of MHC class I molecules, induction of tumor cell senescence, and death with immunogenic features. They also synergize with cytotoxic drugs that are already used in the clinic. Moreover, they have the astounding ability to drive the anti-tumor immune response by modulating the activity of many of its key components, such as NK and B-cells. More importantly, they have a tight association with the action of DCs, the main APCs, and orchestrators of the IS, which generate highly potent tumor-specific CTLs. In addition, conventional treatments such as radiation and chemotherapy rely on type I IFN signaling to promote the elimination of transformed cells. However, the vast range of biological effects and complex networks of signaling and feedback loops triggered by type I IFNs are complicating factors that need to be elucidated to circumvent the issues of toxicity and find very specific, effective targets to drive our attention to. Emerging fields such as gene therapy and nanomedicine are promising areas that could effectively harness the potential of type I IFNs and develop more applicable technologies in the future.

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T-Cell Immunotherapy: From Synthetic Biology to Clinical Practice

12

Dina Schneider and Rimas J. Orentas

Contents

12.1 Introduction

With the completion of the human genome project, continued advances in gene vector technology, and new insights into the generation of differentiated cell populations from stem-like precursors, we are about to enter an era of unprecedented innovation in the application of biological therapy for cancer. These advances are based on decades of research that sought to define the fundamental mechanisms of immune cell function, much of it

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in animal model systems. From the first Nobel Prize in Medicine or Physiology granted in 1901 to Emil von Behring for the discovery in the immune serum of what came to be known as immunoglobulin, to the prize in 1996 to Peter Doherty and Rolf Zinkernagel for cell-mediated immune defense, the immune system has been rigorously analyzed, and the function of major immune cell subsets defined. The realization that cytotoxic T-cells can mount specific responses against cancer cells, similar to T-cell cytotoxicity exhibited against virus-infected cells, provided the rationale for the development of both cancer vaccines and the adoptive T-cell therapy. Early evidence of effective T-cell therapy was seen during bone marrow transplantation (the graft-versus-leukemia effect) and in the presence of tumor-infiltrating T lymphocytes (TILs) in melanoma lesions. Specific antitumor T-cell clones could be isolated from TIL, expanded ex vivo, and re-infused into patient. The technical advances in identification, isolation, and ex vivo expansion of tumor-specific TILs, which can then be re-infused into patients, helped make tumor-specific adoptive cell therapy a reality. Furthermore, the technological innovation of conferring antibody-like specificity to cytolytic T-cells by genetically engineering these cells to express a tumor-reactive T-cell receptor (TCR) or a chimeric antigen receptor (CAR) of choice has brought a sea-change to the field of cell-based immunotherapy. An important distinction exists between TCRs and CARs. Recognition of tumor antigen by a TCR requires the antigen to be processed within the target T-cell and presented to TCR in the context of the receptor molecule termed the major histocompatibility complex (MHC) in animals and human leukocyte antigen (HLA) in humans. Thus, the repertoire of antigens that can be presented to TCR is limited by the need for intracellular processing and for presentation in the context of specific MHC/HLA molecules that can be recognized by cytotoxic T-cells of a defined TCR specificity. By contrast, CAR receptors recognize unprocessed tumor surface molecules in an HLA-independent manner. Thus, genetically engineered CAR-T-cells can be redirected to all tumor cells bearing cell surface-expressed tumor-specific antigens. While both approaches are under clinical development, using CAR approach, we can now for the first time synthesize a cellular receptor not found in nature, express it in a recipient cell, and use those cells to cure disease. The high activity of CAR-T-cells and potentially fatal side effects has engendered caution, and the future of applying CAR-based therapy to human disease will depend on rational target selection and increasing the specificity and safety of this approach.

12.2 T-Cell Responses to Cancer

The ability of the immune system to control or eliminate cancer has been a subject of two conflicting hypotheses. The antigenic hypothesis states that cancers arise quite often and [1] that the immune system has the ability to recognize tumor cells bearing aberrant cellular antigens and eliminate them. In this view, cancer immunity is part of healthy somatic homeostasis. The alternative tolerogenic hypothesis states that we see cancer in the clinic because immunity often, or usually, fails. In this scenario, with respect to clinical disease, the immune system is, at best, irrelevant. In view of this, how is the role of the immune system in tumor elimination quantitatively defined?

In one transgenic mouse model of pancreatic cancer, tumors were generated by placing the SV40 virus oncoprotein, large T-antigen, under the control of the insulin promoter. Tumor senescence in this model could be induced via the combined action of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF), and is p16INK4a-dependent [1]. Both TNFR1 and STAT1 were required for the tumor to be responsive to immune control. In this model system, the control of cancer growth was quantifiable and intimately dependent on $CD4^+$ cell-based Th1 immunity. However, in a different model system featuring spontaneous and rare induction of a T antigen-driven tumor, representing a truly autochthonous model, it was demonstrated that spontaneous tumors are inherently tolerogenic [2]. It means that as tumors arise, the immune system is prevented from mounting an immune response. Nevertheless, immunization with tumor antigen prior to the onset of tumors did prevent tumor outgrowth even in this model. These basic observations highlight our current understanding of tumor immuno-surveillance (reviewed in [3]) in which both antigenic and tolerogenic signals play a role in disease recog-

nition and elimination. The discovery of T-cell checkpoint inhibitors and the associated important clinical breakthroughs demonstrate the ability of T-cells to mediate antitumor immunity once tolerogenic signals are inhibited. Checkpoints are inhibitory T-cell molecules, such as CTLA-4 and PD-1, that play a role in physiologic T-cell responses, by preventing extensive and prolonged activation of T-cells. Expression of these molecules on activated T-cells, followed by binding to a specific ligand expressed on the target cell, helps prevent T-cell exhaustion, activation induced cell death, and excessive inflammatory activity. However, checkpoint mechanisms are often hijacked by tumors in order to avoid elimination by activated T-cells. Using checkpoint blockade agents, either anti-CTLA4 antibody, anti-PD-1 antibody, or anti-PD-L1 antibody, tumor-induced inhibition of effector T-cells can be ablated, and significant clinical antitumor responses have been demonstrated using this approach [4, 5]. This indicates in patients that even while an autochthonous tumor may be actively inducing tolerance in T-cells, T-cells are present in the host that have the potential to respond. Once the negative signals are blocked, antitumor immunity can indeed result. These important findings have only increased the drive to develop new adoptive immunotherapy approaches for cancer featuring activated T-cells.

12.3 From Polyclonal to Single-Specificity Effector T-Cells

One of the most informative breakthroughs in adoptive immunotherapy was seen through a direct clinical intervention. Following allogeneic bone marrow transplantation (hematopoietic stem cell transplantation (HSCT)) for leukemia, some patients who relapsed with their disease following HSCT could be treated into remission by the reinfusion of lymphocytes from the original bone marrow donor (donor leukocyte infusion, DLI). For this purpose, the original donor has to be re-contacted for additional leukocytes harvest or the donor cells have to be harvested and consent obtained ahead of time. Despite the logistic complexity, this approach often has therapeutic benefit. The general mechanism by which the infused lymphocytes cause disease regression relies on the fact that while the newly grafted immune system in the patient is donor in origin, the relapsed disease is still derived from the original "self" hematopoietic system, and thus the leukemia is still able to be recognized by the graft as "nonself." The induction of tolerance in the original graft is also clearly demonstrated in this clinical situation, as the immune system that develops in the presence of residual disease is unreactive towards the leukemia—although it bears "patient-self" or "graft non-self" antigens—and relapse occurs. The antileukemic effect seen with infusion of donor leukocytes into the relapsed patient demonstrates that leukemia-reactive cells do reside in the donor repertoire and they are able to effect antileukemic immune responses if they have not been tolerized.

The major toxicity of DLI is graft-versus-host disease (GVHD), which is related to the overall dose of infused T-cells [6]. Toxicity notwithstanding, DLI is able to make a major impact on relapsed chronic myelogenous leukemia but is less effective in other hematologic malignancies, reviewed in [7]. In ongoing effort, different groups are attempting to identify the antigenic specificity of the effector T-cell populations that mediate the antileukemia effect seen in the DLI product. It is hoped that as we learn what the effective cellular immune targets are, we can focus on increasing the frequency of these cells and decreasing the number of cells causing GVHD. Recent studies have demonstrated that TCRαβ cells are responsible for GVHD in haploidentical allografts in leukemic patients [8]. By contrast, TCRαβ-depleted and CD19-depleted haploidentical leukocyte transplants can effectively mediate tumor rejection and are not associated with GVHD [8–10]. This is due to the fact that TCRγΔ T present in the allografts post-depletion are capable of efficient engraftment and potent antileukemic responses [11].

In another approach, leukemia-specific antigens, which would allow precise targeting of leukemic cells by graft leukocytes, are sought out. Termed minor histocompatibility antigens (mHAgs), these antigens represent distinct HLA-binding peptides encoded by polymorphic genes that differ between donor and recipient. Engrafted donor T-cells are thought to be responsible for graft-versus-leukemia effect (GVL) via

recognition of mHAgs. Several mHAgs have already been defined. The first class I-MHC-restricted mHAgs identified were HA-1 and HA-2 [12]. The antigenic entity, encoded by the HMHA1 gene, is a single amino-acid polymorphism that results in a dominant immunogenic peptide for one allele, HA-1 (H), while the HA-1 (R) allele is essentially a "null" phenotype due to unstable HLA-class I binding [13]. Griffioen et al. identified the HLA-DQ presentation of the autosomal gene phosphatidylinositol 4-kinase-type IIβ as a DLI target in a chronic myeloid leukemia (CML) patient receiving DLI [14], reasoning that HLA class II antigens may be less broadly presented throughout normal tissues and thereby less prone to induce GVHD. More recently, four novel HLA-B-restricted and four novel HLA-DR-restricted minor histocompatibility antigens that may mediate GVL reactivity have been identified [15, 16]. This steady progress in uncovering effective immune responses in the context of HSCT is one means to unravel how polymorphisms in commonly expressed genes may be used for antitumor immunity. One caveat is that HSCT is studied in a very unique context. As long as the antigen is restricted to the malignant cells or the original host immune cells, anti-leukemia reactivity can be expected to result. The degree to which antigenic targets are expressed on the non-transplanted host tissues is likely to be a direct correlate of GVHD and remains the major limitation of current approaches.

Another polyclonal T-cell approach to the adoptive immunotherapy of cancer was also developed in the context of HSCT. Prior to the development of anti-CD20 monoclonal antibody (mAb) therapy, the development of Epstein-Barr virus (EBV)-driven post-transplant lymphoproliferative disease (PTLD) was a devastating complication [17]. In these patients, the onset of PTLD was related to the degree of T-cell depletion in the marrow product. In order to counter this, investigators designed methods to expand donor-derived EBV-specific T-cell products and to make their administration part of the HSCT regimen [18]. As in DLI, continued description of the antigens associated with EBV-driven disease, the discovery of other non-viral tumor-associated epitopes, and the refinement of techniques to expand reactive T-cells have led to the continued expansion of adoptive immunotherapy approaches to human cancers [19].

The immunotherapeutic approach with perhaps the greatest demonstrated degree of efficacy, albeit in a restricted group of patients, is the treatment of patients with advanced melanoma with tumor-infiltrating lymphocytes (TILs), yielding 50% overall response and 20% tumor-free survival in patients with relapsed or refractory metastatic melanoma [20]. The ability to culture and expand TILs from tumor biopsy material remains the primary therapeutic bottleneck. However, when the infusion of TILs is combined with lympho-depletion of the host, transferred TILs persist long term and complete cures are seen. The preparative regimens developed for HSCT to deplete the host immune system, that is, the conditioning regimen, have proved essential in creating space for the therapeutic TIL to expand and eradicate melanoma. Whether this space is physical, where niches are made available in the host for the transferred cells to reside and receive growth signals, or it is a potential space created by decreased lymphocyte counts and the subsequent soluble mediators released by the host to increase lymphocyte counts that also increase the number of transferred cells, or an immunologic space wherein negative regulatory lymphocytic or myeloid populations are removed, has yet to be fully resolved and likely all of these factors may contribute. The combination of host conditioning and methodological advances in generation of high-quality effector T-cell populations has opened the door to a completely new universe of therapeutic options. The molecular characterization of individual TIL TCR specificities allowed this approach to be refined even further wherein a retroviral gene vector encoding a single TCR specific for the MART-1 antigen was used for the adoptive immunotherapy of melanoma by T-cells [21, 22]. Additional transgenic TCR specificities presently under clinical investigation include MAGE-A3 and MAGE-A4 for solid tumors [23, 24], NY-ESO-1 for melanoma and multiple myeloma [25, 26], WT1 for myeloid malignancies (NCT01621724, clinicaltrials.gov), HPV-

16 E6 and E7 for HPV-associated cancers ([27], NCT02858310), and thyroglobulin for metastatic thyroid cancer (NCT02390739). Identification of patient-specific autologous neo-epitopes and cognate tumor reactive TCR clonotypes, which can then be used to generate transgenic TCRs, has been accelerated in the recent years with the advent of screening methodologies [28–31]. This is the full logical extension of exploiting single TCR specificities present in the polyclonal TIL population. In summary, the scientific principles of infusing T-cells that have the capacity to recognize and lyse tumor cells have been firmly established. The next step, the creation of chimeric antigen receptors (CARs), allowed for another limitation of T-cell-based therapy, that is, the requirement of peptide–MHC interactions for therapeutic effect, to be side-stepped.

12.4 From MHC to Antibody-Based Recognition: Therapy with T-Cells Expressing CARs

12.4.1 History of CAR Development

In 1987–1989, it was shown for the first time that the binding domains from a hapten-specific antibody could be joined to the constant domains of a TCR and successfully trigger T-cell activation [32, 33]. Using this concept, studies led by Eschar et al. soon demonstrated that ovarian carcinoma cell lines could be lysed by T-cells transduced with a retroviral vector expressing a chimeric antigen receptor (CAR) specific for the folate receptor, in which a single transcript encoded an extracellular antigen-binding motif combined with an intracellular T-cell signaling motif [34]. The specific lysis of tumor cell lines by T-cells engineered to express CARs was greeted with moderate interest, but in hindsight, it was clearly a watershed moment in the history of adoptive immunotherapy. Currently, many different scFv-based CARs have been developed that target tumor-associated antigens (TAAs) from various malignancies, and both antigen-specific cytolytic activity in vitro and antitumor effects in animal models have been demonstrated [35–40].

Compared with T-cell receptor (TCR), one of the advantages of CAR-modified T-cells is that they respond to antigens in a non-MHC-restricted manner and therefore can be used to treat patients with different MHC haplotypes or target tumor cells with downregulated MHC expression. Another feature of CARs is their expanded range of potential targets. CARs can be created which bind not only protein structures but also carbohydrate and glycolipid. Potentially, any cell surface tumor-restricted antigen could be used as target. A novel exception is a newer generation CAR wherein the scFv used to create them is derived from an antibody specific for a peptide–MHC molecule [41, 42].

12.4.2 CAR-T Design

The principal elements used in CAR-T design are depicted in Fig. 12.1a. A first-generation CAR-T molecule is comprised of an antigen-binding domain of choice (i.e., anti-CD19, anti-CD20, anti-CD22, or anti-Sp6, control), usually derived from an scFv, which is linked via an extracellular hinge to the transmembrane domain (often derived from CD8 or CD28) to a signaling domain, usually the ITAM-containing regions of the CD3ζ chain molecule (CD247, part of the TCR receptor complex). Second- and third-generation CARs also contain one or two co-stimulatory domains respectively, which may be derived from CD28, 4-1BB, OX40, or other signaling molecules, and provide additional stimulation or persistence for CAR-T function (Fig. 12.1b). To further refine CAR-T technology, subunit CARs and Tandem CARs were designed, which allow for targeting multiple tumor antigens as a means of mitigating tumor antigen escape. For example, tandem CARs have a "Boolean OR" gate function and can eliminate target cells even if one antigen is lost. Better control of on-target off-tumor toxicity and fine-tuning of CAR-T activation can be approached using a "Boolean AND" gate approach, such that engagement of both CAR binders is required for full functionality (Fig. 12.1c). Linking two scFv domains into one CAR in tandem also requires flexible linkers, such as the oft-used glycine-

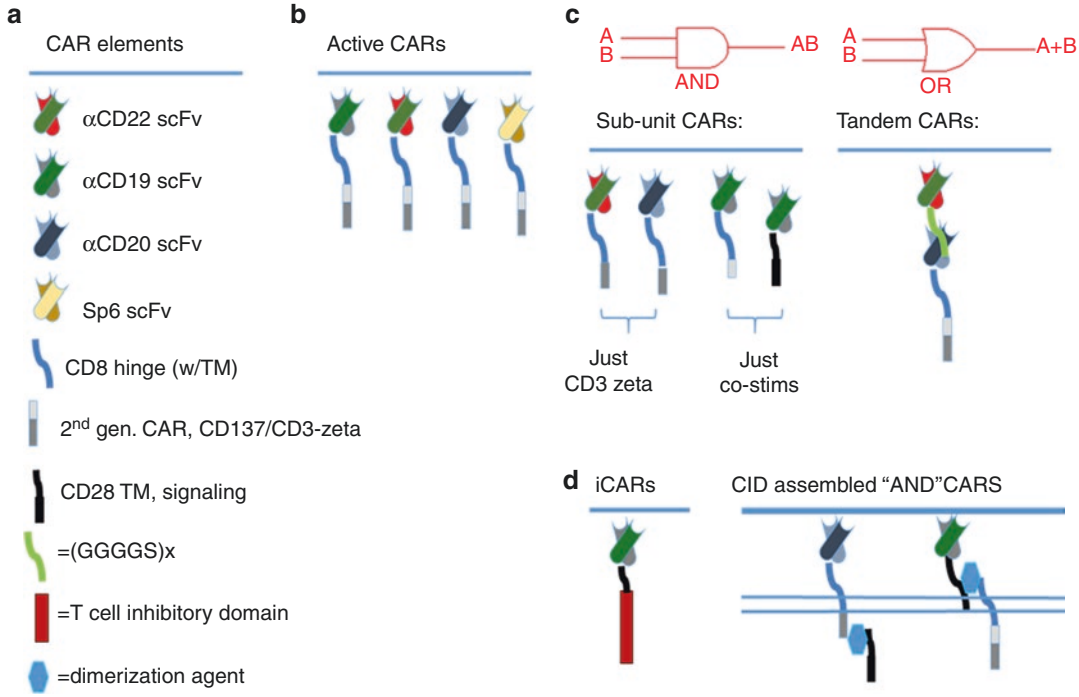

Fig. 12.1 Common CAR-T design elements. T-cells can be designed to express complex CARs created from core design elements (**a**) that include scFv domains that bind antigen (shown are CD22, CD19, CD20, and control Sp6 ScFv), hinge and transmembrane (TM) domains, intracellular signaling domains (linked CD137/4-1BB and CD3-zeta or CD28), linkers to create more complex tandem structures (**c**), as well as inhibitory (**d**) or CID (**d**, chemically induced dimerization) domains. Assembly of a binder with a hinge/TM domain and intracellular signaling domains results in an active CAR (**b**). Splitting activation domains (**c**) can result in a Boolean "AND" gate where the binding of two antigens is required for full activation of the T-cell while combining the binders in one chain creates a Boolean "OR" gate wherein target cells expressing either antigen will induce T-cell activation

serine poly-linker $(GGGGS)_x$, where x = integer repeats, usually less than 5 (Fig. 12.1a). Finally, inhibitory CARs (iCARs) which employ an inhibitory, rather than a stimulatory intracellular signaling domain, may be used as an additional safeguard against unwarranted CAR T-mediated toxicity by inhibiting CAR-T function against normal cells expressing the antigen recognized by the iCAR binding domain (Fig. 12.1d). Switch-controlled CARs (with a CID, chemically induced dimerization domain) require the addition of a synthetic dimerization agent for activation. When the dimerizing agent is present, the cell-surface targeting domain binds with the intracellular signaling domains, and a fully functional CAR molecule is thus assembled. This adds an additional layer of control of CAR temporal activation, resulting in greater safety (Fig. 12.1d).

Advances in CAR-T design and manufacture will require engineering of T-cells in manner that generates a cell product with predictable efficacy, controllable activation, and designed biological distribution. Combining the elements currently available in CAR design will continue to allow for greater potency and engineered control of the CAR-T therapeutic product. As shown in Fig. 12.2, the selection of initial cell substrate, inclusion in the transducing gene vector of auxiliary factors in addition to CAR-T template (such as pro-inflammatory cytokines or factors capable of neutralizing the inhibitory effect of tumor microenvironment), usage of the next generation "gated" CARs with tighter activation controls, and superior target selection will yield significant improvements in adoptive CAR-T therapies in the future.

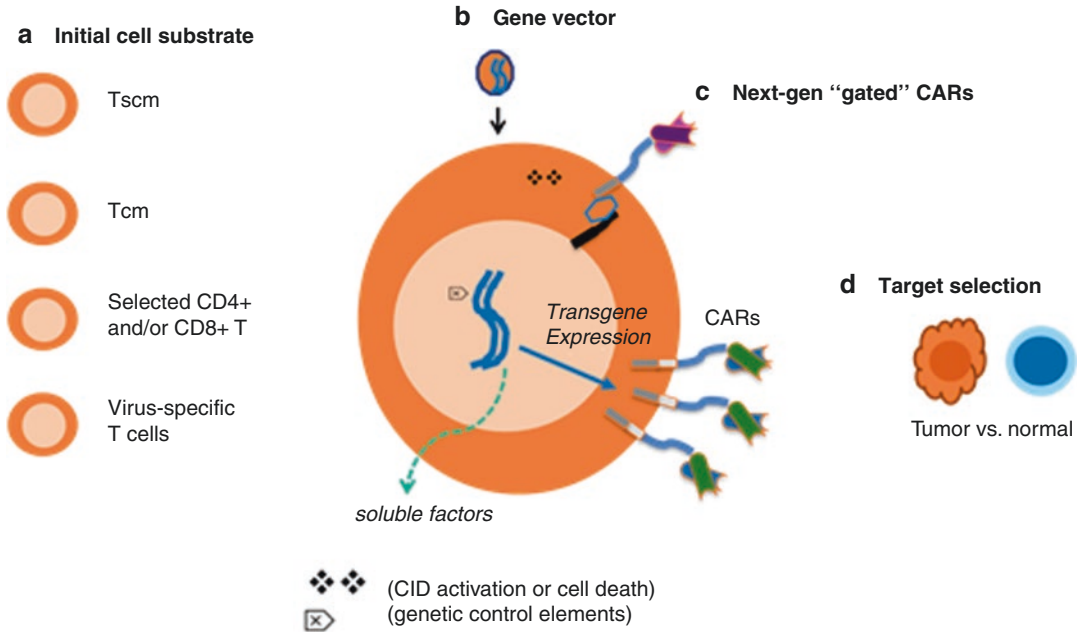

Fig. 12.2 Engineering T-cells to increase product uniformity and function. The creation of an engineered therapeutic T-cells requires optimization by (**a**) choosing the appropriate starting cell population (Tscm, stem cell memory, Tcm, central memory, phenotyping selection of just CD4 and CD8 cells, or virus-specific T-cell populations), (**b**) choosing the appropriate gene vector, (**c**) including enhanced engineering functionality such as CIDs (chemically induced dimerization domains), genetic control elements such as transcriptional switches, and the inclusion of other soluble factors such as binders or cytokines in the transgene package, and (**d**) choosing the appropriate target that is expressed at high levels on the tumor but not on normal tissue

12.4.3 Inclusion of T-Cell Co-stimulatory Moieties

CARs that include only one intracellular signaling motif are called "first generation." Almost always, first-generation CARs include a signaling domain derived from the TCR signaling complex member CD3ζ in their cytoplasmic domain. One notable exception is linkage of the extracellular antigen-binding domain of the CAR to the CD3 ε chain (developed by TCR^2 Therapeutics). While T-cells expressing first-generation CARs demonstrated target cell-specific cytolytic activity in vitro, initial clinical studies were disappointing. The tumor responses were modest and the persistence of the infused cells was limited [43, 44]. A number of factors may contribute to the lack of expansion or persistence of CAR-modified T-cells in vivo, which is notably different from the behavior of adoptively transferred antigen-specific CTLs. One explanation is that T-cell activation requires both *TCR* engagement (signal 1) and co-stimulation provided by antigen-presenting cells (APCs, signal 2). Since tumor cells are deficient in co-stimulatory molecule expression (cell surface glycoproteins such as CD80 or CD86), CAR-redirected T-cells would not experience co-stimulation when engaging with a tumor cell. Moreover, T-cells may not receive tonic activation through the stimulation provided by antigen-presenting cells in secondary lymphoid organs. These deficiencies were overcome in the design of second-generation CARs, in which co-stimulatory signaling domains derived from CD28, 4-1BB, inducible T-cell co-stimulator (ICOS), OX40, or DAP10 were added in addition to the CD3-zeta signaling domain. In murine models, second-generation CARs displayed superior activity over first-generation CARs, showing improved proliferation, survival, and development of memory cells [45–47]. The

enhanced persistence imparted by CARs with two signaling domains has been further confirmed by treating CD19+ lymphoma patients with a mixture of T-cell transduced with either first-generation CD3ζ or second-generation CD28/CD3ζ CD19-CARs [48]. In this clinical study, six patients with B cell lymphomas were simultaneously infused with two autologous T-cell products expressing first- and second-generation CARs targeting CD19. CAR+ T-cells containing the CD28 endo-domain had a strikingly enhanced expansion and persistence compared with CAR-T-cells lacking this endo-domain [48]. Different co-stimulatory molecules may also deliver different signals, resulting in different functional outcomes. When the antitumor efficacy of second-generation CARs constructs with CD28/CD3ζ or CD137 (4-1BB)/CD3ζ were compared using CARs targeting CD22 or CD19 in mouse xenograft models, T-cells expressing CARs including a 4-1BB signal motif led to more robust antitumor activity in vivo [46]. However, in a mesothelioma tumor model, equal antitumor efficacy for CD28 and 4-1BB containing second-generation CARs was seen [49]. In an attempt to further optimize CAR design, several groups have developed third-generation CARs that contain two co-stimulatory domains combined with the CD3ζ chain. However, reported results differ between second- and third-generation CARs. Notably, co-stimulatory endo-domains play a role in CAR-T exhaustion or persistence. CAR CD3ζ chain phosphorylation, triggered by spontaneous clustering of CAR molecules on cell surface, can induce premature exhaustion of CAR-T-cells and limit persistence. In the case of anti-GD2 CAR, the exhaustion was greater if the CD28 co-stimulatory domain was used, in comparison to constructs including the 4-1BB domain [50]. The optimal signaling endo-domains to be included in CAR vectors for conferring optimal T-cell antitumor effects in vivo remains an active field of research, and the variables to be overcome have yet to be fully defined. The challenges may be as varied as the mechanisms by which tumors escape immuno-surveillance.

12.4.4 CAR-T Technological Improvements

12.4.4.1 Safety Switches

A significant effort is dedicated to refining CART therapy in order to make it safer. Concerns associated with CAR safety include "on target/off tumor" toxicity and the cytokine storm related to immune response associated with a large tumor burden. One vector-based option to mitigate these risks is to use a suicide gene to allow the elimination of CAR-T-cells in vivo. One extensively studied suicide gene is the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) system. GCV is activated by HSV/TK forming a monophosphate that is converted into its di- and triphosphate forms by cellular kinases. The triphosphate GCV is then incorporated into replicating DNA, resulting cell death through DNA polymerase inhibition. Bonini et al. utilized this strategy to deplete HSV-TK-expressing allogeneic lymphocytes effectively following HSCT [51]. However, the depletion is not always complete, and the foreign TK protein displays significant immunogenicity [52]. A suicide switch strategy employing modified Fas has been evaluated in vitro and in non-human primate model as well [53].

A more recent approach features inducible caspase 9. When vector-encoded iCaspase 9 is expressed, a pair of inactivate subunits are created. These are induced to form an activate dimer by a small molecule (AP1903), resulting in rapid cell death (as soon as 30 min after drug administration). This approach has been reported to control GVHD in recipients of haplo-identical HSCT [54]. Since the caspase 9 is of human origin, it is likely to be less immunogenic than HSV-TK. As the iCaspase 9 system directly induces cell death, DNA synthesis and cellular replication are not required to eliminate transduced cells, and therefore cell death is much more rapid. Another approach features equipping CAR-T-cells with an extracellular protein tag that can be bound by an injected clinical-grade antibody, leading to CAR-T-cell elimination via CDC (complement-dependent cytotoxicity) or

ADCC (antibody-dependent T-cell cytotoxicity). In one example of this approach, CAR-T-cells are endowed with a truncated epidermal growth factor receptor (tEGFR). This protein is encoded in the same lentiviral backbone as the CAR, the two open reading frames separated by a ribosomal skip sequence (2A) [55]. The tEGFR construct is composed of EGFR ecto-domains III and IV, and the transmembrane domain of the native human EGFR protein, but it excludes the functional dimerization and signaling domains of the native EGFR; thus, it is devoid of ligand binding or signaling activity. The tEGFR epitope is still recognized by the therapeutic antibody Cetuximab, thus enabling in vitro selection and tagging of CAR-transduced cells, and may be utilized as a CAR-T ablation switch. Although clinical efficiency of CAR-T depletion using this approach has not been determined, efficient elimination of tEGFR-tagged CAR-T-cells has been demonstrated in an NSG mouse tumor model [56]. Another protein that has been used as a CAR-T tag is CD20. This protein is amenable to detection by clinical grade anti-CD20 monoclonal antibody Rituximab. T-cells can be transduced either with a whole length CD20 molecule or with a CD20 tag. CD20 mimotopes have also been used pre-clinically to deplete CAR-T-cells in a tumor mouse model [57]. However, unwanted depletion of $CD20^+$ B cells and inadvertent depletion of gene-modified T-cells when treating $CD20^+$ EBV tumors with rituximab are limitations to be considered.

In addition to affording T-cell elimination in vivo, polypeptides tag may be used for identification of CAR-T positive cells by flow cytometry, and for enrichment of CART-cells during production. Such enrichment may be beneficial as it may allow to lower total dose of infused T-cells to be used. Non-transduced T-cells have been postulated to contribute to the toxicities associated with CART treatment, such as cytokine release syndrome (CRS). Along with CD20 and tEGFR tags discussed above, extracellular tags derived from truncated LNGFR, CD34, CD19, CD4, and glycosylphosphatidylinositol-anchored CD90 have been explored for identification and selection of transduced cells [58–61]. The tag approach was further refined in the generation of a short protein sequence combining epitopes of CD20 and CD34 termed RQR8, which is amenable to both CART enrichment and as a suicide switch using either Rituximab or Ofatumumab [57].

12.4.4.2 Deletion of Native Surface Proteins in CART-Cells

In the allogeneic hematopoietic stem cell transplantation setting (HSCT), donor-derived T-cells can be redirected by CAR vectors to achieve clinical response independent of MHC restriction. However, continued cell surface expression of TCRs from an HLA-disparate donor can cause GVHD upon adoptive transfer. In order to generate universal allogeneic CAR-T-cells for multiple recipients, Torikai et al. designed a zinc finger nuclease (ZEF) strategy to irreversibly knock out the endogenous TCRα and TCRβ chains [62]. Their data showed that disrupting endogenous *TCR* expression in CD19 CAR-T-cells did not alter killing of cells expressing the CAR target antigen. A similar strategy was employed by Qasim et al., where universal CAR-T-cells were generated by TALEN-mediated disruption of the TCR α constant chain region in T-cells from an allogeneic donor, thereby abrogating TCR expression and reducing the risk of GVHD [63]. Simultaneously, a second gene was deleted in T-cells in this study. CD52 was also edited using TALEN, thereby enabling selective ablation of leukocytes by anti-CD52 antibody, but sparing the genetically modified CART-cells [63]. For a universal drug product to be utilized, the deletion of HLA genes from the surface of allogeneic CAR-T-cells has also been proposed to prevent rejection by the host.

Other engineering approaches include deletion of checkpoint blockade genes such as PD-1, Tim-3, and Lag-3 to fortify CART-cells against exhaustion induced by tumor cells expressing ligands to these proteins, or endowing resistance from activation-induced cell death (AICD) by deleting the Fas (CD95, APO-1) protein. Multiple T-cell genes can be erased at the same time using the CRISPR/Cas9 system, and the

feasibility of deleting up to four genes in parallel with CART transduction has been demonstrated [64]. Directing CAR19 insertion into TCR-α locus using CRISPR/CAS9 approach was shown to carry a dual benefit in disrupting native TCR expression and enhancing the antitumor activity of the CAR [65]. If successfully expanded to large production scale, these deletion strategies may provide means to generate "universal" CART products to treat multiple patients.

12.4.4.3 Switch-Controlled CARs

Another means to control CAR-T function is to create soluble binding domains that can dissociate from the T-cell-expressed extracellular associative domain, linked to transmembrane and signaling domains. This creates a functional switch, whereby dissociation of the binding domain results in the inability to engage transgene-expressing T-cell.

The use of a soluble switch CAR, such as "biotin CAR," is another approach to making CAR therapy universal. In some studies, CAR-redirected T-cells caused initial tumor regression, but tumor relapse was observed due to the outgrowth of tumor with antigen-loss variants. In order to target tumors with heterogeneous antigen expression, a uniform CAR vector could be used, which expresses extracellular avidin linked to intracellular T-cell activation domains. Transduced T-cells would then be coated with biotinylated antigen-specific binding molecules (termed as biotin-binding immune receptor (BBIR)) [66]. The versatility afforded by BBIRs permits sequential or simultaneous targeting of a combination of distinct antigens. This platform also holds the potential for a high-throughput means to screen and select novel scFvs for the generation of single-specificity CAR constructs [66]. In this vein, a novel modular approach termed UniCAR has been recently developed [67]. UniCAR system consists of a soluble module, which is comprised of a tumor antigen-targeting binding domain fused to a unique peptide epitope E5B9, and an effector module, which is comprised of a T-cell expressing the E5B9-targeting domain fused to transmembrane and intracellular CAR domains. Another group has developed a similar approach termed sCAR-T [68]. In application of this technology, the effector module, that is, CAR-T-cells, will be infused into patients along with one or more soluble module(s) targeting antigens of choice. Since the soluble module has short half-life, the duration of each treatment will be controlled by the length of time when the soluble module is infused, with the goal of controlling potential on target/off tumor toxicity.

12.4.4.4 Reducing CART Immunogenicity

Another potential problem that may arise during CAR-T therapy is immunogenicity of CAR-T sequences to the host. In the worst-case scenario, CAR immunogenicity may lead to CAR-T graft rejection and treatment failure. CAR-induced immunogenicity may also lead to adverse immune reactions in the host, which may be life threatening. In one approach utilizing transiently transfected CAR-T-cells containing murine-derived scFv domain targeting mesothelin (CART-meso), repeat infusions were necessary in order to sustain therapeutic effect. Utilizing this approach in a phase I study of malignant pleural mesothelioma (NCT01355965), multiple infusions of CART-meso cells were administered, and some patients have developed anti-CAR human anti-chimeric antibodies (HACAs) or human anti-mouse antibodies (HAMAs) [69]. Moreover, one patient treated with CAR-meso developed anaphylactic shock following the third infusion of the product, thought to be mediated by IgE antibodies elaborated against CAR-T-cells [70]. Historically, CAR antigen-binding domains were derived from linked mouse Fv heavy and light chain sequences (scFv), and they are one potential source for immunogenicity. A number of pre-clinical and early clinical studies are now using humanized scFv sequences or sequences derived from human antibody libraries [71–73], thus reducing the risk of anti-CAR reactivity in the host. However, even when fully human sequences are used in CAR design, fusion sites between different structural components of the CAR and joining synthetic linkers are potentially immunogenic. Effort has been made to pre-emptively identify immuno-

genic risk of CAR sequences in silico by identifying putative immunogenic peptides that can be presented to $CD8^+$ T-cells in the context of MHC I, and to alter their sequences to abrogate immunogenic potential [72].

12.4.4.5 Mitigating Tumor Antigen Escape

A critical hindrance to lasting cancer remissions in CAR-T therapy is tumor antigen escape. While instances of tumor escape may be attributed to short persistence of CART-cells, phenotypic changes in the tumor, or the inhibitory effect of tumor microenvironment, loss of tumor antigen from tumor cell surface, aka tumor antigen escape, remains a major problem. Despite the short-term success of CAR19 therapy in B cell malignancies, a substantial fraction of all patients relapses with loss of CD19 antigen [74–77]. It has been postulated that tumor antigen escape can be prevented by simultaneously targeting several tumor antigens. Pre-clinical studies have demonstrated the feasibility of combining targeting CD19 and CD20 by tandem CAR constructs containing two scFv antigen-targeting domains linked sequentially in CAR ecto-domain [78, 79]. Schneider et al. have demonstrated that the tandem CAR construct CAR2019, targeting CD19 and CD20 tumor antigens simultaneously via linked targeting domains expressed by the same CAR-T molecule, was less prone to induce CD19 antigen loss on tumor cells than the single targeting CAR19, and that tandem CAR2019 had high antitumor efficacy and favorable toxicity profile as compared to either CAR19 or CAR20 alone, or a mixture of single-transduced CAR19 and CAR20 in a high-tumor-burden NSG mouse model system ([79], NCT03019055, clinicaltrials.gov). Similarly, tandem CARs targeting CD19 and CD123 antigens simultaneously were shown to prevent tumor antigen escape of leukemic blasts in vivo, and were more effective than each CAR individually or a mixture of two CAR-T populations each targeting one antigen [80]. In solid tumors, tandem CARs simultaneously targeting Her2 and IL13Rα2 antigens successfully controlled tumors and mitigated antigen escape in a mouse model of glioblastoma [81]. Furthermore, by virtue of targeting two antigens, tandem CARs had greater level of activation, but not exhaustion, which is a highly desired CAR-T therapy feature, especially in the context of treating solid tumors [81]. Future studies will determine the effectiveness of mitigating tumor antigen escape by simultaneously targeting multiple tumor antigens.

12.4.5 Vectors Used for CAR Expression

Current methods used to introduce DNA or RNA encoding CARs into effector T-cells are built on the approaches that gave success in *TCR* gene transfer and include both viral vector and nonviral delivery systems. Gamma-retroviral vectors have been used as for gene transfer for more than 20 years and include the MFG/SFG, MP71/SF91, and MSGV1 vector systems [82–84]. Genes encoded by these vectors integrate into the host genome and give consistent CAR expression in T-cells and their daughter cells. However, gamma-retrovirus vectors can only infect dividing cells and prefer to integrate near transcriptional start sites, raising concerns about insertional mutagenesis, as had been reported for CD34-expressing bone marrow progenitor cells [85, 86]. Nevertheless, retroviral gene transfer has shown acceptable safety and efficiency for the expression of CAR genes in mature human lymphocytes derived from peripheral blood [87]. To date, there has been no report of insertional oncogenesis or clonal overrepresentation in gene-modified mature lymphocytes harvested from peripheral blood using gamma-retrovirus-based vectors [88]. Lentiviral vectors offer certain advantages over gamma-retroviral vectors. Lentiviral vectors can transduce non-dividing or minimally proliferating cells and therefore are more likely to transduce less differentiated or naïve T-cells. This may be beneficial for therapy as these cell types are thought to undergo less activation-induced cell death and reduced clonal exhaustion, as is seen in more rapidly dividing cell types. Compared with gamma-retroviral vectors, lentiviral vectors also have larger gene

insertional capacity and are at present considered to be less prone to insertional mutagenesis [89].

Transposon-based nonviral gene delivery systems, such as *sleeping beauty* and *PiggyBac* vectors [90–92], also appear to have random genomic integration profiles with acceptable gene transfer efficiency and are currently being developed as vectors for CAR expression in T lymphocytes. These nonviral delivery systems have the potential to greatly reduce the cost of vector manufacture. Some groups have reported that electroporation or nucleofection of RNA yields high levels of CAR expression in transfected lymphocytes [93]. Due to the short half-life of transduced RNA expression post transfer, this approach may require multiple CAR-T-cell infusions to achieve a clinical response. Nevertheless, transient expression approaches to somewhat minimize the safety concerns of CAR therapy caused by genomic vector integration, may limit host toxicity due to transient transcript expression, and may also avoid the requirement for extensive ex vivo activation and expansion, allowing for better persistence of CAR-T-cells in vivo.

12.4.6 Impact of T-Cell Culture and Expansion Techniques

In current clinical trials, human lymphocytes have been activated with agonistic mAb-mediated CD3 stimulation, with or without additional CD28 co-stimulation, prior to transduction with CAR-encoding gene vectors. CAR-modified T-cells then expand to large numbers in high-dose IL-2 culture conditions. This tends to generate very mature T effector (Teff) cells. Growing evidence suggests that "younger" cells (naïve or central memory-like) may better engraft and persist in vivo and have longer-lived antitumor potency [94–96]. A recently defined stem cell-like T-cell population (Tscm) has shown stronger engraftment potential and more effective antitumor activity in adoptive cell therapy in model systems [97]. Alternatively, evidence from other studies demonstrated enhanced efficacy when T-central memory (Tcm) cells were redirected by CARs [98, 99]. Studies are under way to optimize methodologies for isolation of defined cell subsets under good manufacturing practices (GMP) for human clinical trials. For example, enriching T-cell subsets based on the expression of the phenotypic markers CD62L, CCR7, and CD45RO using immunomagnetic beads could be employed. Another challenge is how to expand or maintain a phenotypically younger cell population during in vitro culture. Efforts to explore other gamma-chain cytokines besides IL-2, such as IL-15, IL-7, or IL-21, for the expansion of therapeutic T-cell populations aim to modulate the resultant T-cell phenotypic and functional profiles [100, 101]. One study where $CD4^+$ and $CD8^+_{CM}$ cells were isolated, manufactured separately, and then formulated at a defined $CD4^+$ $CD8^+$ ratio of 1:1 has shown efficacy, successful CART engraftment, and relatively low toxicity, thus paving the way for safer CAR-T regimens [75].

Small molecules known to modulate key metabolic and developmental pathways are also being tested for their ability to restrict T-cell differentiation. These include the mTOR pathway inhibitor rapamycin [102] and the GSK3b inhibitor TWS119 [103]. However, both inhibitors prevent T-cell proliferation in vitro and may not allow sufficient in vitro expansion. The ideal agent would promote Tcm-like or Tscm-like phenotypes (or other selected phenotypes) to be maintained without limiting cell expansion.

In addition to altering the cytokine milieu in vitro during transduced T-cell expansion, the CAR vector itself can also encode cytokine support. This strategy provides autocrine support for T-cell function, proliferation, or persistence and can favorably alter the tumor microenvironment upon therapeutic T-cell infusion. T-cells expressing vector-encoded IL-15 or IL-2 have increased viability and proliferative capacity in vitro despite withdrawal of exogenous IL-2 [104, 105]. IL-7-, IL-12-, or IL-21-secreting T-cells have been used to expand antigen-specific cells in vitro and have demonstrated enhanced tumor killing in animal models [106, 107]. Several groups have reported that CAR-T-cells transduced to also express a conditionally released

IL-12 demonstrated greater antitumor potency than T-cells expressing the CAR alone [108–111]. In these studies, IL-12 was controlled by a nuclear factor of activated T-cells (NFATs) responsive element, which was activated following T-cell activation by engagement of specific CAR ligand [112]. However, this approach requires further refinement, as a clinical study evaluating an inducible IL-12 vector featuring melanoma-specific TIL was recently terminated due to unexpected toxicities and a lack of durable responses (NCT01236573).

As with cytokines, co-stimulatory support with cell surface receptors can be engineered into T-cells independent of the actual CAR. Vectors that encode ligands from the immunoglobulin (Ig) superfamily or the TNF receptor family, including CD80 and CD137L (4-1BBL), are known to enhance T-cell proliferation and cytokine production upon antigen engagement [113]. In order to render CAR-modified T-cell targets more tumor specific, alternative strategies are being developed. Co-expression of two CARs in the same cell that separately deliver T-cell activation signals and co-stimulatory signals to the cell while engaging two distinct tumor antigens is being developed. Kloss et al. demonstrated that T-cells modified by both a CAR targeting prostate stem cell antigen (PSCA) with a suboptimal activation profile and a chimeric co-stimulatory receptor (CCR) targeting a second antigen, prostate-specific membrane antigen (PSMA) resulted in regression of tumor where both antigens are expressed [114]. This combinational antigen recognition strategy is one means to enforce stricter tumor specificity [115]. Strategies like this will become increasingly important as tumors that do not express a single antigen that distinguishes them from host normal tissue are described. In fact, one study was able to rank different pediatric tumors according to the degree of overall difference between their cell surface antigens and those expressed on normal tissue [116]. In this way, bioinformatics will continue to identify target antigens, which subsequently must be analyzed for actual protein expression in tissue arrays.

12.4.7 Clinical Advances in CAR Therapy

When the renowned oncologist and geneticist Wacław Szybalski coined the term “synthetic biology” in 1974, he was referring to the creation of whole genomes [117]. Herein, the term is adopted to refer to the creation of a synthetic protein based upon the understanding of protein subunit function. In this way, a new protein product that has never been encoded as a functional unit in the genome itself is expressed by means of gene vector technology. Insertion of the DNA encoding this unit using a viral gene vector makes this a permanent genomic alteration that will affect the function of the transformed cell for as long as that gene is expressed. To this view, the recent success seen in the clinic with T-cells engineered to express a CAR specific for the B cell antigen CD19 is a key success, bringing together decades of innovation in molecular cloning, viral gene vector development, and T-cell biology.

The treatment of diffuse large B cell lymphoma in adult patients remains a major clinical challenge. To that end, CAR technology specifically focusing on the B cell lineage antigen CD19 was developed. In 2010, Kochenderfer et al. reported the successful treatment of a patient with CD19-specific CAR-modified T-cells and followed up this report with a small trial featuring doses of $0.3–3 \times 10^7$ CAR^+ T-cells/kg. In the follow-up report with anti-CD19 CAR, four of the eight patients treated had durable responses that coincided with prolonged depletion of B cells from the peripheral blood [40, 75, 76, 118–124]. Unique aspects of this trial included the use of a CD28 and CD3ζ chain-driven second-generation signaling package and the administration of IL-2 over 5 days following T-cell infusion. The toxicities seen were associated with high cytokine release and were attributed to interferon-γ and TNF-α release by the infused CAR-expressing lymphocytes. As the group at the NCI in Bethesda, Maryland, was developing these strategies, researchers at the Sloan-Kettering Cancer Center in New York and at the University of Pennsylvania in Philadelphia were developing

their own anti-CD19 CAR approaches [40, 76, 122–124]. Although the initial report by Porter et al. featured only three patients, the clarity of the difference between the immune response mediated by anti-CD19 CAR-T-cells and any effect from preparative or therapeutic chemotherapy was easily seen, and thus had a lasting impact on the field. Anti-CD19 CAR approaches have matured to a point that now a registered CAR19 product, Kymirah (Tisagenlecleucel) by Novartis, recently received Food and Drug Administration (FDA) approval for the treatment of "certain pediatric and young adult patients with a form of acute lymphoblastic leukemia…" (www.FDA.gov, press release of August 30, 2017).

The approach in Philadelphia is unique in the use of a lentiviral as opposed to retroviral gene vector for the transduction of patient lymphocytes and in the use of a 4-1BB (CD137) as opposed to a CD28-based second signaling motif in the second-generation CAR construct. Children receiving 1.4×10^6–1.2×10^7 CAR^+ T-cells/kg had profound antileukemic effects. The infused cells showed an amazing degree of in vivo expansion and were highly active against disease [123]. In the subsequent cytokine storm that followed T-cell infusions, the onset of severe fever was ablated by the administration of anti-IL-6 antibody. On the same day a Keymirah received FDA approval, anti-IL-6 receptor antibody, Actemra (Tocilizumab, Roche/Genentech), was also approved to treat cytokine release syndrome. As experience is gained, the clinical science of adoptive immunotherapy with CAR-modified T-cells will continue to advance, with safer and more predictable patterns of treatment emerging.

The targeting of new B cell lymphoma targets is expected to expand to include other B cell restricted self-antigens such as CD22 [71]. Identifying expendable self-antigens for the treatment of solid tumors remains a serious challenge. Investigators have begun to formulate bioinformatics approaches to identifying antigens restricted to tumors and not expressed on normal self-tissues, but these have yet to be validated directly at the protein expression level, perhaps using frozen of formalin-fixed normal tissue and tumor tissue arrays [116]. A string of on-target but off-tumor (that is reacting to the intended antigen—but finding problematic expression on normal tissue, as opposed to cancerous tissue), toxicities have been seen with T-cells engineered to target MAGE-A3 with TCR vectors, with TCRs against CEA, and with CARs specific for HER2 [24, 125, 126]. The experience with HER2 is especially informative as thousands of patients had received antibody to HER2 with no toxicity reported due to self-reactivity, as seen with CAR-modified T-cells. Thus, even antibody screens on tissue arrays may not be sufficiently predictive of CAR-transduced T-cell activity.

The continuing development of a CAR expression vector for the neuroblastoma antigen GD2 is another example wherein an antibody in current clinical use has been adopted for use in CAR therapy. Use of anti-GD2 antibody therapy made a major impact on the outcome of advanced neuroblastoma patients who had been treated with a bone marrow transplantation regimen, increasing long-term survival by at least 20% [127]. Use of a GD2-specific first-generation CAR by investigators at the Baylor College of Medicine demonstrated that this first-generation less effective vector was safe and showed some indication of antitumor activity [128]. The primary side effect common to various trials with anti-GD2 antibody is peripheral nerve pain, indicating an off-tumor on-target antibody effect [129]. An ongoing clinical trial features a third-generation anti-GD2 CAR, iC9-GD2-CD28-OX40, which is comprised of CD28 and OX40 co-stimulatory domains and an inducible suicide safety switch (NCT01822652), should reveal whether or not this side effect is unique to antibody-based therapy or if CARs amplify this effect.

CAR-T or recombinant TCR function may be further enhanced by combination therapies. Synergistic effects may be achieved by inhibiting tumor growth by chemical means, while at the same time employing CAR-T-cells for active tumor killing. Pre-clinical data indicate that lenalidomide, an antitumor, anti-vasculogenic, and immunomodulatory drug indicated for monotherapy in multiple myeloma and myelodysplastic syndrome, boosts the function of anti-EGFRIII CAR by enhancing immune synapses

between the effector and the target cells [130]. A clinical trial combining anti-BCMA CART-cells with lenalidomide for the treatment of multiple myeloma has recently opened (NCT03070327, www.clinicaltrials.gov). Another ongoing trial is evaluating the concurrent administration of ibrutinib, an inhibitor of Bruton tyrosine kinase (BTK) indicated as a monotherapy in chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), with CAR19 for the treatment of CLL (NCT02640209). Neutralizing the detrimental effects of tumor microenvironment is especially critical in adoptive cell therapy of solid tumors. To this end, checkpoint blockade may be used in combination with tumor-redirected T-cells. The addition of PD-1 blockade to E7 TCR therapy of human papilloma virus-associated cancers is an example of this approach and is under evaluation (NCT02858310).

Clinical trials administering CAR-modified T-cells to patients are increasing rapidly in number, and some have shown promising results. In recent reviews of open clinical trials, CAR-T-cells specific for the following tumor-associated antigens were reported: for hematologic malignancies—CD19, CD22, CD20, ROR1, Igκ, and CD30 for B cell malignancies, CD123, CD33, LeY for AML, BCMA, CD38 and CD138 for multiple myeloma; for solid tumors— PSMA (prostate cancer), mesothelin (pancreatic and ovarian cancers and mesothelioma), FAP (mesothelioma), EGFRvIII (glioma, glioblastoma), EGFR (malignant glioma), CEA (liver metastases, lung, colorectal, gastric, breast cancer), GD2 (neuroblastoma, osteosarcoma, melanoma), GPC3 (hepatocellular carcinoma), HER2 (glioblastoma, sarcoma, glioblastoma multiforme), IL-13Rα2 (Glioma), along with numerous other targets in various stages of development [131, 132]. As with antibody-based therapies, we are entering a golden era for adoptive immunotherapy, and the fruits of many years of investment in basic T-cell biology, gene vector development, cancer biology, and clinical immunology are coming to bear on clinical disease. Continued understanding of how best to culture and engineer T-cells, outlined in Table 12.1, and development of the clinical science of adoptive immunotherapy will prove to be rich areas of investigation and will provide new benefits for cancer patients for many years to come.

Table 12.1 General features to consider in the engineering of effector T-cell populations for adoptive immunotherapy

Primary concerns in the clinical utilization of CAR-modified T-cells
1. T lymphocyte population selection and culture
(a) Selection of starting material (i.e., PBMC, $CD4^+$, $CD8^+$, mixtures)
(b) Mechanism of T-cell activation (OKT3, CD3-CD28 beads)
(c) Cytokines or small molecules included in culture and expansion protocol
(d) Selection of optimal T-cell phenotype (Tcm, Tem, Tscm)
2. Gene vector design
(a) Selection of target antigen (both at the epitope and tissue expression levels)
(b) Creation of binding domain
(c) Inclusion of other T-cell activation motifs beyond CD3-zeta (CD137, OX40, CD28)
(d) Transient versus permanent gene transduction methodology
(e) Evaluation of the need for a "safety switch" feature
(f) Combination therapies

As discussed in the text, both selection and culture of the immune cell population and the specifics of the gene vector design will govern the biology and the anticancer effectiveness of the transferred cells upon infusion into the patient

12.5 Concluding Remarks

The current state of the art in CAR-modified T-cell therapy in the clinic is focused on CD19-specific second-generation vectors that encode a 4-1BB (CD137) and CD3ζ-chain signaling package (see NCT02228096, NCT02445248 at clinicaltrials.gov). Interestingly, because of the high activity of anti-CD19 CARs, the CD28 and CD3ζ-chain signaling package is still highly effective against disease and may be clinically sufficient to approach a cure, especially if used as induction therapy before hematopoetic stem cell transplant (see NCT02614066, NCI-2015-00239, NCT02348216). The combination of CAR-based therapy with lymphodepletion or immune check-

point blockade (such as anti-PD-1 or anti-CTLA4 antibody) demonstrates that we are in a rapidly changing clinical study environment in which new insights towards the effective use of CAR-T-cells against hematologic malignancies will continue to develop. In scenarios where immune activity is potentiated, a less active CAR (at least as defined in the laboratory) may be more desirable. Given the rapid translation of CAR-T-cell therapy into the clinic, where are the next breakthroughs going to come from? First will be with regard to the viral vector technology. Currently lentivirus-based approaches are state of the art. However, this represents a cost and developmental bottleneck; thus, new transfection-based approaches are awaiting development. Second, the ability to define the most effective CAR-T-cell populations with regard to phenotype and the ability to direct their developmental state through cytokines or modification of signal transduction pathways (such as with mTOR inhibitors) will continue to refine current culture techniques and approaches. The goal is to more rapidly define or create T-cell populations that could be infused at lower doses (thus requiring less laboratory effort) while retaining high antileukemic activity. Finally, the demonstration of an effective CAR-based therapy against a solid tumor awaits clinical confirmation. The high degree of normal tissue damage that has been seen in some trials indicates that pathological tissue destruction is indeed possible. However, we do not yet know if it is a paucity of truly tumor-specific cell surface targets or if it is the tumor microenvironment that prohibits clinical antitumor effectiveness. An ongoing trial featuring a third-generation CAR specific for the pediatric tumor-associated antigen GD2 is of interest in this regard. The retroviral vector used in this trial expresses a GD2-specific binding motif and a combination of CD28, CD3ζ, and OX40 signaling motifs (see NCT01822652). This signaling combination is thought to perform similar to the 4-1BB second-generation vectors, where the anti-apoptotic properties of a TNF-receptor superfamily member (OX40, TNFRSF4, or CD137, TNFRSF9) may enhance survival of the transduced cells once they are infused. This vector also encodes an iCaspase-9 safety gene. If this credentialed tumor-specific anti-GD2 scFv fails to make an impact on disease in a CAR setting, this indicates that engineered T-cells alone cannot overcome the solid tumor microenvironment and future successes will hinge on altering this milieu. If the GD2-specific CAR is effective, we will have turned an important first corner in treating solid tumors with engineered T-cells.

As our ability to analyze the tumor microenvironment on a patient-specific basis matures, CARs may be specifically tuned for the solid tumor microenvironment they must encounter. The evasion of negative signals (such as TGFβ or PD-L1), the appropriate chemokine receptor expression for homing to the tumor, the appropriate adhesion molecule expression for tissue invasion, and the maintained durability of response by evading metabolic exhaustion will all play important roles in creating the CAR-T approaches of the future [50, 133, 134].

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13 Role of γδ T Lymphocytes in Cancer Immunosurveillance and Immunotherapy

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13.1 Introduction

The γδ lineage of T lymphocytes was first described in the mid-1980s with reports of a new heterodimeric T-cell receptor that was associated with CD3 [1, 2]. Since then, γδ T-Cells have been extensively studied (albeit considerably less than their αβ counterparts), in a global effort to unravel the mechanisms underlying their development, antigen recognition, activation, and function.

γδ T-Cells are typically regarded as a "bridge" between innate and adaptive immune responses [3, 4]. On one hand, γδ T-Cells may be considered a component of the adaptive immune system as they can somatically rearrange their *TCR* genes to generate great diversity and can selectively expand particular subpopulations upon infection. On the other hand, various γδ T-cell subsets, displaying restricted (oligoclonal) TCR repertoires, can immediately respond to challenge—with little evidence of memory formation—and may thus be considered part of the innate immune system.

A combination of antigen specificity, tissue distribution, and functional properties, rather than in any of these individually, is essential for the pleiotropic γδ T-cell responses [5]. In terms of functional attributes, γδ T-Cells are important providers of cytotoxicity, cytokines, chemokines, and other molecules that can substantially affect downstream immune responses [4]. As a result, the physiological roles fulfilled by γδ T-Cells are varied and include protective immunity against extracellular and intracellular pathogens, tissue healing and epithelial cell maintenance, and—most importantly—tumor surveillance [5]. In the following, the biology of γδ T-Cells will be introduced and their mechanisms of response to tumor cells, resulting in their application in cancer immunotherapy, would be discussed.

Notably, for clarity throughout this chapter, the Vγ gene nomenclature of Heilig and Tonegawa [2] will be used for murine γδ T-Cells and Lefranc and Rabbits [6] for human γδ T-Cells.

13.2 TCRγδ Repertoires and Functions

γδ T-Cells express a unique type of TCR that has been strongly conserved across 400–500 million years of evolution of jawed vertebrates. Despite the *TCRγ* and *TCRδ* genes being highly conserved in terms of general organization, Vγ genes diverge considerably between species: the *TCRγ* locus in mice contains seven commonly utilized genes, as it does in humans (Table 13.1). On the

Table 13.1 Frequency, distribution, and repertoires of γδ T-Cells

Species	V segment usage	Common VγVδ usage	V(d) J diversity	Day of exportation from the embryonic thymus	Distribution
Mouse	Vγ1	Vγ1Vδ6.3 (liver)	High	From E18 onward	Spleen, liver
	Vγ4		High	From E15 onward	Spleen, liver, lung
	Vγ5	Vγ5Vδ1	Invariant	From E15 until E17	Epidermis
	Vγ6	Vγ6Vδ1 (uterovaginal epithelia)	Invariant	From E16 until E18	Liver, lung, uterovaginal epithelia, tongue
	Vγ7	Vγ7Vδ4	Intermediate	Not applicable (extra-thymic development)	Gut epithelia
		Vγ7Vδ5			
		Vγ7Vδ6			
Human	Vδ1		High	Unknown	Spleen, liver, epithelia, dermis
	Vδ2	Vγ9Vδ2	Intermediate	Unknown	Peripheral blood
	Vδ3		High	Unknown	Liver, gut epithelia

contrary, there are 20–30 chicken Vγ chain gene segments and more than 6 Vγ families in skate [3]. The complexity of TCRγδ genes correlates with the abundance of γδ T-Cells: in adult mice, they account for 0.5–2% of peripheral lymphocytes; in human blood, they can range between 1.5% and 15%; whereas in young ruminants, they can account for more than 70% of the peripheral $CD3^+$ cells, declining to 5–25% with age [3].

Even though a great diversity of TCRγδ can be theoretically generated in rodents and humans, the set of TCRs detected on peripheral γδ T-Cells is far more limited. Individual γδ T-cell subsets in particular tissue locations show biased use of certain TCR V gene segments and, in some cases, express "invariant" TCR with identical (canonical) junctional sequences [5] (Table 13.1).

13.2.1 Mouse γδ T-Cell Subsets

Murine γδ T-Cells are generated in the thymus in "developmental waves" that sequentially populate different tissues by regulated expression of appropriate chemokine receptors (Table 13.1). Mouse thymocytes bearing an invariant canonical Vγ5Vδ1 TCR at embryonic day E15–17 are the first to leave the fetal thymus, giving rise to skin-associated dendritic epidermal T-Cells (DETCs); thymocytes bearing a Vγ6Jγ1Cγ1 TCR at E16–18 give rise to the γδ T-Cells in the tongue and reproductive tract; peri- and postnatal thymocytes bearing Vγ1Cγ1 and Vγ4Cγ1 TCRs give rise to systemic γδ T-Cells. This sequential generation of γδ T-Cells at different stages of ontogeny is a fixed developmental program; for example, the disruption of the generation of γδ T-Cells in the early fetal thymus by the administration of an anti-γδ-TCR antibody to pregnant mice resulted in selective absence of DETCs in adult mice [7].

It is thought that the highly restricted TCRs expressed by different subsets of γδ T-Cells enable them to recognize ligands that are specifically expressed in infected or stressed cells in particular anatomical sites where these cells populate. For example, epidermal intraepithelial Vγ5Vδ1 (DETCs) cells have been shown to carry out distinct functions which are not typical of other γδ T-Cells, such as production of keratinocyte growth factor, which plays an important role in wound healing. These cells form a dendritic network which is unique among T-Cells, but similar to that of Langerhans cells, the antigen-presenting cells of the epidermis. In physiological states, DETCs constitute more than 90% of the epidermal T-Cells, with virtually no TCR diversity [8].

Vγ6Vδ1 T-Cells comprise the vast majority of the intraepithelial lymphocytes of the tongue and reproductive tract. These cells seem to play an important role in tissue remodeling at the maternal–fetal interface [9]. Moreover, Vγ6Vδ1 were also shown to mainly produce IL-17 during pulmonary inflammation, thus preventing lung fibrosis [10].

Cells that express the Vγ7 TCRγδ (usually paired with Vδ4 or Vδ5) are typically found as intestinal epithelial lymphocytes (IELs) in gut epithelia and show cytoprotective, immunomodulatory, and antibacterial functions. These protective functions are associated with the production of epithelial cell trophic factors, inflammatory cytokines (e.g., IL-2 and IFN-γ), and cytotoxic molecules [11].

Cells that express Vγ1 and Vγ4 constitute the major peripheral recirculating γδ T-cell subsets of the blood and lymphatics. Vγ1 cells are capable of killing *Listeria*-infected macrophages via Fas/Fas ligand [12] and are also shown to promote mouse chronic granulomatous disease [13]. The Vγ4 population tends to be IL-17 biased, whereas the Vγ1 population tends to produce IFN-γ [14].

13.2.2 Human γδ T-Cell Subsets

Human γδ T-Cells use three main Vδ and at most six Vγ region genes to make their TCRs [3]. Nevertheless, the actual peripheral γδ TCR combinatorial diversity is even more limited because the TCR V region repertoire of human γδ T-Cells, as in rodents, is highly skewed in particular tissue locations [15].

The two main populations of human γδ T-Cells constitute the Vδ1 and the Vγ9Vδ2 subsets. Vδ1 T-Cells are abundant in mucosal tissues, where they are thought to be involved in maintaining epithelial tissue integrity following damage,

infection, or transformation [3]. Vγ9Vδ2 T-Cells dominate (60–95% of all γδ T-Cells) in the blood, where they comprise 1–10% of circulating lymphocytes in healthy adults.

Similarly to mice, the first γδ T-Cells to emerge in the human fetal thymus, which are Vδ1 T-Cells, preferentially populate epithelial tissues, such as the intestine [16]. Vγ9Vδ2 T-Cells derive from a subsequent pool of thymic progenitors. By studying γδ T-Cells from the thymus or peripheral blood of children, it was revealed that the Vγ9Vδ2 pairing makes up only 5% of γδ thymocytes, indicating selective (chronic) expansion of Vγ9Vδ2 T-Cells in the periphery [17]. Such extensive peripheral expansion seems to be driven by antigens present in environmental microbes and certain edible plants which stimulate Vγ9Vδ2 T-Cells during childhood. Of note, this Vγ9Vδ2 pairing is only present in humans and nonhuman primates [3, 18] and therefore has no equivalent in mice.

Vγ9Vδ2 and Vδ1 T-cell subsets differ in several aspects. Most Vγ9Vδ2 T-Cells display a memory phenotype acquired during perinatal life, whereas Vδ1 T-Cells are mainly naive in young adults [19]. Vγ9Vδ2 T-Cells express more cytokines involved in promoting inflammation, such as TNF-α, IFN-γ, and IL-21, and higher levels of CCR5, suggesting that they can home to sites of inflammation [20]. By contrast, Vδ1 T-Cells express higher levels of L-selectin and CCR7, conferring that they can home to non-inflamed tissues. Furthermore, while Vγ9Vδ2 T-Cells react against a set of non-peptidic, phosphorylated compounds ("phosphoagonists"), Vδ1 T-Cells seem to recognize unrelated antigens still poorly defined. In the context of the robust response of Vδ1 T-Cells to cytomegalovirus (CMV) infection, it was suggested that putative antigens are not virally encoded but instead consist of endogenous stress-induced ligands possibly shared by CMV-infected cells and several colon tumors [21]. Finally, Vγ9Vδ2 cells, but not Vδ1 cells, were recently shown to display (upon activation) several features of professional APCs, namely, the capacities to phagocytize and process antigens; to either present antigens on MHC-II or cross-present antigens on MHC-I; to upregulate CD80, CD86, or CD40; and to activate naive αβ T-Cells [22, 23]. The APC function of Vγ9Vδ2 T-Cells adds a new component to the role of γδ T-Cells as a "bridge" between innate and adaptive immunity.

13.3 γδ T-Cell Activation: TCRγδ Agonists

Immunologists have been searching for TCRγδ ligands for about two decades. However, this has proven to be a very difficult task, likely due to the low affinity interactions that prevent biochemical purification of the putative ligands. An important characteristic of γδ T-Cells is that they do not recognize classical TCR ligands (peptides derived from processed proteins) and do not depend on MHC-mediated antigen presentation, which markedly distinguishes them from αβ T-Cells.

It is postulated that γδ T-Cells recognize a diverse set of "stress-associated" molecules, which may be complexed (or not) with an antigen-presenting element (distinct from classical MHC). As more TCRγδ ligands will become elucidated, it will be interesting to determine whether they comprise molecules whose major function is to regulate immunity (as we conventionally view MHC) or molecules with intrinsic function(s) related to cellular dysregulation, for example, heat-shock proteins (HSPs) [4]. Below, the authors review the state of the art on the molecular entities suggested to activate γδ T-Cells in a TCR-dependent manner.

13.3.1 Phosphoagonists (Phosphoantigens)

13.3.1.1 Phosphoagonists Produced by Microorganisms and Eukaryotic Cells

Early in vitro studies indicated that Vγ9Vδ2 T-Cells strongly react in a non-MHC-restricted fashion to inactivated *Mycobacterium tuberculosis* and a variety of other microorganisms, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Yersinia enterocolitica*, and *Francisella tularen-*

sis [24–28]. It was found later that the γδ T-cell-stimulating moiety of microbial extracts was not protein but rather consisted of phosphatase-sensitive low-molecular-weight compounds [28, 29]. Different types of phosphorylated ligands were isolated from *Mycobacteria*, including four structurally related phosphoesters (so-called TUBag [1–4] 1996) [30]. The other identified phosphate-containing antigens were isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). These molecules were collectively termed "phosphoantigens" [30–32].

As a class of compounds, phosphoantigens contain multiple members, either naturally produced or synthetic, able to activate Vγ9Vδ2 T-Cells within a very large range of affinities [33]. The most potent natural phosphoantigen identified to date is a phosphorylated intermediate of isoprenoid biosynthesis pathway, produced by Eubacteria and Protozoa, but not by eukaryotes, called *E*-4-hydroxy-3-methylbut-2-enyl-pyrophosphate (HMB-PP, also known as HDMAPP for hydroxy-dimethylallyl pyrophosphate) [28, 34].

The intracellular mechanisms of HMB-PP-mediated Vγ9Vδ2 T-cell activation were previously described [35]. HMB-PP activates MEK/Erk and PI-3K/Akt pathways with similar kinetics to TCR/CD3 cross-linking using OKT3 (anti-CD3ε mAb) and induces an almost identical transcriptional profile associated with γδ T-cell activation, proliferation, and antitumor cytotoxicity [35].

Antibody blocking and gene transfer experiments showed that Vγ9Vδ2 TCR expression is required for cell activation [25, 36]. Nevertheless, it is still controversial if there is a direct interaction between the Vγ9Vδ2 TCR and phosphoantigens—for which the designation "phosphoagonists" may be more appropriate. In particular, while some studies suggested a direct ligation between Vγ9Vδ2 TCR and phosphoagonists [37, 38], all the attempts to co-crystallize phosphoagonists with the Vγ9Vδ2 TCR have not been successful [39].

Very recently, Scotet and co-workers showed that butyrophilin 3A (CD277/BTN3A) plays a key role in phosphoagonist-induced activation of Vγ9Vδ2 T-Cells in both tumor and infectious contexts and that CD277-dependent activation is conferred by Vγ9Vδ2 TCR [40]. Their work suggests that phosphoagonist may interact more directly with CD277 than the Vγ9Vδ2 TCR. How Vγ9Vδ2 T-Cells may detect phosphoagonist-induced changes of CD277 remains to be determined. These changes could be sensed directly by Vγ9Vδ2 TCR; however, the authors failed to demonstrate cognate interactions between recombinant Vγ9Vδ2 TCR and CD277 [40]. Alternatively, CD277 might promote recruitment of other molecules that interact with the Vγ9Vδ2 TCR, such as ecto-F1-ATPase [41, 42].

13.3.1.2 Phosphoagonist Intermediates of Isoprenoid Biosynthetic Pathways

Isoprenoids are essential metabolites, important for cellular and intercellular biology, and are produced by all living organisms. They constitute a diverse structural family, comprising ubiquinones, sterols, terpenes, carotenoids, gibberellins, and taxoids. All these compounds are synthesized through the same precursors, the IPP, and its isomer DMAPP. IPP can be synthesized via two different biosynthetic pathways. Archaebacteria, few Eubacteria, and most eukaryotes synthesize IPP from acetyl CoA through the mevalonate pathway (MVA) [43]. Cyanobacteria, algae, plastids, and most Eubacteria (including *M. tuberculosis*) produce IPP in a different way, through a carbohydrate-based route referred to as methylerythritol phosphate or 1-deoxy-*d*-xylulose-5-phosphate (MEP pathway or DOXP pathway respectively) [44]. Which of these two pathways, MEP or MVA, have evolved first remains unknown, since MEP only exists in bacteria and plastids where it provides most primary isoprenoids instead of the MVA used by *Archae* [45]. Both pathways can be used simultaneously by some bacterial species, but for different roles, MAP for primary metabolism and MVA for secondary metabolites [46].

Vγ9Vδ2 T-Cells recognize metabolites of isoprenoid synthesis generated by the MEP pathway in certain pathogenic microorganisms but not by the mevalonate pathway in other bacteria and mammalian cells. HMB-PP has a 1000-fold stronger stimulating activity of Vγ9Vδ2 T-Cells than IPP, probably due to its nonhuman origin

[28, 32]; this may allow the efficient detection of infected cells producing very small amounts of microbial phosphoantigens, while preventing activation by normal cells that express basal levels of the weak stimulatory mammalian metabolites. Moreover, the high potency of HMB-PP as a stimulator of Vγ9Vδ2 T-Cells correlates with the γδ T-cell stimulatory activity of the bacteria exploiting the MEP but not the MVA pathway (e.g., *M. tuberculosis* and *Escherichia coli*) [47]. To a lesser extent, the synthetic bromohydrin pyrophosphate (BrH-PP) is also considered as a strong activator of Vγ9Vδ2 T-Cells and is frequently used in experimental procedures [33].

In plants and yeast, regulation of the MVA pathway occurs at the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) level [48]. High levels of farnesyl pyrophosphate (FPP), sterols, or phenylalanine inhibit HMGR activity. In mammalian cells, the HMGR activity is inhibited by statins [49] and phenylalanine [50] or by a feedback inhibition with aminobisphosphonate-induced FPP accumulation [51]. The HMGR activity and, thus, the whole MVA pathway are increased in various cancer cell types, such as leukemia, non-Hodgkin lymphoma (NHL) [52], and mammary and lung adenocarcinoma [53, 54].

13.3.2 Aminobisphosphonates

In 1999, Kunzmann et al. discovered that several patients with multiple myeloma (MM) treated with the well-established osteoporosis inhibitor pamidronic acid (pamidronate) presented significantly high numbers of blood-borne γδ T-Cells [55]. Later, it was shown that pamidronate activates γδ T-Cells in vitro to secrete cytokines (IFN-γ), proliferate, and exhibit strong cytotoxicity against various cancer cell lines [37]. Importantly, the bioactivity of aminobisphosphonates like pamidronate required the presence of accessory "antigen-presenting cells" (APCs) treated with this drug prior to the assay with the γδ T-Cells [36]. A wide variety of tumor cell lines pretreated with aminobisphosphonates could efficiently activate Vγ9Vδ2 T-Cells to proliferate and produce cytokines in a TCR-dependent manner [56]. Zoledronate and ibandronate are more potent than pamidronate in promoting Vγ9Vδ2 T-cell activation [57].

It is well known that, in order to activate Vγ9Vδ2 T-Cells, aminobisphosphonates must be internalized and exert a statin-sensitive effect, namely, inhibiting the endogenous MVA pathway [32]. Thus, aminobisphosphonates cause a pharmacological inhibition of the mevalonate pathway in the treated cells leading to IPP accumulation. More precisely, aminobisphosphonates are inhibitors of the farnesyl pyrophosphate synthase (FPPS), an enzyme acting downstream of IPP along the pathway [32]. Of note, non-aminobisphosphonate inhibitors for osteoporosis such as etidronate or clodronate neither inhibit the MVA pathway nor enable Vγ9Vδ2 T-cell activation.

13.3.3 Alkylamines

Similarly to aminobisphosphonates, alkylamines were shown to inhibit FPPS activity. Thus, Vγ9Vδ2 T-Cells can be activated through accumulation of phosphoagonists in alkylamine-treated cells. Alkylamines are structurally composed of nonphosphate short alkyl chains bearing a terminal amino group. Prototypic bioactive alkylamines are ethylamine and sec-butylamine, present in wine and green tea and produced by certain plants and bacteria. *Listeria monocytogenes*, *Bacteroides fragilis*, *Proteus morganii*, *Clostridium perfringens*, and *Salmonella typhimurium* produce alkylamines in concentrations able to activate Vγ9Vδ2 T-cell responses [58]; contrary to phosphoagonists, they only work in the millimolar range (compared to nanomolar to picomolar for phosphoagonists). The activated Vγ9Vδ2 T-Cells then release abundant Th1-type cytokines and for this reason, it is thought that alkylamine-rich diets may contribute to prevent (Th2-driven) food allergies [49].

13.3.4 Protein Ligands

13.3.4.1 Self-Ligands

Several self-proteins thought to report cellular "stress" have been shown to activate γδ T-Cells via the TCR [15].

T10/T22

T10 and T22 are murine nonclassical MHC class I molecules expressed by highly activated cells that have been shown to bind specifically to two TCRγδ molecules (G8 and KN6) in surface plasmon resonance experiments [59, 60]. The crystal structures of these murine TCRγδ complexed with T10/T22 have also been solved [60]. So far, these are the only structural evidences for direct binding of TCRγδ to its ligand. Although MHC-I related, T10 and T22 do not present peptides or lipids, being instead recognized as intact proteins via contacts with an extended complementary-determining region (CDR)3 loop of TCRγδ [60–62]. T10-/T22-specific γδ T-Cells represent 0.4–0.6% of the peripheral γδ T-cell pool of naive mice [59]; however, this reactivity is not conserved in humans (where T10 and T22 do not exist).

F1-ATPase

The human Vγ9Vδ2 TCR was shown to bind to Ecto-F1-ATPase, a form of the mitochondrial ATP synthase (ATPase) ectopically expressed at the cell membrane. This ligand was identified by screening monoclonal antibodies capable of inhibiting the recognition of tumor cell lines by Vγ9Vδ2 T-Cells in vitro [41]. F1-ATPase is recognized by Vγ9Vδ2 TCR in a complex with the serum protein apolipoprotein A1 (ApoA-1). These components seem involved in endogenous phosphoantigen presentation, considering the ability of ecto-F1-ATPase to bind and present triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) [63]. ApppI is an intracellular nucleotidic metabolite containing an isopentenyl moiety that accumulates in aminobisphosphonate-treated cells. ApppI can specifically activate Vγ9Vδ2 T-Cells, but not in its native form; it requires processing by a nucleotidic pyrophosphatase (NPP), which releases IPP and adenosine monophosphate (AMP). In this regard, ApppI should represent an inactive storage form of phosphoantigens that can only bind to ecto-F1-ATPase upon cleavage by NPP and generation of IPP [63].

However, the biological relevance of this interaction is still being addressed. It is possible that mitochondrial antigens could be an alerting signal that indicates the status and fate of the cell. On the other hand, the interaction between these molecules could be justified by the specific microbial origin of mitochondria, carrying antigens similar to modern microbes.

ULBP4

The nonclassical MHC class Ib protein, ULBP4, was detected on the cell surface of Epstein–Barr virus (EBV)-infected cells as well as on colon, ovarian, and liver cancer cells, suggesting a role in anti-infection and antitumor immunity. Immobilized soluble ULBP4 was shown to bind directly to soluble Vγ9Vδ2 TCR and to stimulate the activation of Jurkat Vγ9Vδ2 TCR transfectants (lacking NKG2D expression) [64]. Furthermore, ULBP4 ligation induced proliferation, cytokine production, and cytotoxic activity of human ovarian and colonic carcinoma-infiltrating Vγ9Vδ2 T-Cells in vitro. However, blocking experiments indicated that both Vγ9Vδ2 TCR and NKG2D are involved in ULBP4 recognition [64], raising questions about the hierarchy between NKG2D and Vγ9Vδ2 TCR in γδ T-cell activation and target recognition (Table 13.2).

Table 13.2 Expression of NKG2D in lymphocyte subsets

Cell type	Mouse	Human
NK cells	100%	100%
$CD8^+$ T-Cells	Before activation: absent	Before activation: ≈100%
	After activation: ≈100%	After activation: ≈100%
$CD4^+$ T-Cells	Rare or absent	Normally absent
γδ T-Cells	Spleen (Vγ4 and Vγ1): ≈25%	Blood (Vγ9Vδ2) ≈ 100%
	IELs (Vγ7): absent	Blood (Vδ1) ≈ 100%
	Skin DETCs (Vγ5Vδ1): ≈100%	IELs (Vδ1) ≈ 100%

MICA

Dual recognition of tumors and infected cells is achieved by human Vδ1 cells, as TCR-dependent responses toward both epithelial cell-derived tumors and infected cells have been shown [21]. MHC I chain-related peptide A (MICA) has been proposed as an important tumor antigen, with recognition of MICA-positive tumor cells by Vδ1 lymphocytes infiltrating colon carcinomas [65–67]. Nevertheless, the very low affinity of MICA–Vδ1TCR interactions estimated by surface plasmon resonance analyses raises doubts about the functional relevance of MICA recognition by Vδ1 TCRs [68].

EPCR

Recently, a human Vγ4Vδ5 clone was shown to directly bind endothelial protein C receptor (EPCR), which allowed γδ T-Cells to recognize both endothelial cells targeted by CMV and epithelial tumors. EPCR is a major histocompatibility complex-like molecule that binds lipids analogously to the antigen-presenting molecule CD1d [69].

Heat-Shock Proteins (HSPs)

Because of their role as sensors during cell stress or transformation, HSPs (heat-shock proteins) were initially proposed as antigenic targets for γδ T-Cells. Some members of HSPs were shown to be upregulated on tumors, where γδ T-Cell had infiltrated, suggesting HSP-65-dependent recognition of tumor cells by Vγ9Vδ2 T lymphocytes [46, 70]. Also, HSP-60 was shown to be recognized by Vγ9Vδ2 T-Cells [71] and promote their expansion [72].

13.3.4.2 Non-Self-Ligands

Tetanus toxoid, a strong immunogen derived from a protein, the tetanospasmin of *Clostridium tetani*, was the first defined antigen reported to be capable of stimulating γδ T-cell responses [73, 74]. Others that followed include viral proteins such as glycoprotein I from herpes simplex [75] and staphylococcal enterotoxin A [76]. More recently, the defined mycobacterial protein ESAT-6 was found to stimulate γδ T-Cells [77], and this may not be the only mycobacterial protein recognized by γδ T-Cells [78].

13.4 γδ T-Cell Activation: Costimulatory Molecules

T-cell activation depends not only on TCR triggering but also on signals from several additional receptors, commonly referred to as costimulatory molecules. Although these mechanisms have been extensively studied for conventional αβ T-Cells, they are less well established for γδ T-Cells [79].

13.4.1 CD27

CD27 is a member from the TNF-receptor superfamily that plays critical roles on γδ T-cell activation, particularly in response to viral and tumor challenge [80]. The ligand for CD27 is CD70, and the interaction between these molecules provides a potent second signal for cytokine production, induction of activation markers, and proliferation of primed and unprimed peripheral blood lymphocytes [81].

The authors have shown that the expression levels of CD27 define two stable subsets of γδ T-Cells in naive C57BL/6 mice [14, 79]. The majority of γδ T-Cells in the spleen, lymph nodes, and various tissues are $CD27^+$ and secrete IFN-γ upon activation. By contrast, IL-17 is only produced by their $CD27^-$ counterparts. Interestingly, these distinct phenotypes are "preprogrammed" in the thymus, as early as in embryonic stages [14, 82]. Moreover, CD27 stimulation (using soluble recombinant CD70) in fetal thymic organ cultures favored the development of IFN-γ^+ γδ T-Cells [14].

In the periphery, CD70–CD27 interactions provide survival and proliferative signals that control TCRγδ-driven activation. Thus, CD27 signaling activates the noncanonical NF-κB pathway and enhances the expression of antiapoptotic and cell cycle-related genes in murine γδ T-Cells [79, 83, 84].

In humans, an average of 80% of Vγ9Vδ2 T-Cells express CD27 [83] including both naive and central memory cells [85]. Upon activation with phorbol myristate acetate (PMA) and ionomycin, the vast majority of $CD27^+$ Vγ9Vδ2 T-Cells produce IFN-γ, whereas less than 1% produce IL-17 [83]. A recent work performed by the authors demonstrated that CD70–CD27 interactions enhanced survival and proliferation of phosphoantigen-activated Vγ9Vδ2 T-Cells and promoted their Th1-like responses (i.e., the secretion of IFN-γ and TNF-α) [83]. Thus, a major role of CD27 costimulation in Vγ9Vδ2 T-Cells appears to be the protection from activation-induced cell death (AICD) following phosphoantigen-mediated (TCR-dependent) stimulation [83]. Interestingly, CD70 is strongly induced in phosphoantigen-activated Vγ9Vδ2 T-Cells, which may therefore provide their own CD27 ligands during immune responses.

13.4.2 CD28

CD28, the receptor for B7.1 (CD80) or B7.2 (CD 86), is the primary costimulatory receptor for αβ T-Cells. CD28 signaling has been shown to produce both qualitative and quantitative changes leading to lower activation thresholds and enhanced αβ T-cell functions. CD28 signaling promotes proliferation, survival, and cytokine production of $CD4^+$ and $CD8^+$ T-Cells, and such responses are frequently impaired in *Cd28*$^{-/-}$ mice [86].

CD28 is upregulated upon activation in murine γδ T-Cells and it is expressed by 40–60% of freshly isolated human peripheral blood γδ cells [79, 87]. Although some reports suggested that CD28 costimulation promotes the proliferation of peripheral γδ T-Cells, other biological processes appeared to be CD28 independent [79].

The authors have recently revisited the role of CD28 costimulation in γδ T-cell activation. It was observed that CD28, constitutively expressed on freshly isolated lymphoid γδ T-Cells, promoted γδ T cell survival and proliferation in both mice and humans. Thus, γδ cell expansion was significantly enhanced by CD28 receptor agonists but abrogated by B7 antibody-mediated blockade [87]. Mechanistically, it was shown that the induction of IL-2 production is a major and specific function of CD28 (but not CD27) costimulation in γδ cells, which are known to strongly benefit from IL-2 signals for their expansion [35, 88]. The fact that γδ cells can produce high levels of IL-2 strictly upon CD28 costimulation defines important rules for their expansion in situ. Of note, CD28-deficient mice displayed reduced [relative to wild type (WT) controls] numbers of total or activated γδ cells following *Plasmodium berghei* infection, which was not phenocopied in CD27-deficient animals. This demonstrates that the two costimulatory pathways play independent roles in γδ T-cell activation in vivo [87]. Most importantly, CD28-deficient mice failed to expand both IFN-γ^+ and IL-17^+ γδ T-Cells in response to *Plasmodium* parasites [87], which contrasted with the selective effect of CD27 on IFN-γ-producing γδ cells [84]. Regarding the latter, the authors further showed that CD28 acts nonredundantly and synergistically with CD27 in their activation and expansion following malaria infection [87].

13.4.3 Fc Receptors: CD16

NK cells are able to detect IgG antibody-coated cells through the FcγRIIIA (CD16) cell-surface receptor and to exert antibody-dependent cell cytotoxicity (ADCC) and cytokine production. Specifically, higher cytolytic activity and early IFN-γ production are functional properties of $CD56^{dim}CD16^+$ NK cells [89]. CD16 is coupled to the CD3ς and FcRγ signal transduction proteins bearing immunoreceptor tyrosine-based activation motifs (ITAMs). Besides NK cells, a subset of Vγ9Vδ2 T-Cells has been shown to express CD16. CD16 upregulation is associated with terminal differentiation into effector cells of both αβ and γδ T-Cells. Interestingly, Angelini et al. showed that this phenotypic differentiation was associated with decreased Vγ9Vδ2 TCR signaling that paralleled enhanced CD16-mediated T-cell activation [90]. The mechanisms underlying the balanced contribution of TCR versus

CD16 signaling along γδ T-cell functional differentiation remain unclear. Nevertheless, experiments led by Lafont et al. have highlighted the role played by CD16 engagement in γδ T-Cells. Indeed, cross-linking of CD16 on Vγ9Vδ2 T lymphocytes initiates intracellular signaling events similar, although significantly delayed, to those occurring following TCR activation. Moreover, as observed with the TCR activation process, CD16-triggered TNF-α production can be efficiently inhibited by the coincident ligation of CD94/NKG2A [91].

Recently, the activation of Vγ9Vδ2 T-Cells with the synthetic phosphoantigen BrH-PP was shown to improve the efficacy of cancer immunotherapy by the therapeutic mAb rituximab (RTX). Thus, combination of BrH-PP with RTX increased Vγ9Vδ2 T-cell binding and ADCC activity against $CD20^+$ lymphoma cells in vitro. Moreover, a regimen combining RTX, BrH-PP, and IL-2 activated Vγ9Vδ2 T lymphocytes and enhanced B-cell depletion from blood and lymph nodes of cynomolgus macaques [92].

13.5 γδ T-Cell Activation via Natural Killer Receptors (NKRs)

13.5.1 NKG2D

Natural killer group 2 member D (NKG2D) is an activating C-type lectin receptor expressed on the surface of NK cells, $CD8^+$ T-Cells, and γδ T-Cells [93] (Table 13.2). NKG2D activation is best described in NK cells, where its cross-linking (on murine NK cells) was shown to trigger several effector mechanisms, such as Th1 cytokine production (IFN-γ, GM-CSF, TNF-α) and the release of cytotoxic granules [94, 95].

NKG2D itself does not possess signaling capacity. In humans, NKG2D exists on the cell surface complexed with the DAP10 adaptor protein that contains a YxxM motif which, upon tyrosine phosphorylation, couples the receptor complex to the PI3K/Grb2-Vav pathway [96, 97]. Murine NKG2D is encoded by two splice variants [98]. The long isoform (mNKG2D-L) associates only with DAP10, whereas the short isoform (mNKG2D-S) associates with DAP10 or DAP12 [98, 99].

Several mechanisms are known to regulate the cell-surface expression of the NKG2D receptor, including the differential action of particular cytokines. Thus, TGF-β1 [100–102] and IL-21 [103] lead to downregulation of NKG2D expression on NK and $CD8^+$ T-Cells. By contrast, IL-2 and IL-15 signals increase NKG2D surface expression [104, 105] by upregulating DAP10 mRNA and protein synthesis. Interestingly, it was shown that TCR ligation in $CD8^+$ T-Cells also upregulates NKG2D/DAP10 cell-surface expression [106], which may underlie a costimulatory function for NKG2D in $CD8^+$ T-Cells.

The role of NKG2D in T-Cells remains controversial, as some authors argue that NKG2D has solely a costimulatory function, whereas others defend that NKG2D signals can activate T-Cells in the absence of TCR engagement. Thus, for human $CD8^+$ T-Cells, various reports showed that NKG2D-DAP10 can mediate cytolysis independent of TCR engagement when cells are exposed to IL-15 or high-dose IL-2 [105, 107–109]. Specifically for γδ T-Cells, some studies reported the ability of Vγ9Vδ2 T-Cells to trigger effector responses through NKG2D stimulation alone [110, 111]. However, others have failed to show any Vγ9Vδ2 T-cell NKG2D-induced activation without coincident TCR stimulation [112, 113]. In particular, it was recently shown that NKG2D triggering per se could not produce calcium fluxes in γδ T-Cells, but its co-engagement with TCR/CD3 significantly augmented the intensity of calcium responses, which also translated into enhanced cytotoxicity (while not affecting IFN-γ production) [113].

The ligands for NKG2D belong to the MHC class Ib protein family (also known as nonclassical MHC), which are usually upregulated on transformed, stressed, or infected cells. The MHC class Ib molecules are structurally related to class Ia proteins in that they show typical (α1–α2) MHC fold on a single polypeptide, which, in the case of Ib, does not obligatorily paired with β2-microglobulin. Furthermore, although many *MHC Ib* genes are located in the MHC locus,

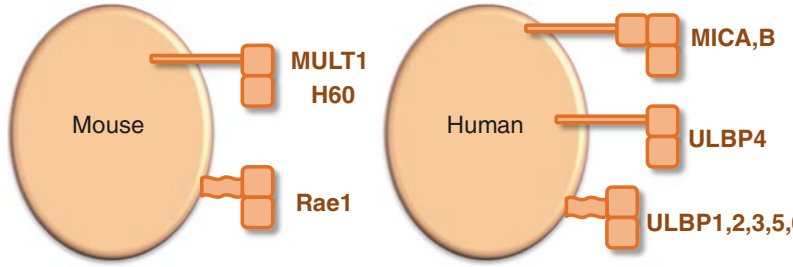

Fig. 13.1 Mouse and human NKG2D ligands. All NKG2D ligands have α1 and α2 domains with structural homology to MHC class I, and MICA and MICB have also a α3 domain. By contrast with MHC class I, none of the NKG2D ligands associate with β2-microglobulin or bind peptides. MULT1, H60, MICA/B, and ULBP4 are transmembrane-anchored type I glycoproteins, whereas Rae1 and ULBP1, ULBP2, ULBP3, ULBP5, and ULBP6 bind to cell membrane by a GPI anchor

they tend to be oligomorphic, with few alleles present in the population (with the notable exception of MICA/B), which markedly contrasts with the extensive polymorphism of class Ia [114]. MHC class Ib molecules can work as ligands for particular types of TCRs or NK receptors, most notably NKG2D [114].

Mouse NKG2D binds to retinoic acid early transcript (Rae1), histocompatibility antigen 60 (H60), and murine UL16-binding protein-like transcript 1 (MULT1) (Fig. 13.1). Human NKG2D binds to MHC I chain-related (MIC) peptides A and B (MICA and MICB) and to UL16-binding proteins (ULBP, members 1–6) (Fig. 13.1) [114, 115]. MICA/B, ULBP4, H60, and MULT1 are transmembrane proteins, while ULBP1, ULBP2, ULBP3, ULBP5, and ULBP6 and Rae1 localize to the cell surface using glycosylphosphatidylinositol (GPI) linkages [93, 115]. None of the NKG2D ligands bind to peptide or lipid antigens but rather interact directly with the receptor. In addition, NKG2D ligands do not associate with β2-microglobulin [93] in contrast to some other members of the MHC class Ib family (e.g., HLA-G or CD1d).

NKG2D ligands are usually induced by a variety of signals associated with cellular stress, namely, oxidative stress, ionizing radiation, DNA-damaging agents, viral infections, and intracellular bacterial infections [116]. Nonetheless, the various NKG2D ligands have distinct patterns of expression, indicating that they cannot be considered simply redundant in function.

Despite the marked differences in their amino acid sequences, the different ligands interact with NKG2D in similar fashion, and the receptor does not seem to undergo marked conformational changes to accommodate different ligands [117]. So far, there is no evidence that the different ligands induce qualitatively distinct biological effects in responding cells, though this remains a possibility. Minimally, the various ligands would be predicted to differ quantitatively in their effects based on the marked differences in their affinity for NKG2D. At present, the relevance of such differences has not been documented.

The murine ligands Rae1 and H60 are rare in healthy adult tissues, but their transcription is strongly induced in keratinocytes after their exposure to carcinogens in vivo [118], and they are overexpressed in the cutaneous papillomas and carcinomas that subsequently develop, as well as in various tumor cell lines [98, 119]. The expression of Rae1 or H60 by target cells was shown to enhance cytolysis and the production of IFN-γ by cytotoxic T lymphocytes (CTLs) [120] and γδ T-Cells [118] leading to tumor rejection in vivo. Moreover, transduction of Rae1, H60, or MULT1 into NK-cell-resistant target cells made them susceptible to NK-cell-mediated killing and stimulated IFN-γ secretion [120, 121].

In contrast to other mouse ligands (Rae1 and H60), MULT1 is expressed at marked levels by various normal cells at the mRNA level [122], but cell-surface expression is low or has not been documented. For example, C57BL/6 thymocytes

contain high levels of Mult1 mRNA but stain poorly with NKG2D tetramers [123]. However, MULT1 is expressed at functional levels on the cell surface of numerous tumor cell lines, indicating that these molecules might be regulated at a level other than transcription [123].

The human MICA and MICB proteins show restricted and low expression in healthy tissues but are strongly induced by cellular stress (including heat shock) and transformation. In addition, they accumulate in various tumor cell lines, particularly those of epithelial origin [66, 124]. Upregulation of MICA and MICB expression by these cells seems to result from activation of heat-shock transcription elements in the promoters of the corresponding genes, an event known to accompany transformation [66]. Interestingly, heat-shock elements have not been implicated in regulating the expression of Rae1, H60, MULT1, or ULBPs. Atypically for MHC Ib molecules, the MIC genes are highly polymorphic consisting of 61 MICA and 30 MICB alleles [93].

Whereas the membrane-bound form of MICA provides stimulatory signals to killer lymphocytes, soluble forms that shed from the cell surface may downregulate surface NKG2D and impair tumor cytolysis, constituting an important immune evasion mechanism [125, 126]. Moreover, NKG2D ligands can be expressed by tumor-released exosomes [127] that promote downregulation of surface NKG2D expression by NK and $CD8^+$ T-Cells. Interestingly, a similar phenomenon occurs in human placenta to avoid immunosuppression during pregnancy [128].

Distantly related to the MIC proteins are the members of the ULBP family. In contrast with Rae1 or MICA, ULBPs are expressed at significant levels in a wide range of healthy tissues and cell lines of both epithelial and non-epithelial origin [129, 130]. Ectopic expression of ULBP1 or ULBP2 on murine EL4 or RMA tumor cells elicits potent antitumor responses in syngeneic C57BL/6 and SCID mice, recruiting NK, NKT, and T-Cells to the tumor [131]. Similarly, tumor cells that are insensitive to NK cells can be lysed effectively when transfected with ULBPs [132]. Moreover, tumor cell susceptibility to current first-line treatment to NHL, rituximab (anti-CD20 mAb), was shown to greatly depend on ULBP1–ULBP3 expression [133].

We have demonstrated that ULBP1 is a non-redundant determinant of hematological tumor susceptibility to Vγ9Vδ2 T-Cells [134]. By using loss- and gain-of-function studies, the authors have shown that ULBP1 expression on leukemia and lymphoma cell lines is required and sufficient for Vγ9Vδ2 T-cell recognition [134]. Moreover, leukemic B-Cells were also shown to express ULBP3 that is recognized by Vδ1 T-Cells, the other major subset of human γδ T-Cells [135].

Furthermore, epithelial tumors, such as ovarian and colon carcinomas, which express low or undetectable levels of ULBP1 [110], seem to rely on ULBP4 for Vγ9Vδ2 T-cell recognition [64].

Cancer cells can also shed proteins of the ULBP family. ULBP2 is secreted both from tumor cell lines and primary tumor cells from patients and sera-soluble ULBP2 was shown to have poor prognostic value in melanoma patients [136]. Other studies also correlate NKG2D ligand expression with cancer clinical prognosis; for example, loss of ULBP1 in hepatocellular carcinoma correlates with tumor progression and early recurrence [137], whereas expression of MICA/B and ULBP2 in breast cancer is an independent prognostic parameter for relapse-free period [138].

The expression of human NKG2D ligands seems to be modulated by proteasome regulation. For example, in head and neck squamous cell carcinoma (HNSCC), bortezomib (an approved drug for treatment for plasma cell myeloma) and other proteasome inhibitors with distinct mechanisms of action dramatically and specifically upregulated ULBP1 mRNA and cell-surface protein expression. In different types of tumors, such as hepatocellular carcinoma, low-dose proteasome inhibitor drugs caused upregulation of MICA and MICB, but not ULBP1–3 [139]. In contrast, other reports showed that several proteasome inhibitor drugs increased ULBP2 levels on Jurkat surface T-Cells, whereas MICA, MICB, ULBP1, ULBP3, and ULBP4 were not affected [140].

Moreover, both murine and human non-tumor cell lines may upregulate NKG2D ligands in response to DNA-damaging agents and DNA

synthesis inhibitors. Activation of the DNA damage pathway is frequently activated in tumor cell lines, possibly due to the greater genomic instability of these cells compared with transformed cells [116].

Other mechanisms of NKG2D ligand expression regulation include differences in promoter sequences of the several ligands [141]; cytokine treatment, for example, TGF-β decreased transcription of MICA, ULBP2, and ULBP4 in human gliomas [142, 143] and IFN-γ decreased MICA message levels in melanoma [144]; and induction of p53, which lead to upregulation of ULBP1 and ULBP2 at the tumor cell surface [145].

An open question in the field is why there are so many ligands for the NKG2D receptor. It is possible that the several ligands stimulate NKG2D positive cells to respond to different forms of stress because they are capable of being expressed independent of each other [129, 130, 141] and because they engage NKG2D with different affinities, suggesting that NKG2D ligands may not be functionally equivalent. In any instance, NKG2D is clearly a key determinant of tumor immunosurveillance, since NKG2D-deficient mice show increased growth of epithelial and lymphoid tumors in two transgenic models of de novo tumorigenesis [146].

13.5.2 NKG2A

As previously shown for NK cells, most human Vγ9Vδ2 T-Cells express several inhibitory NK receptors, including killer Ig-like receptors (KIR), leukocyte Ig-like receptors (LIRs), and lectin-like receptors, such as the NKG2A/CD94 heterodimer.

The NKG2A/CD94 heterodimer is regarded as a crucial complex molecule for the inhibition of γδ T-cell responses [147]. Most of these inhibitory NKRs decrease the killing of target cells expressing high levels of either classical or nonclassical MHC molecules. Due to the broad cellular distribution of some Vγ9Vδ2 TCR agonists such as IPP, which are upregulated on transformed cells, MHC class I-specific inhibitory NKR may selectively downregulate recognition of healthy cells by Vγ9Vδ2 CTL [118, 120, 148]. Accordingly, masking of inhibitory NKRs increases Vγ9Vδ2 T-cell killing of several hematopoietic and non-hematopoietic tumors [149].

13.5.3 Natural Cytotoxicity Receptors (NCRs)

Although TCR and NKG2D play central roles in the activation of γδ T-Cells, their response to tumors may involve other receptors, such as natural cytotoxicity receptors (NCRs), including the activating receptors NKp30 [150], NKp44 [151, 152], and NKp46 [153, 154].

NKp30 is encoded on chromosome 6 and has no homology with NKp44 and NKp46, which are encoded on chromosomes 6 and 9, respectively [150]. Notably, NKp30 is a pseudogene in mice, with the exception of the wild strain *Mus caroli* [155]. A functional but low level of NKp30 protein is expressed in resting peripheral chimpanzee NK cells [156]. Several studies have shown that NKp30 is a major activating receptor involved in tumor cell lysis by NK cells. IL-2 [157] and IL-21 [103] induce NKp30 upregulation, whereas TGF-β downregulates NKp30, leading to impaired NK cytotoxicity [158]. Additionally, an NKp30-dull phenotype was shown to be acquired during leukemia development in acute myeloid leukemia (AML) [158, 159] and breast cancer [160] patients. This downregulation is possibly a mechanism of escape from innate immunity.

A recent study conducted by the authors demonstrated that human Vδ1 T-Cells can be selectively induced to express NKp30, NKp44, and NKp46 [161]. Importantly, specific gain-of-function and loss-of-function experiments showed that NKp30 makes the most important contribution to TCR-independent leukemia cell recognition. Moreover, the Vδ1 NKp30^{+} subset is able to target primary hematological tumors highly resistant to fully activated Vγ9Vδ2 PBLs [161].

Several groups have shown the constitutive expression of NKp30 ligands on tumor cells by assessing the binding of soluble NKp30 [162]. However, only one ligand (*B7-H6*) was demonstrated to be clearly involved in NKp30-mediated

tumor cell recognition [163]. *B7-H6* is a surface protein similar to other members of the B7 family. In contrast to B7.1 and B7.2, that recognize both CD28 and CTLA-4, *B7-H6* is not promiscuous, since it does not bind to any other CD28 family members or other NCRs [163]. Similar to NKp30, but in contrast to other B7 members, a functional *B7-H6* gene is missing in *Mus musculus*.

B7-H6 transcripts have not been detected in most normal adult tissues, consistent with the absence of the protein on circulating cells, isolated from healthy individuals. In contrast, *B7-H6* surface expression is observed in a restricted panel of tumor cell lines from various origins including lymphoma, leukemia, melanoma, and carcinoma as well as on primary tumor blood cells [163]. The pattern of *B7-H6* expression, which appears so far to be limited to tumor cells, is another example of stress-induced self-recognition by NK cells [164]. However, in pilot experiments, treatment of some NKp30 ligand-negative tumor cells with a panel of DNA-damaging agents had no major effect on *B7-H6* expression.

NKp44 is a type I transmembrane protein non-covalently associated in the plasma membrane with a disulfide homodimer of DAP12 (a transmembrane accessory protein that contains an ITAM, which provides intracellular activation signals) [151, 152]. The NKp44 molecule is expressed on the surface of IL-2 stimulated, but not on resting human NK cells, and therefore is referred to as an activation-induced triggering receptor [152]. Anti-NKp44 mAb can reduce NK-cell cytotoxicity toward certain tumor target cells, thereby indicating that these targets express the appropriate ligands for the receptor [151]. However, the identity of NKp44 ligands on tumors is currently unknown.

NKp44 seems to be involved in Vγ9Vδ2 cytotoxicity against MM cell lines lacking expression of NKG2D ligands. However, the percentage of NKp44$^+$ γδ T-Cells in culture was very low [165], thus raising the question about the biological importance of NKp44 expression on Vγ9Vδ2 T-Cells. Nonetheless, it seems like NKp44 is important for Vδ1$^+$ γδ T-Cells, as gain-of-function and loss-of-function experiments demonstrate that NKp44 is also a functional receptor in activated Vδ1$^+$ T-Cells and mediates tumor cell killing [161]. Importantly, a synergistic effect between NKp30 and NKp44 (with no additional effect of NKp46) was observed [161]. The authors are currently exploiting the potential of NCR$^+$ Vδ1$^+$ T-Cells in cancer immunotherapy.

13.5.4 DNAM-1

Another important NK receptor is DNAX accessory molecule-1 (DNAM-1 or CD266), a transmembrane glycoprotein that associates with LFA-1. Its ligands include poliovirus receptor (PVR) and Nectin-2. In NK cells, DNAM-1 has a role in tumor cell recognition together with NCRs and to a lesser extent with NKG2D [166]. Decreased expression of DNAM-1 has been observed in NK cells from AML patients [158, 167]. In mouse, DNAM-1 is a crucial component of T-cell-mediated immunological surveillance and partially contributes to NK-mediated lymphoma rejection [168].

Importantly, the human Vγ9Vδ2 T-cell subset expresses DNAM-1, and upon recognition of ligands expressed by hepatocellular carcinoma cells, DNAM-1 signals were shown to increase Vγ9Vδ2 cell cytotoxicity and IFN-γ secretion [169]. Furthermore, a recent report demonstrated that Vγ9Vδ2 T-Cells efficiently killed autologous AML blasts dependent on DNAM-1 and TCR signals. The DNAM-1 ligands, PVR and Nectin-2, were expressed by the targeted AML blasts [170].

13.6 Tumor Cell Recognition by γδ T-Cells: TCRs Versus NKRs

Studies on hematological tumors have highlighted the major role played by activating NKRs in tumor cell recognition by human γδ T-Cells. This was observed for both Vγ9Vδ2$^+$ and Vδ1$^+$NKp30$^+$ T-cell subsets, in which NKG2D and/or NKp30, rather than the respective TCRs, mediated leukemia/lymphoma cell recognition [134, 161].

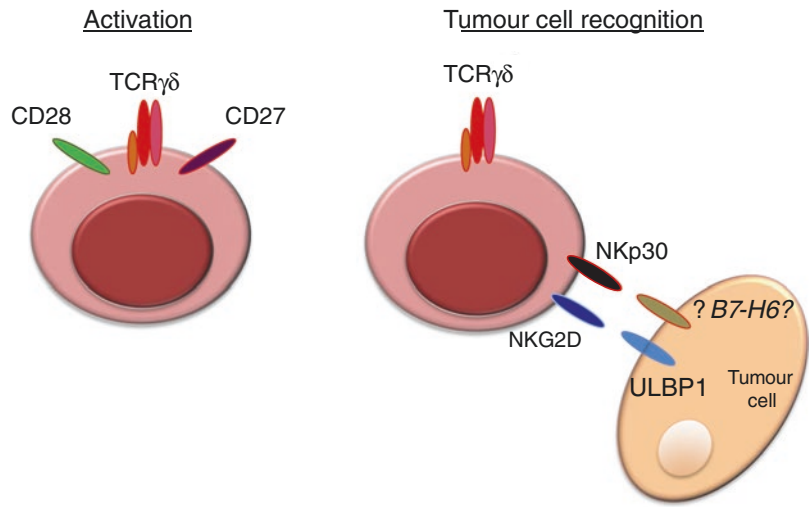

Fig. 13.2 Tumor cell targeting by γδ T-Cells is two-step process of activation and subsequent tumor cell recognition. γδ T-cell activation requires TCRγδ signaling plus costimulation (CD27 and CD28), whereas tumor cell recognition involves natural killer receptors such as NKG2D and NKp30 (and DNAM-1) that bind counter-ligands (over) expressed on tumor cells

Some other groups have suggested that γδ T-Cells recognize tumor targets through TCR interactions with self-ligands overexpressed by tumor cells and simply use NKR signals to fine-tune their activation threshold (reviewed in [5, 171–173]). In this scenario, TCR-mediated activity would be tightly regulated by an interplay between activating and inhibitory NKRs [171].

Building on these considerations, the authors' current working model includes two stages of γδ T-cell activation/differentiation and tumor cell recognition (Fig. 13.2). First, γδ cells are potently activated by (mostly unknown) TCRγδ ligands in the presence of IL-2. This, which can be achieved for Vγ9Vδ2 cells using (microbial or synthetic) phosphoagonists (plus IL-2), endows them with potent cytolytic (and cytokine-secreting) function but requires a subsequent phase of target identification, namely, for discrimination between tumor and healthy cells. We propose this is mainly determined by activating NKRs that bind stress-inducible proteins which selectively accumulate on the surface of tumor cells. Of note, the segregation of these two processes (activation vs. tumor cell recognition) in experimental systems requires pre-activation of γδ T-Cells (through the TCR) before testing them against tumor targets. More importantly, we believe the integration of these two phases will be the key for success of γδ cell-based protocols in future cancer clinical trials.

13.7 γδ T-Cell Responses to Tumors

13.7.1 Antitumor Properties

γδ T-Cells can kill transformed cells, through pathways that involve the engagement of death-inducing receptors, such as CD95 (also known as FAS) and TNF-related apoptosis-inducing ligand receptors (TRAILR), and the release of cytotoxic effector molecules, such as perforin and granzymes [173]. Murine IELs, activated DETCs, and human Vγ9Vδ2 cells primarily express granzymes A and B at levels substantially higher than conventional $CD8^+$ T-Cells. Moreover, a significant fraction of Vγ9Vδ2 cells express intermediate levels of CD16 and thus γδ T-Cells can improve antibody-dependent cell cytotoxicity (ADCC) [174].

The importance of murine γδ T-Cells in tumor immunosurveillance was first described in 2001 by a seminal paper from the Hayday lab. They showed that γδ-deficient mice were highly susceptible to multiple regimens of cutaneous carcinogenesis. Moreover, they observed that the γδ T-cell response in WT mice was determined by NKG2D recognition of Rae1 and H60 molecules, expressed by skin tumor cells. This work further revealed that γδ T-Cells not only inhibited the early stages of papillomas development but also limited their progression to carcinomas [118].

In the murine B16 melanoma model, γδ T-Cells were shown to infiltrate tumor lesions already at day 3 posttransplantation and to provide a critical early source of IFN-γ [175]. By using bone marrow chimeras and fetal liver reconstitution experiments, the authors showed that IFN-γ production by γδ T-Cells seems to be required to control the growth of both methylcholanthrene (MCA)-induced tumors and B16 melanoma tumors. This ability of γδ T-Cells to produce IFN-γ was crucial for the subsequent αβ T-cell activation and differentiation. Thus, depletion of γδ T-Cells resulted in significantly reduced IFN-γ production by both $CD4^+$ and $CD8^+$ T-Cells upon challenge with tumor lysates [175]. The direct comparison of protective properties of γδ T-Cells and αβ T-Cells was addressed in other chemical carcinogen-induced tumors, namely, squamous cell carcinoma [176]. While papilloma development was comparable in WT and *Tcrb*$^{-/-}$ mice, it was highly accelerated in *Tcrd*$^{-/-}$ and in the double-knockout mice, *Tcrb*$^{-/-}$ *d*$^{-/-}$. This study revealed that γδ T-Cells are strongly protective, whereas the contribution of αβ T-Cells for tumor progression control is more modest [176].

Subsequent studies also using carcinogen-induced skin tumors reinforced the nonredundant antitumoral role of γδ T-Cells [177–179]. Moreover, by backcrossing *Tcrd*$^{-/-}$ mice with transgenic adenocarcinoma mouse prostate cancer (TRAMP) mice, Liu and colleagues showed that γδ T-Cells limit the development and progression of spontaneously arising mouse prostate cancer [180]. The authors also assessed the possibility of developing an adoptive cell therapy, by treating TRAMP-C2 subcutaneous tumor-bearing mice, with adoptively transferred γδ T-Cells. Treated mice with supraphysiological numbers of WT γδ T-Cells develop measurably less disease compared with untreated mice [180].

γδ T-Cells were also characterized as prototypic antitumor mediators in B-cell lymphomas. Peng and colleagues showed that B-cell lymphomas arose with higher frequency in Fas mutant lpr mice that were additionally deficient for γδ T-Cells [181]. Moreover, γδ T-Cells were present in great numbers around B-Cell tumor masses in the spleens of *pfp*$^{-/-}$ mice [182]. Also, in this work, both γδ T-Cells and NK cells were shown to display potent cytotoxicity against spontaneously arising MHC class I-deficient B-Cell lymphomas.

Studies in mice (Table 13.3) have thus provided important clues to the physiological roles of γδ T-Cells, but owing to the differences between mouse and human γδ T-cell subsets, these studies have not generally predicted the behavior of human γδ T-Cells [5].

This notwithstanding, both main subsets of human γδ T-Cells, Vγ9Vδ2 and Vδ1 cells, have been shown to lyse a broad range of tumor cell lines in vitro. The $V\gamma9V\delta2^+$ subset has been more widely studied than the Vδ1 subset, probably due to the easiness of isolation, as they comprise most of the γδ-PBLs. They have been shown to display potent cytotoxicity toward several cell lines of different origins, including breast cancer [183], colon and nasopharyngeal carcinomas [184],

Table 13.3 Mouse tumor models implicating γδ T-Cells in tumor immunosurveillance

Spontaneous tumors	Chemical carcinogen-induced tumors	Transplantable tumor cell lines	Tumor type	Reference
	MCA, DMBA + TPA	PDV	Skin fibrosarcoma	[118]
			Squamous cell carcinoma	
	MCA	B16-F0	Skin fibrosarcoma	[175]
			Squamous cell carcinoma	
	DMBA + TPA		Squamous cell carcinoma	[176]
b2m$^{-/-}$			Spontaneous B-cell lymphomas	[182]
pfn$^{-/-}$				
TRAMP × *Tcrd*$^{-/-}$			Prostate carcinoma	[180]
	DMBA + TPA		Squamous cell carcinoma	[177]

MCA methylcholanthrene, *DMBA* dimethylbenzanthracene, *TPA* 12-*O*-tetra-decanoylphorbol, *β2m* β2-microglobulin, *pfn* perforin, *TRAMP* transgenic adenocarcinoma mouse prostate cancer

melanoma [185], pancreatic adenocarcinomas [185], and particularly a large number of hematopoietic cell-derived tumors [186, 187], including Daudi cell line derived from Burkitt's lymphoma [48, 188–190], and recently also toward cancer stem cells [191, 192]. However, the frequency of Vδ2 cells within lymphocytes infiltrating solid tumors is generally low, even within Vγ9Vδ2-suscepible tumors, such as renal and colon carcinomas [184, 193].

Another important antitumor effect is the induction of IFN-γ-producing Vγ9Vδ2 T-Cells in vivo. Multiple antitumor effects have been attributed to IFN-γ, including direct inhibition of tumor growth or more indirect effects such as the upregulation of MHC class I molecules and blocking of angiogenesis [194]. Interestingly, a significant negative correlation between the serum levels of the angiogenic factors like vascular endothelial growth factor (VEGF) and IFN-γ was found in cancer patients treated with aminobisphosphonates [195].

Conventional mouse models cannot be used to explore the possible antitumor activity of Vγ9Vδ2 cells in vivo, due to the lack of homologous TCR and thus the reactivity to phosphoantigens. However, xenogeneic immune deficiency (SCID) mouse models of human tumors have been established and revealed the efficacy of Vγ9Vδ2 T-Cells against several human tumors in vivo [35, 185, 196–202]. Pre-activated adoptively transferred human Vγ9Vδ2 T-Cells localized to tumors [197], increased survival, and inhibited tumor growth [35, 185, 197, 199, 201]. Vγ9Vδ2 T-Cells are also active against freshly isolated tumor cells from patients with follicular B-cell lymphoma or B-cell chronic lymphocytic leukemia (B-CLL) [203]. Similarly, a high survival rate is obtained when Vγ9Vδ2 TCR^+ tumor-infiltrating lymphocytes (TILs) (expanded from human colorectal tumors in vitro) are transferred into Daudi cell-bearing BALB/c nude mice compared with the transfer of αβ TCR^+ TILs or mice without treatment [204].

Although less studied, Vδ1 T-Cells are also promising targets for cancer immunotherapy. Vδ1 tumor-infiltrating lymphocytes from colorectal cancer were shown to lyse autologous and allogeneic colorectal, renal, and pancreatic tumor cell lines [205]. Moreover, circulating Vδ1 cells from chronic lymphocytic leukemia patients were able to lyse B-CLL cells expressing ULBP3 [206]. By contrast, with their Vγ9Vδ2 counterparts, Vδ1 cells are quite frequent within T-Cells infiltrating solid tumors [193, 205, 207, 208].

The authors have also recently demonstrated that Vδ1 antitumor properties can be enhanced by their culture in the presence of PHA and IL-2 [161]. Fully activated Vδ1 cells display stronger cytotoxicity against B-CLL cells than the corresponding Vδ9Vδ2 counterparts, which was attributed to the selective induction of NCR expression in Vδ1 cells [161].

Interestingly, Vδ1 cells share reactivity toward CMV-infected cells and tumor intestinal epithelial cells [21]. This dual recognition also seems to be a characteristic of the Vγ4Vδ5 clone [69]. Willcox and colleagues demonstrated that Vγ4Vδ5 TCR binds directly to endothelial protein C receptor (EPCR) and that is expressed in both endothelial cells targeted by cytomegalovirus and epithelial tumors [69].

13.7.2 Pro-Tumor Properties

The potent antitumoral properties of γδ T-Cells have been widely shown for more than 15 years. This notwithstanding, some recent studies imply a pro-tumorigenic role for γδ T-Cells, for example, γδ T-cell depletion reduced papilloma incidence [209] and breast tumor-infiltrating γδ T-Cells suppressed naive and effector T-cell responses and blocked maturation and function of dendritic cells (DCs) [210]. Moreover, intratumoral γδ T-Cells represented the most significant independent prognostic factor for assessing the severity of breast cancer compared with the other known factors. Intratumoral γδ T-Cells were positively correlated with $FOXP3^+$ regulatory T-Cells but negatively correlated with cytotoxic $CD8^+$ T-Cells in breast cancer tissues [211].

Peng and colleagues have shown that human Vδ1 cells derived from breast cancer biopsies inhibited the maturation and function of dendritic cells and suppressed proliferation and IL-2

production of CD4+ T-Cells in vitro [210]. Thus, a pro-tumor role of γδ T-Cells may be linked to immunosuppressive functions that need to be further characterized.

Alternatively, the controversial pro-tumor function of γδ T-Cells may rely on their production of IL-17, based on a study that showed that murine IL-17-producing γδ T-Cells promoted tumor growth in a murine fibrosarcoma tumor model [212]. However, murine IL-17-producing γδ T-Cells were reported to be necessary for Bacillus Calmette-Guerin (BCG) treatment of bladder cancer [213] and for chemotherapeutic efficacy in subcutaneous tumor models [214]. Actually, the role of IL-17 in tumor surveillance is itself paradoxical. IL-17 production has been associated with enhanced tumor development/progression in murine models of intestinal [215], skin [216], bladder [217], and ovarian carcinoma [218]; but, by contrast, IL-17-deficient mice were more susceptible to the development of lung melanoma [219] and lung metastasis [220].

A recent work performed by the authors suggests that γδ T-Cells promote tumor progression in a mouse model of ovarian cancer (unpublished data). The authors observed that γδ-deficient mice displayed decreased tumor burden compared with wild-type mice. Interestingly, a selective expansion of IL-17-producing γδ T-Cells in the peritoneal cavity of tumor-bearing mice was observed; therefore, the authors are investigating if γδ T-Cells promote ID8 tumor progression through the production of IL-17.

Several functions of IL-17 in the tumor microenvironment seem to contribute to tumor progression. Apart from a minor direct effect on the proliferation and survival of tumor cells (as not all tumor cells express the IL-17 receptor and respond to IL-17), the major pro-tumor function of IL-17 in inflammation-associated cancer cells seems to rely on its proangiogenic properties on the surrounding endothelial cells and fibroblasts [221]. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators [222, 223], including VEGF, which markedly promotes inflammatory and tumor angiogenesis.

A more detailed characterization of γδ-TILs, in a wider set of preclinical tumor models, is required to clarify the role of IL-17-producing γδ T-Cells in tumor immunosurveillance. This should take into account the two functional γδ T-cell subsets recently identified: CD27+ γδ T-Cells produce IFN-γ but no IL-17, whereas IL-17 production is restricted to CD27− γδ T-Cells [14].

13.8 γδ T-Cell Modulation in Cancer Clinical Trials

Several features of γδ T-Cells make them attractive targets for cancer immunotherapy: abundant IFN-γ secretion; potent, broad, and MHC-unrestricted cytotoxicity; and the availability of clinical grade agonists for Vγ9Vδ2 T-Cells. Vγ9Vδ2 T-Cells can be directly activated in vivo with TCR agonists or can be expanded in vitro and then reinfused into patients (adoptive cell therapy) [224] (Fig. 13.3). Clinical grade agonists used so far include the synthetic phosphoagonist bromohydrin pyrophosphate (BrH-PP) and the aminobisphosphonates pamidronate and zoledronate. In most clinical trials, recombinant IL-2 (rIL-2; a fundamental cytokine for γδ T-cell expansion) was used in combination with TCR agonists (Table 13.4).

The antitumor activity of γδ T-Cells was first tested in a clinical trial in 2003 in which rIL-2 was administered to patients combined with pamidronate for the treatment of NHL and MM [225]. The combination of pamidronate and low-dose rIL-2 was well tolerated and partial responses were observed in 33% of the patients. Aminobisphosphonates were originally developed as therapeutic drugs for osteoporosis but are increasingly used for cancer therapy due to their antiangiogenic and proapoptotic properties [241], as well as their properties of activating Vγ9Vδ2 T-Cells.

Several clinical trials followed, with most of them relying on an alternative strategy consisting of the adoptive transfer of in vitro-expanded Vγ9Vδ2 T-Cells with aminobisphosphonate (zoledronate, pamidronate, and BrH-PP) [224]. Zoledronate (the most used aminobisphosphonate) is efficient at expanding in vitro γδ T-Cells

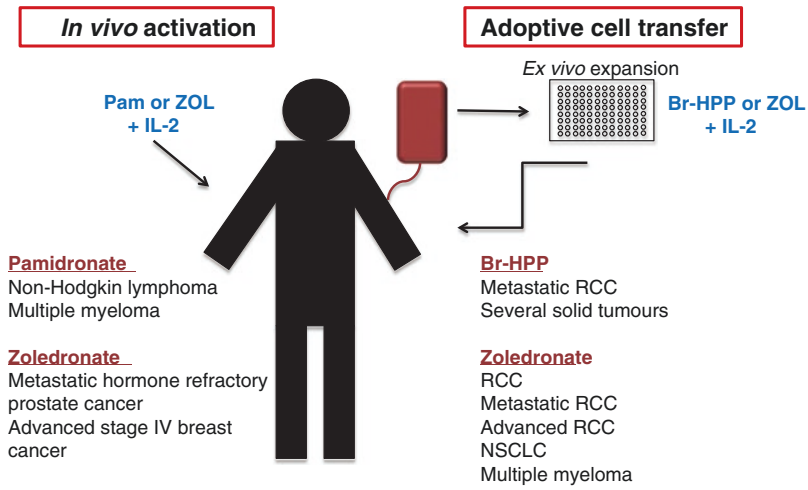

Fig. 13.3 Vγ9Vδ2 T-cell-based clinical trials. Strategies used in clinical trials include in vivo activation or adoptive transfer of ex vivo expanded γδ T-Cells with aminobisphosphonates (pamidronate or zoledronate) or phosphoantigens (BrH-PP), in combination with IL-2. *RCC* renal cell carcinoma, *NSCLC* non-small cell lung cancer, *ZOL* zoledronate, *BrH-PP* bromohydrin pyrophosphate

from patients with different diseases [233] and its efficacy was tested in clinical trials in patients with MM [234], renal cell carcinoma [231, 242], non-small cell lung cancer [235, 238]. These studies revealed no serious treatment-related adverse effects and demonstrated efficient expansion of Vγ9Vδ2 T-Cells [231] and inhibition of tumor growth [234]. However, the objective responses have been generally quite modest (Table 13.4).

Due to the potent activation properties of HMB-PP, this phosphoagonist seems a potential alternative to use in the clinic. In preclinical models, HMB-PP injection in macaques induced a prolonged major expansion of circulating Vγ9Vδ2 T-Cells with cytotoxic properties [243]. In clinical studies, there has been a complete remission in a metastatic renal cell carcinoma patient [237]. The patient underwent six monthly cycles of autologous γδ-PBLs, activated and/or expanded in vitro with HMB-PP plus rIL-2, combined with the infusion of zoledronate plus low-dose rIL-2. This response was associated with a sharp increase in IFN-γ-producing Vγ9Vδ2 T-Cells following adoptive transfer, and the patient has been disease-free for 2 years without any additional treatment.

Globally, the clinical trials completed to date (summarized in Table 13.4), particularly those stimulating γδ T-Cell in vivo, have shown objective responses in the range of 10–33%. While in some patients there was clearly insufficient expansion of Vγ9Vδ2 T-Cells [225, 227, 228], in other patients, this could not explain for the absence of objective response. A general disadvantage of autologous γδ T-cell-mediated immunotherapy is the frequent impaired function of γδ T-Cells in cancer patients. This phenomenon has been described in certain chronic infectious diseases such as HIV infection or tuberculosis, although the cause of this γδ T-cell anergy is not fully understood [244, 245]. Recent data obtained with other lymphocyte subsets suggest that tumor-derived PDL1/2 signals may be responsible for the inhibition of PD-1$^+$ T-Cells [246, 247]; nevertheless, these findings need to be further investigated [248]. Current γδ T-cell-based treatments, although feasible and safe, have obvious limitations. It is therefore critical to further clarify the basic mechanisms of γδ T-cell responses to tumors and to successfully modulate their activity in the clinic.

Table 13.4 Cancer immunotherapeutic approaches based on Vγ9Vδ2 T-cell activation

Immunotherapy	Cancer type	Treatment	*N*	% PD	% SD	% PR	% CR	Reference
In vivo administration of bisphosphonates	Refractory low-grade non-Hodgkin lymphoma and multiple myeloma	PAM + rIL-2 (d6–d8) without preselection	10	80	10			[225]
		PAM + rIL-2 (d1–d6) with preselection	9	44	22	33		
	Advanced breast and prostate cancer	ZOL	9	ND	ND	ND	ND	[226]
	Metastatic hormone-refractory prostate cancer	ZOL	9	78	11	11		[227]
		ZOL + rIL-2	9	33	44	44		
	Advanced stage IV breast cancer	ZOL + rIL-2	10	70	20	10		[228]
	Metastatic RCC	ZOL + rIL-2	6	ND	ND	ND	ND	[229]
	Advanced RCC, malignant melanoma, and AML	ZOL + rIL-2	21			25% (AML patients)		[230]
Adoptive transfer of Vγ9Vδ2 T-Cells expanded and activated in vitro	Advanced RCC	BrH-PP + rIL-2	7	ND	ND	ND		[231]
	Metastatic RCC	BrH-PP + rIL-2	10	40	60			[232]
	Solid tumors	ZOL + rIL-2	25	24				[233]
	Multiple myeloma	ZOL + rIL-2	6	ND	ND	ND	ND	[234]
	Non-small cell lung cancer	ZOL + rIL-2	10	63	37	0		[235]
	Solid tumors	BrH-PP + rIL-2	28	ND	ND	ND		[236]
	Metastatic RCC	ZOL + rIL-2	1				100 ($N = 1$)	[237]
	Non-small cell lung cancer	ZOL + rIL-2	15	60	40			[238]
	Solid tumors	ZOL						[239]
		– chemotherapy	5	40	40			
		+ chemotherapy	20	30	5	15		
	Solid tumors	ZOL + rIL-2	18	61	17	11	6	[240]

PD progressive disease, *SD* stable disease, *PR* partial remission, *CR* complete response, *RCC* renal cell carcinoma, *AML* acute myeloid disease, *PAM* pamidronate, *ZOL* zoledronate, *ND* not determined

13.9 Concluding Remarks

Over the past decade, various studies have reported encouraging results to target γδ T-Cells for cancer immunotherapy [224]. However, despite these important findings, various major questions remain unanswered. For instance, it will be very important to decipher the full repertoire of tumor antigens involved in γδ T-cell recognition and to find additional determinants of tumor cell killing. γδ T-Cells express a very diverse panel of inhibitory and activating receptors that directly impact on their activation state and function (Fig. 13.4). However, we still lack a dynamic picture of the receptors elicited along tumor-induced γδ T-cell activation, as well as a deep understanding of the interplay between the numerous signaling cascades induced upon sequential or concomitant receptor engagement [79].

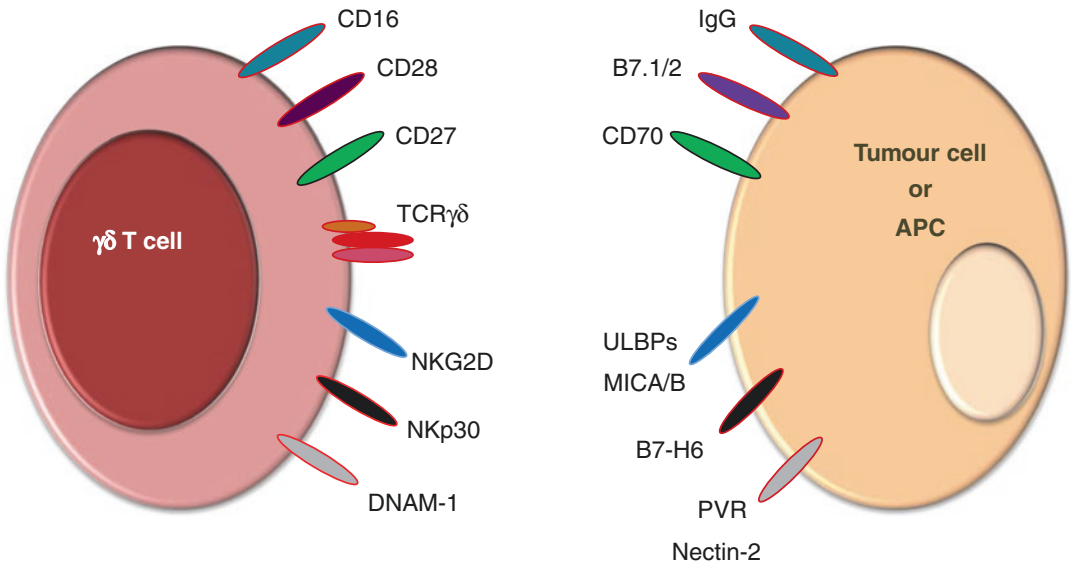

Fig. 13.4 Receptors involved in γδ T-cell activation and tumor cell recognition. T-Cells use their signature TCR to recognize antigens and cellular immune responses whose magnitude depends on the integrated engagement of a series of other surface receptors, including CD27, CD28, CD16, and natural killer receptors, such as NKG2D and DNAM-1

It will be very important to determine exactly how phosphoagonists trigger Vγ9Vδ2 TCR-mediated activation. One important recent study showed that intracellular accumulation of phosphoantigens is associated with membrane reorganization of CD277 molecules (BTN3A), which in turn leads to Vγ9Vδ2 T-cell activation [40]. Moreover, Harly and colleagues also described agonist and blocking CD277-specific antibodies that could be used for immunotherapeutic modulation of Vγ9Vδ2 T-cell responses toward tumor cells.

We believe that preselection of patients will increase the success of γδ T-cell-based clinical trials. Thus, patients with leukemia or lymphoma expressing ULBP1 [134], or ovarian epithelial carcinoma or colonic carcinoma expressing ULBP4, presumably will benefit the most from Vγ9Vδ2 T-cell therapy [64]. Also, additional work has identified a panel of ten genes encoding cell-surface proteins that segregated with "susceptible" versus "resistant" hematological tumors [249].

Nonetheless, the "anergy" of repeatedly challenged phosphoantigen-treated Vγ9Vδ2 T-Cells reported in vitro and in clinical trials [225, 227, 232] constitutes a serious obstacle to phosphoantigen-based immunotherapies. This acquired anergy may be caused by inhibitory receptors expressed on Vγ9Vδ2 T-Cells, as it was seen for PD-1 on $CD8^+$ T-Cells [250], but other mechanisms are also likely to be involved. Importantly, the promising results with PD-1 blockade in cancer clinical trials [251] suggest that its combination with Vγ9Vδ2 T-cell agonists may hold the key to improved success.

The absolute need for exogenous IL-2 administration in cancer patients has become the major drawback for the later stages of development of phosphoantigen therapies [232]. In vivo administration of IL-2 (a very pleiotropic molecule) has a very deep impact on the patients' immune system and unpredictable consequences concerning Vγ9Vδ2 T-cell activation. For example, the authors revealed that Tregs (which are highly sensitive to IL-2) can inhibit γδ T-cell proinflammatory functions in mice [252] and other studies have shown this in humans [253]. Studies with αβ T-Cells struggled with the same problem, although only a few trials have omitted IL-2 infusions

[254]. As previously described, phosphoantigens alone cannot sustain Vγ9Vδ2 T-cell activation and very low levels of IL-2 lead to incomplete cell activation. Thus, the ex vivo activation of γδ T-Cells for adoptive cellular immunotherapy, avoiding IL-2 infusions, clearly seems to be a more attractive strategy. Still, nonresponsive (NR) patients are typically excluded from Vγ9Vδ2 T-cell-based adoptive immunotherapy trials, owing to the impossibility of increasing the number of cells in vivo or ex vivo. The reason for this is not yet understood, although autologous DCs pretreated with zoledronate induced some expansion of Vγ9Vδ2 T-Cells in NR patients [255].

The antitumor properties of adoptively transferred γδ T-Cells can also be improved during in vitro expansion. This could be achieved, for example, through addition of IL-15 (which may increase cytolytic properties and tumor reactivity of γδ T-Cells through upregulation of NKG2D signaling) or IFN-α (which may increase TNF-related, apoptosis-inducing, ligand-dependent killing of tumor cells). Moreover, transduction of γδ T-Cells with tumor-specific TCRs, or chimeric tumor-specific antigen receptors [256], will enlarge the tumor cell recognition pattern of γδ T-Cells.

On the other hand, the authors have demonstrated that Vδ1 T-Cells may be an important alternative to Vγ9Vδ2 T-Cells. A novel, highly cytotoxic subset of Vδ1 T-Cells that express NCRs has been characterized [161]. Interestingly, Vδ1 T-Cells were numerically enriched and displayed enhanced cytotoxicity when compared to their Vδ2 counterparts in a collection of 74 primary cutaneous melanomas [208]. Moreover, the authors' most recent work demonstrated that Vδ1 T-Cells, but not Vδ2 T-Cells, express CCR2 and migrate to CCL2, whose expression is strongly deregulated in multiple human tumor types [257]. We are now pursuing with preclinical studies to apply Vδ1 T-Cells (expressing NCRs) in cancer immunotherapy. Of note, no clinical trial based on Vδ1 T-Cells has been conducted to date.

The in vivo efficacy of γδ T-cell-based immunotherapies can also be improved by using combinatorial regimens with chemotherapy. For example, prior lymphodepletion (similarly to the protocols applied before bone marrow transplantation) may sustain γδ T-cell proliferation and survival after adoptive transfer protocols. Moreover, along with the studies in mice [214, 258], γδ T-Cells seem to be highly beneficial after chemotherapy-induced tumor cell death.

Finally, it was observed that despite their promise for cancer immunotherapy, γδ T-Cells may, under certain conditions, display pro-tumor functions. Moreover, γδ T-cell infiltration is associated with poor survival of breast cancer patients [211]. These findings raise interesting questions for future investigation: Are there distinct pro-tumor versus antitumor γδ T-cell subsets? Do these differentially infiltrate tumor types? Does the tumor microenvironment manipulate the balance between pro-tumor versus antitumor γδ T-cell subsets? If so, can we intervene to tip the balance toward antitumor γδ T-Cells?

It is hoped that the collective efforts in developing novel γδ T-cell-based immunotherapy protocols will offer an alternative treatment to patients affected by cancer, particularly by preventing disease relapse upon failure of conventional treatments.

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14 Adoptive T-Cell Therapy: Optimizing Chemokine Receptor-Mediated Homing of T-Cells in Cancer Immunotherapy

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14.1 Introduction

Cancer immunotherapy has emerged in the last decade as a promising strategy to prevent cancer by use of the immune system. Several approaches have been developed [1–5]; these include administration of immunostimulatory agents, highly specific monoclonal antibodies (mAbs), cancer vaccines, and cell-based therapies. Cancer immunotherapy can be broadly divided into three major branches: (a) immunostimulatory interventions, which enhance existing immune responses, (b) anticancer vaccines (including protein, peptide, and cell-based vaccines), which stimulate an immune response against the cancer, and (c) adoptive cell transfer based therapy,

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which involves the administration of immune cells capable of directly attacking the tumor [6].

Immunostimulatory interventions include systemic administration of lymphocyte targeting growth factors such as interleukin-2 (IL-2), pro-immunogenic cytokines such as interferon alpha (IFN-α), or chemotherapeutics that selectively deplete immunoregulatory cell populations. Immunotherapy with high-dose interleukin-2 (IL-2) can mediate long-term survival only in a small percentage of patients [7]. Combination biochemotherapy is administered frequently and can also result in modest objective responses, but with no substantial improvement on overall survival compared with chemotherapy alone [8]. In the last few years, the use of monoclonal antibodies directed towards T-cell-associated immunosuppressive molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA4) and programmed cell death protein 1 (PD-1), have revolutionized oncology treatments, providing tumor regression and durable responses also in patients with advanced diseases. However, only some patients responded to these treatments [7, 9–11]. Some anticancer agents that are currently used in the clinic also mediate immunostimulatory effects by selectively inhibiting/killing immunosuppressive cells such as $Foxp3^+$ regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs). These include, for instance, antibody-based agents or kinase inhibitors mediating both the cytotoxic/cytostatic effect on tumor cells and the vessel network and the stimulatory effect on the immune system [12, 13].

Adoptive cell therapy (ACT) has emerged as an effective form of immunotherapy, with rates of complete durable responses (in specific clinical settings) as high as 40% [14, 15]. Notably, ACT must be conceptually differentiated from other cell-based immunotherapies, including the reinfusion of autologous DCs (dendritic cells) pulsed ex vivo with tumor antigens or tumor cell lysates (aimed at eliciting an anticancer T-cell response in vivo) and the infusion of allogeneic T and NK cells (aimed at obtaining a powerful and hopefully curative graft-versus-disease effect) [16, 17]. Immunotherapy using autologous T-cells has de facto emerged as a powerful treatment option for patients with metastatic melanoma. These it includes the adoptive transfer of autologous tumor-infiltrating lymphocytes (TILs), T-cells transduced with high-affinity T-cell receptors against major tumor antigens, and T-cells transduced with chimeric antigen receptors composed of hybrid immunoglobulin light chains with endodomains of T-cell signaling molecules.

In this chapter, the authors will briefly review the scientific rationale behind ACT and discuss the recent advancement and studies evaluating various aspects of T-cell adoptive transfer in current oncological settings.

14.2 T-Cell Infiltration Correlates with Prognosis

Activated effector T-cells move through tissues, scan for MHC (Major Histocompatibility Complex) peptide complexes that convey further activation signals through their antigen receptors (TCRs), and are capable of indirectly sensing a variety of signals that can alert them against potentially threatening pathogens; the same applies to their responses to cancer. Tumor-specific T-cells are capable of directly recognizing antigens presented by specialized antigen-presenting cells (APCs) and also on the surface of tumor cells [18]. Tumors contain a variable number of tumor-infiltrating lymphocytes, whose importance is highlighted by their prognostic value in human cancer [19]. T-cells traffic to areas where their target antigens are expressed and can produce cytokines, chemokines, and angiogenic factors that affect tumor growth. T-cells that mediate effective antitumor responses may also directly mediate cytotoxic responses against tumor cells, either through their expression of apoptosis-inducing molecules or through the release of cytotoxic granules [19]. Mature differentiated $CD8^+$ T-cells and some types of $CD4^+$ T-cells release proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor (TNF), which enhance the immune response by upregulating the expression of MHC class I and MHC class II molecules on both tumor cells and tumor-resident APCs. $CD4^+$ T-cells are capable of activating and regulating many aspects of innate and adaptive immunity, including the function of cytotoxic $CD8^+$ T-cells.

Besides, they can also engage and authorize APCs, which in turn recruit additional T-cells and promote the activation of the innate immune system [20]. On the contrary, in other tumors, like melanoma, the protective role of TILs is compromised by the high proportion of Tregs that downregulate the activation and expansion of tumor reactive lymphocytes [21].

It has been shown using genetic and histological analysis of a large cohort of colorectal cancer patient biopsies that both the type and the location of immune cell infiltrate predict improved patient survival. Specifically, patients whose tumor centers or invasive margins were highly infiltrated with T-cells had the best predicted survival. In contrast, patients with stage I tumors containing few or no infiltrating T-cells had a prognosis similar to metastatic stage IV patients, even though they originally presented with minimally invasive disease [22]. Other studies also show that in some tumors, particularly in colon carcinoma, the presence of TILs is a strong predictor of the clinical outcome. Higher $CD3^+$ TIL densities, colonic site, and absence of nodal involvement were significantly associated with a lower risk of metachronous metastasis [23].

Many studies examining other cancers reached similar conclusions, consequently defining a better picture in which the immune infiltrate, also defined as the immune score, correlates with improved prognosis [24].

Indeed, increased antitumor response has been shown to correlate with higher leukocyte infiltrate in mice and humans [25–29]. Aiming to increase the trafficking of T-cells to tumors may indeed result in more effective antitumor responses. The generation of an effective immune response is a complex series of events involving threat recognition, antigen presentation by specialized cells in lymphoid tissues, and clonal expansion of antigen-specific T-cells [30, 31]. After their generation, antigen reactive T-cells need to move from lymph nodes and migrate to the site of threat and penetrate the affected tissue. Trafficking of T-cells to particular sites is in itself a multistage process involving rolling and arrest on endothelium, followed by extravasation and penetration of tissue.

The critical steps of arrest and tissue penetration are dependent on selectin and integrin expression on endothelium and lymphocytes [32] and the interaction between chemokines, secreted by tissues, and chemokine receptors expressed on the surface of T-cells [33–35].

14.3 Adoptive T-Cell Therapy

The treatment of patients with cell populations that have been expanded ex vivo is called adoptive cell transfer (ACT). Cells that are infused back into a patient after ex vivo expansion ($>10^{10}$ cells in some cases) can traffic to the tumor and mediate its destruction. Immunotherapy based on the adoptive transfer of tumor-specific lymphocytes isolated from excising tumor masses (such as TIL expanded with T-cell growth factor interleukin-2 (IL-2) ex vivo), or of genetically engineered T-cells, has a rich history dating back to several decades ago [36–38]. The transfusion of lymphocytes, referred to as adoptive T-cell therapy, is being tested for the treatment of cancer and chronic infections. Adoptive T-cell therapy has the potential to enhance antitumor immunity, augment vaccine efficacy, and limit Graft-versus-Host Disease (GVHD). This form of personalized medicine is now in various early- and late-stage clinical trials. These trials are currently testing the best strategies to infuse tumor-infiltrating lymphocytes, CTLs, Th (T helper) cells, and Tregs [39, 40].

To date, one of the most powerful immunotherapies against metastatic melanoma has been ACT using autologous ex vivo expanded TILs adoptively transferred back into patients. This strategy for the treatment of metastatic melanoma patients was initially described in 1988 [41] and has since yielded dramatic results with greater than 50% clinical responses [42], many of which are lasting for years in recent clinical trials [14, 43–47]. Although ACT with TILs has delivered promising results in phase 1 and 2 trials at the Surgery Branch, NCI, USA [45, 46], it is not currently possible to treat every patient with metastatic melanoma with this strategy due to several reasons, including lack of an available tumor for surgical harvest, inability to isolate and grow viable TIL, or inability to show robust, specific effector function of isolated TIL. The latter could potentially be due to impairment of

T-cell effector function due to "immune checkpoint" mechanisms. Indeed, combination therapy with anti-CTLA4 (NCT 01701674) has been recently demonstrated to be a feasible approach to improve ACT therapy in patients with metastatic melanoma [48].

Alternative investigative protocols have thus evolved in an effort to address these limitations. Use of genetic engineering to create antigen-specific effector T-cells from peripheral blood lymphocytes may be an option for those patients without tumors amenable to surgical resection or for patients in whom viable TIL cannot grow from their tumors [49–55].

More recently, other forms of ACT using engineered T-cells have entered clinical testing. These include T-cells propagated from peripheral blood mononuclear cells (PBMCs) expressing cloned recombinant T-cell receptor (TCR) chains recognizing epitopes from shared tumor-associated antigens (TAAs) [54, 56], or expressing chimeric antigen receptors (CARs) composed of immunoglobulin variable regions recognizing tumor antigens. These immunoglobulin regions are fused to signaling domains of the TCR and of co-stimulatory molecules, such as CD28 and CD137/4-1BB [57, 58].

The pace of research in autologous T-cell-based therapies for melanoma has increased dramatically over the last decade with new target antigens and an increased number of clinical trials testing both TIL and TCR- or CAR-transduced T-cells [59]. Improved molecular biology techniques have also increased enthusiasm and feasibility for testing genetically engineered T-cells. Recent advances in cellular immunology and tumor biology are guiding new approaches to adoptive T-cell therapy. For example, the use of engineered T-cells is being tested as a strategy to improve the functions of effector and memory T-cells, and manipulation of the host to overcome immunotoxic effects in the tumor microenvironment has led to promising results in early-stage clinical trials. Challenges that face the field must be addressed before adoptive T-cell therapy can be translated into routine clinical practices.

14.4 Challenges in Adoptive T-Cell Therapy

Despite the frequent detection of circulating tumor antigen-specific T-cells, either spontaneously or following active immunization or adoptive transfer, immune-mediated cancer regression occurs only in a minority of patients. In addition, although some ACT patients achieve long-term disease-free survival, most patients still suffer relapses [60]. Furthermore, the requirement of a large number of laboratory expanded T-cells ($>1 \times 10^{10}$) makes ACT a costly and labor-intensive treatment [61]. One important limiting factor for ACT is the inefficient migration of T-cells into tumor tissue.

By labeling T-cells before ACT, it has been shown that the number of adoptively transferred T-cells migrating to the tumor microenvironment correlates positively with clinical response [49]. However, this analysis also showed that the trafficking efficiency of transferred T-cells was extremely low [62]. Therefore, strategies aimed at improving the migration of T-cells to tumor sites are likely to enhance the efficacy of ACT therapy and improve clinical response rates [63].

Homing of effector T-cells to inflamed tissues is thought to depend on various adhesion molecules such as LFA-1 and VLA-4 [29, 64] and also on the activity of specific chemokines [65]. The homing of T-cells toward tumors depends on an intricate network of guiding cues that is only beginning to be understood and involves chemokines secreted from the tumor milieu [66, 67]. The relatively low clinical activity of melanoma vaccines, despite induction of specific T-cell responses detected in the blood, has suggested the possibility of downstream resistance mechanisms at the level of the tumor microenvironment. Current studies indicate that some tumors lack key chemokines that can be critical for recruitment of activated T-cells into metastatic sites. This could represent an important barrier for effective T-cell-mediated rejection of tumors in vivo.

The typical tumor vasculature exhibits disorganized, tortuous, and highly permeable vessels

causing increased interstitial pressure, heterogeneous permeability, and irregular blood flow. This complex tumor vasculature creates a major hurdle for tumor-specific T-cells: it hampers getting in direct contact with the target by crossing the abnormal tumor vessel barrier and interstitium [68]. A more detailed explanation could be that, within the tumor microenvironment, the presence of angiogenic factors such as vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) causes downregulation of intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and CD34 on endothelial cells [69].

More recent studies have shown that VEGF may also induce indirect inhibition of T-cell recruitment via the induction of FasL [70]. As a result, leukocyte–vessel wall interactions are diminished in tumors; effector T-cells, regardless of being induced in vivo by vaccination or adoptively transferred, may thus be impaired in their deployment towards tumor sites from getting in direct contact with target tumor cells. Strategies have been attempted to improve immunotherapy by reducing the endothelial barrier, favoring the penetration of drugs, and improving T-cell infiltration based on the use of angiogenesis inhibitors such as anginex, endostatin, and angiostatin or anti-VEGF reagents such as soluble chimeric VEGF receptor (VEGFR) and anti-VEGF or VEGFR antibodies [71–75]. These drugs transiently normalize the tumor vasculature, pruning away immature and permeable vessels and remodeling the remaining vasculature. In the tumor microenvironment, these drugs can also overcome the endothelial barrier by preventing VCAM and ICAM downregulation, therefore promoting leukocyte infiltration in tumors [69].

The inability of many T-cells to reach the cancer tissue depends also on stromal features, which may form physical barriers, thus impeding T-cell arrival, including that of CTL used in ACT. In the tumor microenvironment, cancer-associated fibroblasts (CAF) display an activated phenotype and continuously deposit collagens and other extracellular matrix proteins, eventually building a dense stroma, such as those found in some lung or pancreatic cancers [76]. High density extracellular matrix formation has several effects, such as the increase in the interstitial fluid pressure and dysfunction of new vessels, both affecting tissue perfusion of blood circulation. Increasing evidence shows that failure to respond to anticancer treatments is associated with a TGFβ signature in fibroblasts. Targeting CAFs with TGFβ-blocking agents decreased fibrosis formation and facilitated T-cell penetration in experimental murine tumors [77, 78]. In both reports, inhibition of TGFβ significantly increased the response to anti-PD-1/PD-L1 immunotherapy, leading to tumor regression.

As anticipated above, another important issue potentially hampering the trafficking ability of adoptively transferred T-cells is the lack of stimuli specifically directing the migration of lymphocytes into tumor tissue, such as chemokines.

14.5 Chemokines

Chemokines were first recognized as a family of small protein molecules, induced by inflammatory conditions, and capable of attracting inflammatory leukocytes (such as monocytes, activated T-cells, and neutrophils) [79]. Chemokines act through transmembrane domain G-protein-coupled receptors to elicit a signaling cascade culminating in directed locomotion. They are classified into four groups (C, CC, CXC, and CX3C) according to the number and spacing of cysteines in a conserved N-terminal motif [65, 80]. In humans, more than 50 chemokines classified into four families according to their nomenclature have been described. Facing these ligands, 19 chemokine receptors have been identified, indicating that one receptor may be associated with several ligands [65]. Two functional types have been defined, namely, the “inflammatory” or inducible chemokines, recruiting effector cells in inflamed tissues, and the “homeostatic” chemokines, constitutively produced by lymphoid or non-lymphoid tissues that control leukocyte traffic under physiological conditions [34, 81–84].

The chemokine system is characterized by redundancy, with some receptors binding several chemokines (e.g., CCR1–CCR5) and others only one (e.g., CXCR4–CXCR6). Some receptors function as "deceptors" or decoy receptors that bind chemokines but do not transmit signals [85, 86]. Though originally identified in the control of leukocyte chemotaxis, especially during infection and inflammation, it is now known that virtually all cells, including tumor cells, express chemokines and chemokine receptors.

The pleiotropy in the chemotactic system is reflected by the diverse physiological and pathological processes it coordinates with, including patterning of neuronal cells in the developing nervous system, homeostatic transport of hematopoietic stem cells, lymphocytes and dendritic cells, inflammatory diseases, tumor growth, metastasis, angiogenesis, and recruitment of macrophages by tumors [66, 67, 87, 88].

Recent characterization of the function of chemokines and chemokine receptors in the immune system has shown how immune cell localization can act as a regulatory mechanisms during both immune responses and tolerance. Tumor cells and the microenvironment constitutively express a variety of chemokines that play a key role in orchestrating the recruitment and positioning of leukocytes, including effector cells with potential antitumor functions. Immune cell recruitment and cell-based systems that may control leukocyte trafficking in cancer immunotherapy are some of the potential areas of focus in the efforts to enhance T-cell immunotherapy against cancer.

However, chemokine action is not restricted to their eponymous function of "cell mobilization" and these molecules are key participants of the cancer-related inflammation [67, 82, 89]. CCL2 and related chemokines contribute to polarizing tumor-associated macrophages (TAMs) in a tissue repair/remodeling, promoting tumor growth [90, 91]. Blocking the CCL2/CCR2 axis in a liver tumor mouse model inhibits monocyte recruitment and macrophage M2-like polarization at the tumor site [92]. Recent clinical trials using chemotherapy in combination with CCL2 inhibition have shown initial antitumor activity in patients with advanced prostate cancer (NCT00992186) and other solid tumors (NCT01204996) [93]. Chemokines have positive effects on tumor cell proliferation/survival and regulate angiogenesis: for instance, CXCL8 is a growth factor for most malignant melanomas and other tumors [94, 95], as well as CCL5 and CXCL12 [96, 97]. It has been shown in vitro that cancer-associated fibroblasts release chemokines CXCL12, CXCL14, CCL2, and CCL5 and a variety of cytokines involved in tumor cell growth, angiogenic and metastatic processes, and immune cell infiltration, as recently reviewed in [98].

14.6 Role of Chemokines in Directing Tissue Trafficking in Tumors

Recent studies highlighted the potential use of chemokines in cancer immunotherapy to improve innate and adaptive cell interactions and recruit effectors into the tumor microenvironment and lymphoid tissues [99]. Some of the most promising chemokine networks for cancer immunotherapy are CCL21/CCL19 and the receptor CCR7, CCL2/CCL3/CCL5/CCL16, and their cognate receptors. The chemokine receptor CCR7 and its ligands CCL21 and CCL19 were first identified for their homeostatic role in directing the migration of mature dendritic cells (DCs) from the periphery to tumor-draining lymph nodes, where they present antigen to naive T-Cells. The latter also use CCR7-mediated mechanisms to enter the T-cell zone [100]. These chemokines have also been shown to chemoattract B-cells and NK cells to the lymph nodes. More recently, ectopic CCL19 and CCL21 expression in the tumor microenvironment has been used to bring naive lymphocytes and mature DCs together in a pseudo-lymph node for cancer immunotherapy [101].

In 2000, the first studies using recombinant CCL21 as a monotherapy for preclinical tumor models demonstrated a potent immune-mediated antitumor response that led to complete eradication of lung carcinoma tumors [102]. This response was found to be $CD4^+$ and $CD8^+$ lymphocyte dependent with significant DC infiltration into tumors and tumor-draining lymph nodes.

Similar studies by Vicari et al. showed that mouse CCL21 exerted antitumor effects. This was mediated through its angiostatic effect, whilst activation of $CD8^+$ T and possibly NK cells also lead to reduced implantation of CCL21-transduced CT26 colon carcinoma cells [103]. Furthermore, CCL19 transduction of murine breast carcinoma cells led to the rejection of tumors in a NK and $CD4^+$ T-cell-mediated manner [104]. In addition to its use as a monotherapy, CCL21 has been included in combined immunotherapy protocols. Studies using murine B16 melanoma lysate-pulsed DCs, modified to produce CCL21, demonstrated the ability of this chemokine to enhance the antitumor effects of DC vaccination [105, 106].

The chemokines CCL2, CCL3, and CCL5 have overlapping roles in regulating the migration of multiple subsets of innate and adaptive immune cells. Upon binding of CCL2, CCL3, or CCL5 to their cognate receptors (CCR2, CCR1, and CCR5, respectively), immature DCs, monocytes, and memory and T effector cells extravasate from the vasculature and enter peripheral sites of inflammation or infection [107–109]. The broad chemotactic actions of these proteins have made them important components of cancer immunotherapy strategies aimed at increasing immune cell infiltration into tumors. To this end, CCL2, CCL3, and CCL5 used in monotherapy or in combination therapy have been shown to induce both tumor regression and immunity to the subsequent tumor challenge in multiple preclinical models, as described by Homey et al. [101]. Chemokine receptor CCR5 is involved in T-cell migration; post-IL-12 treatment, upregulation of mRNA expression of CCR5 has been seen in splenic T-cells as well as of ligands for CCR5 (MIP-1α and MIP-1β) in tumor masses. Administration of a synthetic CCR5 antagonist TAK-779 to tumor-bearing mice during IL-12 immunotherapy prevented T-cell migration and tumor regression. Anti-CCR5 antibody was found to inhibit T-cell migration in the lymphoid cell migration assay. Similarly, human tumors made to overexpress CCL5 were more capable of attracting T-cells in mouse xenograft 2-photon imaging experiments [110]. These results indicate a critical role for CCR5 in the induction of T-cell migration to tumor sites after IL-12 treatment [111]. CCL5 was also found to be effective when used as a monotherapy or in combination immunotherapy protocols. Aravindaram et al. demonstrated that B16/gp100 primary tumors and lung metastasis in C57BL/6JNarl mice are strongly suppressed in murine models treated with gp100 vaccination and CCL5 therapy, which induces more potent splenocyte cytotoxic activities toward B16/gp100 cells [112]. Higher levels of IL-4, IL-6, IFN-γ, and TNF-α along with longer survival times are seen in mice treated with recombinant CCL5 protein and GM-CSF-transduced tumor cell vaccines when compared with mice treated solely with GM-CSF-transduced vaccines [113]. CCL5 and FLT3L combined with a DNA vaccine have also been shown to inhibit tumor growth in hepatitis B viral antigen HBc-expressed B16 melanoma model [114]. Lapteva et al. created an Ad-RANTES-E1A vaccine, which utilizes a recombinant oncolytic adenovirus expressing CCL5 that induces primary tumor regression and blocks metastasis in mammary carcinoma murine models [107].

Parker et al. showed enhanced tumor growth inhibition and greater levels of $CD4^+$ and $CD8^+$ T-cell infiltrates in murine flank neuroblastoma treated sequentially with HSV-1 expressing IL-12 and HSV-1 expressing CCL2 when compared with either treatment alone [115]. Furthermore, Nagai et al. demonstrated that vaccinations with human malignant glioma constitutively secreting CCL2 in nude mice induced tumor infiltration by NK cells and monocytes [116]. Similar results were found in studies using CCL3. Hirose et al. showed that nude mice given subcutaneous injections of Chinese hamster ovary cells genetically modified to secrete CCL3 demonstrated greater tumor growth inhibition and greater neutrophilic infiltration when compared with controls [117]. Cao et al. demonstrated that CCL3-recruited DCs, transduced with a tumor antigen gene, induced a strong CTL response and effectively eliminated established tumors and prevented metastases [118].

Among CXC chemokines, CXCL9 and CXCL10 are considered the main attracting stimuli for TIL, which express high levels of the cog-

nate receptor CXCR3. Increased expression of these chemokines can elicit antitumor responses correlated with increased infiltration of CD4 and CD8 lymphocytes [119]. The importance of CXCL9 and CXCL10 in the recruitment of TIL at the tumor site is also supported by observations in human tumors characterized by the abundance of TIL, such as gastric and colorectal carcinoma [120, 121]. In these tumors, TILs predominantly express CXCR3, and significant levels of CXCL9 and CXCL10 are produced mainly by myeloid cells in the stroma. In line with these findings, a recent study demonstrated that lack of CXCL9 and CXCL10 produced by $CD103^+$ dendritic cells (DCs) was the cause of poor infiltration of effector T-cells into tumors [122].

TIL can be recruited through the production of CX3CL1, a transmembrane and secreted chemokine also named Fractalkine. CX3CL1-overexpressing neuroblastoma cells are capable of inducing migration, adhesion, and IFN-γ secretion by immune effector cells [123]. High expression of CX3CL1 was positively correlated with good prognosis and the number of TILs in colorectal carcinoma [124]. The chemokine CXCL16, also a transmembrane protein, can contribute as well to the recruitment of TIL in carcinomas. CXCL16 was found overexpressed by reactive astrocytes and glioma cells [125], neuroblastoma, pancreatic ductal adenocarcinoma [126], and breast carcinoma [127].

It has been reported that ionizing radiation therapy markedly enhanced CXCL16 secretion by mouse and human breast cancer cells, which recruited $CXCR6^+$ effector T-cells [128]. CXCL16 has been described as a positive prognostic marker in renal [129] and in colorectal carcinoma, where tumors with high CXCL16 expression had an increased number of $CD4^+$ and $CD8^+$ cells and a better prognosis than the weak CXCL16 expression group [130]. On the contrary, in prostate cancer CXCL16 expression has been correlated with poor prognosis [131]. Similarly, the presence of fibroblasts with enhanced CXCL16 expression correlates with aggressive tumor phenotype and higher protumoral innate cell infiltration in patients with triple negative human breast cancer [132].

In spite of these positive effects for an antitumor immune response, chemokines also play a major role in enhancing the accumulation of immune suppressor cells responsible for promoting tumor growth. As regulators of cell migration, chemokine networks are frequently usurped by cancer cells for facilitating tumor growth and metastasis, suppressing antitumor immune responses, regulating angiogenesis, and influencing the formation and spread of metastases [67, 83]. Expression of chemokines by tumors may also have immunomodulatory effects resulting in decreased immunogenicity of the tumor [133, 134] or desensitization of chemokine receptors on T-cells [135]. CCL2 was shown to be overexpressed by tumor-associated fibroblasts in breast cancer and greater CCL2 and CCL5 levels in the tumor microenvironment correlated with the accumulation of macrophages and more advanced disease [136]. Similarly, Zhang et al. demonstrated multiple roles for CCL2 in promoting prostate cancer growth, including modulation of TAM migration and promotion of osteoclast maturation, as well as direct effects on prostate cancer cell proliferation, migration, and invasion [137].

In the tumor microenvironment, CXCL12 functions as an anti-inflammatory chemokine that skews the polarization of antigen-specific Tregs and IL-10-producing DCs/monocytic cells to restrain the inflammatory process and suppress antitumor immunity [138, 139]. CCL2 and CCL3 have been shown to increase the infiltration of Tregs, myeloid-derived suppressor cells (MDSCs), and TAM [140–143]. Furthermore, $Foxp3^+$ regulatory T-cells migrate to the paracortical areas of peripheral lymph nodes in a CCR7-dependent manner [144].

On the whole, while chemokines are instrumental to direct tumor infiltration by immune effector cells, they may also contribute to the recruitment of suppressor cells that hamper antitumor immune responses and promote tumor tolerance. Immunotherapeutic strategies using depletion or inactivation of suppressor cell populations in addition to chemokine-based stimulation of antitumor immunity may thus prove especially effective.

14.7 Overexpression of Chemokine Receptors in Engineered Lymphocytes to Be Used for Cancer Immunotherapy

Adoptive T-cell immunotherapy with tumor-infiltrating lymphocytes or genetically modified T-cells has yielded important results in some cancers. However, T-cells need to traffic properly into tumors to adequately exert therapeutic effects. One approach to improve antitumor immunity is to increase the infiltration of immune cells into the tumor or facilitate the movement of antigen-presenting cells (APCs) to tumor-draining lymph nodes to prime naive T and B lymphocytes. The chemokine receptor pattern expressed by T lymphocytes depends on their differentiation and activation state and is influenced by the tumor microenvironment. Through specific antigenic priming, naive T lymphocytes differentiate into memory/effector cells, downregulate the receptors for homeostatic chemokines such as CXCR4 and CCR7, and upregulate those for the inflammatory chemokines according to the type of polarization: CCR1, CCR2, CCR3, and CCR4 for a Th2 response and CCR5 and CXCR3 for a Th1 response [145].

Furthermore, after T-cell activation, the chemokine receptor expression can be transiently modulated, thus acquiring new migratory capacities [90, 146]. Engineering T-cells by methods such as introduction of chimeric antigen receptor or introduction of co-stimulatory signal gene has indeed produced impressive results in adoptive T-cell-based cancer immunotherapy. Likewise, introduction of chemokine receptor gene into T-cell engineering may also become an important aspect of improving the process of T-cell immunotherapy. Advances in the genetic modification of T-cells and understanding of leukocyte trafficking can make it possible to afford the opportunity of engineering T-cells to express any one or combination of receptors and thus potentially direct their migration to a predetermined target (Fig. 14.1). Expression of the chemokine receptor CXCR4 into T-cells may be useful to target CTL to bone marrow for the treatment of leukemias or metastatic tumors growing in the milieu of marrow stromal cells that produce CXCL12, the ligand for CXCR4 [147]. Similarly, introduction of CXCR5 or CXCR2 to T-cells might be used for targeting CTL to follicular lymphoma cells producing CXCL13 or melanoma cells producing CXCL1, respectively [148, 149].

The published data regarding overexpression of chemokine receptors on T-cells directing antitumor effector T-cells to tumor sites are still limited. It was found, for example, that CCL2 and CCR4 play a role in T-cell chemoattraction by melanoma in vitro [150] and that tumor infiltration of T-cells is strongly associated with high CXCL9 and CXCL10 expression in melanoma in in situ hybridization studies [151]. CXCL12 is shown to enhance T-cell migration toward melanoma in vitro [152], but it also causes chemorepulsion in other systems [153]. The selective expression of chemokine receptors by different subsets of T-cells can determine specific trafficking of these subsets to tissues expressing the appropriate chemokine. Thus, for example, CCR7, expressed by naïve T-cells, facilitates migration to lymph nodes where the ligands for this receptor, CCL21 and CCL19, are produced [154]. The expression of chemokine receptors by T-cells and chemokines at sites of antigenic challenge determines the specific traffic of lymphocytes. For example, the ligands for CXCR3, CXCL10, and CXCL9 [155], which can be expressed by activated monocytes, fibroblasts, keratinocytes, and endothelial cells [156], may enable cells bearing CXCR3 to traffic preferentially to IFN-γ-producing inflammatory sites.

Recent evidence regarding the hierarchy of chemokine receptors that are involved in tumor antigen-specific T-cell trafficking has shown that in melanoma CXCR3 has a necessary and nonredundant role in lymphocyte homing compared to CCR2 and CCR5, which are nonessential [132]. However, it has been demonstrated that melanoma and other types of solid tumors do not express sufficient levels of CXCR3 ligands as well as CCL2, CCL4, and CCL5 [157]. Yet, the complete T-cell/tumor chemotactic network is still to be explored, as well as the pattern of chemokine receptors on clinically derived ex vivo

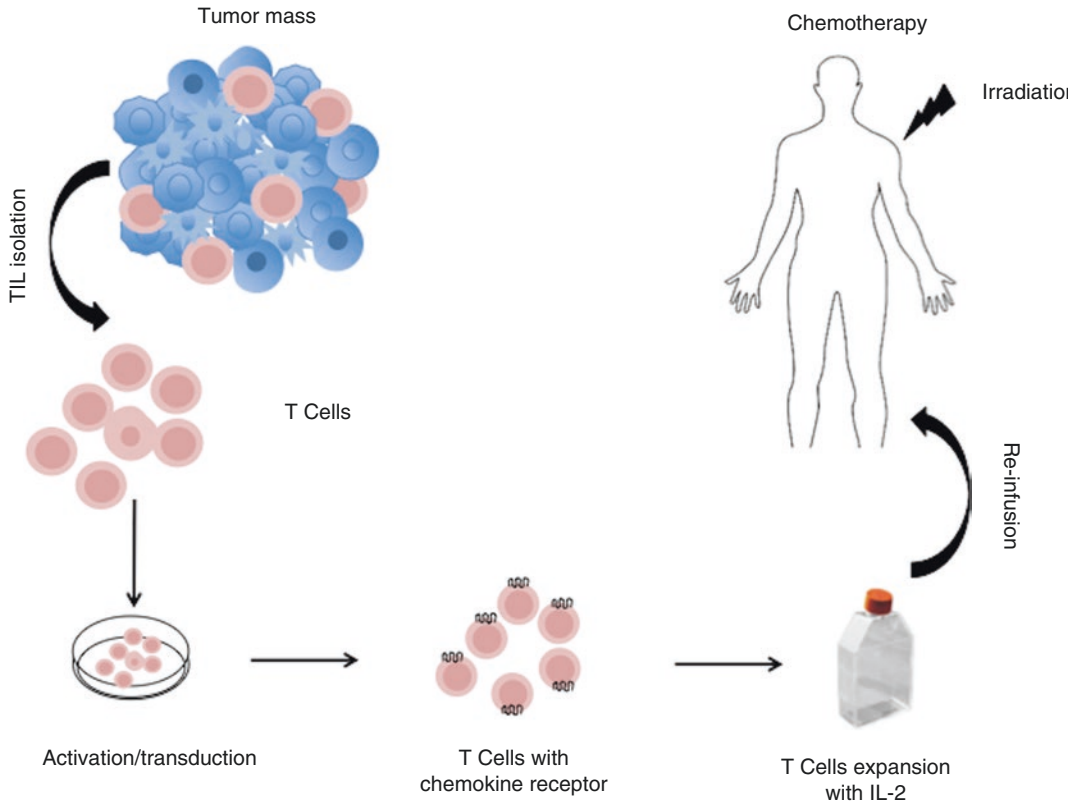

Fig. 14.1 Schematic representation of adoptive T-cell transfer therapy using T-cells genetically modified with chemokine receptors. Tumor mass is excised from the patient and TILs (tumor-infiltrating lymphocytes) are isolated from the tumor. TILs are cultured in IL-2 and transduced with the chemokine receptor matching with the ligand abundantly produced by tumor cells. Chemokine receptor positive T-cells are selected and expanded in culture using medium enriched with IL-2 and other homeostatic cytokines such as IL-15 and IL-7. Expanded modified T-cells are infused back into patient after lymphodepletion, with better homing potential and effective tumor cell killing

cultured T-cells. Our understanding of how to exploit chemotactic signals in order to manipulate reactive T-cells to better reach tumor sites is far from being complete.

Tumor-reactive T-cells do not necessarily express the appropriate receptor for chemokines produced at the site of tumors, as discussed earlier. For example, CXCL1 is produced by a large percentage of melanomas [158], but its receptor, CXCR2, is expressed only in a small subset of T-cells [159]. In a study to identify which chemokines are produced by cancer cells and which chemokine receptors are expressed by cultured T-cell, CXCL1 and CCL5 were identified in a series of human tumor cell lines and fine needle aspirates; in addition, it was determined that several chemokine receptors are expressed by cultured human T-cells, including CCR1, CCR2, CCR4, CCR5, CXCR3, and CXCR4. Activated lymphocytes may also be a source of chemokines; in a strategy to direct T-cells toward chemokines expressed by tumors, CXCL1 was chosen because it was produced by tumors but not by T-cells themselves. The absence of CXCL1 by T-cells may be an important requisite for trafficking to tumors because endogenous chemokine production may block or cause downregulation of chemokine receptor on T-cells. However, T-cells did not express the receptor CXCR2. To compensate for this, T-cells

were transduced with a retroviral vector encoding CXCR2. T-cells expressing CXCR2 were responsive in vitro toward both recombinant protein and tumor-derived chemokine. Furthermore, it was demonstrated that CXCL1 was able to induce the secretion of the proinflammatory cytokine IFN-γ by transduced T-cells, thereby extending the possibility of antitumor functions in modified T-cells. This study demonstrates the feasibility of redirecting the migration properties of T-cells toward chemokines secreted by tumors [149].

Several approaches have been applied to decipher the mechanism causing the unsuccessful migration and homing of effector T-cells to the tumor microenvironment. Methods such as Affymetrix gene expression profiling on a series of metastatic melanoma biopsies were performed to reveal T-cell-associated transcripts that could be of potential use. The presence of lymphocytes also correlates with the expression of defined chemokine genes. In this approach, a subset of six chemokines (CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10) was confirmed by protein array and quantitative reverse transcription PCR to be preferentially expressed in tumors that contained T-cells. Corresponding chemokine receptors were found to be upregulated on human $CD8^+$ effector T-cells, and transwell migration assays confirmed the ability of each of these chemokines to promote migration of $CD8^+$ effector cells in vitro. Screening by chemokine protein array identified a subset of melanoma cell lines that produced a similar broad array of chemokines. These melanoma cells recruited human $CD8^+$ effector T-cells more effectively when implanted as xenografts in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice in vivo. Chemokine blockade with specific antibodies inhibited migration of $CD8^+$ T-cells. This study suggests that lack of critical chemokines in a subset of melanoma metastases may limit the migration of activated T-cells, which in turn could limit the effectiveness of antitumor immunity [160]. The majority of tumors, including neuroblastoma, produce the chemokine CCL2. In one recent study, forced co-expression of chemokine receptor CCR2b, along with chimeric antigen receptor specific for the tumor-associated antigen GD2, enhanced the tumor trafficking of activated T-cells [161]. As a result, adoptively transferred T-cells co-modified with both CCR2b and GD2-CAR had greater antitumor activity in vivo.

To better understand the importance of homing of the adoptively transferred T-cells to all tumor sites in a sufficient number, a similar study was done exploiting endogenous chemotactic signals in order to manipulate and enhance the directional trafficking of transferred T-cells toward melanoma. Based on chemokine profiling of 15 melanoma cultures, it was shown that CXCL1 and CXCL8 are abundantly expressed and secreted from melanoma cultures. However, the complementary analysis on 40 melanoma patient-derived tumor-infiltrating lymphocytes (TILs) proved that the corresponding chemokine receptors are either not expressed (CXCR2) or expressed at low levels (CXCR1). Using the in vitro transwell system, it was demonstrated that TILs preferentially migrate toward melanoma and that endogenously expressing CXCR1 TIL cells are significantly enriched among the migrating lymphocytes. The role of the chemokine receptor CXCR1 was validated by the enhanced migration of CXCR1 engineered TIL cells toward melanoma or recombinant CXCL8. Cytotoxicity and interferon secretion activity were unaltered by CXCR1 expression profile.

Taken together, these results mark CXCR1 as a candidate for genetic manipulations aiming to enhance the trafficking of adoptively transferred T-cells [162]. This approach is complementary and potentially synergistic with other genetic strategies designed to enhance antitumor potency. In a similar study, the introduction of chemokine receptor CXCR2 gene into tumor-specific T-cells was shown to have enhanced localization to tumors and improved antitumor responses against melanoma expressing chemokines CXCL1 and CXCL8 [61]. The chemokine CXCL16 also plays an important role in T-cell-mediated antitumor immune responses: mice lacking CXCR6, the receptor for CXCL16, displayed reduced recruitment of activated effec-

tor T-cells in breast tumor tissue and impaired tumor regression [128]. A similar study was done to suggest that the capacity of adoptively transferred T-cells to home to tumors may be, in part, dictated by the species and amounts of tumor-derived chemokines, in particular CCL2 [163].

The chemokine CCL2 is highly secreted by malignant pleural mesotheliomas, but the corresponding chemokine receptor, CCR2, is minimally expressed on activated human T-cells genetically transduced with a chimeric antibody receptor (CAR) directed to the tumor antigen mesothelin (mesoCAR T-cells). The chemokine receptor CCR2b was thus transduced into mesoCAR T-cells using a lentiviral vector and the modified T-cells were used to treat established mesothelin-expressing tumors. CCR2b transduction led to CCL2-induced calcium flux and increased transmigration, as well as augmentation of in vitro T-cell killing ability. A single intravenous injection of 20 million mesoCAR CCR2b T-cells into immunodeficient mice bearing large, established tumors (without any adjunct therapy) resulted in a 12.5-fold increase in T-cell tumor infiltration by day 5 compared with mesoCAR T-cells. This was associated with significantly increased antitumor activity. This study concluded that CAR T-cells bearing a functional chemokine receptor can overcome the inadequate tumor localization that limits conventional CAR targeting strategies and can significantly improve antitumor efficacy in vivo [164].

In one of the most recent studies, the introduction of chemokine and receptor axis CCL2/CCR2 is shown to potentiate in vivo anti-lung cancer reactivity mediated by $CD8^+$ T-cells [165]. WT1 is a well-known tumor antigen expressed to various degrees by human lung cancer cells and the small cell lung cancer cell line used as a target that produces a high amount of chemokine CCL2. Lymphocytes were engineered to co-express both WT1-specific TCR and chemokine receptor CCR2 not only via CCL2-tropic tumor trafficking but also via CCL2-enhanced WT1-responsiveness. Based on this observation, the clinical feasibility of this strategy for adoptive immunotherapy against human lung cancer can be addressed in the future.

Similar in vivo experiments have also managed to demonstrate enhancement of T-cell recruitment to the tumor by transduction of the chemokine receptor CCR4 in two different models of cancer [166, 167]. A common feature of the studies highlighted so far is the attempt at enhancing T-cell recruitment to an injected tumor of known characteristics. An eventual translation to the clinic will require facing the hurdle of dealing with unknown tumors or metastatic sites. To tackle this, a recent study performed chemokine receptor-modified T-cell therapy in the transgenic adenocarcinoma of mouse prostate (TRAMP) mouse model of spontaneous prostate tumor and metastasis. Simulating the unknown variability that may occur in the clinic, the metastatic sites were analyzed in order to identify chemokines that were sufficiently differentially expressed at the target site. This enabled the selection of the most suitable chemokine receptors (in this case CCR2) to be used for a tailored modification of therapeutic cytotoxic T-cells. Indeed, the subsequent therapy with these cells led to improved homing and quantitatively more efficient antitumor response [168]. In principle, such an approach could be adapted to any tumor its or metastatic site from which a biopsy can be obtained prior to treatment, so as to tailor the T-cell therapy specifically to the patient.

Another innovative strategy attempted recently involves generating a recombinant chemokine receptor CXCR4 with optically activated domain. The photoactivatable chemokine receptor enabled targeted T-cell migration to mouse melanoma sites on which light was applied. While clearly limited to accessible tumor sites, this extraordinary example demonstrates how versatile the chemokine/chemokine receptor axis can become in a context of improving tumor immunotherapy [169].

One potentially crucial chemokine in a tumor context is CX3CL1 or Fractalkine, having an important role in leukocyte migration. Neuroblastoma cells overexpressing Fractalkine

are capable of inducing migration, adhesion, and IFN-γ secretion by immune effector cells [123]. The role of this chemokine/receptor pair CX3CL1/CX3CR1 has been well established in glioblastoma, an aggressive tumor of the central nervous system, and in the adenocarcinoma of the pancreas [170, 171]. Recent studies by our group show overexpression of Fractalkine in colorectal cancer assessed in human clinical samples [172]. Fractalkine/CX3CL1 is a proinflammatory chemokine that chemoattracts and activates $CX3CR1^+$ leukocytes such as $CD8^+$, $CD4^+$, and γδ T lymphocytes, natural killer (NK) cells, dendritic cells (DCs), and monocytes. Leukocyte trafficking is modulated by multiple signal transduction pathways, including CX3CL1–CX3CR1 signaling [173]. High expression of CX3CL1 was positively correlated with good prognosis and the number of TILs in colorectal carcinoma [124]. High expression of CX3CL1 by tumor cells correlates with a good prognosis and increased tumor-infiltrating $CD8^+$ T-cells, NK cells, and DCs in breast carcinoma [174]. The choice of the chemokine receptor CX3CR1 to enhance the homing potential of adoptively transferred T-cells has recently been studied in mouse tumor models. In a humanized mouse xenograft model of injectable colorectal cancer, human CX3CR1-transduced T-cells were demonstrated to have enhanced tumor homing and led to reduced tumor growth [175]. This study highlighted the feasibility of re-directioning T-cells via chemokine receptor transduction. Moreover, the lack of transduced T-cell recruitment in cancer cell lines overexpressing the ligand CX3CL1 suggested that circulating levels of the cognate ligand of CX3CR1 could interfere with the homing of transferred T-cells, eliminating the efficient chemotactic gradient necessary to reach the tumor site [175]. All these findings highlight the translational feasibility of approaches focused on improving ACT via more specific homing. Currently, a phase 2 clinical trial is recruiting patients with advanced melanoma (NCT01740557) to define safety and efficacy of treatment with chemotherapy in combination with transduced T-cells expressing CXCR2 receptor and nerve growth factor receptor, a specific highly expressed melanoma antigen [176].

14.8 Concluding Remarks

Over the past decade, it has become clear that the adoptive transfer of ex vivo expanded antigen-specific cytotoxic T lymphocytes promotes sustained antitumor effects in patients. Because of this compelling clinical evidence and the concomitant development of methodologies for robust gene transfer to human T lymphocytes, the field has rapidly evolved, offering new opportunities to extend T-cell-based therapies [177]. To exert a therapeutic effect, adoptively transferred tumor-specific cytotoxic T lymphocytes must traffic to sites of tumor burden, exit the circulation, be directed exactly to tumor tissue, and deeply infiltrate the microenvironment. Several strategies have now been implemented to enhance the efficacy of ACT. The development of targeted small molecules, mAbs, and biological therapies that modify the microenvironment, for instance, to overcome the fibrotic physical barriers to normalize the cancer vasculature and favor T-cell infiltration, have opened new perspectives. With the notion that chemokines play a major role in directing effector cell trafficking during antitumor immune responses, they hold great potential in cancer immunotherapy. Studies in experimental tumor models and cancer patients clearly demonstrate the potential of chemokine immunotherapy to increase immune cell infiltration of tumors and suggest that future trials should seek to incorporate chemokines or their receptors into therapy protocols. The possibility of developing novel strategies aimed at improving T-cell homing to tumors used alone or in combination with other treatments, such as checkpoint blockade immunotherapy, may prove to be more efficient and holds great promises in oncology (Fig. 14.2).

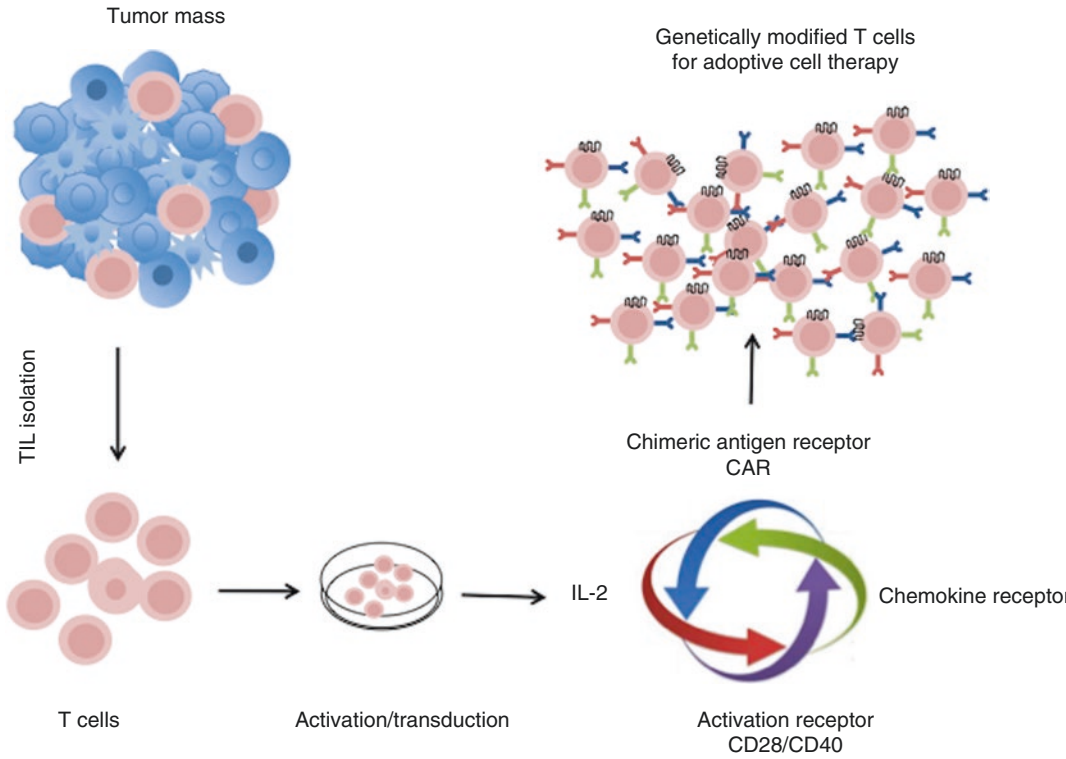

Fig. 14.2 Optimizing adoptive T-cell transfer therapy using T-cells genetically modified with multiple factors. Tumor mass is excised from the patient and TILs (tumor-infiltrating lymphocytes) are isolated from the tumor. TILs are genetically modified with the chemokine receptor matching with the ligand abundantly produced by tumor cells along with CAR specific to the antigen expressed by tumor cells as well as with enhanced T-cell activation signal genes such as CD28 or CD40. Modified T-cells are selected and expanded in cell culture using the medium enriched with IL-2. These personalized engineered cells can be used as the starting material for ACT that can be combined with therapeutic vaccination and with checkpoint inhibitors to enhance their antitumor activity

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Monoclonal Antibodies for Cancer Immunotherapy

15

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15.1 Introduction

Immune system patrols the body not only to identify and eliminate invading pathogens but also to keep the cancer cells under surveillance. As internal mirrors, antibodies (Abs) continuously monitor subtle changes in the quantity and/or structure of the cell surface markers to recognize the altered molecules, commonly created during tumorigenesis. Accordingly, monoclonal antibodies (mAbs) have been proven as robust treatment modalities for many malignant diseases. Although Abs possess diverse clinically relevant mechanisms of action to control cancer progression, there are still several drawbacks to their functions. To overcome these shortcomings, engineering techniques have attempted to generate novel Ab constructs with superior features such as higher stability and binding affinity, and more effective tissue penetration. Furthermore, antibody–drug conjugates are considered as new potential therapeutic approaches for solid tumors and lymphomas, and antibodies with immunomodulatory effects have also recently obtained promising clinical benefits [1]. Apart from the continuously growing number of US Food and Drug Administration (FDA)-approved anticancer mAbs, there are still plenty of Abs waiting to be

clinically authorized. This chapter concerns the major elements that should be considered in the development of Ab-based antitumor modalities.

15.2 Structural and Functional Features of Antibodies

Immunoglobulins (Igs) also called Abs are highly specific, antigen-reactive proteins in the immune system, which recognize and eliminate foreign antigens (Ags). Generally, each milliliter of normal human serum contains approximately 10^{16} Ig molecules. There are five classes (isotypes) of Igs (IgM, IgG, IgE, IgA, and IgD) in every individual. From a biotechnology perspective, IgG is the most important class of Ab commonly utilized as a therapeutic tool in clinical applications. The particular ability of IgG in performing crucial functions such as induction of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) along with neutralization of pathogens has made it the best therapeutic choice among Ig isotypes.

All Ab isotypes, in their monomeric form, are Y-shaped tetrameric proteins consisting of two identical heavy (H, ~50 kDa), and two identical light chains (L, ~25 kDa)—with covalent (disulfide) and non-covalent bonds conferring remarkable rigidity [2]. Both L and H chains contain variable (*V*) and constant (*C*) domains. An Ig light chain contains only one *V* domain (V_L) and one *C* domain (C_L), whereas a heavy chain has one *V* domain (V_H) and three or four *C* domains (C_H1–C_H4).

The structural characteristics of Abs account for their binding versatility, binding specificity and biological activities. The classical structure of Igs consists of two fragment antigen-binding (Fab) regions, one hinge region and one fragment crystalline (F_c). Each Fab is composed of one *C* domain and one *V* domain of a heavy chain (V_H1–C_H1) associated with a complete light chain (V_L–C_L), and accounts for specific binding of Ab (paratope) to a unique epitope. Thus, the arms of an Ab confer the versatility and specificity of responses a host can raise against Ags.

The hinge region, that is a short segment made of the region between C_H1 and C_H2 domains of both heavy chains, links the Fab and Fc regions of an Ig molecule. This proline- and cysteine-rich region allows for segmental flexibility of the Fab arms and Fc portion relative to each other, which is vital for Ag binding and effector functions of Igs.

Fc, as the tail region of IgG, is composed of C_H2 and C_H3 domains of both heavy chains. This piece of Ig mediates effector functions including ADCC and CDC. Moreover, Fc determines serum half-life of an Ab molecule through interaction with the neonatal Fc receptor (FcRn). This pH-dependent binding prolongs half-life of human IgG1 from 1 day to up to several weeks. Immunoglobulins are glycoproteins, with glycans associated especially with their Fc region. Fc domain glycosylation contributes in supplying sustainability and modulates features like ligation to Fc receptors [3]. In case of an IgG molecule, there is a conserved N-linked glycosylation site located at asparagine (Asn)-297 on each of C_H2 domains. The glycans retain the binding ability of IgG to Fc gamma receptors (FcγRs) on effector cells [4].

15.3 Natural Antibodies in Cancer

There are currently many mAbs that have been approved for the treatment of various tumor types [1]. One major challenge in this regard is to find proper tumor-specific Ags. In fact, most of the thus far produced mAbs bind to molecules that are not exclusive to tumor cells [5]. One potential solution might be achieved through investigating the already existing immune responses provided by different arms of the immune system and in particular natural Abs.

Natural Abs, mainly produced by B-1 lymphocytes, are found in circulation of normal individuals in the absence of apparent immunization or infection. Nevertheless, there is evidence proposing gut microbial flora as the potential source inducing the production of these Abs. Natural Abs serve as a rapid first-line defense mechanism recognizing mainly carbohydrate epitopes of microbial pathogens. These Abs are not affinity matured since they are encoded by a set of germ line variable genes with a limited repertoire [4]. IgM constructs large amount of such polyreactive Abs, and IgA and IgG confere lesser amount [6].

Numerous tumor-specific monoclonal natural Abs have been isolated from either normal individuals or cancer patients [7–9]. An intriguing feature of these Abs is their preferential binding to post-translationally modified carbohydrate Ags that are unique to transformed cells [7, 10, 11]. In fact, by modifying certain carbohydrate structures on their surface, tumor cells try to hide from humoral immune responses [12, 13]. However, this modification renders tumor cells easy targets for naturally occurring Abs.

Gangliosides, which are membrane bound carbohydrate antigens, regulate transmembrane signaling, which are vitally involved in tumor cell proliferation, invasion, and metastasis. It has been shown that gangliosides are inversely correlated with half-life of Abs. Naturally producing antibodies against ganglioside GM2 in melanoma have been demonstrated to associate with increased half-life. Lewis y (Ley), also known as CD174, exhibits a carbohydrate blood group antigen, which is overexpressed on the surface of neoplastic gastrointestinal tissues and possesses procoagulant and angiogenic functions [14].

Glycoproteins are regarded as the second category of carbohydrate antigens that bind to membrane. There are numerous specific glycoproteins, which undergo modifications during glycosylation after transformation of malignant cells. Mucins like MUC1 and MUC4, which both are in membrane-bound forms, are among such glycoproteins [14].

Heat shock proteins (HSPs) are also another classification of membrane conjugating molecules with altered glycosylation patterns on tumor cells [14]. Heat shock proteins serve to preserve the perfect folding of cellular proteins in normal cells [15, 16], and their overexpression or modification functions in favor of tumors causing higher drug resistance and malignancy level [17, 18]. The glucose-regulated protein 78 kDa (GRP78), is a member of the HSP family with a modified glycosylation pattern, which has been detected in various cancers including gastric [19], lung [20], and breast [21] cancers. An anti-GRP78 natural Ab, called SAM-6, was isolated from a patient with gastric cancer [22]. This Ab was shown to exclusively bind to an isoform of GRP78 specifically expressed by malignant cells. Interestingly, treatment of murine models of pancreatic cancer with SAM-6 culminated in diminished tumor weight and size along with increased incidence of apoptosis in treated tumors [22, 23]. SAM-6 has been shown to exert its antitumor impacts through an intracellularly triggered apoptosis pathway that resembles the conventional intrinsic or mitochondria-mediated pathway [24].

Post-translational modification (PTM) in glycosylation patterns has also been reported for decay acceleration factor (DAF or CD55) that serves to protect host cells from complement-associated lysis [25, 26]. Stomach carcinoma cells express this altered isoform of DAF to guard themselves against complement-mediated fatal effects. This, however, has been shown to make them ideal targets for a natural mAb called SC-1, which was isolated from a stomach cancer patient [27, 28]. According to the results of several in vitro and in vivo studies, binding of SC-1 to the modified isoform of DAF promotes apoptosis in stomach cancer cells [10, 27, 29–31]. Furthermore, in a set of clinical studies, intravenous injection of primary stomach cancer patients with SC-1 led to tumor regression and apoptotic effects that were exclusively observed in tumor tissues [30, 32, 33].

Nearly all cancer-associated epithelial cells express a growth factor receptor known as a new variant of cysteine-rich fibroblast growth factor receptor (CFR-1). Interestingly, this receptor has been reported to possess a tumor-restricted carbohydrate epitope that is recognized with a natural mAb called PAM-1 [11, 34, 35]. Akin to its aforementioned counterparts, PAM-1 reacts with a carbohydrate epitope that has undergone a modified glycosylation process restricted to malignant cells. In addition to inducing apoptosis in cancer cells, PAM-1 has also been applied to detection of precursor lesions and/or primary stages of cancers such as breast, squamous cell, colon, and stomach cancers [11, 34, 35].

Neural growth factor (NGF) has been shown to have a pivotal role in growth and metastasis of several cancers including breast cancer, squamous cell carcinoma of the esophagus, malignant melanoma, and prostate cancer [36–39]. Injection of certain human cancers with intravenous immunoglobulin (IVIg) has led to favorable antimetastatic results [40–42]. Interestingly, one study reported the existence of anti-NGF natural Abs in IVIg commercial batches. These Abs were able to hin-

der growth and differentiation of PC-12, a prostate cancer cell line [43]. Furthermore, IVIg has been shown to reduce migrating ability of two prostate cancer cell lines, DU-145 and PC-3, due to the existence of anti-NGF natural Abs [44]. Therefore, natural anti-NGF Abs can be considered as potential candidates to be used in the future diagnostic or therapeutic preclinical and clinical trials.

In general, there are many published reports supporting the potential roles natural Abs can play in fighting against cancers [7, 9, 10, 35]. Additionally, tumor Ag-specific natural Abs isolated from normal individuals and cancer patients can be used to identify novel Ags that are exclusive to tumor cells. These Abs could also be considered as specific tools for diagnosis of early stages and precancerous lesions of various tumors [24].

15.4 Finding an Appropriate Antibody Target for Cancer Therapy

15.4.1 Characteristics of a Favorable Cell Surface Antigen

Any alteration in Ag expression by tumor cells could be regarded as a potential candidate for Ab therapy. An ideal target Ag should have an abundant, homogenous, and exclusive expression on tumor cells, along with no or low expression on normal cells [45, 46]. More importantly, it should both play a vital role in tumorigenesis and be expressed on cancer stem cells in the vast majority of human cancers [1]. Furthermore, a perfect target should be highly immunogenic [47], and should be found in all or most subgroups of patients.

If targeting of a tumor-associated receptor is desired, then it is preferred to focus on a receptor that uses a signaling pathway not hired by other surface molecules. Furthermore, target receptors should have minimal secretion from tumor cells since secreted Ags can bind the circulating mAbs and neutralize their binding to the surface of cancer cells.

In Ab-based studies that aim at enhancing ADCC and/or CDC, optimal results could only be expected when the resultant Ag–Ab complexes are not rapidly internalized. This way, the Fc portion of the therapeutic mAb would be more available to immune effector cells and/or complement proteins. By contrast, proper internalization is desirable for Abs that deliver toxins into cancer cells, and for those focusing on downregulation of cell surface receptors [1].

15.4.2 Classification of Cancer Antigens

At first, based on their expression pattern, tumor Ags were classified into two categories: tumor-specific antigens (TSAs), which are associated only with tumor cells, not any other cell, and tumor-associated antigens (TAAs), which are not exclusively expressed by cancer cells. In fact, these classifications are far from perfect because many molecules that were known as tumor-specific Ags are now found to be expressed on some normal cells as well. Thus, the current tumor Ag classification systems are mostly developed based on molecular structure, source, and function of Ags (Table 15.1) [48, 49].

15.4.3 Target Identification Approaches

Several efficient methods have been promoted to identify the potential differences between tumor and non-tumor cell lines and/or tissues at the DNA, mRNA, protein, or Ab reactivity levels. Several major techniques used for the discovery of tumor antigens are briefly described below.

15.4.3.1 Genomics

Cancer-related alterations in genome include silent mutations (e.g., deletions and insertions) [50, 51], gene amplification [52], and larger scale defects such as chromosomal translocations [53]. Today, gene amplifications or deletions as well as chromosomal translocations are detected using several techniques such as comparative genomic hybridization (CGH) [54, 55] and spectral karyotyping (SKY) [56–58]. Amplification of *HER2* gene is known as the first solid tumor-associated genomic aberration, which led to the successful development of trastuzumab [59].

Table 15.1 Classification of cancer antigens

Ag category	Examples	Expression in cancer
Tissue differentiation Ags	Mclan-A/MART-1, gp100, tyrosinase, TRP-1, TRP-2	Melanoma
	PSA	Prostate carcinoma
	Prostate-specific membrane Ag (PSMA)	Prostate carcinoma
	MUC-1	Particular adenocarcinomas
	MUC-16 (CA-125)	Mainly ovarian cancer and also in endometrial cancer, fallopian tube cancer, lung cancer, breast cancer, and gastrointestinal cancer
	EpCAM	Various carcinoma types
	Gangliosides (GM2, GD2, GD3)	Melanomas, small cell lung cancer, and neuroblastoma
	CD5	T-cell leukemia/lymphoma
	CD19, CD20, CD21, CD25, CD37	B-cell lymphoma
	CD30	Hodgkin lymphoma
	CD33, CD45	Acute myeloblastic leukemia
	CAMPATH-1 (CDw52)	Lymphoid malignancies (T and B cell)
Oncofetal Ags	CEA	Expressed on several gastrointestinal malignancies and adenocarcinomas
	AFP	Hepatocellular carcinoma, germ cell tumors, and metastatic cancers of the liver
	β-hCG	Germ cell tumors and choriocarcinoma
Cancer-testis Ags	MAGE 1, 3, 12, NY-ESO, BAGE, GAGE, LAGE	Various tumors
Viral Ags	Human papillomavirus 16 E6 and E7 proteins	Cervical and anal cancers
Growth factor receptors	EGFR	Lung, glioma, breast, head, and neck tumors
	ERBB2	Breast, ovarian, stomach, and endometrial carcinoma
	CD140b (PDGFRB)	Various tumor types
Stromal Ags	Fibroblast activation protein (FAP)	Colon, breast, lung, head, and neck carcinoma
	Tenascin, metalloproteinases	Colon, breast, lung, head, and neck carcinoma
Vascular Ags	Endosialin	Breast cancer, colon carcinoma, neuroblastoma
	Vascular endothelial growth factor (VEGF)	Metastatic colorectal cancer, non-small cell lung cancer (NSCLC), metastatic breast cancer, glioblastoma, metastatic renal cell carcinoma
	αVβ3	Melanoma and prostate cancer

15.4.3.2 Transcriptomics

Two approaches commonly employed to analyze global gene expression in tumors include microarray analysis and serial analysis of gene expression (SAGE). Microarray is based on the hybridization of fluorescently-labeled sequences (probes or targets) to their complementary sequences [60, 61]. Complementary DNA (cDNA) microarray has been used to identify the frequency of elevated tumor Ag expression, for instance, in acute myeloid leukemia (AML) [62]. In 1995, Velculescu et al. [63] described SAGE as a sequencing-based method for gene expression profiling, which facilitated the global and quantitative characterization of a transcriptome.

Although DNA microarray is an excellent method for rapid screening of large numbers of samples and genes, it can only examine the already-identified sequences. In contrast, SAGE does not require prior knowledge, and represents an unbiased, comprehensive representation of transcripts [64]. Furthermore, SAGE can quantitatively identify low-abundance transcripts and detect relatively small differences in their expression [65]. Nonetheless, it is expensive and time-consuming [66] and requires relatively high amounts of RNA samples [67].

15.4.3.3 Proteomics

Genomic and transcriptomic analyses are indirect methods of protein identification and the number

of transcripts identified by these methods does not necessarily correlate with protein levels [68–71]. In contrast, proteomics can be used as a direct method of searching for cancer-specific Ags. An additional advantage of proteomics is that it can identify differences in post-translational modification (PTM), a potentially important source of tumor Ags formation.

Proteomic evaluations were initiated by two-dimensional gel electrophoresis and subsequent mass spectroscopy (2DE/MS) [72] and were expanded to more advanced methods. 2DE/MS has been widely used for separation of proteins in complex mixtures according to their molecular weight and isoelectric points; and identification of proteins that are differentially expressed in various malignances [73–78]. However, a major drawback of this technique is its inability to provide high throughput.

Other techniques that are used for the expression analysis of proteins include matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (used for investigation of haptoglobin expression in ovarian cancer) [79]; surface-enhanced laser desorption/ionization-time-of-flight/mass spectrometry (SELDI-TOF-MS) (used to study the association of cytosolic ubiquitin and ferritin light chain levels in breast cancer prognosis) [80]; liquid chromatography combined with tandem MS (LC–MS–MS) (used for phosphoproteomic analysis of HeLa cells at various stages in the cell cycle) [81]; and more-quantitative techniques such as isotope-coded affinity tags (ICATe) (used to identify differences in specific protein expression between nipple aspirate fluid samples from tumor-bearing and disease-free breasts) [82]; and isotope tags for relative and absolute quantification (iTRAQe) (utilized for identification of serum biomarkers in metastatic prostate cancer) [83]. Despite the advantages of these methods in identification of low molecular weight and low-abundance protein fractions of the proteome, they fall short of identifying protein–protein interactions.

15.4.3.4 Antibody-Based Technologies

Protein microarray is a high-throughput gel-free method with a tremendous potential to explore the interactions, activities, and functions of proteins. This approach is divided into two major classes: (1) forward-phase arrays (FPAs) in which Abs are arrayed and probed with cell lysates, and (2) reverse-phase arrays (RPAs), where cell lysates are arrayed and probed with Abs [84, 85]. Protein microarray has been utilized to recognize cancer-associated glycan variations on the proteins musin-1 (MUC1) and carcinoembryonic antigen (CEA) in the sera of pancreatic cancer patients [86] or to identify biomarkers of bladder cancer [87].

Serological expression cloning (SEREX) was developed to combine serological analysis with Ag cloning techniques to identify human tumor Ags that elicit high-titer IgG [88]. SEREX is now being used for screening the sera of patients to detect a large range of different solid [89–92] and hematological malignancies [93, 94]. Moreover, SEREX in combination with two dimensional polyacrylamide gel electrophoresis (2D-PAGE) technology created a serological proteome analysis (SERPA) technique [95] through which investigators were able to identify melanoma [96], breast [97], and colorectal cancer Ags [98].

15.5 Molecular Mechanisms Involved in Monoclonal Antibody-Based Therapy

In general, Ab-based approaches are able to damage tumor cells through three mechanisms: direct elimination of tumor cells, indirect immune-mediated targeting of cancer cells, and the targeting of tumor stroma and vasculature system [1].

15.5.1 Direct Tumor Cell Elimination

Growth factor receptors that are overly expressed on tumor cells have been targeted by many therapeutic Abs that act through the blockade of ligand binding and/or abrogation of signal transduction [99]. Epithelial growth factor receptor (EGFR) family members have been the focus of several studies. For instance, HER2 is a member of the EGFR family with no identified ligand and Abs targeting this molecule have been shown to prevent receptor dimerization [100].

Trastuzumab, that is applied to the treatment of invasive breast cancers with overexpression of HER2, acts through prevention of receptor dimerization, along with activation of immune responses [101]. Moreover, pertuzumab, another anti-HER2 mAb, has been shown to bind to a site different from that of trastuzumab and inhibit receptor dimerization [102]. Notably, a combination of trastuzumab and pertuzumab has shown promising antitumor results in preclinical models [103]. Cetuximab, a chimeric EGFR-specific mAb, could inhibit ligand binding and prevent receptor dimerization [104]. Further efforts are underway to target similar molecules such as HER3 and HER4 [105, 106].

The receptor tyrosine-kinase-like orphan receptor 1 (ROR1) has been suggested as a survival factor for certain cancers such as chronic lymphocytic leukemia (CLL) [46, 107], lung cancer, adenocarcinoma [108], and breast cancer [109]. Ab targeting of this transmembrane receptor by several studies has culminated in tumor cell elimination through the induction of apoptosis and necrosis [110–112]. A very recent study showed the role of ROR1 in survival of melanoma cell lines. Utilization of anti-ROR1 mAbs in this research could effectively induce apoptosis in the cell lines, proposing ROR1 as a potential target for future melanoma therapies [113].

15.5.2 Harnessing the Potential Capacity of Immune System to Eliminate Tumors

Due to their indispensable antitumor roles, immune responses have long been the focus of many Ab-based therapeutic strategies. The so far designed mAbs exert their antitumor effects through various immune-mediated mechanisms: ADCC, CDC, promoting Ag cross-presentation and targeting of immunomodulatory receptors (Fig. 15.1).

15.5.2.1 Antibody-Dependent Cell-Mediated Cytotoxicity

FcγR-dependent interactions are known to induce either stimulatory or inhibitory signals. FcγRIIIa as an activating receptor is expressed by dendritic cells (DCs), macrophages, natural killer (NK) cells and neutrophils, and is essential for NK-mediated ADCC [114]. Within the process of ADCC, activation of immune cells—commonly natural killer (NK) cells—leads to target cell lysis through binding of IgG to surface of target cell [115]. There is an ensemble of results from both murine experiments and clinical trials establishing ADCC involvement in antitumor effects of certain mAbs. The relationship between Ab treatment and ADCC was confirmed by the study showing that rituximab (anti-CD20) and trastuzumab were less efficient in FcγR-deficient mice compared to the wild-type ones [116]. Further support was provided by the study reporting high response rates to rituximab in follicular non-Hodgkin lymphoma (NHL) patients with certain polymorphisms in the FcγRIII encoding gene [117].

Notably, a recent promising approach has been to enhance ADCC through making modifications to the Fc domain of an Ab molecule. Accordingly, an anti-CD20 Ab with enhanced affinity for FcγRIIIA could significantly increase ADCC in comparison with the original Ab and rituximab [118].

15.5.2.2 Complement-Dependent Cytotoxicity

The potential capacity of IgG subclasses to activate the classical complement pathway ending in target cell lysis and immune cell recruitment has been harnessed by several studies with the aim of eliminating tumor cells. Indeed, there is compelling evidence highlighting the relationship between complement activation and therapeutic efficacy of antitumor mAbs. During cancer therapy, mAbs bind to complement proteins, culminating in direct cell cytotoxicity, which naturally occurs as CDC [119]. A preclinical therapy model showed that the antitumor impact of anti-CD20 mAb (rituximab) was thoroughly abrogated in C1q-deficient mice [120]. Consistently, complement depletion culminated in decreased protective effect of rituximab in a murine model of human B cell lymphoma [121]. The majority of so far clinically-approved antitumor mAbs has been shown to activate ADCC and the complement pathway.

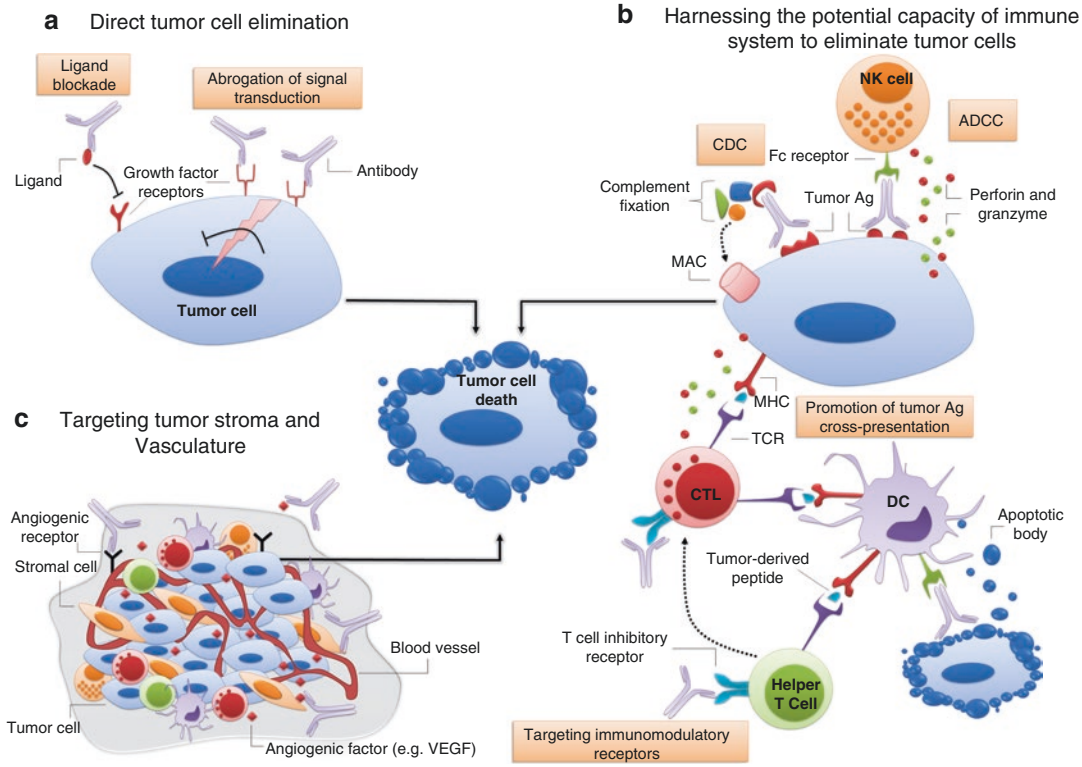

Fig. 15.1 Major mechanisms of tumor cell elimination by monoclonal antibodies. (**a**) Direct elimination of tumor cells is often elicited by abrogation of signal transduction via growth factor receptors (e.g., members of the epithelial growth factor receptor family) and/or blockade of ligand-receptor binding. (**b**) Indirect killing of tumor cells can be achieved through binding of activatory Fc receptors on immune effector cells (e.g., natural killer cells) to the Fc portion of antitumor antibody promoting antibody-dependent cell-mediated cytotoxicity (*ADCC*); or activation of complement compartments on the F_c fragment of antibody leading to formation of membrane attack complex (*MAC*) and tumor cell osmotic lysis. Additionally, antibody-coated apoptotic tumor cells or apoptotic bodies that are produced following ADCC can be engulfed and presented by dendritic cells (*DCs*) to tumor-specific T-cells. Antibodies blocking T-cell inhibitory receptors (e.g., CTLA-4 and PD-1) or those stimulating activatory T-cell receptors (not shown) can also indirectly improve the outcome of antitumor responses. (**c**) Monoclonal antibodies can also be used to antagonize receptors or ligands of tumor vasculature system, and/or to target tumor stromal cells and their products. *Ag* antigen, *CDC* complement-dependent cytotoxicity, *CTL* cytotoxic T lymphocyte, *MHC* major histocompatibility complex, *NK* natural killer

15.5.2.3 Promotion of Tumor Antigen Cross-Presentation

It is well established that Ag cross-presentation by DCs plays a pivotal part in generation of T-cell responses following Ab therapy. In fact, DCs can present tumor Ag-derived peptides in the context of MHC-I molecules and stimulate tumor-specific $CD8^+$ T-cells [122, 123]. The association between Ab therapy and induction of T-cell immunity was demonstrated by two studies indicating that the use of mAb increased cross-presentation of tumor Ags and cytotoxic T lymphocyte (CTL) generation [124], and that cross-presentation was enhanced following the blockade of FcγRIIB, an inhibitory receptor [125].

In general, antitumor mAbs are known to promote T-cell responses through two distinct mechanisms. Firstly, Ab-mediated ADCC leads to apoptotic tumor cell generation and peptides derived from these cells might subsequently be engulfed and presented to specific T-cells by DCs [126]. Secondly, Ab-coated apoptotic tumor cells can be phagocytosed, through FcγRs, and sent to the cross-presentation pathway ending in effec-

tive tumor-specific T-cell responses [124, 126]. However, one should bear in mind that DCs can mediate both immunostimulatory and immunomodulatory responses depending on the tumor microenvironment [127]. Thus, it is recommended to employ Ab-based antitumor strategies in combination with approaches that target suppressive agents of tumor microenvironment.

15.5.2.4 Targeting Immunomodulatory Receptors

The interaction of T-cell stimulatory or inhibitory receptors with their ligands on antigen presenting cells (APCs) or certain tumor cells determines the outcome of tumor-specific immune responses [1]. Therefore, the exertion of mAbs that target "immune checkpoints" (molecules on T-cells) has received widespread attention by several therapeutic studies [128].

Inhibition of pathways involved in checkpoints, such as programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1), or cytotoxic T-cell lymphocyte associated protein-4 (CTLA-4), can reverse tumor-associated immune repression, which facilitates immune cell responses against tumors with clinically beneficial effects in approximately 20% of individuals [129]. Among these receptors, CTLA-4 has gained increasing credibility owing to the promising preclinical and clinical results. This T-cell receptor suppresses activated T-cells through binding to CD80 (B7.1) and CD86 (B7.2). One study showed that blocking of CTLA-4 on both effector and regulatory T-cell compartments contributed to the antitumor activity of anti-CTLA-4 Abs [130].

Data obtained from preclinical studies has provided the foundation for production of two clinically-approved anti-CTLA-4 mAbs (ipilimumab and tremelimumab). Ipilimumab (anti-CTLA-4, Yervoy®) owes its clinical approval to a pivotal study indicating that treatment with this mAb results in improved overall survival of patients with metastatic melanoma, and this is considered as a remarkable advancement [131]. However, one should be cautious about employing CTLA-4 blockade in general, since it has been shown to exert a series of toxic side effects called immune-related adverse effects (irAEs) [131, 132]. Likewise, blockade of another T-cell inhibitory receptor, namely PD-1, via a fully humanized monoclonal antibody (mAb) against PD-1 (Nivolumab; also known as MDX-1106), has led to favorable antitumor responses [133, 134] and additional PD-1 targeting Abs are being investigated [135, 136]. Anti-PD-1 reactivates "exhausted" T-cells through binding to PD-1 expressed on them [129]. Pembrolizumab was approved by FDA for the treatment of patients with previously untreated metastatic non-squamous non-small cell lung cancer (NSCLC) and metastatic melanoma [134].

PD-L1 overexpression, indicated by several clinical studies, has been attributed to a poor prognosis in several types of tumors such as bladder cancer, renal-cell carcinoma, esophageal cancer, gastric cancer, pancreatic cancer, ovarian cancer, and hepatocellular carcinoma. By expressing PD-L1, tumors can evade host immune surveillance, which inversely modulates immune responses through interacting with PD-1 molecule expressed on T-cells. Atezolizumab (anti-PD-L1 antibody, Tecentriq®) was also approved for therapy of unresectable bladder cancer and NSCLC in 2016 [134].

Some other agents determined for targeting immunoregulatory pathways are also under investigation that include antagonists of inhibitory checkpoints, such as TIM-3 and LAG-3. Additionally, some others have been designed against costimulatory molecules on immune cells, like CD40, CD137 (4-1BB), GITR, and OX-40 [137].

Urelumab (anti-4-1BB antibody) is a fully humanized IgG4 monoclonal antibody that has agonistic roles on T-cell activating receptor, CD137, that has shown encouraging antitumor efficacy in phase I clinical trials [126, 137, 138]. Urelumab specifically binds to and stimulates CD137-expressing immune cells, which then initiates an immune response, particularly a cytotoxic T-cell response, toward cancer cells [139]. On a cautionary note, high doses of this Ab can result in toxic effects, and studies with lower less toxic doses are currently underway [1]. Recently, an investigation was accompanied with achieving

optimum urelumab dosage alongside with representations of immunologic activity, and was well tolerated [137]. Encouraging results upon employing Abs with agonistic impacts on CD40 have also been noted in the literature [135]. Among other CD40 agonists are checkpoint inhibitor mAbs, like anti-OX40 [140].

15.5.3 Targeting Tumor Stroma and Vasculature

Factors that support angiogenesis as well as those that form the extracellular matrix play an indispensable role in tumor survival [141–143]. Therefore, targeting tumor microenvironment has been shown to be of great therapeutic value in preclinical and clinical settings [144].

Vascular endothelial growth factor (VEGF), secreted by many solid tumors, supports tumor angiogenesis by binding to its receptor on endothelial cells. A combination of chemotherapy and anti-VEGF mAb (bevacizumab) is clinically approved for therapy of patients with colorectal, breast, and non-small cell lung cancers (NSCLCs) [143]. Ab-targeting of VEGF receptor (VEGFR) has also been investigated by several studies. Ramucirumab, an anti-VEGFR2 mAb, showed potential antitumor impacts in a murine cancer model [145]. Consistently, targeting of VEGFR-1 by a fully human mAb showed favorable preclinical results [146].

As for many therapeutic mAbs, the growing use of bevacizumab resulted in the emergence of bevacizumab-resistant tumors due to the upregulation of alternative angiogenic factors such as platelet-derived growth factor (PDGF), which supports the growth of blood vessels through binding to its receptor (PDGFR) [147]. In fact, the addition of an anti-PDGFR mAb to anti-VEGFR-2 therapy showed promising antitumor results in preclinical models, introducing an efficient solution for the treatment of bevacizumab-resistant tumors [148].

Cancer cells often press tissue stromal cells into service to provide a more hospitable microenvironment. In addition, cancer-associated fibroblasts (CAFs), as the most frequent cell population in tumor microenvironment, have a crucial role in growth and metastasis of solid tumors. Hence, approaches that target CAFs and/or molecules secreted by them have recently gained momentum [149]. For instance, a mAb directed against fibroblast activation protein (FAP), produced by CAFs, elicited robust antitumor responses in a phase I clinical trial in patients with advanced or metastatic FAP-positive colorectal cancer and NSCLCs [142].

15.6 Engineered Antibodies

Two features of mAbs that have made them interesting drug candidates are high target specificity and organization into distinct structural and functional domains. These features have facilitated protein engineering of intact Abs by a variety of methods to suit for diverse therapeutic applications. Antibody engineering techniques have attempted to optimize the therapeutic efficacy of untouched Abs, and to overcome their shortcomings by creating novel Ab structures with features such as decreased immunogenicity, optimized stability, higher binding affinity, effective tissue penetration, modified Fc function, recruiting effector players of immune system, rapid renal clearance, and ease of production. Notably, advances in molecular biology have made it possible to go beyond optimization and in fact have created entirely new Ig domain-based structures, not found in nature, which can be tailored to achieve favorable results. A number of approaches have been developed to explore novel antibodies, including hybridomas, which are genetically engineered mice harboring human immunoglobulin sequences, and phage display. Each method has pros and cons; as a result, antibody discovery researchers will try several strategies simultaneously toward targeting a particular molecule [129]. This section describes Ab engineering (Fig. 15.2) as a way of generating optimized therapeutic Abs with improved effector functions.

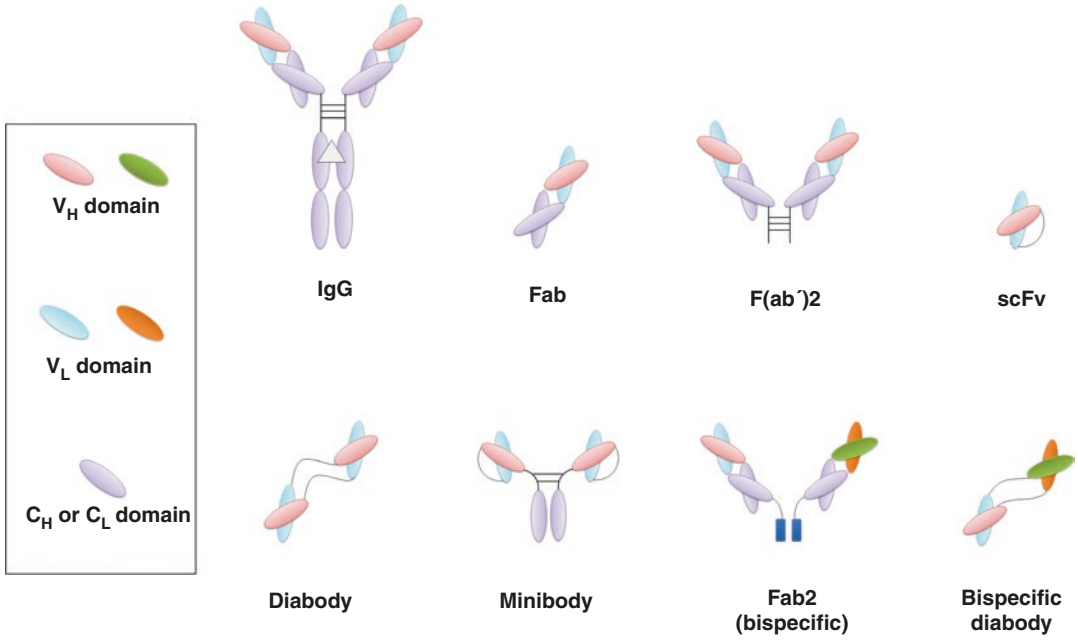

Fig. 15.2 Schematic representation of different antibody fragments with therapeutic applications. Fragment antigen-binding (Fab) and F(ab′)2 may be generated by papain or pepsin digestion of intact IgG, respectively. Other types of antibody fragments can be produced using antibody engineering methods. Single-chain fragment variables (scF_{vs}) are composed of V_H–peptide linker–V_L (or vice versa). Diabodies are homodimers of scFvs, covalently linked by a short peptide linker. Minibodies consist of two scFv–hinge–CH3 chains covalently connected by disulfide bonds. Bispecific antibodies, in general, consist of variable fragments of two different antibodies. Fab2 and bispecific diabody are two examples of bispecific structures. The triangle on the intact IgG indicates carbohydrates covalently attached to heavy chains

15.6.1 Murine Monoclonal Antibodies

Murine mAbs are entirely derived from mice using hybridoma technology, which involves the fusion of immortalized myeloma cells with B cells from immunized mice [150–154]. However, injection of humans with murine Abs induces the generation of human anti-mouse Abs (HAMA) that always target the injected murine mAb and, therefore, were not appropriate for therapies in chronic time periods [129]. Not only can these HAMA remove murine Abs upon repeated administrations, but also the formation of antibody-HAMA-complexes has shown end in mild to severe allergic reactions [155]. Therefore, major shortcomings of intact murine Abs have limited their clinical applications related to immunogenic problems and diversities between the immune systems of humans and rodents [156, 157]. Molecular biology and protein engineering settled this disadvantage in order to develop more human-like mAbs that have low immunogenicity [129].

Although the first mAb approved for clinical applications was a murine IgG2a Ab (OKT3, or muromonab; 1986) [158], many technical efforts were soon made to develop a second-generation mAb appropriate for human administration. Currently, murine Abs serve mainly as radioisotope-labeled agents aiming at targeted killing of tumor cells.

Technical advances in recombinant protein engineering, transgenic mice, and phage display have promoted the development of chimeric, humanized, and fully human mAbs. This has helped overcome the limitations of intact murine mAbs and resulted in creation of more effective therapeutic agents [159–161].

15.6.2 Chimeric and Humanized Monoclonal Antibodies

The desire to produce murine Abs with less immunogenicity in humans, and more immunologic efficacy, led to the production of various types of mAbs, such as chimeric, and humanized mAbs [48, 162, 163]. Chimeric mAbs are produced through hybridizing the antigen binding Fab regions from murine to backbone of human immunoglobulin, which is called "chimerisation" [129]. Such Abs are 75% human and much less immunogenic compared to the intact rodent ones, because interspecies immunodominant Ig epitopes are frequently located within the CH2 and CH3 domains of the Fc region [164]. Chimeric antibodies that have been approved are Erbitux® (cetuximab), Remicade® (infliximab), and Rituxan® (rituximab) [129]. Humanized mAbs, on the other hand, are constructed via engrafting of hypervariable regions of peptide binding loops from mouse (also named complementarity determining regions (CDRs)) onto human Abs rendering them 85–90% human, with less immunogenicity than chimeric Abs [129, 164]. Herceptin® (trastuzumab), a "humanized" antibody was extracted from a murine hybridoma and then underwent "humanization" process, through which except than binding site to the HER2 antigen was altered to a human sequence [129]. It is of note, however, that the binding affinity of the humanized mAbs is often weaker compared to parent murine mAbs. Therefore, additional manipulation needs to be made to humanized Abs to improve their affinity and specificity. These alterations are typically achieved by introducing mutations by methods like chain-shuffling randomization in the CDRs of Abs [165, 166]. In fact, the majority of currently approved Abs used in oncological applications and those used in advanced clinical trials are of humanized construct.

15.6.3 Fully Human Monoclonal Antibodies

To further reduce the immunogenicity of chimeric or humanized mAbs, both of which still contain some murine fragments, fully human mAbs were constructed [156, 167]. Replacement of mouse Ig variable and constant domains with those of the human effectively reduces the incidence of anti-antibody response (AAR) hypersensitivity reaction [168]. While some humanized mAbs are currently under studying for human clinical applications, Panitumumab® and Adalimumab® have been marketed for therapeutic purposes [169].

Transgenic mice (bearing human Ig germ line loci) and phage display (the display of Ab fragments on filamentous bacteriophages), as two of the well-established technologies for production of human mAbs, are reviewed here.

15.6.3.1 Human Monoclonal Antibodies from Transgenic Mice

A new approach for the development of fully human mAbs is the creation of a mouse strain engineered to produce a large repertoire of human Abs. Such mice are generated by introducing human Ig gene segment loci into the germ lines of mice deficient in Ab production [170]. Interestingly, VDJ recombination and somatic hypermutation of the human germ line Ab genes are carried out in a normal fashion in these mice, thereby producing high-affinity Abs with completely human sequences differing just in glycosylation patterns [171]. Such murine strains may serve as a source of high-affinity human mAbs generated against a broad spectrum of Ags, including those of the human. Development of genetically engineered mice facilitated production of fully humanized antibodies, such as Ofatumumab, Vectibix® (panitumumab), and ipilimumab [172] (Table 15.2).

15.6.3.2 Human Monoclonal Antibodies Created Through Phage Display Technology

Another important strategy uses synthetic (human) antibody libraries that are presented on the surface of phage or yeast, which is beneficial for targeting less immunogenic antigens [129]. Phage display was first described by George P. Smith [173] in 1985, when he demonstrated that a foreign DNA fragment can be fused to the

Table 15.2 Monoclonal antibodies approved by FDA for cancer therapy

Generic name[a]	Brand name/ company	Targeted antigen	Antibody construct	FDA-approved indication	Approval date
Trastuzumab	HERCEPTIN/ Genentech	ERBB2	Humanized	Breast cancer, metastatic gastric or gastroesophageal junction adenocarcinoma	1998
Bevacizumab	AVASTIN/ Genentech and Roche	VEGF	Humanized	Metastatic colorectal cancer, non-squamous non-small cell lung cancer, metastatic breast cancer, glioblastoma, metastatic renal cell carcinoma	2004
Cetuximab	ERBITUX/ Bristol-Myers Squibb	EGFR	Chimeric	Head and neck cancer and colorectal cancer	2004
Panitumumab	VECTIBIX/Amgen	EGFR	Human	Metastatic colorectal carcinoma	2006
Ipilimumab	YERVOY/ Bristol-Myers Squibb	CTLA-4	Human	Unresectable or metastatic melanoma	2011
Pertuzumab	PERJETATM/ Genentech	ERBB2	Humanized	Metastatic breast cancer	2012
Conjugated antibodies: solid malignancies					
Ado-trastuzumab emtansine	KADCYLA/ Genentech	ERBB2	Humanized	Metastatic breast cancer	2013
Naked antibodies: hematological malignancies					
Rituximab	Mabthera/Roche, Rituxan/Roche	CD20	Chimeric	Non-Hodgkin lymphoma, chronic lymphocytic leukemia	1997
Alemtuzumab	Campath/Genzyme	CD52	Humanized	B-cell chronic lymphocytic leukemia	2001
Ofatumumab	Arzerra/Genmab	CD20	Human	Chronic lymphocytic leukemia refractory to fludarabine and alemtuzumab	2009
Conjugated antibodies: hematological malignancies					
Brentuximab vedotin	ADCETRIS/Seattle Genetics	CD30	Chimeric	Refractory Hodgkin lymphoma, systemic anaplastic large cell lymphoma	2011
^{90}Y-labeled ibritumomab tiuxetan	ZEVALIN/IDEC Pharmaceuticals	CD20	Murine	Relapsed or refractory, low-grade or follicular B-cell non-Hodgkin lymphoma, previously untreated follicular non-Hodgkin lymphoma	2002
Tositumomab and ^{131}I-labeled tositumomab	Bexxar/ GlaxoSmithKline	CD20	Murine	Rituximab-refractory non-Hodgkin lymphoma	2003

[a]Certain suffixes are used in generic names of monoclonal antibodies that are used as medications: -momab (murine), -ximab (chimeric), -zumab (humanized), or -mumab (human)

gene encoded for pIII coat protein of a filamentous phage and expressed as a fusion protein on the virion surface. A few years later, McCafferty [159] verified that a single-chain fragment variable (scFv) can be presented on a phage surface as a functional protein, while retaining its capability for antigen binding [174]. Today, this is a well-established technology for the development of novel fully human Abs. Phage display can mimic the immune system by creating large libraries of Ab genes and selecting for binding to desirable Ags. Exploration for specific antibody fragments with good affinities is possible upon biopanning the phages. The aim of this process is to enhance the efficacy of antigen-specific scFv, for increasing the affinity of scFV toward antigens, along with enhanced specificity [175]. Depending on the Ab source, there are several types of libraries: immune, naïve, and synthetic libraries. Immunized and naïve phage

libraries are constructed through isolating the peripheral lymphocytes from immunized and non-immunized donors, respectively [176]. To create fully synthetic libraries, germ line Ab gene segments, VH, DH, and JH or *V*κ/*λ* and *J*κ/*λ* are cloned and arranged combinatorially in vitro to reconstitute genes encoding complete VH and VL chains [171]. Although, currently, there is no FDA-approved anticancer therapeutic mAb produced by phage display technology, several of such mAbs are in clinical development [177].

15.6.4 Antibody Fragments

The development of fully humanized Abs was a major breakthrough in therapeutic application of Abs. However, the large size of mAbs together with the presence of the Fc portion may be disadvantageous in some settings since it limits Ab penetration into tumor, especially in the case of solid tumors [178]. In fact, tissue penetration is known as a vital parameter in therapeutic settings, and often severely restricts the complete efficiency of the treatment [45, 179]. In addition, the long half-life of Abs, which is related to their Fc portion, is not appropriate for applications such as radioimmunotherapy or imaging as it may result in irradiation of healthy tissues and high background, respectively [180]. Antibody engineering offered new methods for overcoming these shortcomings, which are discussed below.

Antibody fragments including Fab, scFv, diabodies, and minibodies can be produced by elimination of the whole constant region or removal of a part of Fc or its entire portion from Ab [164]. In fact, better renal clearance and improved tumor penetration made such fragments attractive alternatives to the whole Ab molecule for radiotherapy and/or imaging application [181]. The biodistribution of intact radiolabeled chimeric mAb U36 (125I-cMAB U36) and its radiolabeled-recombinant fragment, 125I-F(ab′)2, was compared in nude mice bearing head and neck xenograft tumors. Results demonstrated better tumor penetration and superior tumor-to-blood ratio for the latter [180]. Another study demonstrated acceptable tumor uptake of 111In-panitumumab F(ab′)2 in the athymic mice bearing LS-174T xenografts, suggesting this fragment as a promising candidate for imaging of HER1-positive cancers [182].

scFv fragment (27 kDa) contains the variable domains of one heavy and one light chain linked by a flexible linker and is capable of retaining the binding activity of the full Ig molecule in a monovalent fashion [183]. However, the main disadvantage of scFv is its too short serum half-life (~2 h) compared to the intact Abs (1–2 weeks), which may necessitate a successive administration of the molecule for achieving a proper response [164]. Interestingly, the intracellular expression of anti-Ras neutralizing scFv induced cell death in tumor cells expressing oncogenic Ras [184]. In a preclinical in vitro study, scFv-PEG-lipid conjugate, as an anti-HER2 liposome-inserting agent, was applied to HER2-overexpressing cancer cells [185].

Diabodies are homodimers of scFvs, covalently linked by a short peptide linker of four amino acids [186]. This kind of Ab fragment is a bivalent, medium-size (55 kDa) molecule with a higher avidity and superior tumor retention as compared to a single scFv. Engineered Ab fragments, such as diabodies, and scFv-Fc, have been successfully employed for immuno-positron emission tomography (immunoPET) imaging of cancer cell surface biomarkers in preclinical models [187]. Larger fragments such as minibody (scFv-CH3; 80 kDa) [188] and scFv-Fc (110 kDa) [189] fusion proteins can exhibit even higher tumor uptakes. The longer serum half-life of these species improved their localization and allowed for longer exposure of the target tissue to the Ab fragment. In this regard, genetically engineered minibody and diabody displayed rapid, high-level tumor uptake coupled with rapid clearance from the circulation in the athymic mice bearing LS174T human colon carcinoma [190].

15.6.5 Bispecific Antibodies (BsAbs)

Different modifications have been applied to conventional therapeutic Abs in order to improve their clinical efficacy. Accordingly, bispecific Abs (BsAbs) have been devised that simultane-

ously target two different Ags or epitopes on the cell surface [191].

These hybrid proteins can be produced using different approaches such as chemical cross-linking, quadroma technology by somatic fusing of two different hybridoma cell lines [192], genetic techniques through recombinant DNA technology (knobs-into-holes strategy) [193]. Conjugating to two different antigens simultaneously confers a vast spectrum of applications, such as NK cells or T-cells to cancer cells, inhibition of two different signaling pathways, dual targeting of diverse disease-involved molecules, and delivering the desired molecule to targeted sites [194].

BsAbs present numerous beneficial aspects: (1) unlike combination monoclonal antibody strategy, BsAbs can lead specific immune effector cells to the vicinity of tumor cells in order to increase the efficacy of tumor cell killing. (2) Through interacting with two different antigens on the cell surface rather than one, BsAbs have the potential to enhance specificity of binding. (3) In comparison to the development of single antibody-based agents in combination strategies, BsAbs confer a chance to decrease the cost with respect to development, clinical trials implementation, and controlling reviews. (4) BsAbs will confer the opportunity to blocking of two different pathways at the same time that play specific or shared functions in the disease pathogenesis [194].

Until recently, synthesis of bispecific mAbs has been encountering difficulties [129]. Today, Ab engineering is capable of producing a wide variety of BsAbs with any antigen-binding combination, and molecular weight, as well as a predictable serum half-life. F(ab′)2 heterodimer, various types of bivalent and trivalent scFvs, and tetravalent BsAb (including Ab-scFv, dimeric miniantibodies, and dimeric antibody-Fc molecules) are some examples of engineered BsAbs in this category [195].

Frequently, BsAbs have been designed to simultaneously bind tumor markers and effector cells. Effector cells such as T-cells are activated via CD3, while others like NK cells, macrophages, and neutrophils are generally activated through FcγRIIIa, b, and FcγRIIa [196, 197]. In fact, there are many BsAbs with one arm specific to CD3 on cytotoxic T-cells and the other arm specific to a tumor Ag such as EGFR [198], HER2 [199], CA-125 [200], or CD20 [201]. Such BsAbs have been administrated in the immunotherapy of NHL, breast, ovarian, and prostate cancers. In 2009, the first bispecific trifunctional antibody, catumaxomab (Removab®), was approved for the therapy of malignant ascites in cases with EpCAM-positive tumors [1]. This bispecific T-cell engager (BiTE) antibody binds simultaneously to both EpCAM on human adenocarcinomas and CD3 on cytotoxic T-cells. The immunological reaction is triggered against tumor cell through binding of BiTE to T lymphocyte and target cell, and binding of heavy chains to an APC like a DC, macrophage, or NK cell [202, 203].

Blinatumomab, a recombinant bispecific tandem scFv molecule (bispecific T-cell engager, BiTE) directed against CD3 and CD19, is undergoing clinical trials and has demonstrated promising results in phase I and II studies in acute lymphoblastic leukemia (ALL) and NHL patients [204, 205]. Aside from approved catumaxomab (anti-CD3 and anti-EpCAM) and blinatumomab (anti-CD3 and anti-CD19), many more BsAbs are now in various phases of clinical development.

Although at the beginning of BsAb development T-cells received considerable interest, the attention of recent studies is shifting onto the employment of NK cells. T-cells are known as highly motile cells with robust tumor infiltration capacity. However, to become fully activated, these cells need to interact with co-stimulatory molecules such as B7 on APCs, and this is considered a major drawback to T-cell-based modalities [164].

In addition to activation of immune effector cells, BsAbs could be utilized in combination with cytotoxic agents resulting in accumulation of highly active but nonspecific payloads in desired tissues. Recently, recombinant bispecific immunotoxins were produced through fusing a tandem scFv to the catalytic or translocation domain of diphtheria toxin [206–208]. These immunotoxins were directed against CD19 and CD22 and showed improved efficacy against

murine xenograft models of B cell malignancies and metastases [206–208].

On the other hand, another major escape mechanism of tumor cells may through down regulation of antigens that are target of antibody and deterring from recognition in the process of treatment. Several clinical trials have demonstrated that anti-CD19 chimeric antigen receptor T-cells (CART19) possess therapeutic potency against malignancies with relapsed B-cell. Nonetheless, a recent clinical trial of CD19 CAR T-cell therapy reported complete response in 90% of cases, whereas 11% of them finally presented relapsed tumors with CD19-negative status. Every additional antigen that had the possibility to be recognized via the CAR T-cells reduced the chance of antigen escape through selective proliferation of antigen-negative tumor cells and spontaneous mutation. As a result, combination of bispecific antibodies for production of T-cells recognizing multiple antigens is considered as a promising approach to prevent antigen escape. Development of the first bispecific CAR T-cells was occurred to inhibit the antigen escape process of malignant B cells, through which simultaneous recognition of both of CD19 and CD20 molecules was carried out via these CAR T-cells [209, 210].

15.6.6 Antibody Fusion Constructs

Antibody molecules in the fusion constructs are generally used to direct therapeutic agents such as toxins [211], cytokines [212], drugs [213], and radioisotopes [214] to the tumor microenvironment. The rationale behind this approach is the direct and specific delivering of higher concentrations of cytotoxic agents to tumor tissues, while avoiding damage to normal cells [215]. In fact, several potent drugs such as auristatins [216] and maytansinoids [217] (inhibitors of microtubule assembly) or emtansin [218] (a microtubule polymerization inhibitor) have been utilized in fusion with Abs in cancer therapy. Trastuzumab emtansine is an antibody-drug conjugate consisting of a maytansine derivative (DM1) conjugated to the FDA-approved trastuzumab [219]. Trastuzumab-DM1 has recently been shown to inhibit tumor growth via induction of apoptosis, ADCC, and mitotic catastrophe in a trastuzumab/lapatinib (a kinase inhibitor used in breast cancer therapy) resistant murine model [220].

Aside from drugs, various cytokines (e.g., IL-2, IFN-γ, TNF-α, and GM-CSF) have been investigated as therapeutic agents in conjugation with Abs as explained by their immunomodulatory and antitumor effects. At present, several immunocytokines are undergoing phase I and II clinical trials, and are close to FDA approval [221–223]. One therapeutic approach has combined a humanized Ab recognizing ED-B (extradomain B of fibronectin) with IL-12 [224]. This conjugated Ab has been evaluated in a phase I study in malignant melanoma and renal cell carcinoma (RCC) patients [224]. Moreover, Ab-IL-2 fusion proteins have been used in several phase I clinical trials to treat melanoma and neuroblastoma [225–227].

Tumor-targeted delivery of radioisotope agents in the form of radioimmunoconjugates is believed to improve its antitumor activity and safety. To minimize toxic effects, the conjugates are commonly designed based upon Abs with short serum half-lives. The only radioimmunotherapy agents licensed by the FDA are yttrium-90 (^{90}Y)-ibritumomab tiuxetan and iodine I 131 tositumomab. Either of these radioimmunoconjugates targets CD20, and each has been associated with potent responses in patients with relapsed NHL, or those with tumors resistant to rituximab [228].

15.6.7 Improvement in Antibody Function

Modifying Abs to improve their function has been a very active area of Ab engineering. Several strategies such as modulating the Fc carbohydrate, and/or protein sequences to enhance immune mediator functions, and altering half-life characteristics are instances of this concept. The existence of oligosaccharides and in particular the N-linked oligosaccharides at Asn-297 in the CH2 domain of IgG1 is crucial for binding to FcγR as well as complement fixation [229–231].

Two independent studies have demonstrated that lack of the fucose moiety from carbohydrate on Asn-297 significantly improves the binding of Ab to FcγRIII and ADCC [232, 233].

Altering protein sequence can be considered as another strategy to improve Ab function. Directed modification of amino acids within the Fc region of Ab leads to alteration of Ab half-life or enhancement of immune-mediated effector functions. A mutated Fc was able to decrease IgG affinity for FcRn, leading to shorter serum half-lives and thus rapid clearance of IgG-toxin or IgG-drug complexes [234]. However, for some therapeutic applications, increasing the half-life is favorable, as it would reduce the need for repetitive injections of the Ab to achieve a therapeutically relevant serum concentration. In one study, utilizing human IgG1 mutants with increased binding affinity to human FcRn led to a 2.5-folds increased serum half-life compared to the wild-type Ab [235].

Monoclonal Abs elicit effector functions following interactions of their Fc portion with various Fc receptors [1]. Hence, increasing the affinity of this interaction by engineering methods can play a major part in the efficacy of Ab-based therapies. Shields et al. determined several amino acids, located on the CH2 domain, as being important in IgG1 binding to FcγR [236]. The binding of IgG1 to FcγRIIIa, the major receptor mediating ADCC by NK cells, was 51% higher when alanine mutations were made at Ser298, Glu333, and Lys334. Notably, this mutant resulted in greater NK-mediated ADCC compared to a higher concentration of native IgG1 [236].

15.7 Evaluation of Antibody Efficacy

15.7.1 Preclinical Evaluations

Preclinical evaluation of Abs aims at predicting their potential pharmacologic and toxicologic effects in humans.

Different kinds of antitumor activities are evaluated by in vitro tests including inhibition of growth (e.g., trastuzumab [237, 238]), inhibition of metastasis or angiogenesis (e.g., bevacizumab [239, 240]), induction of apoptosis (e.g., rituximab [241, 242]), and induction of secondary immune functions such as ADCC (e.g., trastuzumab) [237, 238] or CDC (e.g., rituximab) [241].

The in vivo preclinical studies, on the other hand, can provide valuable information about product-specific dose level, dosing regimen, route of delivery, treatment duration, pharmacokinetics, pharmacodynamics, toxicity [243, 244], and sensitization to chemotherapy [245] or radiotherapy [246].

Choosing the most relevant animal model is a critical step for successful preclinical safety evaluation of a mAb [247–249]. The species- and target-specific nature of mAbs often rules out the use of rodents and in some cases makes it difficult to find the appropriate species. A non-human primate, if ethically justified, could be regarded as the species of choice for human/humanized mAbs [243]. To achieve a thorough assessment, some prefer to use different models including mouse, rat, and monkey as in a study of humanized-anti CD40 mAb (SGN-40) [250].

15.7.2 Clinical Evaluations

Valuable information on the whole procedure of clinical safety evaluation of mAbs has been provided by various regulatory agencies. In 1997, FDA released a revised version of "Points to Consider (PTC) in the Manufacturing and Testing of Monoclonal Antibody Products for Human Use." This document presents a useful guideline for designing a clinical safety evaluation program of mAbs in areas such as dose estimation, pharmacokinetic evaluation, and immunogenicity consideration [244].

A critical step in the clinical evaluation of a therapeutic mAb is to assess its biodistribution, which is the ratio of Ab access to the tumor vs. normal tissues [142, 251, 252]. This step is essential for predicting Ab toxicity [252, 253], defining an appropriate Ab dose regimen, and determining the potential impacts of Ag saturation when using high Ab doses. Scott et al. used

a model of a clinical trial that incorporated biodistribution, pharmacokinetic, and pharmacodynamic evaluations with toxicity assessment [251] to the first-in-human clinical trials of several anticancer Abs [142, 251–253]. Further pharmacodynamic assessment methods, such as computerized tomography with magnetic resonance imaging, plasma-based protein, cell and genomic analyses, and tumor biopsies can also be used to evaluate the clinical efficacy of newly designed mAbs [254].

15.8 Clinically-Approved Monoclonal Antibodies

At the beginning of the twentieth century, Paul Ehrlich postulated "magic bullet" as a tool for specific targeting of diseases [255]. His hypothesis became practical with the development of an efficient method for generation of mAbs, in 1975, by Kohler and Milstein who are laureates of the Nobel Prize in Physiology or Medicine in 1984 [150, 256]. Since then, these molecules have been known as ideal tools for therapy and imaging applications [164]. In this regard, mAb-based therapy of cancer has been used as a new therapeutic modality that has rapidly been adapted in many cancer types [257] and also received a great deal of interest by pharmaceutical companies. This interest has partly been stimulated due to the well-defined safety, efficacy, and quality of mAbs, and also because physicians and patients have clearly accepted mAbs as innovative therapeutics [156].

In 1982, for the first time, a therapeutic mAb was successfully used to treat B-cell lymphoma patients [258]. Consequently, Ehrlich's magic bullet hits the target by introducing rituximab (1997) and trastuzumab (1998) as the first chimeric and humanized FDA-approved mAbs for cancer therapy, respectively [255]. Since 1997, 13 mAbs including 7 mAbs specific to solid tumors and 6 mAbs specific to hematological malignancies have received FDA approval (Table 15.1). Here, we provide an overview of trastuzumab, bevacizumab (applied for solid tumors), and rituximab (applied for hematological malignancies) as instances of the most successful therapeutic mAbs in clinical oncology [1].

15.8.1 Trastuzumab

Overexpression of human epidermal growth factor receptor-2 (HER2, c-erbB-2/neu, HER2/neu) is reported in approximately 15–20% of human breast cancers and is associated with a more aggressive disease and poor disease-free survival [259–261]. Trastuzumab (Herceptin®) is a humanized mAb against human epidermal growth factor receptor 2 (HER2) and is considered as the pioneer in modern movement of mAb-based therapy of solid tumors [129]. Trastuzumab is a recombinant humanized mAb (rhumAb 4D5) reacting with an extracellular region of HER2 protein and inhibiting growth of the breast cancer cell line, SKBR-3 [262]. In a pivotal phase III clinical trial on metastatic breast cancer (MBC) patients with HER2 amplification, addition of trastuzumab to the chemotherapy regimen was associated with a few months delay in disease progression (median, 7.4 vs. 4.6 months), a higher rate of objective response (50% vs. 32%), a longer duration of response (median, 9.1 vs. 6.1 months) and survival (median, 25.1 vs. 20.3 months) [263]. Subsequently, four major international studies corroborated that trastuzumab either following or in combination with chemotherapy could reduce the risk of relapse and death by approximately 50% and 33%, respectively, in HER2-positive early breast cancer patients [264].

Although trastuzumab is accepted as the standard drug in the breast cancer therapy, its use has commonly led to favorable results in a small portion of human breast cancers [259–261]. In addition, up to 40% of patients with MBC do not respond to trastuzumab-based regimens and in those who respond, the median progression time is less than 1 year [265, 266]. Moreover, acquired trastuzumab resistance is a serious concern ending in disease progression [266, 267]. Notably, due to HER2 expression on cardiomyocytes, cardiac toxicity issues such as symptomatic congestive heart failure have been observed in some of the patients receiving trastuzumab therapies

[268, 269]. In general, these shortcomings call for creation of novel and improved Ab-mediated therapies for MBC. The murine parent of trastuzumab, namely MuMAb4D5, was demonstrated to be inefficient on normal cells or tumor cells lacking the upregulation of HER2. Pertuzumab, recently FDA approved new humanized mAb, blocks HER2 dimerization through binding to a separate epitope on HER2 [129, 265]. The major achievement of HER2 program was that mAbs have a potential in treatment of solid tumors and that tyrosine kinase oncogenes could be regarded as feasible cancer targets [129]. Pertuzumab in combination with trastuzumab and docetaxel is a standard of care for patients with previously untreated MBC [269].

15.8.2 Bevacizumab

As mentioned earlier, vascular endothelial growth factor (VEGF) is a proangiogenic molecule with a critical role in tumor metastasis [270]. Bevacizumab is a humanized mAb that inhibits VEGF activity and is mainly used in combination with chemotherapy for the treatment of many types of advanced cancers such as colorectal cancer, RCC, NCLCs, ovarian cancer, and glioblastoma [271–277]. The addition of bevacizumab to cytotoxic chemotherapy has improved response rates and survival of patients with metastatic colorectal cancer (mCRC) [278]. Moreover, in a phase III trial, the increase in overall survival of mCRC patients attributable to bevacizumab was 4.7 and 2.1 months following first-line and second-line therapies, respectively [279, 280]. Bevacizumab-based therapy resulted in improved clinical responses in other malignancies as well. For instance, incorporation of bevacizumab to a chemotherapy regimen produced a 2 months clinically relevant improvement in overall survival in NSCLCs compared to chemotherapy alone [276].

Regardless of the utility of several FDA-approved mAbs for cancer treatment, the therapeutic application of mAbs for solid tumors encounters several problems, which are discussed in Sect. 15.11. Compared with solid tumors, targeting of hematological malignancies has proven less complicated because mAbs have easy access to malignant cells allowing for administration of lower Ab doses to achieve potent therapeutic results. Here, rituximab is addressed as the first mAb approved for the treatment of hematological malignancies.

15.8.3 Rituximab

Rituximab is a chimeric mAb specific to CD20, the first Ag targeted for therapeutic purposes and expressed by more than 90% of B-cell lymphomas [281]. mAbs, which were approved initially, were designed to target those membrane proteins that were commonly expressed on both hematologic malignancies and their related immune cell precursors. This mAb was able to abrogate both cancer and normal cells [129]. Randomized studies have demonstrated that rituximab induces reasonable antitumor responses in patients with various lymphoid malignancies of B-cell origin, including indolent (e.g., follicular lymphoma (FL)) and aggressive (e.g., diffuse large B-cell lymphoma (DLBC)) forms of NHL (NHL), and CLL. Non-comparative studies have also shown an activity in all other lymphomas [281–283].

A multicenter phase II study on relapsed low grade FL patients showed an overall remission rate of 48%, (including 6% of complete response (CR)), and a median progression time of 13 months following rituximab therapy [284]. In untreated FL patients, utilization of rituximab as the first-line therapy along with maintenance therapies led to the improvement in the overall response rate from 47% (7% CR) after initial treatment to 73% (37% CR) following maintenance treatment [285]. Consolidation therapy with 90Y-ibritumomab tiuxetan, which targets CD20, in the first remission of advanced-stage FL, increased the 8-year overall progression-free survival rate from 22% to 41%. Interestingly,

the median time for the next treatment step was 8.1 years for ^{90}Y-ibritumomab vs. 3.0 years for control [286].

Furthermore, utilization of rituximab in combination with fludarabine and cyclophosphamide led to a significant improvement in the overall survival in CLL patients. Consistently, single-agent rituximab was efficient, even in patients with treatment-refractory or poor-prognosis CLL so that the overall response rate was 90.9% with a complete remission rate of 63.6%. Moreover, the median progression-free survival was 28.5 months, and the median duration of response was 26 months [287]. Nonetheless, administration of rituximab as a single agent to CLL has limited clinical activity inasmuch as it generally does not eradicate leukemia from the marrow. However, when employed in combination with chemotherapy, rituximab can improve the survival of patients relative to that of those treated with chemotherapy alone. Subsequently, FDA approved the use of rituximab in combination with fludarabine monophosphate and cyclophosphamide in previously untreated and chemotherapy-treated CD20$^+$ CLL [288].

15.8.4 Therapeutic Monoclonal Antibodies Approved by Non-FDA Organizations

Apart from those authorized by FDA, there are mAbs that are approved outside the United States for cancer therapy (e.g., catumaxomab and nimotuzumab) [289, 290]. For instance, catumaxomab, a trifunctional Ab specific to epithelial cell adhesion molecule (EpCAM) on tumor cells, CD3 on T-cells, and Fcγ receptors on accessory cells was approved by the European Union for the treatment of patients with malignant ascites generated by EpCAM-positive carcinomas [291]. Moreover, nimotuzumab, a humanized mAb against EGFR, was developed in Cuba and is approved to treat patients with head and neck cancer, glioma, and nasopharyngeal cancer in more than 20 countries in Asia, South America, and Africa [289, 290, 292].

15.9 Monoclonal Antibodies Currently Undergoing Clinical Trials

The current research is mainly focused on innovative mAbs to novel targets in order to overcome the current limitations of mAb therapy. Currently, approximately 350 mAbs are available with potential utilization for various disorders. Historically, about 50% of these Abs recognize tumor Ags [293]. Although most of these mAbs are in initial development stages, more than 100 anticancer mAbs are being evaluated in different phases of clinical trials [294]. Hence, in near future the number of approved mAbs is expected to rise significantly, which could help to improve the outcome of cancer patients by overcoming the current therapeutic limitations. This section briefly introduces some antitumor mAbs that are currently undergoing clinical trials. Several of the mAbs in trials try to provide an opportunity for the treatment of untreatable cancers through targeting of novel tumor Ags. For instance, intetumumab, a humanized mAb against human αV integrin, has been successfully tested in phase I/II clinical trials as the first-line treatment in patients with metastatic castration-resistant prostate cancer [295, 296].

Some innovative mAbs target the well-validated Ags that were previously targeted with the approved mAbs, such as necitumumab (a fully human IgG1, passed phase I of clinical trial in advanced solid malignancies); and nimotuzumab (a humanized IgG1, passed phase I of clinical trial in NSCLC), which both bind specifically to EGFR [297–299]. Some newly designed mAbs in this category are those attempting to improve the functionality of previously-approved mAbs. For instance, obinutuzumab (GA-101), a glycoengineered humanized mAb, binds with high affinity to CD20 type II epitope, resulting in the induction of much stronger ADCC and superior cell killing properties compared to rituximab [300, 301]. Moreover, a phase I/II clinical trial demonstrated that GA-101 has a similar safety profile comparable to that of rituximab, and exhibits promising efficacy in patients with

relapsed/refractory CD20-positive lymphoid malignancies [301–303].

Furthermore, there are mAbs designed to bridge cancer and immune cells. A BsAb, named blinatumomab, with dual specificity for CD19 and CD3, potentially engaged cytotoxic T-cells for redirected lysis of tumor cells [304]. Consistently, blinatumomab therapy led to a higher degree of in vitro lysis of human lymphoma cells, and was efficient at much lower concentrations compared to rituximab [305]. A phase II trial indicated that blinatumomab could induce complete long-lasting remission in B-lineage ALL patients with persistent or relapsed minimal residual disease (MRD). According to the results, blinatumomab administration induced a 76% MRD response rate defined as MRD negativity within four cycles of treatment [204, 306].

European Medical Agency (EMA) and FDA are evaluating avelumab, which is an anti-PD-L1 human IgG1 mAb, for the treatment of metastatic Merkel cell carcinoma. This mAb is also currently under assessment through phase III trials for patients with other cancer types, such as renal cell, non-small cell lung, gastric, ovarian, and urothelial cancers [307].

FDA in October 2016 approved olaratumab (Lartruvo®), which is a human IgG1 mAB targeting platelet-derived growth factor receptor α (PDGFRα), for the treatment of soft tissue sarcoma. ANNOUNCE study, an ongoing phase III trial, which evaluates olaratumab/doxorubicin combination compared with doxorubicin alone in advanced or metastatic soft tissue sarcoma patients, resulted in further support for authorization to be continued [307].

Finally, drug conjugates such as immunotoxins and antibody drug conjugates (ADCs) are another class of mAbs under clinical investigation. Moxetumomab pasudotox, which is a recombinant immunotoxin composed of the Fv fragment of an anti-CD22 mAb fused to a 38-kDa fragment of Pseudomonas exotoxin A, passed phase I clinical trial with safety and activity in relapsed/refractory hairy cell leukemia (HCL) [308]. Furthermore, this mAb is being evaluated in phase I trials in patients with CLL, B-cell lymphomas, and childhood ALL [309].

Gemtuzumab ozogamicin (Mylotarg®), a humanized IgG4 CD33 mAb linked to the toxin calicheamicin, is the first clinically validated cytotoxic immunoconjugate, which targets the CD33 antigen, found on leukemic blast cells in more than 80% of patients with AML, as well as normal myeloid cells. Bistranded DNA damage by calicheamicin results in the death of the myeloid cell but does not affect pluripotent stem cells. After 10 years of approved clinical use of GO, it was withdrawn from market in June 2010, because subsequent follow-up trials failed to demonstrate the supporting data suggesting clinical efficacy and significant benefits over conventional cancer therapies. In early 2017, it was reintroduced into the market based on several investigator-led clinical trials and results of Pfizer' clinical trial, the phase III, open-label, randomized trial enrolled 280 newly diagnosed AML patients [310–313].

Successful construction of clinically effective ADCs and advancements in ADC linker design and conjugation technologies are reflected by recent approval of brentuximab vedotin (Adcetris®) for CD30-positive Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (ALCL) and trastuzumab emtansine (Kadcyla®) for metastatic breast tumors overexpressing HER2/neu [314–318].

15.10 Combinational Monoclonal Antibody-Based Modalities

A brief review of the so far published data on cancer therapy reveals that a single method, such as Ab-based therapy, per se would not be efficacious enough to eradicate the fully armed tumor cells. Hence, in recent years researchers have employed multimodality approaches, which utilize more than a single antitumor agent [4, 319, 320]. This section describes the studies that have examined the effectiveness of combining Ab-targeting with additional common antitumor strategies.

15.10.1 Combination with Chemotherapy

Chemotherapy is one of the methods widely used in combination with Ab therapies to treat various cancers. This method is known to support antitumor immune responses via inducing tumor cell death, eliminating Tregs, and/or making tumor cells more sensitive to lysis by CTLs. Ab-targeted strategies, on the other hand, are believed to render tumor cells more susceptible to chemotherapeutic drugs [321, 322]. An anti-EGFR mAb in combination with chemotherapy could improve overall and/or progression-free survival compared to each agent alone, in patients with mCRC [323]. Moreover, the combination of AZD8055, a rapamycin analogue, and a CD40 agonist mAb, was employed to treat a murine model of metastatic RCC. Notably, the mixture provoked a robust antitumor response in terms of increased infiltration, stimulation, and proliferation of NK cells and $CD8^+$ T-cells in metastatic areas compared with what was observed following the use of each treatment alone [324].

Nevertheless, to achieve potent antitumor results one must take into account the probable factors affecting each of the strategies used in a combination therapy approach. For instance, although generally effective, anti-EGFR mAb combined with chemotherapy would be of no therapeutic value if used to treat patients bearing *KRAS* mutant tumors [323, 325].

15.10.2 Combination with Radiotherapy

Radiotherapy, similar to chemotherapy, has extensively been used in combination with antitumor Abs. The traditional perception of radiotherapy function as a cytocidal weapon decreasing tumor metastasis has recently been shifted to that of a potent adjuvant helping immunotherapy. Radiotherapy is accompanied with immunological effects on tumor cells, including a promoted production of cytokines and peptides, comprising radiation-specific peptides, and an overexpression of adhesion and MHC Class I molecules [326]. Additionally, current evidence suggests that ionizing radiation per se can successfully induce immunogenic cell death leading to effective activation of antitumor immune responses [327, 328]. However, it should be noted that induction of a potent immunogenic cell death depends upon each tumor's intrinsic features as well as the genetic polymorphism for certain genes in each host [329, 330].

Additional proimmunogenic mechanisms have been shown to be promoted by ionizing radiation. For instance, chemokines including CXCXL9 and CXCL10, involved in T-cell recruitment, were released following radiotherapy of different tumors [331–333]. Interleukin 1β and TNF-α are examples of proinflammatory cytokines induced by radiation [331, 334, 335]. Moreover, sublethal doses of radiation have been shown to enhance the expression of certain molecules on tumor cells rendering them more susceptible to recognition and killing by tumor-specific T-cells [328]. On the other hand, radiation therapy has been reported to induce several immunosuppressive mechanisms instead of immune stimulation. There is evidence that radiation activates the latent form of TGF-β, an immunomodulatory cytokine involved in tumor progression [336, 337]. Moreover, radiotherapy has been indicated to induce tolerogenic properties in macrophages [338, 339]. Furthermore, an increase in the number of Tregs has been reported in some patients receiving radiation as an antitumor modality [340, 341].

Hence, radiation has the capacity to induce either proimmunogenic or immunosuppressive responses. In most cases, favorable impacts of radiotherapy dominate over the unfavorable ones. However, this is insufficient to thoroughly shift the balance of immune responses against tumor cells in the absence of accompanying immunotherapies [328].

In fact, promising results have been obtained by several preclinical studies that have combined radiotherapy with Ab targeting. Antibody blockade of CTLA-4 combined with local radiation in a murine model of breast cancer significantly increased the survival rate due to the induction of effective T-cell responses, whereas radiotherapy

alone could only delay tumor growth, and anti-CTLA-4 mAb by itself was completely ineffective [328]. Consistently, the metastasis of poorly immunogenic colorectal and mammary carcinomas was successfully inhibited by a combination of radiation and anti-CTLA-4 mAb in mice [342]. Targeting of co-stimulatory molecules, such as CD137 (critical receptor on T-cell surface), CD40 or OX40 with immunomodulatory antibodies and ionizing radiation has resulted in several other beneficial antitumor effects [343–346]. Interestingly, the combination of radiotherapy and anti-CTLA-4 Ab has also led to promising results in clinical trials [347]. In a case report of melanoma, treatment of the patient with ipilimumab (anti-CTLA-4 Ab) following radiation [348] could mimic the successful results previously observed in murine models [328, 342].

Nonetheless, to exploit the full potential of this type of combination to treat cancers entails the establishment of standard radiation regimens, which can result in effective domination of proimmunogenic over immunosuppressive responses. To this end, investigators are recommended to test different doses and frequencies of radiation in combination with each immunotherapeutic method for every cancer type and choose the optimal combination strategy [328, 342, 349].

15.10.3 Combination with Other Immunotherapeutic Methods

Antibody-based therapeutic methods have also been used together with other immunotherapeutic strategies to outsmart tumor-associated evasion mechanisms. For instance, anti-4-1BB mAb, as a $CD4^+$ T-cell adjuvant, was applied together with in vitro activated antitumor T-cells to a murine model of microscopic pulmonary metastasis. The combination was advantageous over Ab administration or adoptive T-cell therapy alone. In fact, anti-4-1BB mAb served as an efficacious adjuvant through augmenting the antitumor function of transferred T-cells and resulted in persistence of infiltrated effector T-cells [350]. However, one major disadvantage of using anti-4-1BB mAb is its toxic effects in higher doses. To overcome this issue, one study employed a combination of lower doses of anti-4-1BB and tumor lysate-pulsed DCs for the treatment of liver metastatic colon cancer. This nontoxic combination strategy resulted in a significant increase in tumor rejection comparable to the level obtained with higher toxic doses of anti-4-1BB alone [351]. In a very recent study, T-cells, engineered to express a type of tumor-specific MUC-1 receptor, were adoptively used to target prostate cancer cells. However, the vaccine efficacy was hindered by the heterogeneous expression of MUC-1 by tumor cells. Interestingly, the addition of a type of conventional anti-androgen mAb to the treatment regimen could improve the antitumor effects in vitro [352]. These examples substantiate the advantage of employing alternative immunotherapeutic approaches along with Ab-based modalities to obtain more potent and less toxic antitumor responses.

15.10.4 Other Combinational Approaches

In addition to the aforementioned more popular combination approaches, researchers have examined the efficacy of employing several less-known modalities. For instance, a combination of Abs against two growth factors, secreted by human pancreatic cell lines, was successfully used to improve the efficacy of chemotherapy in pancreatic cancer patients [353]. Moreover, in a recent murine model of breast cancer, a recombinant protein with the capacity to bind to epithelial cell junctions was used as a partnering treatment for anti-EGFR-mAb. Interestingly, the cell junction opener protein could improve the intratumoral penetration of mAb culminating in robust antitumor responses [354].

Overall, with regard to Ab-based antitumor strategies, data obtained from preclinical and clinical studies corroborate that combinatorial approaches are undoubtedly superior to simple utilization of a mAb alone. Designing the most efficacious approaches entails gaining a precise understanding of the cellular and molecular

events underlying the interaction between the combined methods. Notably, the mAb of interest needs to be used in combination with a range of successful immunostimulating methods to choose the best partnering agent.

15.11 Current Limitations in Monoclonal Antibody-Based Therapies

15.11.1 Tumor Escape

It often occurs that patients with the same cancer type respond differently to a certain Ab-based strategy. This could be in part attributed to the diverse mechanisms tumor cells use to escape immune responses [355]. Here, we describe major mechanisms underlying tumor resistance to Ab-based modalities.

One reason for the resistance to mAb therapy in most cancer patients might be the presence of agents that inhibit CDC [356]. Protectin (CD59) inhibits homologous CDC by preventing formation of the membrane attack complex, thereby inhibiting cell lysis [357]. In fact, a great deal of evidence indicates that CD59 is highly effective in protecting NHL, melanoma, and CLL cells from antibody-mediated CDC and up-regulation of CD59 is an important determinant of sensitivity to Ab treatment in such cancers [358, 359].

Tumor cells might circumvent ADCC via expression of NK cell inhibitory molecules such as HLA-G, a non-classical HLA class I [360], which is known to be expressed on melanoma and other malignancies [361–363]. Interestingly, rituximab-mediated NK cell lysis depends on the HLA class I expression level on B-lymphoma cells [360].

To evade Ab-mediated therapies, tumor cells can downregulate the expression of Ags targeted by mAbs. Intriguingly, high receptor expression is known to be associated with a favorable response to trastuzumab. However, due to target receptor downregulation following Ab therapy, a proper response may not always be achieved [1]. Similarly, acquired rituximab resistance in B-cell lymphomas following exposure to rituximab has been associated with reduced levels of CD20 [364–366].

Masking of target proteins on tumor cells is another tumor escape mechanism. Resistance to trastuzumab was associated with increased expression of the membrane-associated glycoprotein MUC-4, which was shown to bind and sterically prevent HER2 from binding to trastuzumab [367–369].

Tumor resistance to Ab targeting might occur because of the induction of compensatory or alternative signaling by other cell surface receptors. Cetuximab (anti-EGFR mAb)-resistant tumors have been shown to escape Ab treatment through increased expression of G-protein coupled receptors [355, 370]. Furthermore, resistance to cetuximab treatment in colorectal cancers is often related to point mutations of *KRAS* and its downstream signaling molecules (e.g., BRAF) [371–374].

15.11.2 Relatively Low Single Agent Activity

Although numerous therapeutic mAbs have been approved for clinical use, in most cases, the overall response to a single mAb remains low. Accordingly, mAbs are commonly used in combination with other treatment modalities to achieve more favorable results (discussed in Sect. 15.10).

Protecting antibodies that interfere with clearance mechanisms through binding to the Fc domain of the neonatal Fc receptor—namely FcRn—has increased the serum half-life of antibodies (2–4 weeks in circulation). When extended function of a drug is required for a patient, this long half-life along with less frequent dosing is usually more desirable. Dosing of antibody is typically performed intravenously or subcutaneously. Due to immediate antibody degradation in the gut, oral administration is not recommended. Furthermore, pristine blood–brain barrier does not allow the therapeutic antibodies to pass in favorable quantities [129].

15.11.3 Low Tissue Penetration

Molecular size plays a key role in tumor penetration of therapeutic mAbs, and in fact, the diffusion rate inversely correlates with the cube root of molecular weight. Therefore, mAbs, as large molecules, would have difficulty diffusing into solid tumors, resulting in increased resistance of larger tumors to mAb-based modalities [375].

Using mAbs with high affinity can further diminish tumor penetration of Abs, a factor called "binding site barrier effect" [376]. In fact, there are several reports verifying that very high affinities can lead to suboptimal antitumor responses [377, 378]. The tight binding of mAbs to their Ag targets on the outer surface of solid tumors hampers their deeper penetration into tumor mass. Therefore, development of mAbs with optimal affinities for tumor Ags would result in efficient antitumor responses. However, achieving robust clinical responses mandates the consideration of several factors including Ag density, internalization, association, and dissociation rates; therefore, it is not always easy to develop perfect mAbs.

15.11.4 Fc–Fc Receptor Interactions and Associated Limitations

Elimination of tumors using mAbs that promote ADCC meets several challenges. First of all, a successful ADCC process requires a high affinity between Fc of a mAb and its receptor on effector cells; this is a major problem since a high percentage of the population expresses low affinity variants of the Fc receptor [117]. It has been shown that the presence of a valine (V) at position 158 of FcγRIIIa/CD16a instead of a phenylalanine (F) improves the FcR affinity for IgG [379, 380], and this replacement is shown to correlate with improved responses to rituximab therapy [117, 381].

Secondly, the glycosylation pattern of the Fc fragment of a mAb can be of major importance when working with therapeutic mAbs. In particular, the C_H2 domain of IgG1 is glycosylated (Asn-297) and this has been shown to have a key role in modulating the interaction of Fc with FcγRIIIa, thereby affecting the Ab efficacy. More specifically, the presence of fucose residues in the carbohydrate moiety has been reported to end in decreased ADCC efficiency [233].

A third challenge in front of ADCC triggering approaches is that there are a large number of IgG molecules in patients' sera, which compete with therapeutic mAbs in binding to FcRs. Specifically, IgG concentration in serum is 8–17 mg/mL, 66% of which is allocated to IgG1 molecules that can interact with FcγRIIIa. This explains why the effective mAb dosage needed for in vivo applications is much more than what is needed for in vitro ADCC experiments, which are performed in the absence of serum IgGs [382].

Finally, the affinity of mAbs for an inhibitory Fc receptor, called FcγRIIb, can significantly affect the outcome of an ADCC-based Ab therapy. FcγRIIb, expressed by several immune cells including DCs, macrophages, B cells, and neutrophils, is known as a negative regulator of immune responses [383]. In fact, signaling through this receptor keeps the potentially harmful immune reactions under control. This, however, poses a challenge to Ab therapy of tumors in which fully activated antitumor immune responses are desired. There is in fact evidence that binding of certain therapeutic mAbs to FcγRIIb leads to decreased therapeutic efficacy [164].

15.11.5 High Production Cost

Most therapies need high Ab doses over a long period of time, which requires large amounts of purified product per patient. In fact, therapeutic Ab production poses the costly process of establishing large mammalian cell cultures and extensive purification steps to companies, and ultimately places heavy financial burdens on cancer patients. Hence, improvement in alternative culture systems (e.g., microorganisms or plants) might lead to substantial reduction of production cost in the near future [384, 385].

15.12 Concluding Remarks

Despite the prominent role of the cellular arm of immune system in fighting against cancer, there is a great deal of evidence substantiating the effectiveness of the humoral immune system for cancer therapy. Not only can Abs directly destroy cancer cells, but also they can prevent tumor outgrowth and deliver radiation and/or powerful cytotoxic drugs to the tumor site. With this aim in view, many anticancer mAbs targeting different epitopes in several malignancies have opened their ways into the clinic, and there is rapid progress in discovering novel Ab targets for cancer therapy. Several engineering attempts have been evaluated in the development of improved therapeutic antibodies with the aim of promoting their efficacy and safety as antibody-based therapies. These attempts comprise antibody chimerization, humanization, and the development of fully human antibodies. mAbs are being investigated for new applications and, currently, manipulated for simultaneous targeting of two or more targets, conferring enhanced therapeutic efficacy. Due to the diverse evasion mechanisms of cancer, the application of Ab-based immunotherapeutic approaches per se may not be sufficient to overwhelm cancer outgrowth. Hence, Ab-based combinational cancer treatment modalities have been the focus of many recent investigations.

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Toll-Like Receptor Pathway and Its Targeting in Treatment of Cancers

16

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16.1 Introduction

The innate immune system has been shown to be responsible for the diagnosis and reaction to pathogens, leading to inflammatory response and accumulation of professional phagocytes to the site of invasion [1]. Also, it has been reported that innate immune response is significantly associated with changes in cellular metabolic signaling pathways [2]. In addition, the innate immune response has been found to be crucial for stimulation of adaptive immune response against pathogens by formation and presentation of antigens and the production of mediators that are needed in combination to induce T cell- and B cell-mediated responses [3].

Toll-like receptors (TLRs) are transmembrane pathogen recognition receptors (PRRs) that recognize various pathogen-associated molecular patterns (PAMPs), such as bacterial lipoproteins (TLR2), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (ssRNA) (TLR7 and 8), and cytosine-phosphorothioate-guanine (CpG) DNA (TLR9) [4]. In addition to TLRs, intracellular NOD-like receptors (NLRs) are also involved in human immunity. NLRs are intracellular innate immune detectors of microbial and other dangerous signals [5]. NLRs that contain NALP, NOD1, and NOD2 have been found to be involved in several signaling pathways, leading to regulation of production of proinflammatory cytokines, including interleukin-1β (IL-1β) and IL-18. Moreover, NLRs play important roles in the induction of cell death [6]. Additionally, NLRs can discriminate between pathogens which break cellular and mucosal barriers and nonpathogenic microorganisms, therefore providing a functional benefit over TLRs to work as sentinels of the innate immune system at mucosal levels [7]. It has been reported that NODs are also involved in immune response against tumors. Although simultaneous targeting of TLRs and NLRs has been found to be effective in the induction of CD4+ and CD8+ T cell function, leading to suppression of tumor growth [8], NOD's targeting/triggering effects on tumors are not adequately stated. Hence, we decided to review the role of TLRs in tumorigenesis and discuss the prospect of TLRs in the treatment of cancers.

Activation of various TLRs may lead to complete opposite results, such as anti- or protumor effects. TLR role is cell specific, and the varied outcome of TLR function originates from difference of TLR stimulators in combination with other microenvironmental factors. It has been found that TLR4 and TLR9 activation leads to tumor cell escape from immune system attack, promoting tumor growth. In contrast, triggering of TLR3 on breast cancer cell promotes antiproliferative signaling. Besides, TLR3 expression in head and neck cancer (HNC) induces tumor aggressive behaviors [9].

It has been found that chronic inflammation may lead to cancer initiation [10]. TLR has been recognized as not only being responsible for secretion of proinflammatory cytokines but also for the upregulation of metalloproteinase and integrins, thereby promoting tumor cell invasion and metastasis [11]. Among tumorigenesis cytokines, IL-6 has been shown to play a crucial role in the differentiation, angiogenesis, proliferation, and apoptosis of several cell types [10]. Initially, it has been thought that TLRs are present only on immune cells; however, recently, it has been understood that TLRs also have important functions in human cancers (Table 16.1). Later, it has been discovered that TLRs promote proinflammatory cytokines, leading to tumor growth and chemoresistance. However, various differential

Table 16.1 Expression of TLRs in several cancer cells

Cancer type	TLRs expressed
Basal cell carcinoma	TLR7
Breast cancer	TLR2, 3, 4, 5, 7, and 9
Brain cancer	TLR2 and 4
Colorectal cancer	TLR2, 3, 4, 5, 7, and 9
Cervical cancer	TLR3, 4, 5, and 9
Esophageal squamous cell carcinoma	TLR3, 4, 7, and 9
Gastric cancer	TLR2, 4, 5, and 9
Human head and neck squamous cell carcinoma	TLR4
Hepatocellular carcinoma	TLR2, 3, 4, 6, and 9
Laryngeal cancer	TLR2, 3, and 4
Lung cancer Liver (HCC)	TLR2, 3, 4, 7, 8, and 9 TLR4
Melanoma	TLR2, 3, 4, and 7
Ovarian cancer	TLR2, 3, 4, and 5
Oral squamous cell carcinoma	TLR2 and 4
Pancreatic carcinoma	TLR2, 3, 4, 7, and 9
Prostate cancer	TLR3, 4, and 9

pro- and antitumor effects have been recognized for TLRs [12]. In addition, the recent studies showed TLR can have a prognostic value. Overexpression of TLR7 and TLR5 is associated with worse overall survival in HPV-positive patients with oropharyngeal squamous cell carcinoma [13]. On the other hand, low expression of TLR9 in triple-negative breast cancer defined a very aggressive tumor subtype [14].

16.2 TLRs Play Important Roles in Human Carcinogenesis

In addition to bacterial and viral components, TLR expression increases in response to inflammation by-products and cellular injury, namely, damage-associated molecular patterns (DAMPs) [15]. Even though TLR7 activation shows antitumor responses in various tumors, including basal cell carcinoma (BCC), breast cancer, and melanoma, it has been postulated that overexpression of TLR7 promotes pancreatic carcinogenesis through mediating several complex pathways [16]. TLR7 is significantly upregulated in both neoplastic ductal epithelial and inflammatory cells, whereas it is undetectable in human normal pancreata. Also, it has been found that TLR7 expression is associated with tumor progression [17]. TLR7 plays important roles in pancreatic carcinogenesis by upregulation of intrapancreatic Notch, MAPK, and NF-κB signaling pathways [17, 18]. It has been discovered that Notch signaling pathway exacerbates inflammation and therefore regulates human pancreatic cancer initiation and maintenance [19]. The NF-κB and MAPK signaling pathways also have proinflammatory effects, mediating TLR7-stimulated pancreatic carcinogenesis [17]. In contrast to TLR7 effects on the pancreas, the expression of TLR4 has been shown to suppress lung carcinogenesis [20], whereas TLR2 expression leads to lung and gastric tumor cell progression [21, 22]. Although TLR7 has been considered responsible for intrapancreatic inflammation and fibrosis, destructing exocrine and endocrine organs, its pancreatic carcinogenesis is dependent on baseline levels of inflammation [23]. Moreover, it has been speculated that Kras oncogene is necessary for TLR7-mediated pancreatic carcinogenesis, because no changes have been found in cell cycle regulation and tumor suppressor genes in TLR7-promoted pancreatitis [17]. Collectively, it seems that TLR7-induced pancreatic carcinogenic changes on Kras-transformed cells are secondary to direct effects on peritumoral inflammatory cells, rather than being direct effects of TLR7 stimulation [17].

In addition to TLR7, TLR4 is also involved in colorectal cancer (CRC) tumorigenesis but independent of the presence of baseline inflammation. TLR4 is expressed on CRC cells regardless of the tumor stage [24]. It has been suggested that TLR4 activation is crucial for dysplasia [25]. LPS-stimulated TLR4 activates phosphatidylinositol-3′-kinase (PI3K), leading to phosphorylation of phosphoinositides and, therefore, phosphorylation and activation of Akt. It has been found that PI3K/Akt pathway is expressed in CRC in a stage-dependent fashion [24]. Altogether, TLR7 agonists have been discovered as novel therapeutic approaches for the treatment of BCC and melanoma [26]. However, TLR7 ligation plays opposite roles in pancre-

atic cancer, indicating the importance of TLR7 signaling blockade in the prevention and treatment of malignancy. More evidently recent data shows a stage-dependent upregulation of both TLR7 and TLR8 expression in pancreatic cancer. TLR7 and TLR8 expression increases tumor cell proliferation and promotes chemoresistance in human pancreatic cancer [20, 27]. Also, targeting of TLR4 signaling pathway in CRC may prevent tumor initiation [12].

16.3 TLR Regulates Tumor-Induced Immune System Response

It has been found that almost all tumor cell lines express single or more commonly multiple TLRs, with TLR4 expression as the highest (Table 16.1). Hsp70 has been found to be highly expressed by tumor cells, playing a ligand role for TLR4. Hsp70-/LPS-mediated TLR4 overexpression leads to the production of nitric oxide (NO) and cytokines such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF), tumor necrosis factor-α (TNF-α), IL-6, and IL-12 p40 [28]. It has been postulated that TLR4 expression is responsible for immune suppression (Fig. 16.1). LPS-stimulated TLR4 expression inhibits T cell proliferation. Also,

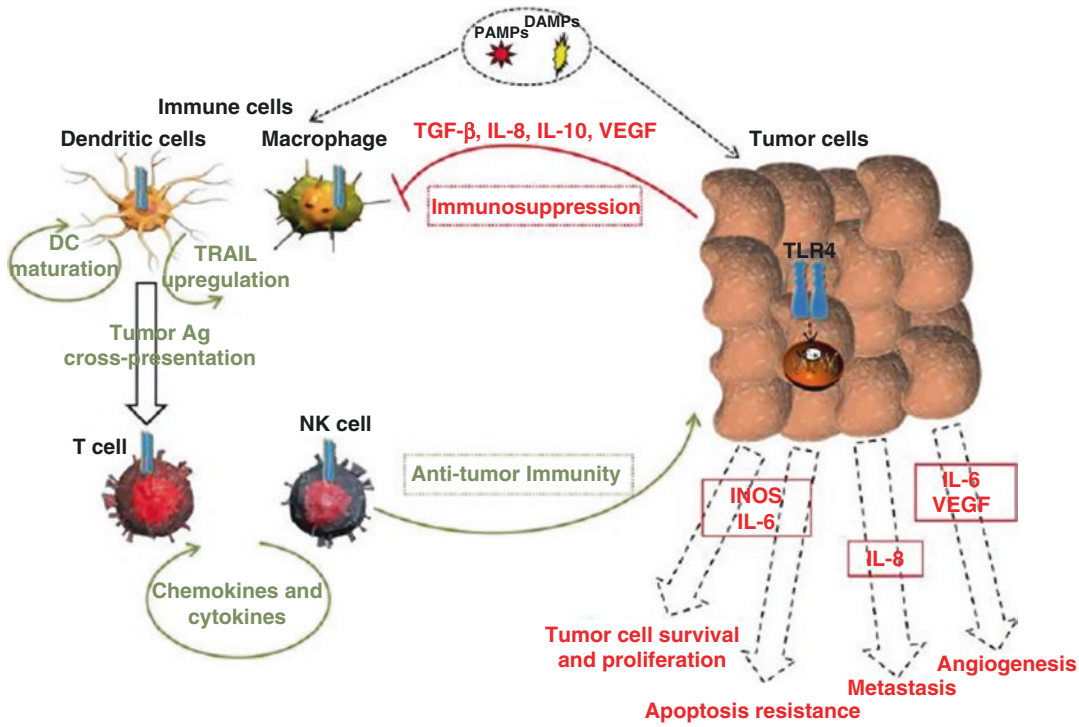

Fig. 16.1 Role of TLR4 signaling in cancer. TLR4 is widely expressed on both immune and tumor cells. TLR4 signaling in cancer is considered a double-edged sword with both pro- and antitumor consequences. TLR4 signaling on immune cells (depicted on the left-hand side in green color) enhances antitumor immunity by cytokine/chemokine upregulation, DC maturation, and function. TLR4 is also responsible for efficient tumor antigen cross-presentation. Alternatively, TLR4 signaling on tumor cells (depicted on the right-hand side in red color) increases their tumorigenic activity

TLR4-mediated NO suppresses T cell activation [29]. In addition, TLR4-induced IL-6 promotes impairment of dendritic cell (DCs) maturation and activation of natural killer (NK) T cells and can also influence NK cell anergy [30]. Furthermore, IL-12 inhibits the generation of allogenic or tumor-specific CTL, contributing to the immune suppression [31].

TLR4 also has an important role in chronic induction of IL6 and activation of STAT3 which has a significant effect on uncontrolled cellular proliferation [32]. On the other hand, upregulated TLR4 increases B7-H1, B7-H2, and CD40 levels but decreases Fas expression on tumor cells, thereby leading to cancer cell escape from immune system surveillance and CTL attacks [28]. Therefore, TLR4 plays an important role in the protection of tumor cells from the immune system response (Fig. 16.1); nonetheless, it has been suggested that TLR4 function is necessary for DC maturation and CD4+ CD24+ regulatory T cell blockage [33].

TLR4 is highly expressed in both cell membrane and cytoplasm of human oral squamous cell carcinoma (OSCC) [34]. The expression is associated with tumor cell differentiation, and TLR4 level is significantly higher on well- and moderately differentiated tumor cells when compared to poorly differentiated cancer cells. LPS-stimulated TLR4 activates both NF-κB and p38 MAPK pathways, leading to the massive production of IL-6, IL-8, and VEGF. IL-6 is considered as a principal biomarker of poor prognosis in several human cancers [34]. Higher levels of IL-6 can lead to tumor progression, resistance to apoptosis, chemoresistance [35], tumor angiogenesis, and tumor invasion [36]. IL-8 plays anti-apoptotic roles and promotes tumor metastasis [37]. VEGF is involved in angiogenesis and immunosuppression and also suppresses DC number and differentiations [38]. These results indicate the crucial effects of TLR4 signaling in human OSCC survival and metastasis, therefore suggesting the importance of novel approaches targeting TLR4 signaling pathway for OSCC treatment.

Although TLR2, TLR3, and TLR4 are expressed in normal primary melanocytes, they are significantly overexpressed on most melanoma cell lines [39]. The presence of TLRs on normal melanocytes plays important roles in the recruitment of innate immune cells. Overexpression of TLRs in melanocytes leads to chronic inflammation, thereby increasing the risk of tumor development and progression [40]. Upregulated TLR2, TLR3, and TLR4 promote production of proinflammatory cytokines (TNF-α, IL-1, IL-6, and granulocyte colony-stimulating factor (GCSF)) and chemokines (CCL2 and CXCL10). Also, these TLRs stimulate the secretion of IL-10 and cyclooxygenase-2 (COX-2) (inflammatory factor) [39]. Higher levels of TNF-α induce IL-6 and CCL2 synthesis, leading to the tumor progression. Also, TNF-α regulates infiltration of leukocytes in cancers by chemokine modulation [41]. Besides, CCL2 and CXCL10 promote escalating inflammation and immunity in melanoma cancer [42]. Additionally, TLR3 triggers NF-κB-mediated upregulation of inflammatory molecules and recruits leukocytes, promoting anticancer immune responses [43]. TLR4 is found to be highly expressed in breast cancer cells. It has been found that targeting of TLR4 signaling by TLR4AsiRNA leads to significant inhibition of breast cancer cell proliferation. Also, inhibition of TLR4 interrupts its downstream signaling pathway, leading to the strikingly depressed levels of IL-6 and IL-8, and, therefore, attenuates tumor cell survival by decreasing their resistance to cytotoxic T lymphocyte (CTL) and natural killer cell (NKC) attack. These results suggest that targeting of TLR4-mediated signaling pathway by TLR4AsiRNA is a novel promising strategy for breast cancer treatment, although this inhibition may promote other cancers, including lung cancer [44]. Thus, manipulation of TLR4 should be done with precise attention considering its possible interactions. Additionally, LPS-stimulated TLR4 upregulation promotes NF-κB signaling pathway and contributes in the production of inflammatory cytokines (including IL-6 and IL-8), VEGF, and granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to tumor progression and development of myeloid-derived suppressor cell (MDSC) [45]. MDSC can promote chronic inflammation and also immune suppression by stimulation of regulatory T cell function [46].

On the other hand, flagellin-stimulated TLR5 leads to the production of various chemokines such as epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), macrophage inflammatory protein 3α (MIP3α), monocyte chemotactic protein-1 (MCP-1), macrophage-derived chemokine (MDC), IL-6, Gro-α, and osteoprotegerin, which are involved in monocyte, leukocyte, and neutrophil attraction [47]. TLR5-induced infiltration of immune cells, including neutrophils, suppress proliferation marker PCNA, promoting strong antitumor response through tumor necrosis and inhibition of tumor growth [47]. Thus, flagellin-induced TLR5 expression can be used as a novel therapeutic approach for human breast cancer.

New technology allows for evaluation of tumor-associated antigens (TAAs) to develop vaccines for the treatment of cancer. However, most of these are poorly recognized by immune system. In result, vaccines containing these antigens require the inclusion of potent immunological adjuvant. Monophosphoryl lipid A (MPL) is only approved TLR4 agonist for human use which is tested in clinical trials as a cancer vaccine adjuvant [48, 49].

16.4 TLR Targeting May Inhibit Cancer Cell Proliferation

TLR7 expression suppresses phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [17]. Suppressed levels of PTEN lead to PI3K/Akt pathway activation and increased level of TGF-β, mediating phosphorylation and activation of STAT3 [50]. STAT3 acts as a proinflammatory marker and central to neoplastic progression in pancreatic tumor [51]. TGF-β promotes cancer invasion [52], and PI3K/Akt signaling pathway stimulates tumor cell proliferation, thus leading to tumor progression [53]. Also, it has been suggested that TLR4 has proproliferative roles. It has been found that human head and neck squamous cell carcinoma (HNSCC) expresses almost all TLRs for its own benefit. TLR4 has been shown to be highly expressed in well- and moderately differentiated HNSCC but weakly present on poorly differentiated cells [45]. It has been suggested that well-differentiated cells contain higher amounts of bacteria and bacterial products, thereby leading to higher expression of TLR4. LPS-induced expression of TLR4 can phosphorylate Akt, thus increasing tumor cell proliferation [45]. CADI-05 is a potent TLR2 agonist. The recent randomized trial showed patients with squamous non-small cell lung cancer who received CAD1-05 in addition to chemotherapy had a better median survival [54].

16.5 TLR Triggering Can Promote Antitumor Response

It has been reported that TLR5 is overexpressed in gastric cancer cell, leading to strong antitumor immune response and suppression of tumor growth [55]. In contrast, early activation of TLR5 has been shown to promote tumor growth in mouse mammary cells. High levels of TLR5 have been found in invasive ductal carcinoma cells, whereas moderate expression is observed in medullary carcinoma and invasive lobular carcinoma. Flagellin-induced expression of TLR5 in breast cancer cells increases phosphorylation of IκB, ERK, JNK, STAT1, and STAT3, leading to the induction of inflammatory cytokine (such as TNF-α, IL-1β, IL-6, and IL-8) mRNA. This flagellin-stimulated cytokine production leads to decreased level of proteins contributed in the cell cycle and inversely increased level of CDK inhibitor 27, thereby inhibiting breast cancer cells proliferation and colony formation. However, it has been found that flagellin fails to induce cancer cell apoptosis [47]. In addition, TLR expression can have an effect therapeutic response. A recent study showed TLR3 pathway rather than the cytoplasmic pathway plays more significant role on enhancing the therapeutic effect of radiation [56]. TLR polymorphism can also predict the response of cancer to chemotherapy. An association was found between TLR7 rs3853839 and progression-free survival among patients with metastatic colorectal cancer who received cetuximab-based chemotherapy in two independent clinical trials. The results were suggesting that this polymorphism predicts the efficacy of cetuximab [57].

16.6 Regulatory Effects of TLRs on PI3K/Akt Signaling Controlling Tumor Progression

Akt has been known to promote cyclinD1 and c-Myc expression by targeting the kinase PI3K/Akt mammalian target of rapamycin (mTOR), which leads to proliferation of various cancer cells [58]. Also, Akt inhibits GSK-3b phosphorylation and therefore suppresses β-catenin nuclear translocation [59]. In addition, Akt regulates cell death through decreasing levels of pro-apoptotic molecules, such as caspase-9, p53, NOXA, and PUMA [60]; however, it inversely regulates increasing anti-apoptotic molecule levels including XIAP, Bcl-xL, and Mcl-1 [61]. Moreover, Akt functionally suppresses both p21Wsf1/Cip1 and p27Kip1 that are negative regulators of the cell cycle [62]. Furthermore, the presence of phosphorylated Akt has been reported to be associated with advanced stages of tumor and poor clinical prognosis [63].

Several TLRs have been detected on human prostate cancer cells. TLR3 and its ligand polyinosinic-polycytidylic (poly(I:C)) acid negatively regulate Akt-mediated pathways in human prostate cancer cells. Poly(I:C) dephosphorylates Akt and therefore impairs PI3K/Akt pathway, leading to the inhibition of cell proliferation by downregulation of cyclin D1 and c-Myc and upregulation of p21Wsf1/Cip1 and p27Kip1 [64]. Also, poly(I:C) increases β-catenin translocation into the nucleus [59, 64]. The PI3K/Akt pathway has also been found to play potent roles in CRC progression and metastasis. TLR4 is responsible for the activation of PI3K/Akt pathway and therefore promotion of tumor progression. Moreover, it has been reported that TLR4 targeting can prevent liver metastasis and burden of the tumor [65]. However, TLR4 pathway targeting seems to be a novel valuable therapeutic approach for the prevention of CRC progression and metastasis.

16.7 TLR-Mediated Hypoxia-Inducible Factor 1 (HIF-1) Expression Leads to Tumor Progression

It has been found that HIF-1 is involved in tumor progression [12]. In hypoxic conditions, HIF-1α stabilizes and binds HIF-1β, leading to the active form of HIF-1 [66], but, in normoxic situations, oxygen-sensing prolyl hydroxylases degrade HIF-1α and keep its level low [67]. Poly(I:C)-induced TLR3 increases the specific I.3 isoform of HIF-1α expression and HIF-1 complex nuclear accumulation in normoxic environment. TLR3's effect on the enhancement of HIF-1α expression is based on the increase of HIF-1α translation rather than prevention of its degradation [68]. Higher levels of HIF-1α have been detected in prostate cancer bone metastasis indicating the importance of HIF-1α in prostate tumor prognosis [69]. It has been reported that poly(I:C)-stimulated TLR3 leads to the upregulation and nuclear translocation of HIF-1α in more advanced prostate cancer cells. Overexpressed HIF-1 increases VEGF secretion [12]. VEGF promotes neovascularization in hypoxic tumor space, leading to tumor progression [70]. HIF-1α complex upregulates anti-apoptotic genes including Bcl-xL, survivin, and MCL-1 [71]. Moreover, the complex impairs caspase-3 function, inhibiting TLR3-mediated apoptosis of progressed prostate cancer cells. However, forcing the upregulation of the HIF-1α-isoform 3 in less aggressive prostate cancer cells can lead to HIF-1 complex nuclear accumulation secondary to the poly(I:C) stimulation. It seems that differential expression levels of HIF-1α in different stages of prostate cancer cells regulate the tumor cell's response to TLR3 stimulation [12]. However, HIF-1α level should be precisely regulated through changes in TLR signaling pathway.

16.8 Role of TLRs in Tumor Cell Lysis and Apoptosis

TLR3 and TLR7 have been found to be effective in increasing γδ T cell cytotoxicity and cytokine production [72]. It has been reported that γδ T cells play important roles in tumor cell lysis by massive production of IFN-γ and TNF-α. Also, γδ T cells

secrete perforin, granzymes, and TNF-α apoptosis-stimulator ligands, mediating tumor cell lysis [73]. The cytotoxic effect of γδ T cells increases in response to poly(I:C)-stimulated TLR3 overexpression. Additionally, γδ T cell-secreted cytotoxic mediator levels increase in tumor cells secondary to poly(I:C)-induced TLR3 overexpression and imiquimod-stimulated TLR7 upregulation. In the presence of γδ T cells, poly(I:C)-mediated TLR3 activates NF-κB p65 and caspase signaling, leading to IFN-β production and apoptosis [74]. Imiquimod-induced TLR7 also increases MyD88 and NF-κB signaling pathways, leading to caspase pathway activation and therefore resulting in tumor cell death [72].

It has been reported that the activation of killer receptor NKG2D, which binds to the stress-inducible MHC class I chain-related antigens (MIC) A/B and UL16-binding proteins (ULBP) 1–4, is crucial for the cytotoxic activity of γδ T cells [75]. Poly(I:C)-stimulated TLR3 leads to the production of TNF-α and, therefore, CD54 expression [76]. Although imiquimod-induced TLR7 decreases MHC class I molecules on tumor cells, imiquimod fails to increase CD54 levels. The presence of CD54 and NKG2D may increase the ability of γδ T cell-mediated tumor lysis. These results indicate that several pathways are involved in tumor cell lysis [72]; nevertheless, it seems that TLR3 and TLR7 are involved in the cytotoxic function of γδ T cells, and proper regulation of these TLRs may bring new treatment hopes for cancer patients. TLR7 activation also leads to the induction of STAT3, which occurs simultaneously with increasing proliferative and anti-apoptotic genes such as c-Myc and Bcl-Xl [17]. It has been reported that a high c-Myc level acts as a prognostic factor in advanced pancreatic tumor, and also its level is associated with poor survival in patients suffering from pancreatic cancer [53]. On the other hand, TLR7 upregulation impairs G1 phase control by downregulation of cyclin D1 and also increasing cyclin B1, leading to the G2 to M phase transition [17].

It has been suggested that tumor cell's resistance to the drug-induced apoptosis originates from TLR4-mediated Akt phosphorylation. On the other hand, it is reported that TLR4 leads to the translocation and binding of p65 subunit of NF-κB to DNA, thereby leading to the inhibition of cisplatin-induced apoptosis and NK cell-mediated tumor lysis. Also, TLR4-activated NF-κB, MyD88, and IRAK4 are associated with tumor progression, as these factors play anti-apoptotic and inflammatory roles. In addition, TLR4 has been considered responsible for tumor cell resistance to chemotherapy, suggesting TLR4 pathway targeting as an important novel treatment strategy for HNSCC [45]. During the targeting of the TLR4 signaling pathway, beneficial effects of TLR4 stimulation should be harnessed while eliminating the possible negative ones (Fig. 16.1). Therefore, it has been speculated that TLRs work like a double-edged sword, stimulating host immune reaction against tumor on one hand and promoting tumor progression on the other.

Moreover, poly(I:C)-induced expression of TLR3 promotes cancer cell apoptosis by caspase upregulation, with the induction of p53 and its pro-apoptotic target NOXA. In addition to apoptosis induction by poly(I:C), the ligand can induce autophagy that is cytoprotective toward apoptosis, indicating the inverse association of apoptosis and autophagy [64].

16.9 TLRs Are Involved in Tumor Metastasis

It has been accepted that the upregulated expression of TLR3 leads to increased chemokine (C–C motif) ligand 5 (CCL5) and IL-6 levels. It has been suggested that cancer cell migration and perineural invasion is mediated by TLR3-induced CCL5 and IL-6 [9]. CCL5 increases matrix metalloproteinase 9 (MMP-9) and, therefore, inhibits T cell antitumor response, leading to angiogenesis and tumor growth [77]. On the other hand, activated NF-κB stimulates genes that are involved in cell differentiation, cell invasion, and anti-apoptotic protein production, such as HIF-1α [12] and apoptotic protein-2 inhibitor [78]. It has been speculated that higher levels of TLR3 in breast and intestinal malignancies and HNC are strongly associated with tumor invasion

and metastasis [79, 80]. The administration of bafilomycin A1 (BA1) which antagonizes TLR3 leads to decreased levels of CCL5 and IL-6, therefore controlling tumor aggressive behavior [80]. Also, TLR4 activation has been found to be responsible for apoptosis resistance in ovarian cancer cell [81]. These results highlight the importance of TLR targeting in the prevention of tumor progression and metastasis. Furthermore, upregulation of COX-2 has been found to be associated with an aggressive type of melanoma cancer. Interestingly, Goto et al. have found that TLR-mediated signaling pathway (MyD88 and NF-κB) is also responsible for melanoma tumor cell migration [39]. These results show that TLRs play principal roles in the progression of melanoma cells, thereby suggesting the beneficial effect of targeting TLR signaling pathways in discovering a novel therapeutic approach for melanoma. It has been reported that TLRs are also involved in cancer recurrence and metastasis [65]. Tumor resection is a choice treatment; however, 30% of patients with grade III CRC and 10% of patients with grade I/II suffer from recurrence 5 years after curative surgery [82]. It has been found that surgical resection can induce local recurrence or distant metastasis [83]. Recently, it has been suggested that systemic inflammation and postoperative infection are associated with CRC recurrence [84]. TLR4 has been found to be highly expressed in patients with liver metastasis and poor clinical outcome [85]. Upon infection, LPS-induced upregulation of TLR4 leads to physical interaction of PI3K with MyD88, leading to phosphorylation of Akt and, therefore, β1 integrin activation, which is the main subunit for collagen binding. LPS-stimulated TLR4 and β1 integrin are responsible for endothelial adhesion by enhancing cancer cell's binding mostly to type I/IV collagen and less to fibronectin and laminin [86]. Additionally, TLR4-mediated signaling promotes hepatic involvement and liver metastasis [87]. Another study in murine models emphasized the role of TLR4/MYD88-driven neutrophilic inflammation initiated by HMGB1. This study showed UV irradiation not only causes tumor-initiating genomic alterations in melanocytes but also promotes their perivascular expansion and metastatic dissemination release from UV-damaged keratinocytes using TLR4/MYD88 pathway [88].

Although few studies have found that TLR4-induced cascade plays proliferative and anti-apoptotic roles in cancer cells, leading to cancer metastasis [89], the same results were not obtained in other studies [65]. This LPS-induced signaling suggests a novel therapeutic target for preventing recurrence or metastasis in patients who were treated by curative resection of colorectal cancer. Three targeting approaches such as TLR4 targeting by eritoran, PI3K inhibition by PI 103, and β1 integrin functional blockage by anti-β1 integrin antibody have been suggested. Since PI3K and β1 integrin play important roles in several normal processes and also LPS-induced TLR4 signaling-mediated events in cancer cells, TLR4 targeting strategy seems to be a better therapeutic approach in patients with CRC [62]. Thus, targeting of TLR4 signaling pathway can be beneficial for patients both with and without postoperative infection.

Even though TLR3 upregulation has proven to be beneficial for prostate cancer treatment, certain TLRs, such as TLR9, should be downregulated because of its boosting effects on cancer progression and invasiveness [90]. Thus, manipulation of TLR pathways should be performed meticulously in order to prevent improper interactions.

16.10 Concluding Remarks

Several studies have provided convincing evidences that TLRs play crucial roles in human cancers. The upregulation of some TLRs leads to tumor progression and therefore increasing of tumor metastasis. On the other hand, certain TLRs inhibit proliferative signaling pathways, leading to tumor regression. Interestingly, TLRs play critical roles in the regulation of tumor cell apoptosis and resistance to chemotherapy, indicating the importance of precise regulation of TLR signaling pathways. Since various TLRs promote contrary effects, their pathways should either be targeted or triggered based on tumor cell type and TLRs expressed. These facts highlight the key point that

TLR functions like a double-edged sword. Thus, TLR expression should be regulated meticulously to bring promising therapeutic possibilities for patients suffering from cancers.

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Recent Advances in the Use of NK Cells Against Cancer

17

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Contents

17.1 Introduction

In the recent past, cancer immunotherapy was focused on adaptive immune cells such as $CD8^+$ T cells and their antitumor cytotoxic capabilities. More recently, due to increased understanding of the biology and function of innate immune cells in tumors as well as technical advances, natural killer (NK) cells have emerged as an exciting new option for targeting tumor cells. In this chapter,

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we will introduce important facts about NK cells that are required in order to understand their function in the tumor microenvironment, and we will then proceed to recent clinical studies utilizing NK cells to fight cancer. Cancer immunotherapy using NK cells is progressing rapidly, and initial results, both preclinical and clinical, are very promising.

17.2 NK Cell Basics

NK cells are lymphocytes of the innate immune system, well known for their role in immunosurveillance and defense against virally infected or malignant cells. NK cells complement T cell immunity in their ability to recognize transformed cells without prior sensitization [1]. Human NK cells can be defined by their expression of the cell surface marker CD56. $CD56^{bright}$ NK cells are referred to as the immunoregulatory subset and precede the $CD56^{dim}$ subset in maturity [2, 3]. The $CD56^{dim}$ population represents the majority of NK cells in peripheral blood (90%), and this subset is highly cytotoxic. Overall, NK cells make up 10–15% of peripheral blood mononuclear cells (PBMCs) in the circulation [4]. From the circulation, they are able to extravasate into inflammatory peripheral sites containing malignant cells.

17.2.1 How Do NK Cells Become Activated to Kill?

Once in contact with malignant cells, NK cells can be activated to kill tumor cells through several different mechanisms. Cytokine activation of NK cells requires priming from factors such as interleukin-15 (IL-15), an important cytokine in the survival, development, and activation of NK cells [5–7]. Several other cytokines are also known to activate NK cells including IL-2, IL-12, and IL-18 [8, 9]. In addition to cytokines, NK cell activation is regulated by the expression of activating or inhibitory receptors present on the NK cell's surface. Whether or not an NK cell kills its target is determined by the balance of these receptors and the density of their corresponding ligands. NK cells kill target cells which lack inhibitory ligands, such as MHC class I molecules, on their cell surface. In this way, it is ensured that NK cells do not harm healthy cells which express MHC I but only those in which MHC I has been downregulated [10]. In humans, the two main groups of inhibitory receptors include the killer immunoglobulin receptors (KIRs), which bind to HLA class I, and CD94-NKG2A/B, which recognizes HLA-E [11]. The loss of a single MHC class I allele can lead to the induction of NK cell lysis of tumor cell targets, a process which is known as "missing self" NK cell activation [12]. Unlike what was initially believed, NK cells are capable of overcoming the inhibitory signals delivered by MHC class I molecules by recognizing activating ligands upregulated on target cells. In general, activating ligands are not expressed on untransformed cells to prevent autoimmunity. However, when cells become transformed, stress caused by DNA damage can upregulate activating ligands, causing the cell to become a target for NK cell destruction [13]. This type of NK cell activation is known as "stress-induced self" activation [12]. A well-known example of an NK cell activating receptor is NKG2D. The ligands for NKG2D, which include MHC class I polypeptide-related sequence A and B (MICA and MICB), are stress-inducible proteins [12]. The DNA damage response, which occurs during tumorigenesis, causes the upregulation of these ligands, relaying signals to the NK cell to cause tumor cell destruction. Another important group of NK cell activating receptors are the natural cytotoxicity receptors (NCRs). This family includes the receptors NKp44 and NKp46, of which the corresponding ligands on tumor cells have yet to be discovered [12], and NKp30 which recognizes B7-H6 expressed by tumor cells [14].

Upon activation, NK cells are able to kill tumor cells directly through the release of cytotoxic granules containing perforin and granzyme, through antibody-dependent cellular cytotoxicity (ADCC) and death receptor ligands on their surface such as TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand [1]. ADCC is a mechanism which results in the destruction of

antibody-coated cells by NK cells [15]. NK cells express the FCγRIII (also known as CD16) which binds to the Fc portion of IgG on target cells and causes cell lysis. TRAIL and Fas ligand also bind to their corresponding receptors on tumor cells and cause cell death. Activation of NK cells can also cause the release of IFN-γ, a critical cytokine for tumor control. IFN-γ acts indirectly to induce type I immune responses in the surrounding environment as well as directly on cancer cells themselves [11]. The direct mechanism of IFN-γ on cancer cells still remains to be determined.

17.2.2 Why Should NK Cells Be Targeted as Anticancer Agents?

The supporting evidence which demonstrates that NK cells play an important role as anticancer agents comes from both mouse and human research. Using transgenic mouse models that lack NK cells or their activation receptors, it was revealed that these cell types are vital in cancer immunosurveillance [16]. For instance, in a model of spontaneous epithelial and lymphoid malignancy, the absence of the NK cell activating receptor NKG2D resulted in defective tumor surveillance and an increase in tumor growth [17]. The importance of NK cells in early tumorigenesis was also shown in a Her2/neu transgenic mouse model generated on a perforin-deficient background [18]. In this model, NK cells and perforin reduced the onset and number of mammary tumors growing in the Her2/neu model.

In humans, the importance of NK cells in tumor surveillance is mostly derived from correlative studies [9]. For instance, in an 11-year follow-up study, it was found that low NK cell cytotoxicity in peripheral blood lymphocytes correlated with an increase in cancer risk [19]. In addition, the presence of NK cells within several different cancers, including squamous cell lung cancer, gastric cancer, and colorectal cancer, has been shown to be a positive prognostic factor for these patients [20–22]. It has also been found that not only can NK cells kill many human cancer cell lines, they are also capable of killing human melanoma cells that have the characteristics of cancer stem cells [23]. From these studies, it is clear that there is a correlation between the presence of NK cells in a tumor and a positive clinical benefit for cancer patients and that NK cells have the potential to kill parts of tumors resistant to other therapies. However, it has also become evident that not only is the presence of NK cells important but their phenotype and functional status is equally significant to net clinical outcome.

17.3 Challenges Involved in Targeting NK Cells

The importance of NK cells in controlling cancer growth has been clearly defined. However, scientists face many challenges when targeting NK cells in the fight against cancer, because tumors develop a slew of different strategies to avoid NK cell attack. Some of these challenges include low NK cell numbers and altered homing into malignant tissues as well as low NK cell activity in cancer patients. Despite the many challenges involved in targeting NK cells to efficiently kill tumor cells, novel immunotherapeutic strategies which may overcome these obstacles are under investigation.

17.3.1 How Many NK Cells Are in Cancer Patients and Tumors?

A major challenge in the study of intratumoral NK cells has been that very limited numbers of NK cells can be detected and extracted within established tumors [24]. This is consistent with research that has demonstrated that NK cells are decreased in a variety of different cancer patients including head and neck cancer, breast cancer, and chronic myelogenous leukemia [25, 26]. The low numbers of NK cells observed have been linked to a mechanism of spontaneous NK cell apoptosis in the circulation of these patients, particularly in the $CD56^{dim}$ population. $CD56^{dim}$ NK cells are defined as having preferential homing abilities for inflammatory sites; therefore, an

increase in apoptosis in this population would greatly decrease the ability of NK cells to accumulate within tumors and contribute to tumor cell elimination [3]. As the number of NK cells decreases with tumor growth, cytotoxicity and cytokine secretion are reduced as well. In addition, the ability of these NK cells to interact with and activate other innate and adaptive immune cells within the tumor is lost.

In animal studies, tumor growth has been linked to decreased lymphopoiesis, which results in a reduction in overall NK cell numbers [27]. In addition to overall low NK cell numbers, distant tumor growth has been found to have significant effects on NK cell maturation [28]. NK cells from mice challenged with several tumor lineages have been shown to undergo a maturation arrest in the bone marrow leading to a decrease in mature, functional NK cells that can produce IFN-γ in the periphery. In human studies, it has been shown that advanced breast cancer patients have an increased proportion of immature NK cell subsets in their peripheral blood [3]. Similar findings were found in patients with non-small cell lung carcinoma (NSCLC), where a majority of tumor-infiltrating NK cells had a $CD11b^{-}CD27^{-}$ phenotype, indicative of inactive and immature cells [29]. Interestingly, the presence of these immature NK cells had an impact on clinical outcome for NSCLC patients, as the frequency of these cells correlated with increasing tumor stage and size. These studies stress that a deeper understanding of the ability of tumors to alter the NK cell educational process in cancer patients is required. This knowledge will be crucial to effectively utilizing these cells for future immunotherapies.

Low numbers of NK cells in tumor samples from cancer patients can also be attributed to inefficient homing of the NK cells to malignant tissues [30]. This is particularly evident in patients with large solid tumors, where NK cell therapy represents an extraordinary challenge. In these patients, it is very difficult to adoptively transfer or activate enough NK cells to home to one or multiple tumors and impart meaningful effects on tumor growth [15]. There is a greater chance of directing NK cells to malignant tissues in patients with minimal disease or those that have already undergone surgery or chemotherapy to eliminate any residual tumor cells [15]. The goal of any NK cell cancer immunotherapy should involve two parts: to increase the number of NK cells in malignant tissues and to activate them to a sufficient level so that they can suppress tumor growth.

17.3.2 What Is the Functionality of NK Cells in Tumors?

It has also become apparent from clinical evidence that the activity of NK cells from cancer patients is greatly reduced. There are multiple mechanisms in place which fully activate NK cells toward tumor cell destruction. In addition to recognizing cells which lack MHC class I, NK cells require multiple stimulatory signals to achieve maximal response. These include the co-activation of various activating receptors present on NK cells with their corresponding ligands on the surface of tumor cells [15]. However, NK cells from human tumors have a reduction in the expression of activating receptors. Instead, these altered NK cells have an increase in the expression of inhibitory receptors—known to reduce NK cell activity. For instance, the progression of human breast cancer has been associated with a reduction in the function of tumor-infiltrating NK cells in comparison to peripheral blood NK cells [24]. Tumor-infiltrating NK cells were found to display a decrease in the expression of activating NK cell receptors (such as NKp30, NKG2D, DNAM-1, and CD16) and an increase in inhibitory receptors (such as NKG2A). Importantly, the NK cells displaying this altered phenotype had reduced cytotoxic capabilities. This altered NK cell phenotype has also been described in patients with NSCLC, where the local tumor microenvironment drastically impairs the ability of NK cells to degranulate and produce IFN-γ, rendering them less tumoricidal and indirectly supportive to cancer growth [31]. Similarly, in another study on NSCLC, the majority of NK cells infiltrating the tumor displayed a $CD56^{bright}$ phenotype and were less capable of tumor cell

killing compared to peripheral blood or normal lung tissue NK cells [32]. Defective expression of activating receptors has also been a hallmark of metastatic melanoma [33] and acute myeloid leukemia (AML) [34], suggesting that this altered phenotype is a common feature of the antitumor immune response. If novel NK cell immunotherapies are to achieve clinical responses in patients, they have to find a way to increase the expression and maintenance of activating receptors on NK cells at the tumor site.

Why is it that when NK cells arrive at the tumor site, they lose their activity? Like all other immune cells, NK cells can change their characteristics based on the factors present within their environment. Within human tumors, NK cell inhibition can be mediated by interactions with neoplastic cells, T-regulatory cells, myeloid cells, or stromal cells [35]. Each of these cell types can express or release inhibitory factors, which can have profound effects on NK cell activity. For instance, the immunosuppressive cytokine TGF-β has been found to inhibit the expression of activating receptors NKp30 and NKG2D on human NK cells, thereby decreasing their killing ability [36]. TGF-β levels are often found to be elevated in cancer patients, including lung and colorectal cancer patients, and this is associated with a weakened NK cell immune response [37]. It was previously found that an inverse correlation exists between NK cell activation and T-regulatory cell expansion in tumor-bearing patients [38]. These findings were explained by a mechanism linked to the expression of membrane-bound TGF-β on T-regulatory cells causing direct inhibition of NK cell effector functions and NKG2D expression. These data suggest that minimizing T-regulatory cell numbers or the levels of TGF-β in the tumor could constitute a novel way to activate NK cells. PGE_2, a small lipid molecule, has also been found to modulate NK cell antitumor responses. It has been demonstrated that PGE_2 directly suppresses cytotoxicity and IFN-γ production by human NK cells [39]. Furthermore, the tryptophan catabolite, L-kynurenine, generated by the enzyme indoleamine 2,3-dioxygenase (IDO) has immunomodulatory properties which can have drastic effects on NK cells. L-Kynurenine can interfere with the cytokine-induced upregulation of NKp46 and NKG2D, thereby modulating NK cell cytotoxic capacity [40].

In addition to being suppressed by factors within their environment, NK cells themselves can also upregulate immunoregulatory molecules such as programmed cell death-1 (PD-1). In a human study, it was found that NK cells from multiple myeloma (MM) patients expressed increased levels of PD-1 compared to healthy donor NK cells [41]. The direct interaction between PD-1 on NK cells and its corresponding ligand PD-L1 on tumor cells resulted in reduced NK cell function against MM tumor targets [41]. These examples allude to the fact that the most promising therapeutic approaches will involve combination therapies which include the activation of endogenous or adoptively transferred NK cells with removal of the suppressive signals that inhibit them.

As there is abundant evidence of an altered intratumoral NK cell state, it was hypothesized that these altered NK cells induce a unique gene expression signature distinct from NK cells found in healthy tissues. To examine this idea, researchers flow sorted NK cells isolated from non-tumoral and tumoral lung tissues from NSCLC patients and used microarray analysis to determine gene expression changes [42]. It was found that intratumoral NK cells have a unique transcriptional signature induced by the tumor microenvironment. This transcriptional signature suggests that NK cells which initially arrive at the tumor site become activated and then eventually exhausted after tumor cell recognition. In addition to an altered gene expression state, new evidence is arising which promotes the idea that NK cells are not only nonfunctional within tumors but that they might be able to support tumor growth through the release of pro-angiogenic factors. Tumors from patients with NSCLC were isolated and analyzed for their expression of pro-angiogenic factors [43]. Flow cytometric analysis of NK cells from these tumors revealed that these cells produced vascular endothelial growth factors (VEGF), placental growth factor (PlGF), and interleukin-8 (IL-8). Induction of pro-angiogenic factors was mediated by TGF-β,

as exposure to the immunosuppressive cytokine caused upregulation of VEGF and PlGF in NK cells from healthy subjects. In addition, NK cells from the ascites fluid of ovarian cancer patients were found to have an enriched $CD56^{bright}CD16^{-}$ immunoregulatory and poorly cytotoxic phenotype [44]. This phenotype is similar to that of decidual NK cells, which have functions that support tissue remodeling and angiogenesis [45]. Ascites fluid directly induces this phenotypic change as incubation of healthy donor PBMCs in ascites fluid enhanced this $CD56^{bright}CD16^{-}$ NK cell population and downregulated expression of other NK cell activation receptors [44]. Further research into the pro-angiogenic phenotype of NK cells and the impact they have on tumorigenesis is needed in other cancer types.

17.4 Cancer Immunotherapies Involving NK Cells

As outlined, there is extensive evidence that NK cells are capable of killing tumor cells both in animal models and in human studies. This has led to a high degree of interest in using NK cells as an immunotherapy over the last 20 years. While there have been many disappointing results and challenges, there are also many studies that indicate we are finally gaining enough knowledge about NK cells to design trials with much higher levels of success. Herein, the historical journey of NK cell-related immunotherapy will be outlined followed by the newest and most exciting studies in the field. Since cancer patients lack high numbers of NK cells and possess poorly activated NK cells, a natural idea to remedy this would be to transfer activated NK cells to these patients. One of the largest barriers to successful therapy with NK cells has been the production of large numbers of activated cells. Thus, the technological advances that are and will be extremely important for the area of adoptive cell transfer (autologous and allogeneic) will be discussed. In addition, the role of NK cells in monoclonal antibody (mAb) therapies and the status of systemic cytokine treatments to increase NK cell responses will be addressed.

17.5 Adoptive NK Cell Transfer

17.5.1 How Can We Produce Large Numbers of Activated NK Cells?

The main barrier to performing large clinical trials involving NK cell adoptive transfer has been the ability to produce large numbers of activated NK cells under good manufacturing practice (GMP) conditions. NK cells do not grow easily in culture and it has been difficult to produce large numbers of them. Different sources have been used to grow NK cells including the most common, human PBMCs (patient or donor derived), as well as NK cells derived from umbilical cord blood (UCB) or human stem cells. New knowledge regarding NK cell survival, proliferation, and activation has been employed to expand NK cells to the highest numbers possible while still ensuring that they possess a phenotype capable of killing tumor cells. In addition, advances in technology have allowed the upscaling of production. Multiple studies have been published over the last 10 years. These can be subgrouped into those involving cytokines, feeder cell lines, or artificial antigen-presenting cells (aAPCs).

Cytokines such as IL-2 and IL-15 have long been known to support NK cell proliferation, survival, and/or activation [5–7, 46, 47]. Thus, they were a natural starting point for this technology. Klingemann and Martinson [48] published an early study in which lymphocytes were isolated from PBMCs and underwent CD56 positive selection via magnetic bead technology [48]. Cells were then cultured in the presence of IL-2 or IL-2 + IL-15. While there was expansion during the second week, it was variable and high levels of $CD3^{+}CD56^{+}$ NKT cells were produced. While the cells in the IL-2/IL-15 combination treatment were highly cytotoxic, the NK cells produced were mostly CD16 negative [48]. Another group performed a similar protocol, in which $CD3^{+}$ cells were removed and the remaining cells were cultured overnight with IL-2 [49]. While these initial studies were a good starting point, they were limited by the poor expansion capability of NK cells under these conditions.

Further advancement in the field came with the addition of irradiated feeder cells to the protocols. In the majority of these studies, NK cells were isolated from PBMCs via immunomagnetic bead treatment to deplete $CD3^+$ cells and enrich $CD56^+$ cells. The cells were then subsequently cultured with irradiated feeder cells at a ratio of 1:10 (NK/feeder). In two similar studies, NK cells were purified from PBMCs via this method, and the immune cells that remained after selection were irradiated and cultured with NK cells [50, 51]. In addition, the cytokines IL-2 ± IL-15 and an anti-CD3 mAb (OKT3) were added. After 2–3 weeks, the cells were harvested and had expanded between 117- and 300-fold [50, 51]. The clinical potential of this method was demonstrated in a recent study that utilized patient NK cells to mimic an autologous transplant setting and then used either patient feeder cells or donor feeder cells to stimulate NK cells [51]. Patient NK cells incubated with healthy donor feeder cells were able to expand more and had increased purity (93.8% $CD56^+CD3^-$) [51]. Another variant of this method is the use of allogeneic irradiated feeder cell lines. For example, Berg et al. utilized an irradiated Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (EBV-LCL) as feeder cells to expand NK cells (with the addition of IL-2) [52]. After 28 days of culture, the NK cells expanded 300–1000-fold and had high cytotoxicity [52]. IL-21 is another gamma chain cytokine involved in NK cell proliferation [53]. Recently, Granzin et al. have further enhanced EBV-LCL-based expansion with the addition of IL-21 [54]. They achieved a striking 10^{11}-fold NK cell expansion following 6 weeks of culture which were able to inhibit melanoma tumor growth in a xenograft model.

An alternate feeder cell line that has been used frequently in GMP manufacturing of NK cells is a variant of the K562 cell line, which has been modified to express the membrane-bound form of IL-15 attached to the CD8α receptor and human 41BBL (K562-mbIL15-41BBL) [53–56]. When NK cells from either patients or healthy donors were cultured with irradiated K562-mbIL15-41BBL cells and IL-2, there was rapid expansion of the NK cells (in 7 days, expanded median 21.6-fold). After a final $CD3^+$ depletion, NK cells had high levels of activation and were able to kill tumor cells in vitro and in a xenograft model [55]. While the success of these protocols was impressive, further modifications have been made to improve upon them. Gong et al. modified the K562-mbIL15-41BBL cells to also co-express MICA, an NKG2D-activating ligand [57]. After 24 days of culture with this feeder cell line, the NK cells expanded by 550-fold and had increased activation and cytotoxicity compared to those cultured with the original K562-mbIL15-41BBL cells [57]. Another breakthrough came in an attempt to optimize the signals that NK cells require ex vivo to propagate. In this case, a new K562-based cell line was created, termed an aAPC [58]. Researchers engineered the K562 cell line to express FcγRI, B7-2, and 41BBL and added either mbIL-15, mbIL-21, or both [58]. When the irradiated K562 cell line that included mbIL-21 (K562-mbIL21) was cultured with PBMCs and IL-2 (no selection, 1:2 ratio PBMC/aAPCs) for 21 days, they expanded by 47,967-fold (825-fold expansion with the IL-15 construct) [58]. This level of expansion was higher than ever reported before for NK cells and was attributed to the fact that IL-21 signaling promotes an increase in telomere length and prevents the senescence that NK cells usually reach [58]. Not only were these cells highly cytotoxic, they also had an increased ability to perform ADCC [58]. Others have also used these aAPCs to produce NK cells from human stem cells [59] and ovarian cancer patient ascites fluid [60] and have shown their therapeutic effectiveness against xenograft models of human cancer [61, 62].

Since these aAPC expansion methods hold potential given the high fold expansion and cytotoxicity of the resulting expanded NK cells, methods to sustain or further expand these NK cells in vivo following infusion were needed. To address this, recently a particle-based method of NK cell expansion has been developed which utilizes closed plasma membrane vesicles produced from the plasma membrane of K562-mbIL15-41BBL (PM15) or K562-mbIL21 (PM21) cells [63]. The PM21 particles induced the highest fold expansion ex vivo (over 100,000-

fold expansion) of NK cells from healthy donors by 28 days of culture and were also effective in expanding NK cells from leukemia patients. Furthermore, they were able to induce NK cell expansion in vivo by infusing PM21-preactivated PBMCs along with PM21 particles into immunocompromised NSG mice. These PM21 particles hold potential for clinical application as they can be stored, enabling use as an off-the-shelf product, and negate the need for additional safety measures required with the use of irradiated tumor cell lines. Importantly, they can be infused in vivo along with NK cell treatment to support in vivo NK cell expansion and persistence.

As can be imagined, the ability to grow large-scale cultures of NK cells in a GMP facility is also dependent on practical technologies. The methods currently used to grow NK cells include tissue culture flasks, cell culture bags, and bioreactors. A study attempted to expand NK cells in all three of these conditions and compare the resultant products [64]. Interestingly, the cells grown in the closed system or fully automated bioreactor were more cytotoxic than those grown in flasks and had higher NKp44 levels [64]. This method would be ideal if NK cell therapy becomes increasingly employed, as it is less labor intensive and can produce even higher levels of NK cells in a similar time frame. However, it might not be able to be used in all protocols, as certain NK expansion methods cannot be performed in a closed system.

Another major barrier to the large-scale use of NK cell adoptive therapy has been an inability to utilize frozen NK cells. Several recent reports using the previously mentioned expansion protocols have assessed the viability of these cells. Berg et al. found that expanded NK cells could be frozen and when thawed had decreased activating receptors and cytotoxicity. However, their activity could be restored with IL-2 treatment [52]. Others found that NK cells could be successfully expanded from frozen $CD34^+$ umbilical cord blood samples [65, 66]. Recently, it was reported that NK cells produced via the feeder cell line K562-mbIL15-41BBL or the aAPC K562-mbIL21 method could be frozen, even long term, and still function well when thawed [56, 65, 67]. These reports give hope that certain centers could produce expanded NK cells (either autologous or allogeneic) and ship them to smaller centers, allowing more patients the opportunity to receive these novel treatment options.

17.6 Autologous Transfer of NK Cells

The initial clinical trials involving NK cell transfer were autologous in nature and involved the use of IL-2 both in vivo and in vitro. These trials were based on the observation that IL-2-activated patient NK cells cultured with matched autologous melanoma cell lines demonstrated high cytotoxic activity [68]. In several phase I/II trials, patients were treated with IL-2 and their lymphocytes were subsequently harvested by leukapheresis. Patient lymphocytes were then cultured for several days in vitro with IL-2 before these lymphokine-activated killer (LAK) cells were reinfused back into the patient [69–73]. After LAK cells were infused into the patient, IL-2 was administered again systemically. Examination of the LAK cells revealed that the cells with cytotoxic activity against tumor cells were NK cells, not T cells [69]. These trials took place in patients with advanced colon, breast, lung, ovarian, pancreatic, renal cell, and melanoma cancers and overall had disappointing results [70–73]. In addition, some reported treatment-related deaths due to high-dose IL-2 [71]. A few trials attempted to transfer autologous NK cells generated by IL-2 ex vivo treatment as a post autologous stem cell transplant treatment and found that although it was well tolerated and there was increased NK cytolytic function, there were no real clinical improvements for the patient [74, 75]. In a subsequent trial, patients with metastatic melanoma and renal cell carcinoma (RCC) received autologous transfer of IL-2-activated NK cells after lymphodepletion [76]. In this trial, PBMCs were depleted of CD3 cells and the resultant cells were cultured with irradiated autologous PBMCs as feeder cells, IL-2, and OKT3 (and anti-CD3) for 21 days [76]. The IL-2-activated NK cells achieved high lytic activity in vitro; however, once the cells were transferred to the patients, no clinical responses were observed. In these patients, the expression of NKG2D on the transferred NK cells was lowered and the re-iso-

lated NK cells could not lyse tumor cells in vitro unless they were restimulated with IL-2.

After these disappointing results, the field shifted gears and began to concentrate on allogeneic NK cell adoptive transfer, which will be discussed in the following section. Nevertheless, researchers are still working on novel ways to increase clinical responses after autologous NK cell transfer. As further research was conducted on IL-2, it came to light that perhaps the use of this cytokine decreased the effectiveness of autologous NK cell therapy. While IL-2 activates NK cells, it has also been shown to increase T-regulatory cells in vivo which, as mentioned, can negatively regulate antitumor NK cell responses [77, 78]. In fact, in an animal model of lung cancer, depletion of T-regulatory cells improved the outcome of NK cell adoptive transfer [79]. We will discuss the possibility of other cytokines to support NK cell activation in another section. Thus, researchers have started to employ new methods to expand NK cells, including aAPCs. A preclinical paper was published which utilized the K562-mbIL21 aAPC previously described [58, 67]. Researchers were able to expand NK cells from children with neuroblastoma by 2363 ± 443-fold. These cells expressed high levels of the activating receptors NKG2D and CD16 resulting in greater cytotoxicity against neuroblastoma cells lines as well as in a xenograft model of neuroblastoma [67]. These promising preclinical data instigated a clinical trial of autologous expanded NK cells for neuroblastoma treatment which is currently underway (NCT02573896). Another recent preclinical study demonstrated that NK cells from the peripheral blood of breast cancer patients could be as efficiently expanded with K562-mbIL21 as NK cells from healthy donors [80]. Furthermore, these expanded breast cancer patient NK cells demonstrated potent and comparable cytotoxicity as healthy donor expanded NK cells against triple-negative breast cancer cell lines and autologous primary breast cancer cells and were able to prevent breast cancer engraftment in a xenograft model. In a translational study that used a patient-derived xenograft model of human ovarian cancer, NK cells from the peripheral blood or ascites fluid of ovarian cancer patients showed striking anti-tumour efficacy after K562-mbIL21 expansion, as the expanded NK cells eliminated large macroscopic tumours, improved survival time 3-5 fold, and were as effective against the patient's own tumour in vivo as NK cells expanded from healthy donor blood [61]. These studies suggest that NK cell expansion via K562-mbIL21 may activate NK cells enough to overcome the impaired function and susceptibility to inhibition previously reported with autologous NK cells. If these results can be translated into the clinic, they will provide new hope for the area of autologous NK cell transfer. There will likely be many more clinical studies published in the near future based on this platform.

17.7 Allogeneic Transfer of NK Cells

As mentioned, NK cells are negatively regulated by MHC I expression on target cells (KIR on NK cell and HLA class I allele on target cell). In 2002, Ruggeri et al. published a seminal study that revealed that this fact can be exploited [81]. If NK cells possessing a KIR that recognizes a particular HLA molecule are transferred into a host lacking that HLA allele, they will have increased cytotoxicity against cells lacking that particular HLA allele. This is known as donor vs. recipient NK cell alloreactivity [81]. For instance, 112 leukemia patients received a hematopoietic transplant with either KIR ligand incompatibility or not (from an HLA haplotype-mismatched family donor) [81]. It was found that receiving NK cells from an alloreactive donor increased 5-year event-free survival by 55% over those who received nonalloreactive NK cells in AML [81]. It also simultaneously prevented graft-versus-host disease (GVHD) and decreased rejection [81]. This was a huge development in the field of adoptive NK cell therapy as it could explain some of the failures of autologous NK cell transfer. The next development was described in a non-transplant setting where allogeneic PBMCs were taken from haploidentical related donors, enriched for NK cells, and cultured overnight in IL-2 [49]. These were then infused into 19 poor prognosis AML patients after they underwent a high-dose immunosuppressive regime [49]. Remission was achieved in 5 of 19 patients and

the NK cells expanded in vivo [49]. Success in these early studies led to a plethora of similar clinical trials both in hematological cancers [82–85] and solid tumors [83, 85–87]. While some early studies found success with enriched but not expanded alloreactive NK cells [82, 84], others at the phase II level proved non-beneficial [85].

For solid tumors, one consideration for improving therapeutic efficacy is the route of NK cell administration. For instance, Geller and colleagues had failed to see a clinical effect for IL-2-activated allogeneic NK cells against breast and ovarian cancer following intravenous (IV) NK cell infusion to patients [88]. Given this, they turned to a preclinical xenograft model of ovarian cancer to assess whether administration of NK cells directly into the tumor environment via intraperitoneal (IP) infusion could enhance the antitumor efficacy of NK cells. They found that NK cells persisted via this route of delivery (with supporting IL-2 administration) and were effective in reducing tumor burden [88]. These results instigated an ongoing clinical trial of IP delivery of NK cells for patients with ovarian cancer (NCT02118285) and highlight potential advantages of administering NK cells directly to the tumor site.

There have been several preclinical studies using the newest methods of NK cell expansion (feeder cells lines—irradiated allogeneic PBMCs, K562-mbIL15-41BBL, K562-mbIL21, the additive OKT3) and the testing of their efficacy in various solid tumor xenograft models [62, 89–93]. For example, NK cells were transferred after their expansion with K562-mbIL15-41BBL into a xenograft model of myeloma. These NK cells were found to have high levels of activating receptors (NKG2D) and inhibited tumor growth and were found to still proliferate after a month in the tumor (with IL-2 systemic treatments) [90]. This study indicates that NK cells can persist in the host and remain active. One benefit of using aAPC-expanded NK cells is that the generation of large numbers of NK cells via expansion is efficient enough to enable repeated administrations of therapeutic NK cell doses which could further improve the therapeutic effect. Indeed, studies by Poznanski et al. and Hermanson et al. administered repeated infusions of K562-mbIL21 expanded NK cells in xenograft models of human ovarian cancer and found that the expanded NK cells reduced tumor burden and improved survival of the mice [61, 62]. Collectively, the results indicate that generating large numbers of activated NK cells with the latest techniques may be very useful and efficacious in NK cell adoptive transfers.

As a result of these promising preclinical results, currently clinical trials are underway to assess the efficacy of the adoptive transfer of aAPC-expanded NK cells, particularly for the treatment of hematologic malignancies (NCT02123836, NCT01904136). Preliminary results from one trial (NCT01904136) which administered three doses of K562-mbIL21 expanded NK cells following HaploSCT for patients with myeloid malignancies indicate that high doses of expanded NK cells (up to 3×10^8 cells/kg body weight) were safe and therapeutically effective, as expanded NK cells reduced relapse rate and improved survival of patients [94].

17.8 NK Cell Lines for Allogeneic Adoptive Transfer

The development of NK cell lines for adoptive transfer into cancer patients is a highly attractive option for its ease of use and its ability to expand NK cells to high numbers. The most established NK cell line used thus far has been the NK-92 line, which was established from a 50-year-old male with non-Hodgkin lymphoma [95]. This cell line is dependent on IL-2 for growth and is cytotoxic against tumor cell lines, primary tumor cells, and xenograft tumor models [95, 96]. The cytotoxicity can be attributed to the lack of inhibitory KIRs on these NK cells [97]. This cell line has been approved for use in clinical trials, and a GMP method is available which can expand these cells by 200-fold in 2 weeks [97, 98]. In a phase I trial conducted on 12 patients with refractory RCC and melanoma, escalating doses of NK cells from 1×10^8 to $3 \times 10^9/m^2$ were administered [90]. There was only mild toxicity at the highest dose and some response (one mixed response, one partial response, one survived) [99]. New cell lines are also being established that have even higher levels of cytotoxicity than NK-92 to improve results in clinical trials [100]. Another benefit to an NK cell

line is the ability to manipulate it genetically to improve its performance. Several recent studies have created NK-92 variants, such as a cell line that expresses a chimeric antigen receptor (CAR) which is the scFv fragment of a CD20-specific antibody connected to the CD3ζ chain to signal in the cell [101]. It is able to efficiently kill $CD20^+$ targets normally resistant to NK killing [101]. Similar results have also recently been demonstrated for CD19-specific CAR-modified NK-92 cells [102]. Another NK-92 variant expresses a CAR that targets an antigen overexpressed in neuroblastoma called disialoganglioside [103]. This type of innovative NK cell line may be very useful in the future as the NK cells can be activated through regular mechanisms or via their new receptor. Genetic manipulation is not limited to NK cell lines as several reports have shown that NK cells isolated or expanded ex vivo from PBMCs can also be manipulated to express CARs specific to tumor antigens such as Her2 or CD19 or to express chemokine receptors such as CCR7 to promote migration of the NK cells to the lymph node [104–106]. Strategies targeting chemokine receptors on NK cells may be able to overcome inefficient homing of NK cells to tumors in certain cancer types. While relatively high transduction efficiencies have now been obtained, primary NK cells tend to only transiently express CARs, and thus one outstanding challenge in the field is to maintain CAR expression [107]. As these advances improve results in preclinical models, genetic manipulation may prove to be a powerful tool for NK cell therapies. Currently, clinical trials are underway to assess the adoptive transfer of CD19-directed CAR NK cells derived from various sources, including NK cells expanded from PBMCs via K562-mbIL15-41BBL (NCT01974479), IL-2 activated (NCT01974479), or umbilical cord blood derived (NCT03056339), for the treatment of B cell malignancies.

17.9 NK Cells, ADCC, and mAb Therapy

Multiple mAbs to tumor antigens have been approved for use in humans and have become a commonly used immunotherapy proven to be quite efficacious. Initially, the methods by which these mAbs worked were a hot area of debate. The mystery was partly solved when an important paper in the field showed that Fc receptors on either monocytes/macrophages, neutrophils, or NK cells were key molecules in the ability of mAbs to function against tumors [108]. Herceptin (trastuzumab (TZB)) was unable to protect from $Her2^+$ breast cancer cells in a xenograft model when Fc receptor γ was knocked out [108]. As mentioned, Fc receptor γ is a key molecule involved in ADCC. Further studies revealed that NK cells express CD16 (FcγRIII), an activating receptor that binds to the Fc region of IgG1 and is able to trigger ADCC [109, 110]. Others have shown that in cancer cell lines resistant to NK cell killing, the addition of a mAb allows NK cells to perform ADCC on resistant tumor cells [111, 112]. After these studies were published, researchers began to view mAb treatment in a new light. They found that in patients that respond to TZB therapy, there are increased levels of NK cell activity and ADCC in comparison to those that do not respond [113]. In addition, they found that in both Rituxan (rituximab (RXB)) and TZB mAB therapy, patients with certain polymorphisms in the FcγRII and FcγRIIIa had a better objective response rate and progression-free survival [114, 115]. This was also related to an increased ability of their PBMCs to kill tumor cell lines via ADCC [115]. One study demonstrated the critical role of NK cells in mAB-mediated cytotoxic activity by demonstrating that depletion of NK cells abrogated the cytotoxic effect of anti-CD20 mAb against chronic lymphocytic B cell leukemia [116]. Clinical trials using the antibody farletuzumab that targets folate receptor alpha to treat ovarian cancer further highlighted the important role of NK cells in mediating the antitumor effects of mAB therapy. While farletuzumab showed promising results in phase I [117] and II [118] clinical trials, no overall significant difference in progression-free survival was observed between farletuzumab and placebo groups in a phase III trial (NCT00849667). However, when patients were stratified based on levels of the biomarker CA-125, which is known to potently inhibit NK cell function, an improvement in progression-free and overall survival was observed in the treatment arm compared to placebo in patients with

lower CA-125 levels [119]. These results suggest that mAb therapies mediate antitumor functions via NK cells, but that they may require combination with additional therapies to overcome a highly immunosuppressive environment. Once the contribution of NK cells and ADCC to mAb therapy success became known, it opened up a whole new area of ways by which we may be able to improve upon its efficacy.

The use of combination strategies to increase ADCC of tumor targets by NK cells has been reviewed recently [120]. Here, we shall discuss several strategies that seem promising. First of all, it has been shown that in cancer patients with advanced disease, NK cell numbers are decreased and their phenotype is altered [3, 25, 27, 33, 34]. One of the most obvious strategies to overcome this issue would be to transfer highly activated allogeneic or autologous NK cells at the same time as mAb therapy in cancer patients. Several preclinical models have indicated that when NK cells are activated, they are capable of killing cancer cells in conjunction with mAb therapy [110, 112]. The expression of CD16 on activated NK cells which are to be used in conjunction with the mAb is important, as not all expanded NK cells will express this molecule [110]. While the tumour microenvironment is known to downregulate CD16 expression on NK cells, expanded NK cells were shown to sustain high expression of CD16 in the ovarian cancer tumour microenvironment, suggesting the potential for combining expanded NK cells with antibody therapy [61]. Preliminary clinical trials are underway to assess the combination of expanded NK cells with mAb therapy (NCT02805829, NCT02030561). The optimal activation of NK cells and the dosing amount and schedule still remain to be determined. When these factors have been worked out, this combination strategy may prove to be an extremely promising therapy.

Another way to improve mAb therapy is to alter the antibody itself. In an interesting clinical trial (NCT01221571), researchers created a tetravalent bispecific antibody (CD30XCD16A) that has two binding sites for the tumor antigen (CD30) and two binding sites for CD16 on NK cells [121]. In their in vitro studies, this antibody was able to restore NK cell cytotoxicity to patient NK cells that were previously nonfunctional [121]. The phase I trial results show that while all patients had progressive disease upon the start of treatment, antibody treatment activated NK cells in the peripheral blood of patients and 8 of 13 patients experienced tumor regression following treatment [122]. Another strategy that can be employed is to improve the binding of the Fc to the activating FcγR by changing the protein backbone of the antibody. Kellner et al. designed a humanized Fc domain-engineered, affinity-matured CD19 antibody (MOR 208) [123]. In vitro, against cell lines and primary isolates of ALL and utilizing in vivo xenograft models, this antibody was more effective at triggering ADCC via NK cells than the original antibody [123]. In an autologous setting, patients with NK cells were capable of killing their own tumor cells when this MOR 208 was utilized [123]. Another possible way to improve mAb therapy is to perform sequential antibody therapy. Kohrt et al. published an interesting study in which they combined TZB mAb with an agonistic antibody to CD137, which was upregulated on NK cells after TZB treatment [124]. This combination decreased tumor growth in a xenotransplant model using patient breast tumors by increasing ADCC of tumor cells [124].

Lastly, cytokines may play a role in enhancing NK cell activation/numbers and increasing the efficacy of mAb therapy. It was shown that peripheral blood NK cells from advanced cancer patients are capable of performing ADCC in the presence of tumor mAb after in vitro activation with either IL-2 or IL-15 [125]. There is no question that cytokines play an indispensable role in the ex vivo activation of NK cells. It is also possible that cytokines may be useful via systemic administration. These would include cytokines such as IL-2, IL-15, IL-18, and IL-21 that have all been found to affect NK cell activation. The usefulness of these cytokines will be discussed in the next section.

17.10 Cytokines and Promoting NK Activation/Stopping Inhibition

IL-2 was the first cytokine approved for use in humans against melanoma and renal cell carcinoma. While it is known to have the ability to

stimulate immune cells such as NK cells and T cells, it has had very disappointing results in the clinic. There have been multiple phase II trials with IL-2. While a small percentage of cancer patients do respond (response rate 14–16%), it induces severe acute vascular leak syndrome in some patients [126–128]. In addition, it has come to light that IL-2 increases T-regulatory cells, which are highly undesirable in any anticancer therapy [77]. There are several other class I gamma chain cytokines that have garnered interest in cancer immunotherapy due to their effects on immune effector cells. These include IL-15 and recently IL-21.

IL-15 was discovered almost 20 years ago and was soon found to be a factor that promotes the survival, proliferation, and activation of NK cells [5–7, 129, 130]. It was very quickly compared to IL-2 and found to be just as good, if not better, at promoting proliferation and cytotoxicity of NK cells [131–133]. Recently, a study comparing IL-2 and IL-15 activated NK cells demonstrated that IL-15 stimulation confers improved and sustained NK cell cytotoxic activity and survival following cytokine withdrawal as compared to IL-2 activated NK cells [134]. In many animal models, IL-15 has been shown to have strong antitumor effects [135–137]. Unlike IL-2, IL-15 does not increase T-regulatory cells [138]. IL-15 appears to have low toxicity in primate studies and is effective at increasing NK cells [138–140]. The wait for these results to be translated into clinical trials was delayed due to the difficulties encountered in generating large amounts of GMP quality IL-15. Recently, one clinical trial demonstrated that IL-15 administration to patients with metastatic renal cell carcinoma or melanoma induced NK cell tissue redistribution and efflux from blood, followed by NK cell hyperproliferation in the blood, and resulted in a reduction in marker lesions in five patients; however, dosing strategies and toxicity reduction still require optimization [141]. Phase I/phase II trials with recombinant IL-15 in combination with NK/lymphocyte cell infusions were initiated (NCT01385423, NCT01369888, NCT01337544); however, two of the trials were terminated early due to complications such as toxicities. Thus, while IL-15 holds promise as a potent antitumor cytokine and NK cell activator, further work needs to be done to optimize its antitumor effects while minimizing toxicity.

IL-21 was discovered as a cytokine that is similar in structure to IL-2 and IL-15 and plays a role in the proliferation and maturation of NK cells [59]. In contrast to IL-2, IL-21 inhibits the differentiation of T-regulatory cells and does not promote vascular leak syndrome [128, 142, 143]. It has been safely used in multiple phase I and phase II studies with metastatic melanoma or renal cell carcinoma [144–146]. It has been shown to have antitumor activity and is able to boost antitumor NK cell responses [144–146]. IL-21 stimulation of expanded NK cells or patient NK cells in the presence of mAb to tumor antigens has been shown to increase NK cell cytolytic activity against tumor cells [147]. Promising preclinical results such as these have led to the use of IL-21 in conjunction with cetuximab (mAb to EGFR) in a recent phase I trial, which had promising results [148]. While the use of cytokines alone is unlikely to produce enough of an effect on immune cells to eliminate tumors, clinical trials are moving in the right direction. The use of cytokines in combination with adoptive transfer of NK cells or the use of mAb protocols will likely increase the effectiveness of these treatments.

While monokine stimulation can activate NK cells, cytokines are capable of synergizing when used in combination to even further activate NK cells. For instance, different combinations of IL-2, IL-12, IL-15, IL-18, and IL-21 rapidly and potently activate NK cells and increase expression of the high affinity IL-2 receptor (CD25) and IFN-γ production [149]. Stimulation with a combination of IL-18 and IL-12 synergistically enhances NK cell IFN-γ production and degranulation as compared to stimulation with each cytokine alone [150]. Furthermore, administration of IL-18 with IL-12 was shown to overcome NK cell anergy in MHC class I-deficient tumors and improve survival of tumor-bearing mice [151]. As a result, combined cytokine stimulation has attracted attention as a strategy to more highly activate NK cells and improve their function in immunosuppressive tumor environments.

Combined stimulation with IL-12/IL-15/IL-18 has had particular success as a strategy to highly activate NK cells for cancer immunotherapy. This cytokine combination induces highly elevated and sustained levels of IFN-γ production and increased cytotoxicity by murine NK cells and freshly isolated or K562-mbIL21 expanded human NK cells [152–156]. IL-12/IL-15/IL-18 pre-activated NK cells also have high expression of CD25 and, as a result, have been shown to persist in vivo with sustained effector function without requiring exogenous IL-2 administration, as they were able to survive off of picomolar concentrations of IL-2 produced by $CD4^+$ T cells [153]. Given the toxic side effects of exogenous IL-2 administration, this ability to persist without exogenous IL-2 administration is attractive for clinical application. Interestingly, IL-12/IL-15/IL-18 pre-activated NK cells also exhibit memory-like properties as they have been shown to persist long term (up to 3 months in mice) and have enhanced responsiveness following restimulation and have thus been termed "cytokine-induced memory-like (CIML) NK cells" [153, 154]. CIML NK cells have had much success in preclinical cancer models. Indeed, Ni and colleagues demonstrated that a combination of CIML NK cells with radiation therapy reduced tumor growth in a mouse lymphoma model, whereas IL-2 or IL-15-activated NK cells failed to demonstrate a therapeutic effect [153]. Furthermore, in a xenograft model of human leukemia, CIML NK cells were significantly better at controlling tumor growth than control (IL-15 pre-activated) NK cells and significantly improved survival [155]. Given this promising preclinical data, a phase I clinical trial was conducted in which CIML NK cells were administered to leukemia patients. In this trial, five of nine patients experienced a clinical response, four of whom experienced complete remissions [155]. These data indicate that synergistic cytokine pre-activation of NK cells may enhance therapeutic effect and overcome the limitations of poor persistence and loss of effector function in vivo of monokine-activated NK cells.

Another way to enhance the activity of NK cells against tumor cells is to block inhibition of the NK cells. As mentioned, a major concern surrounding endogenous NK cells in cancer patients is that tumor cells and their surrounding microenvironment possess strategies to downregulate NK cell activity. Therefore, simultaneously targeting immunosuppressive molecules while attempting to adoptively transfer NK cells or provide mAb therapy would be extremely advantageous for patients. For example, when a KIR on an NK cell comes into contact with a cell expressing an HLA I molecule that it recognizes, it sends an inhibitory message to that NK cell. Researchers have made a human mAb against KIR 2DL1, 2, and 3 (the inhibitory KIRS) [157]. This antibody (1-7F9 or IPH2101) is functional in cell lines and in vivo models, allowing NK cells to kill cells expressing HLA I molecules that would normally prevent their activation [157]. This has proceeded to phase I trials in MM and AML and has proven to be safe and tolerable [158, 159]. Another mAb against PD-1 (CT-011), an inhibitory molecule on NK cells that can be bound by tumor PD-L1/2 has been proven safe in a phase I study and has now entered phase II trials [160]. Lastly, TGF-β is frequently produced in the tumor microenvironment and can negatively regulate NK cell activity [36, 37]. While there have been concerns about using a mAb to TGF-β due to its tumor-promoting and tumor-suppressing abilities, phase I trials have begun with a GC-1008 antibody (fresolimumab) [161]. In 29 malignant melanoma and RCC patients, this antibody was well tolerated [161]. In addition to safety, the trial demonstrated initial evidence for antitumor effects as a partial response or stable disease was observed in some patients [162]. For certain tumor types that express high levels of TGF-β, this may be an important additional therapy when considering NK cell immunotherapy.

17.11 Concluding Remarks

NK cell immunotherapy is on the brink of becoming a major lifesaving therapy. The development of technologies and methods to increase NK cell expansion and activation from both patient- and donor-derived sources has made adoptive therapy, either autologous or allogeneic, a very

attractive option. We are no longer limited by the low numbers of poorly activated NK cells present in cancer patients. In addition, NK cells can be genetically manipulated to make them even more directed toward the tumor with CARs. One challenge that remains is the adoptive transfer of enough NK cells to home to large tumors. While preclinical studies report that adoptively transferred NK cells can persist and are found in the tumor (especially with the new expansion protocols), there is still room for enhancement. The possibility of genetically modifying NK cells to express chemokine receptors may be an interesting addition. Furthermore, delivering NK cells directly to the tumor site may overcome this obstacle. The knowledge we have gained in learning how mAbs work to kill tumors has led to revolutionary ideas in regard to combination therapies—mAb with adoptive NK transfer and cytokines. There is also the option of genetically engineering the mAb to increase its effectiveness. We have, at least in preclinical models, been able to increase the activation of NK cells by blocking inhibitory molecules such as KIRs, PD-1, and TGF-β. These therapies may be able to subvert the effect of the tumor on NK cell deactivation and emerging results from clinical trials indicate potential therapeutic effects. In addition, it also appears as if the freezing of NK cells, either before or after expansion, is no longer a large consideration. This paves the way for certain centers to become specialized in producing GMP quality NK cells that can be administered to patients elsewhere.

While we have made advances in many of the challenges faced in NK cell immunotherapy, there is still the need for basic research on the interactions of NK cells and the tumor microenvironment. One area that still remains unknown is exactly what the NK cell requires to kill tumor cells most effectively. For example, the role of IFN-γ production by NK cells in tumor cell death is still a gray zone. Is it direct, is it indirect, or both? It has been shown that IFN-γ from NK cells is extremely important for their antitumor activity in melanoma lung metastasis, but exactly how it is necessary is unknown [163]. If basic researchers continue to investigate questions such as these, it may lead to knowledge which will help stimulate NK cells in such a way as to produce the most effective antitumor activities. In addition, it may mean that for certain tumor types, NK cells expressing certain activating receptors or death receptors or the ability to produce certain cytokines may be more effective.

Now that there are many tools to promote effective NK cell responses against tumors, the next step will be to figure out which therapeutic combinations will be most effective for certain patients and cancers. It is also possible that in patients with preexisting conditions, some immunotherapies should be avoided. This leads to the idea of a personalized medicinal approach, which will match the benefit a person will receive from a particular therapy with his/her tumor characteristics. For example, if a patient's tumor expresses HER-2 and they have high circulating levels of TGF-β, it may indicate that they should receive TZB, anti-TGF-β antibody, and an infusion of allogeneic NK cells (with IL-15 in vivo). Research should proceed with clinical trials involving various combination therapies. However, to be able to perform personalized medicine, further research needs to be conducted on potential biomarkers which can be used to determine the most effective therapy for an individual. While the hope for NK cell immunotherapy is very high, we still need time to determine the most successful therapeutic combinations and apply them on a large scale. The next 10 years will be very exciting and progressive as the current early findings move their way into practice.

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18 Dendritic Cell Vaccines for Cancer Therapy: Fundamentals and Clinical Trials

Graziela Gorete Romagnoli and Ramon Kaneno

Contents

18.1 Introduction

Mobilization of the immune system for the generation of an effective lymphocyte response against tumor tissue is one of the goals of immunotherapy. It implies the necessity of a coordinated participation of the innate and adaptive immunity mechanisms in order to both trigger an effective response against tumor cells and preserve the host from an autoimmune response. In this aspect, dendritic cells (DC) perform a fundamental role in linking the innate defenses to the specific responsiveness by lymphocytes.

The very first report on dendritic cells was published in 1868 by Paul Langerhans who found branched skin cells by gold staining (called Langerhans cells), whose "dendritic" extensions of plasmatic membrane resembled nervous cells [1]. A century later Prunieras [2] coined the expression "dendritic cells" for the Langerhans cells and proposed that they can capture antigens and are involved in primary defense against pathogens. However, the key contribution toward the morphological, phenotypical, functional identification and classification of dendritic cells

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as a new population of leukocytes was given by Steinman and Cohn, whose seminal reports from 1973 to 1978 are considered the beginning of a new era in this research field [3–7].

There are two main DC populations: the conventional myeloid-derived DC (cDC or mDC) and the plasmacytoid DC (pDC) [8]. These two populations show some differences in their morphology, and in their multifunctional role in the immunosurveillance and regulation of the immune system [9, 10], being usually discriminated through a wide phenotypical variation of surface markers. Conventional DC are identified by the expression of CD11c, CD1a, or CD83 [11, 12] and maturation markers CD80, CD86, and CD40, among others. These cells are subdivided into $CD1c^+$($BDCA1^+$ cells) and $CD141^+$ ($BDCA3^+$) subsets [13, 14]. Maturation/activation of these cells is characterized by the expression of CD80, CD86, CD40, and CCR7 [8]. Co-receptors ICOSL, TNFSF4, and TNFSF8 as well as receptors for IL-2, IL-1, IL12, and IL-18 are also found under maturation [15]. Differently, pDC are featured by the lack of CD11c and positivity for CD303 (BDCA 2 or CLEC4C) AND CD123 (IL-3 receptor). They also express CD68, CD45RA, and ILT3 [16].

DC are the main professional antigen-presenting cells (APC) and perform a continuous surveillance and recognition of the microenvironment of tissues and organs where they are found as immature cells (iDC). In this condition they have a high capacity for capturing soluble and particulate antigens by endocytosis, phagocytosis, and micropinocytocis [3, 11, 17, 18]. The intakes of opsonized and non-opsonized antigens can be mediated by several surface receptors such as FcγR [11], mannose receptor (MR) [19], DC-SIGN [20], type C lectin receptors (DEC-205) [21], as well as *Toll*-like receptors [12, 22]. These antigens are then processed into peptides that are subsequently presented to T lymphocytes in the context of the major histocompatibility complex (MHC) [11, 12, 23].

Immature DC do not have the unique ability for stimulating *naïve* T cells since in this state they do not have the co-stimulatory signals required for T cell activation. Considering that contact between iDC and a specific T cell can drive lymphocytes to cell anergy or induce regulatory cells [24, 25], DC maturation is critical for achieving the balance between effector responsiveness and autotolerance [11].

Pro-inflammatory signals induce not only the migration of iDC to the secondary lymphoid organs but also their maturation and activation. In contrast with iDC, mature DC show reduced endocytic and antigen processing ability while becoming highly efficient presenters of processed antigens for lymphocytes at the T cell sites of lymphoid organs. DC maturation increases the density of CCR7, driving their chemotactic migration toward the T cell populated regions [11, 26].

Maturation is also followed by increased expression of a set of the abovementioned surface markers and by the production of several pro-inflammatory cytokines, such as IL-12, IL-18, TNF-α, IL-23, IL-10, and IFN-α, depending on the stimulating factor [27–29].

Phenotypical and cytokine features of mature DC contribute to the recruitment, interaction, and activation of lymphocytes for the development of an efficient specific response against pathogenic microbes, allergens, and allogeneic tissues [30, 31] and were also evidenced in antitumor response [8]. In fact, it was reported that tumor mass-infiltrating DC are usually suppressed or maintained as iDC in situ. These observations have instigated many authors to try to stimulate infiltrating DC to play a more effective role against tumor cells [32, 33] or to transfer autologous or allogeneic DC after in vitro loading with tumor antigens, thus giving rise to several studies on the feasibility of using DC as therapeutic vaccines for active immunization of cancer patients.

Such studies have benefited from the observation that murine DC can be differentiated in vitro from bone marrow precursors. Further investigations were strongly reinforced by the finding that human DC could be differentiated from peripheral blood monocytes through treatment with adequate cytokine cocktails, usually, a combination of IL-4 and GM-CSF [8, 34–37], while cocktails to promote their maturation largely vary [38–40].

Being the main professional antigen-presenting cells, DC constitutively express both MHC class I and class II antigens on their sur-

face. Classically, endogenous antigens are processed by the cytosolic pathway which resulting peptides are associated with MHC class I molecules, while exogenous antigens are processed by endocytic pathway, providing peptides to be associated with class II molecules. DC have the unique ability to transfer peptides generated by endocytic pathway to the cytosol that is further associated with class I molecules [41]. This feature allows the cross-presentation of exogenous antigens for $CD8^+$ lymphocytes ensuring an effective antigen-presenting function. Then, strategies for improving the expression of these molecules have been proven to enhance the antitumor response triggered by DC vaccines. In this aspect, it was early observed that increasing the expression of MHC class II molecules on DC by transfecting them with MHC class II transactivator genes (CIITA) induces four times more CTL than parental untransfected DC or DC transfected with irrelevant genes [42].

In an early report, even before the flourishing of proposals for DC-based antitumor vaccines (DC vaccine), it was observed that monocyte-derived phagocytic cells could be sensitized by apoptotic bodies obtained by dead tumor cells [43]. Current studies are still using peripheral blood cells to generate human DC and bone marrow cells for murine ones; however, the efficiency of these vaccines appears to be dependent on a number of factors, such as generation of mature DC [44–46], sustained production of IL-12 [47–50], and overcoming of the suppressive microenvironment provided by regulatory T cells [44, 51–54] and myeloid-derived suppressor cells [55–58]. In fact, there is a variety of approaches to generate DC vaccines and it has been observed that each type of tumor has particular features that can hinder the effectiveness of such preparations.

18.2 Strategies for Developing Clinical-Grade DC Vaccines

One of the main issues for the generation of clinical-grade antitumor DC vaccines is choosing the technique for DC loading with tumor antigens. They range from the easier antigen preparation of tumor cell lysates by quick freeze-and-thaw cycles until the generation of tumor-DC hybrid cells or their transfection with tumor nucleic acid. However, there is still no definitive agreement about what strategy is the best.

Results with DC loaded with lysates of tumor cells are controversial since some studies have shown that this approach results in a poor protective role of DC, whereas other authors have successfully prepared DC. Some details can be crucial to the effectiveness of lysate-pulsed DC vaccines. For instance, tumor cell lysate gains properties to stimulate DC maturation (or reduce their suppressive role) whether tumor cells are stressed by heating at 42 °C for 25 min prior to the cell lysate preparation [59, 60]. It is hypothesized that the expression of heat shock proteins by tumor cells can avoid the suppressive effect of cell lysate by increasing DC maturation, an observation corroborated by others [61–63]. Induction of HSPs may be a required feature for increasing the immunogenicity of tumor cells by treatment with chemotherapeutic agents, as well. We observed that low nontoxic concentrations of paclitaxel or doxorubicin are able to alter the expression of a number of genes including the increased expression of HSP70, HSP40, and HSP105 mRNA [60].

Besides heat shock proteins, the main danger-associated molecular patterns (DAMPs or danger signals) ecto-CRT (ecto-calreticulin), HMGB1 (high mobility group box-1), and ATP also increase the immunogenicity of tumor cells and enhance the efficiency of loaded DC [64–66]. Expression of such DAMPs can be efficiently induced by challenging tumor cells with ionizing radiation, photodynamic therapy [67], and chemotherapeutic agents, as well [60, 68, 69].

Cross-priming performed by DC is a phenomenon that can enhance the transference of antigenic peptides through heat shock proteins (HSP), such as gp96 and HSP70 [70–72]. Some HSPs obtained from tumor cells seem to be loaded with tumor antigens and can be internalized by DC through phagocytosis receptors. Such peptides can further be presented in the MHC class I context for inducing a $CD8^+$ response and

subsequent specific attack toward tumor cells [73–76]. Although the use of HSPs seems to represent a good strategy for enhancing the DC loading with tumor antigens [77–79], the clinical application faces some limitations such as the difficulty to construct the HSP-peptide complex and the necessity of a large amount of antigen source for obtaining a sufficient quantity of purified HSPs [80].

Aiming to compare different methods for loading DC with tumor antigens, it was observed that lysate obtained from a homogenate of solid tumor cells showed a poor effect on the ability of DC to stimulate antitumor activity [81]. Stressed tumor cells were obtained by freeze-thaw cycles or by irradiation at 30 Gy, with the irradiation being more useful than a freeze-and-thaw process. However, for these authors, the best method for loading DC was their fusion with live tumor cells. They observed that irradiation of tumor cells at 30 Gy was effective at blocking their proliferative ability and did not affect their usefulness in preparing tumor-DC hybrids. In a phase I study, advanced melanoma patients were vaccinated with $CD34^+$-derived DC pulsed with melanoma peptides. Some of the patients showed peptide-specific DTH response, as well as Melan-A- and gp-100-specific CTL in the peripheral blood [52]. DC loading with tumor-associated proteins or peptides should be preferred in relation to total tumor lysates for the clinical purpose; however, a meta-analysis made by Neller et al. [82] indicate that DC loading with whole tumor lysate shows higher clinical efficacy for diverse cancer types than pulsing them with defined antigens.

One of the limitations for preparing DC vaccine pulsed with tumor lysate is that sometimes the amount of available tumor tissue is not sufficient for repeated applications in the patient. Then, an alternative proposed to overcome this limitation was using tumor nucleic acids in order to induce the expression of tumor antigens by DC themselves. The use of tumor RNA for encoding tumor antigens was first proposed by Nair and Gilboa's group [83, 84], and there is substantial evidence that RNA transfection is a superior method for loading antigens onto DC [85–87]. An important point to consider is that tumor RNA can be amplified through molecular biology techniques so that even a small amount of original RNA can be employed to obtain sufficient material for DC loading. Moreover, both total RNA and selected sequences can be used for DC pulsing to drive the antigen presentation toward a more specific immune response. Finally, RNA shows a safety advantage for DNA, since it cannot be permanently integrated into the host genome.

The strategy of DC transfection with CEA RNA has been used both in murine [88, 89] and human systems [84, 90, 91]. Sakakibara et al. [92] have proposed a method for generating DC vaccines more rapidly by incubating monocytes with GM-CSF and IL-4 for 24 h (Fast DC) transfection with tumor mRNA and cultivation with maturation cocktail for an additional 48 h. The authors observed that mature "fast" DC and standard DC displayed comparable levels of many markers expressed on DC, including HLA-DR, CD83, CD86, CD208, and CCR7. Both were equally able to elicit specific T cell response and IFNγ-secreting T cells, leading to the conclusion that mature "fast" DC are functional antigen-presenting cells (APCs) capable of inducing primary T cell responses.

Vaccination with DC/tumor hybridomas using autologous melanoma or renal carcinoma cells and allogeneic DC is able to change the natural history of the diseases, since it may present stabilization [34] or even regression of metastatic lesions followed by local fibrosis [93]. Whether a patient was unable to fight the tumor development, it is probable that his/her own DC was unable to efficiently process and present relevant tumor antigens to generate specific CTLs. The fact that most tumor antigen peptides are considered to be self-antigens hampers the generation of an effective CTL response. This point of view has led some authors to suggest the use of allogeneic or semi-allogeneic systems to generate DC vaccines. Fusion of allogeneic DC with autologous metastatic colon cancer cells is able to activate both $CD4^+$ and $CD8^+$ T cells in just 24 h, in a higher number than controls, while $CD8^+$ cells are significantly more efficient to lyse target cells [94]. It also can solve some practi-

cal problems such as: (a) it is usually possible to generate a limited sample of autologous DC for vaccination, whereas a higher number of DC could be generated from healthy allogeneic or semi-allogeneic donors; (b) the cellular reactivity triggered by allogeneic or semi-allogeneic DC for allogeneic MHC antigens could facilitate the elimination of escaped tumor variants, as happens in the recipients of semi-allogeneic bone marrow transplantation; and (c) autologous tumor cells are sometimes scarce, which may be overcome by the use of stable tumor cell lines as the source of allogeneic tumor antigens for pulsing autologous DC.

Evaluation of the efficiency of syngeneic, allogeneic, and semi-allogeneic DC has shown that hybrids prepared with allogeneic or semi-allogeneic DC were more effective than syngeneic ones and also worked better as therapeutic vaccines, thus protecting hosts from pulmonary metastasis. Actually, allogeneic and semi-allogeneic DC more effectively induce CTL activity, as well as NK cytotoxicity, and induce higher levels of IFN-γ, by increasing the IFN-γ/IL-10 ratio [95].

The use of exosomes for DC loading has also been proposed by some authors [96–99]. Exosomes are defined as constitutive nanovesicles that can be excited by both tumor and DC displaying a sample of all membrane molecules of original cells [100, 101]. It was observed that vaccination with tumor peptides is more effective when they are carried on exosomes [97, 102]. However, in order to avoid a suppressive effect of tumor exosomes on DC, cancer cells should be submitted to physical stress to increase the expression of danger signals. Regarding this, Dai et al. [61] showed that these nanovesicles can be isolated from heat-stressed tumor cells, culturing them for 43 h at 37 °C, followed by incubation for 1 h at 43 °C. After purification by ultracentrifugation on a discontinuous density sucrose cushion, exosomes were used to induce maturation of monocyte-derived DC. DC loaded with such nanovesicles showed strong upregulation of HLA-DR, CD86, and CD40, as well as the production of IL-12p70 and TNF-α. This technology can be also used for increasing the immunogenicity of tumor cells, since they are able to uptake mature DC exosomes and express themselves, thus activating molecules such as HLA-DR and CD86 [103].

18.3 Routes of Administration

Another fundamental aspect of DC-based immunotherapy is the route of choice for administrating ex vivo prepared DC. Clinical trials have reported various routes of DC administration, aiming to achieve an efficient delivery of cells to the appropriate immune site. DC can be inoculated by intradermal (i.d.), subcutaneous (s.c.), or intranodal (i.n.) routes to deliver loaded cells to regional lymphoid tissues, whereas intravenous (i.v.) methods should be chosen for their systemic distribution. There are also a number of studies showing the feasibility of intratumor (intralesional) inoculation of DC vaccines.

In vivo tracking of s.c.- and i.d.-inoculated DC in multiple myeloma patients revealed their migration to the regional lymph nodes [104]. In fact, the i.d. route seems to be more efficient than s.c. for cell delivery to lymph nodes of patients with metastatic diseases [105]. Although these routes lack DC migration to the spleen, they appear to be more effective for inducing specific antitumor response than the i.v. method [106, 107]. Tracking studies have also revealed that i.v. inoculation promotes DC distribution to the liver, spleen, lungs, and bone marrow. It was observed that DC accumulates in the spleen just 3–24 h after inoculation [106]. Since the majority of relapsing diseases result from metastatic tumor cells, it is reasonable to infer that systemic distribution of DC to the main targets for metastasis (lung, liver, and bone marrow) would be preferred in the protocols developed for preventing them [108–110].

Despite the suppressive microenvironment established at the tumor site, intralesional administration of DC was shown to be feasible, safe, and well tolerated [111–113]. Of course, this choice is limited by the tumor accessibility while Mirvish et al. [114] suggest that in some cases the combination of different routes should be necessary for achieving successful immunization.

Considering the different designs for tumor antigen delivery, as well as the different administration routes, in the next section, we will highlight the clinical experience in relation to selected diseases.

18.4 DC Vaccine for Prostate Cancer

Prostate cancer (PCa) is the second most frequent type of neoplasia worldwide, accounting for more than 903,500 new cases each year [115]. Most patients are successfully treated by prostatectomy or radiotherapy, but about 30% of them relapse [116]. In this aspect, immunotherapeutic approaches become attractive as an alternative treatment, particularly for patients with the advanced disease, since the conventional treatments are merely directed against the symptoms. In addition, its feature of slow progression facilitates the manipulation of the immune system in order to enhance the recognition of tumor antigens.

The first DC vaccine approved by the US Food and Drug Administration (FDA) was called *sipuleucel-T* (Provenge—Dendreon, Seattle, WA, USA) and was developed for castration-resistant metastasis of PCa (for both symptomatic and asymptomatic patients) [117–119]. It is a DC-enriched autologous cell suspension from the patient's own body, prepared by culturing them with a fusion protein called PA2024, which is constituted by the granulocyte-macrophage colony-stimulating factor (GM-CSF) and the prostatic acid phosphatase (PAP), widely expressed by tumor cells. The analysis of disease progression and overall survival in two phase III studies (D9901 and D9902A) found that this vaccine was able to increase the overall survival from 4.5 to 6.7 months [117, 120].

A third phase III trial showed that *sipuleucel-T* improved patient survival time by 4.1 months, with a 22% lower relative risk of death than in control group [121]. Another positive result of these trials is that patients have shown a variable reduction of PSA levels (prostatic specific antigen), the main prognostic marker of this disease [120, 122].

The cellular immune response was also improved by treatment with *sipuleucel-T*, with 73% of patients presenting an adequate lymphoproliferative response, whereas merely 12% of the placebo group showed similar responsiveness [121]. In addition, generation of PAP-specific T lymphocytes was significantly higher in vaccinated patients than in those receiving placebo (27.3% vs. 8.0%), while minimal and well-tolerated collateral effects were also observed [118, 123].

In another successful approach, prostatectomized patients with biochemical relapsing disease were treated with autologous DC pulsed with human recombinant PSA (Dendritophage-rPSA) [124, 125]. Nine out of 24 patients showed 50% reduction in PSA levels whereas 11 others showed less pronounced diminution (6–39%). In addition, 13 patients showed PSA-specific T lymphocyte responsiveness. Six of the patients did not present any sign of circulating tumor cells during a 6-month follow-up. These results are favorable since handling patients with biochemical relapse is still a challenge for oncologists, urologists, and radiotherapists, due to the difficulty of ascertaining the correct location of relapsing disease.

Considering the difficulty of obtaining sufficient amounts of tumor antigens, Fong et al. [125] have proposed the use of xenogeneic murine PAP for loading autologous DC. Six out of 21 patients with metastatic prostate cancer showed stabilization of the disease, with no rise of PSA levels nor the development of PSA-specific T cells.

Preparation of DC/tumor hybrid cells was also tested for prostate cancer. Hybridomas prepared with three different PC cell lines successfully induced an in vitro response in a mixed leukocyte culture by enhancing the IFN-γ production. Results were especially evident when ONYCAP23 and LNCaP were used for fusion (73% and 67%, respectively). Interestingly, the use of ONYCAP23 cells for fusion has induced specific T cell response to different tumor targets [126].

A phase I/II study using DC pulsed with allogeneic tumor cell lysate has demonstrated good tolerance and absence of toxic effects. However, although some patients have presented significant in vitro proliferation of specific antitumor lymphocytes, this approach has not achieved relevant clinical results [127].

18.5 DC Vaccine for Melanoma

The first clinical study on DC vaccines in melanoma patients was published by Nestle et al. [128], who analyzed the efficacy of DC pulsed with HLA-A2-restricted peptides and autologous tumor cell lysates. Two out of six patients presented complete response to vaccination while four of them developed specific DTH response.

Dendritic cells loaded with allogeneic tumor cell lysate and assayed in phase I/II study showed that only 1 out of 15 patients with melanoma treated with autologous iDC pulsed with tumor lysate showed complete remission of metastasis. When the follow-up was discontinued, this patient had maintained an asymptomatic condition for 24 months [129]. In another study, melanoma patients were treated with DC pulsed with melanoma peptides (HLA-A2+) or tumor lysates (HLA-A2−), in association with IL-12, celecoxib, and metronomic doses of cyclophosphamide (phase II study). This association was well tolerated by patients, and 29% of patients with metastasis had the disease stabilized for 7–13.7 months. These patients also showed a higher median overall survival than patients with progressive disease (10.5 vs. 6 months). No significant difference in efficacy was observed between DC loaded with cell lysate or peptides, although no correlation was found between the development of specific immune response and clinical response [130].

Purified gp-100 was also used as tumor-associated antigen for loading DC by varied protocols to prepare vaccines for 97 grade III melanoma patients. Authors observed that 64 of them generated specific T response [131]. Responsiveness to gp-100 can be improved by desialylation of DC surface, since the sialic acid contents inhibit cell maturation/stimulation [132].

The use of autologous tumor RNA for loading DC promotes increased numbers of IFN-γ producing $CD4^+$ cells [133]. This result deserves attention because the strategy of using RNA aims to stimulate $CD8^+$ response; that is, the generation of tumor peptides as a product of transfecting tumor RNA should be processed through the cytosolic machinery. Thus, the effect observed on the activation of $CD4^+$ cells can favor the establishment of memory $CD8^+$ cells [134, 135]. In phase I/II study, Kyte's group showed that administration of RNA-pulsed DC was able to induce a specific DTH reaction and in vitro lymphoproliferative responsiveness as well as IFN-γ production [136].

Cell fusion technology was also applied to melanoma and kidney cancer patients, by fusing autologous tumor cells with allogeneic DC obtained from healthy donors [34, 137]. The measurable clinical response from these patients demonstrated that the disease had been stabilized for a median of 6 months, with no relevant side effects [34].

18.6 DC Vaccine for Colorectal Cancer

DC are constitutive cells of lamina propria and are involved in every local pathological condition. Mechanical disaggregation and enzymatic digestion of intestine specimens of patients with different types of colon disease—including colorectal cancer, Crohn's disease, ulcerative colitis, and nonmalignant, noninflammatory conditions—show that DC correspond to 2% of cells isolated from lamina propria [138]. As to the ability of these cells to stimulate lymphocyte activity, DC-rich suspension induces mixed lymphocyte response (MLR) by T cells. However, tumor-infiltrating DC poorly stimulate T lymphocytes in a primary allogeneic culture (MLR) and are not able to induce significant levels of IL-2 or IFN-γ [138].

The C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin) is involved in the recognition of colorectal cancer cells by DC [139]. Immature DC

within colon tumor tissue expressing DC-SIGN, but not mature DC, interact with tumor cells by binding to Lewis^x and Lewis^y carbohydrate of CEA on in tumor cells. Interestingly, DC-SIGN does not interact with CEA expressed by normal colon epithelium that shows low levels of Lewis epitopes. Therefore, DC interact with human colon SW1116 tumor cells that express aberrantly glycosylated Lewis epitopes (Le^a/Le^b) of CEA and CEA-related cell adhesion molecule 1 (CEACAM1), an interaction that induces the production of immunosuppressive cytokines such as IL-6 and IL-10 [140].

Immunohistochemical analysis of infiltrating cells showed that mature CD83$^+$ DC are found in almost all primary colon carcinoma samples and in some metastases. Heterogeneous infiltration patterns vary from diffuse cells to clustered DC that tend to accumulate around vascular structures and the marginal zone of lymphoid aggregates [141]. Data on maturation markers on DC that infiltrate primary tumors are contradictory. Indeed some authors observed that around 90% of CD83$^+$ cells were double-stained by anti-CD40 or anti-CD86 antibodies, indicating their *in vivo* activation [141], whereas others reported that 64–97% of cells do not express B-7 molecules [142, 143], even after stimulation with TNF-α, IL-4, and GM-CSF [143]. The density of DC at the tumor site was higher in patients with a high proportion of activation markers (CD86 and CD40), suggesting that mature DC can actively migrate to or be activated in the tumor microenvironment under exposure to tumor antigens [141].

Immunization of patients with DC vaccine in phase I/phase II clinical trials showed that the vaccine was effective for 16.7% of patients in the phase I study and for 23% of them in phase II study [84]. Messenger RNA of TAT protein transduction domain and calreticulin increase the immunogenicity of CEA and the effectiveness of mRNA-pulsed human DC. It is interesting that transfection of DC with calreticulin mRNA seems to be associated with activation of CD4$^+$ T cells whereas TAT protein mRNA preferentially stimulates CD8$^+$ cells [144]. Since mRNA represents only up to 5% of total cell RNA, *in vitro* amplification of mRNA was shown to be feasible for producing immunogenically active CEA-encoding mRNA [90].

Instead of using mRNA for known specific antigens such as CEA and Her2/neu, DC transfected with total tumor RNA were able to induce CTL response, while effector cells were able to recognize both the original tumor cell line used for RNA preparation (SW480) and other cell lines, such as HCT-116 (colon cancer) and A498 (kidney cancer) [145]. Supporting this strategy, a clinical trial using total RNA extracted from metastasis tumor cells for pulsing autologous DC, followed by inoculation in the patients (four injections, every 4 weeks), showed an ability to induce specific T response to CEA [146].

We transfected monocyte-derived DC with total RNA of colorectal cancer cells previously submitted to the treatment with low concentrations of 5-fluorouracil and observed that transfection increased the percentage of CD83$^+$, HLA-DR+, CD80$^+$, and CD86$^+$ cells. The functional evaluation showed that they are more efficient than DC transfected with the RNA of non-stressed cells to induce the proliferation of allogeneic lymphocytes and the generation of tumor-specific cytolytic T cells, as demonstrated by IFN-γ production following *in vitro* challenge with target cells [147]. These results were further confirmed *in vivo* in a murine model [69], reinforcing the view that low levels of 5-fluorouracil, as well as paclitaxel and doxorubicin [60], are able to increase the immunogenicity of tumor cells and their ability to prime DC.

Analysis of ten clinical samples of colorectal carcinomas showed that 60% of them overexpressed the antigen EphA2 [148]. Murine DC pulsed with human EphA2 was observed to induce antitumor response against EphA2-transfected MC38 cells. Results have shown that Eph-DC strongly delayed the tumor growth and induced specific CD8$^+$ cells and CD4$^+$ that play a critical role in the antitumor response.

Evaluation of therapeutic DC vaccines prepared with autologous tumor lysates in 58

patients older than 65 years showed that 26 achieved total (1) or partial remission (26) while 30 had stabilization of disease. Among the different kinds of disease, 18 corresponded to colorectal adenoma and decrease of CEA serum levels was found in 24% of the patients, while the expression of other tumor markers as CA199, CA724, alpha-fetoprotein, and neuron-specific enolase decreased in a small number of patients [149].

18.7 DC Vaccine for Nervous Tissue Cancer

The first DC vaccination study in patients with malignant glioma was reported in 2001 by Yu et al. [150], showing increased tumor-specific cytotoxicity in four out of seven patients treated with peptide-pulsed DC. In phase I clinical trial conducted by Sampson et al. [151], 13 patients with glioblastoma (GBM) and 3 with WHO grade III glioma were i.d. inoculated with autologous DC vaccine. Peripheral blood monocyte-derived DC were pulsed with peptide from a mutated region of EGFRvIII conjugated with KLH (keyhole limpet hemocyanin). After three doses, immunization resulted in the restoration of immune responsiveness, which was followed only by grade I or II local reactions at the administration site. The treatment resulted in a median survival time of 110.8 weeks, which was higher than usually observed in patients under other types of therapy such as temozolomide (63.3 weeks, [152]) and carmustine wafers (59.6 weeks, [153]).

Parajuli et al. [154] studied *in vitro* the ability of different DC vaccine strategies to induce T cell response against malignant astrocytomas. Autologous monocyte-derived DC were pulsed with autologous tumor lysate, transfection with total tumor mRNA, or by fusion of DC with tumor cells. The authors concluded that all of the strategies used for pulsing DC efficiently induced T cell cytotoxicity, which was further improved by addition of CD40 ligand [155].

Twelve GBM patients followed in a phase I trial were treated with DC vaccines pulsed with peptides eluted from autologous tumor cells. After three doses, 50% of the patients presented increased immunological response against autologous tumor cells and survival time was higher than historical control data [156].

In a very expressive clinical trial, 56 patients with relapsing GBM were treated with at least three doses of autologous DC loaded with autologous tumor lysate, promoting a 3-month median progression-free survival and a 9.6-month overall survival. Almost 15% of patients presented a 2-year overall survival, although some of them have presented relapse during the follow-up [157]. In a phase II study patients producing increased levels of IFN-γ showed higher overall survival than nonresponders [158].

The polarization of type 1 response can also be achieved by polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC), a type 1 IFN inducer (see more details in the Chapter 11). This product acts on TLR3 [159] to induce the production of IFN-γ, IL-6, TNF-γ, and chemokines including CCL2, CCL5, CCL20, and CXCL10 from astrocyte and microglia [160, 161]. Among the 38 patients with malignant glioma enrolled in the first clinical trial, those inoculated with poly-ICLC showed minimal toxicity associated with the treatment. Sixty-seven percent of the patients exhibited tumor regression or stabilization under radiological evaluation, with a 19-month median survival [162]. The antitumor response was associated with activation of 2′5′-oligoadenylate synthetases, which are antiviral proteins induced by type I IFN [163]. In another study, 30 adult patients with glioblastoma multiforme received poly-ICLC in combination with radiotherapy, thereby demonstrating an advantage in relation to historical studies using radiotherapy alone [164]. Okada's group also analyzed the effect of associating poly-ICLC with DC vaccines generated under IFN-α (called αDC1 by authors), previously shown to be more effective than conventional DC at inducing an antigen-specific CTL response [165].

18.8 Concluding Remarks

Despite their demonstrated effectiveness and promising results, the clinical use of DC vaccines is promising but not definitive. It can be partially explained by the difficulty of establishing a standard effective source of antigens and because several tumor-associated antigens are shared by normal cells. In addition, the increased Treg cells in advanced cancer, as well as other suppressor cells, can hinder the efficacy of a DC vaccine. In fact, even after activation, the autologous DC of breast cancer patients induce higher levels of regulatory T cells (Treg) than DC from healthy donors [166], which determines a low immunogenicity of autologous monocyte-derived DC usually suppressed or induced to tolerance by Treg cytokines.

Reduction of Treg activity by blocking the regulatory molecules CTLA-4 or PD-L1 with monoclonal antibodies can be a good strategy to overcome this obstacle. The FDA reinforced this possibility through its 2011 approval of anti-CTLA-4 (ipilimumab, Yervoy; Bristol-Myer Squibb) for treatment of metastatic advanced melanoma. Treatment was well tolerated by patients and the combination with autologous DC vaccine or peptide-based vaccination was able to develop a significant antitumor response [167, 168].

In conclusion, despite these limitations, promising results are stimulating the search for the best pathways toward improving tumor immunogenicity, the DC antigen-presenting function, the responsiveness of effector cells in the tumor microenvironment, as well as overcoming the tolerogenic or suppressive status of the patient's immune system. Association of different immunotherapeutic approaches or combination of immunotherapy with chemotherapy can open up new avenues for fighting cancer.

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Tumor-Associated Macrophages and Cancer Development

19

Ken-ichi Isobe and Hengyi Xiao

Contents

19.1 Introduction

It has been revealed that tumor-associated macrophages (TAMs) can enhance tumor progression by promoting invasion, migration, and angiogenesis of the tumor [1]. They are often abundantly present in malignant tumors and share multiple features with M2 macrophages, known as alternatively activated anti-inflammatory macrophages with immunosuppressive function [2]. The localization of TAMs in human sample is usually determined by marking the expression of CD163 and CD68 proteins [3–5].

The infiltration of macrophages is largely correlated to poor prognosis of malignant tumors [5–7]. However, various aspects of the accumulation of macrophages in solid tumor tissue remain to be elucidated. One story about this process deems that the repeated inflammation caused by microorganism infection is the major force for the accumulation of macrophages and other inflammatory cells in local, which resultantly affect oncogenesis of tissue cells. Another theory for this process gives priority to the transformed tissue cells, indicating that it is the secretory substances from tumor cells which initiate monocyte migration from blood vessels to tumor site and/or promote the proliferation of tissue macrophages [8]. In this chapter, the correlation between inflammation and cancer will be reviewed at first, and then the information about macrophage ontogeny will be discussed, attempting to summarize the knowledge

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and hints meaningful to further understanding the properties and function of TAMs and helpful to develop tumor therapy.

19.2 Cancer and Inflammation

Pathologists have recognized that tumors often arise at sites with chronic inflammation and that inflammatory cells were always present in biopsied samples from tumors. Galen originally noted this relationship, and Rudolf Virchow reported more evidence in the nineteenth century [1]. Recent molecular and epidemiological studies have led to a general acceptance that inflammation and cancer are correlated [4, 9]. Many triggers of chronic inflammation can increase the risk of cancer development. For example, inflammatory bowel disease is associated with colon cancer, helicobacter pylori with gastric cancer and gastric mucosal lymphoma, and prostatitis with prostate cancer [10].

Two mechanical illustrations have been proposed for the association of inflammation with tumor development. One emphasizes the activation of oncogenes (intrinsic) and another underlies immune cell infiltration which includes the filtration of TAMs, neutrophils, mast cells, and T cells [11]. Although the main focus of this chapter is the second line of understanding, particularly as to TAM filtration, the first mechanical illustration pointing to the inflammation caused by oncogene activation would be briefly discussed here, as clearing up the concept of the inflammatory process triggered by cancer cells (intrinsic) or by immune cells (extrinsic) is important for our comprehension about the role of TAMs in tumorigenesis. The basic concept about "intrinsic" tumor inflammation says that some oncogenes can activate the production of inflammatory chemokines. One example of these oncogenes is RET, a membrane-type protein tyrosine kinase. It is well known that papillary thyroid carcinoma (PTC) is associated with the rearrangement of RET proto-oncogene to form RET/PTC oncogene, while RET/PTC leads to successive MAPK activation and uncontrolled cell proliferation because of its constitutively activated kinase activity [12]. In addition, when exogenously expressed in primary normal human thyrocytes, RET/PTC1 oncogene can evidently induce the expression of a large set of genes involved in inflammation and tumor invasion, including those encoding chemokines (CCL2, CCL20, CXCL8, and CXCL12), chemokine receptors (CXCR4), cytokines (IL1B, CSF-1, GM-CSF, and G-CSF), matrix-degrading enzymes (metalloproteases and urokinase-type plasminogen activator and its receptor), and adhesion molecules (L-selectin) 13 [8]. These RET-induced chemokines act to recruit neutrophils and monocytes from blood vessels; among the recruited cells, monocytes consequently developed into macrophages in the tumor site [13].

As to the "extrinsic" tumor inflammation, it is proposed that chronic inflammatory cell filtration, including TAM filtration, can influence the proliferation and transformation of tissue cells [11]. Macrophages express innate immune receptors called pattern recognition receptors (PRRs), which inspect infection by recognizing conserved microbial features common to various classes of microbes detected [14, 15]. In addition, toll-like receptors (TLRs) on macrophages target a range of microbial ligands, including lipopolysaccharide (for TLR4), lipoproteins (for TLR2), flagellin (for TLR5), unmethylated CpG motifs in DNA (for TLR9), double-stranded RNA (for TLR3), and single-stranded RNA (for TLR7 and TLR8) [16, 17]. The first proof that chronic inflammation induces tumorigenesis comes from the studies for colitis-induced colonic cancer. In the intestine where plenty of bacteria exist, LPS of gram-negative bacteria binds to TLR4 on the surface of immune cells, leading to the activation of NF-κB signaling, a key player in inflammatory processes [18, 19]. Canonical NF-κB pathway acts through the activation of I-κB kinase (IKK) complex, the phosphorylation of I-κBs by IKKβ, the ubiquitin-dependent degradation of I-κBs/p50, and the entrance of NF-κB (p50/p65 or c-rel/p65) dimers to the nucleus [20–22]. On the other hand, alternative NF-κB pathway cascades through IKKα-dependent phosphorylation and cleavage of p100/NFκB2, followed by the formation and nuclear entrance of p52/RelB heterodi-

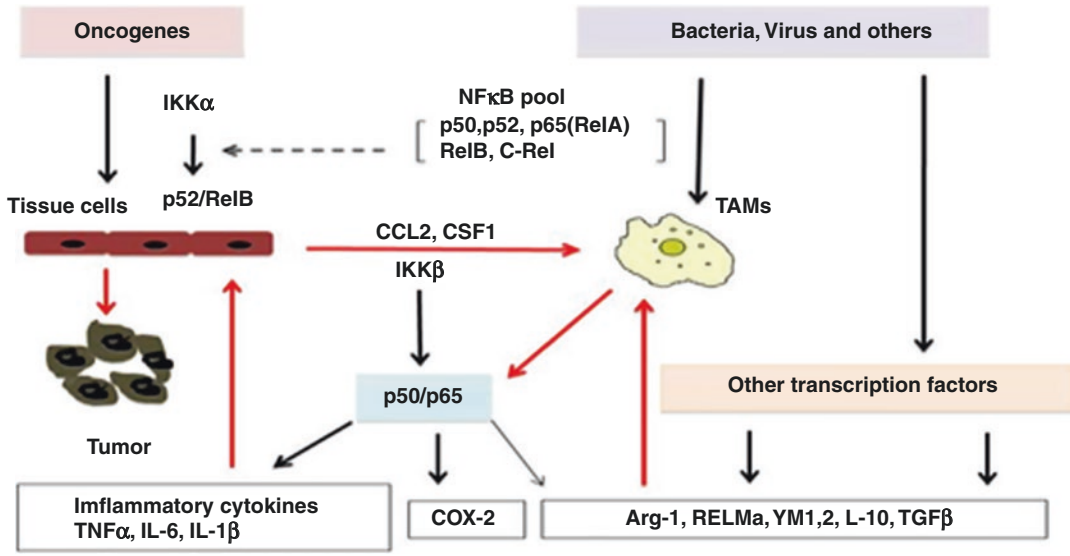

Fig. 19.1 Two mechanisms proposed to explain the association between TAMs and tumorigenesis. (a) A large set of chemokines (CCL2 and others) and cytokines (G-CSF and so on) secreted by tumor cells can promote the recruitment of monocytes in local region and then educate these filtrated monocytes to become TAMs in the location. (b) The inflammatory cytokines produced by TAMs can influence the proliferation of tumor cells. When the factors produced by M2-like TAMs are preponderated, tumor proliferation increases, while the factors produced by M1-like TAMs (reeducated TAMs) are inhibitory for tumor proliferation

mer [23]. In a colitis-associated cancer model, Greten et al. found that deletion of IKKβ in intestinal epithelial cells induced a dramatic decrease in tumor incidence without affecting tumor size; instead, deletion of IKKβ in myeloid cells resulted in a significant decrease in tumor size. They reported that IKKβ depletion in myeloid cells diminished the expression of proinflammatory cytokines which serve as tumor growth factors in this model. They also showed that the oral administration of dextran sodium sulfate disrupted the intestinal endothelial lining, together with the activation of lamina propria macrophages caused by enteric bacteria in the gut. Importantly, they found these activated cells hold active NF-κB pathway and triggered release of inflammatory mediators known to support tumorigenesis. These tumor-promoting inflammatory mediators include COX-2-derived PGE2 and IL-6 [24]. Similar findings were reported in another inflammatory system related to liver cancer [25]. In contrast to inflammatory cytokines, NF-κB were also found to activate the expression of other genes playing roles for tumorigenesis, such as the genes encoding adhesion molecules, enzymes for prostaglandin synthesis (such as COX2), inducible nitric oxide synthase (iNOS), and angiogenic factors. Noteworthy, although noncanonical NF-κB signaling has been shown to be involved in colon inflammation and tumorigenesis, its contribution to tumorigenesis is mainly dependent upon intrinsic mechanism but peripherally upon immune cells (Fig. 19.1) [26].

19.3 Development of Myeloid Lineage Cells Including Macrophages

Tissue macrophages are divided into two types; nonetheless, some overlap exists in surface marker expression between these two types of macrophage [27]. M1 macrophages (classically activated macrophages or inflammatory macro-

phages) act essentially to defend the host from a variety of bacteria, protozoa, and viruses and have roles in antitumor immunity. On the other hand, M2 macrophages (alternatively activated macrophages) exert anti-inflammatory properties and can promote wound healing [28]. From the view of functional features, TAMs are overtly similar to M2 macrophages. Tissue macrophages in adults are usually believed to be recruited from monocytes in blood vessels, while monocytes are derived from hematopoietic stem cells (HSCs) in bone marrow (BM). Two types of monocytes have been classified. LY6C^{hi} monocytes (inflammatory monocytes) expressing CCR2 are recruited to acute inflammatory tissues and become M1 macrophages there [29], whereas LY6C^{low} monocytes (patrolling monocytes) expressing CX3Cl1 are recruited to and become M2 macrophages in tissues usually with chronic inflammation [30]. Recently, the previously believed notion that the origin of adult macrophages stemmed from HSCs in BM has been challenged, since it is reported that macrophages impositioned vested in the yolk sac (YS) from day 8 (E8) in murine embryo [31], whereas definitive HSCs appeared in the hematogenic endothelium of the aorta-gonado-mesonephros region at E10.5 [32–34] and then migrated to the fetal liver [35]. As shown by Schulz et al., YS-derived F4/80 bright macrophages repopulate in adult tissues and turn to liver Kupffer cells, epidermal Langerhans cells, and brain microglia-independent HSCs [36]. Why do macrophages exist during fetal development in limited organs but in almost all adult tissues is an open question. A possible pathway through which macrophages play their role in development is through guiding morphogenesis [37]. A well-studied example is the mammary gland. Mammalian mammary ducts develop multilaminate bulbous termini known as terminal end buds (TEBs) at puberty and during pregnancy. Macrophages are found within the TEB structure, where they phagocytose apoptotic epithelial cells alone with lumen formation [38, 39]. TAMs may have similar properties but play a role in tumor development instead of tissue development. The vertebrate immune system has evolved in concert with parasites, protozoa, bacteria, and virus infection. A situation faced today is that although the parasite infection has decreased largely for human beings, our immune system against parasites still works actively for allergy reaction, wound healing, and others. Herein, the recent discovery about helminth immunity is briefly narrated. Several kinds of cells participating in helminth immunity should be mentioned ahead; the first cell type which must be pointed is T helper 2 (Th2) cells secreting IL4 in gut or lung when helminth infection occurs. The second kind of cells is gut epithelial Goblet cells, which express IL4Ra, secretory mucus, and produces resistin-like molecule-β (RELMβ), an innate protein with direct anti-helminth activity. The third one is M2 macrophages, which own IL4Ra and produce arginase 1, chitinase 3-like proteins 3 and 4 (also known as YM1 and YM2, respectively), and RELMα. Since high arginase activity of myeloid cells coincides with the transport of extracellular L-arginine into cells, causing a reduction of L-arginine in the microenvironment, this decrease in L-arginine would result in T cell hyporesponsiveness [40]. The same thing happens in TAMs. For example, as reported by Rodriguez et al., a subpopulation of mature tumor-associated myeloid cells express high levels of arginase I in 3LL murine lung carcinoma model, and L-Arg depletion by tumor-associated myeloid cells inhibited antigen-specific proliferation of T cells [41]. Despite the high activity of arginase-induced L-Arg depletion, macrophages can convert L-Arg to inducible nitric oxide synthase (iNOS) by other mechanism, which will be discussed later.

Bacterial infection induces macrophage activation, which first recruits neutrophils to the infected site. Neutrophils and macrophages phagocyte the bacteria inside the phagolysosome and kill the bacteria by enzymes inside the lysosome or by reactive oxygen species (ROS) and then produced nitric oxide (NO) radicals. T lymphocytes in regional lymph nodes are stimulated by dendritic cells, followed by the clonal expansion and the migration of these T lymphocytes to infected sites. Among these T cells, Th1 cells produce IFNγ to kill the bacteria inside the

phagocytes; Th17 cells produce IL-17 to recruit more neutrophils to the infected site. However, excessive or continued activities of phagocytes and T cells may induce tissue damages and fibrosis, thereby suppressing tissue regeneration. Early studies showed that macrophages can suppress T cell proliferation by producing NO radicals [42, 43] and indoleamine 2,3-dioxygenase (IDO) [44]. This T cell suppressive function of macrophages is one of TAM characteristics. These macrophages in tumor are specifically called myeloid-derived suppressor cells (MDSCs) [45]. Recently, M2 macrophages have been divided into M2a, M2b, and M2c subgroups according to their inducing stimuli. M2a (induced by exposure to IL-4 and IL-13) and M2b (induced by combined exposure to immune complexes and TLR or IL-1R agonists) exert immunoregulatory functions and drive type II responses, whereas M2c macrophages (induced by IL-10) are more related to the suppression of immune responses and tissue remodeling [46].

19.4 Characteristics of TAMs

Tumor-associated macrophages have been shown to perform a number of different roles in the tumor microenvironment to facilitate tumor progression [37, 47–49], and the density of TAMs in human tumors closely correlates with poor prognosis [5]. TAMs are recruited as monocytes from the bloodstream into tumor tissue. Some chemoattractants produced by both malignant cells and stromal tumor compartments play an important role in this recruitment [50, 51]. For example, stromal- and epithelial cell-produced CSF1 seems the most important chemoattractant working for the recruitment of TAMs to tumor [52], while Csf1 deficiency in macrophages suppressed tumor progression in the mice intestinal cancer model with APC716 mutation [53]. Up to now, various features of TAMs have been identified; however, other features remain to be elucidated. One of these is the close relationship of TAMs and tumor angiogenesis, since TAMs express various angiogenic molecules, including VEGF [54]. Macrophages also promote intestinal cancer by producing TNF, which activates Wnt-catenin pathway essential for tumor progression in intestinal cells [53]. Moreover, TAMs downregulate the expression of major histocompatibility complex class II (MHC II) and their ability of antigen presentation. As for cytokine production, TAMs express COX2-derived prostaglandin E_2, as well as the anti-inflammatory cytokine IL-10 [55]. Murine TAMs express low levels of IL-12 but high levels of M2-specific genes, such as arginase-1 (Arg-1), macrophage galactose-type C-type lectin-2 (Mgl2), Fizz1, and Ym1 [56, 57]. These characteristics are similar to M2 macrophages. However, TAMs express both M1 and M2 markers in certain circumstances, relevant to tumor type and the stage of tumor development. For example, increased expression of inducible nitric oxide (iNOS or NOS2, an enzyme expressed by M1 macrophages) together with elevated levels of Arg-1 (usually expressed by M2 macrophages) was observed in TAMs in CT26 murine colon tumors, Meth A^- sarcoma, and prostate tumors [58, 59]. Meanwhile, TAMs are thought to suppress T cell proliferation or induce regulatory T cells by the expression of IL-10, TGFβ, Arg-1, and prostaglandins [60–63]. These immunosuppressive macrophages are called myeloid-derived suppressor cells (MDSCs). MDSCs are increased in patients with head and neck, breast, non-small-cell lung, and renal cancers [64–66]. Phenotype of murine MDSCs is $CD11b^+$, $Gr\text{-}1^+$, $IL\text{-}4\alpha^+$, and $F4/80^-$.

19.5 "Reeducating" TAMs to Cytotoxic Phenotype

Due to the large population of TAMs existing in many tumors, a therapeutic approach increasing their tumoricidal activity and attempting to activate antitumor immunity would be most appealing. As previously mentioned, NF-κB signaling pathway is important for cancer-related inflammation and malignant progression. Hagemann et al. stated that the infection of TAMs with Adv-IKKβDN to isolated $CD11b^+$ TAMs from ID8 ovarian cancer-bearing mice inhibited NF-κB signaling, and the inactivation of IKKβ in

TAMs also prevented tumor cell invasion through macrophage-mediated tumoricidal activity in vitro. Moreover, they demonstrated that IL-12^{high} IL-10^{low} phenotype of IKKβ-targeted macrophages was associated with decreased expression of arginase-1 and elevated expression of inducible nitric oxide synthase (NOS2). They also showed that adoptive transfer of converted tumor by Adv-IKKβ^{DN} in vivo induced IL-12-mediated increase in NK cells [67]. Another line of evidence revealed that inhibition of COX-2 can prevent breast cancer metastasis. This was recognized based on the fact that the specific inhibitor of COX-2, etodolac, inhibited human M2 macrophage differentiation, as evidenced by the decreased expressions of CD14 and CD163 genes and increased TNFα production. Using a BALB/c breast cancer model, Na et al. found that etodolac significantly reduced lung cancer metastasis, possibly due to the increased expressions of IA/IE and TNFα genes and decreased expressions of M2 macrophage-related genes [68].

19.6 Concluding Remarks

TAMs have been shown to enhance tumor invasion, migration, and angiogenesis by inflammation. Recent progresses to elucidate the molecular mechanisms of the functions of TAMs opened the new ways to treat cancer patients by reeducating TAMs to be tumor inhibitory cells.

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Exosomes: Pros and Cons for Fighting Cancer

20

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Contents

20.1 Introduction

The development and progression of cancer depend on a genetic instability of cells, but also on the interaction of tumor cells with the extracellular matrix compounds and immune cells [1, 2]. Classical cell communication involves a ligand-receptor interaction, being that the ligand can be on the cell surface, free in the extracellular medium, and even in extracellular vesicles (EVs) [3, 4]. EVs have been largely studied on the participation on the intercellular communication due to their ability to deliver bioactive molecules, such as proteins, lipids, miRNAs, mRNAs, and DNA [3–5].

Exosomes (Exo) are the most well-known EVs. They are small lipidic double-layer vesicles, originated from the invagination of late endosomes and raging from 30 to 150 nm [3, 6]. These endosomes are also called multivesicular bodies (MVBs) [3, 6] and fuse with the cell membrane, in order to deliver the intraluminal vesicles to the extracel-

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lular medium, when they become exosomes. Formation of intraluminal vesicles is not an aleatory (random) process of endocytic pathway, being rather coordinated by a group of proteins containing ubiquitin-interaction domains that bind with high avidity to ubiquitinated cargo [3]. This formation can also happen independently of ubiquitination, through ALIX protein, which works as an indirect adaptor to bring transmembrane heparin sulfate proteoglycans into MVBs [3]. Secretion of Exo is also a coordinated process, controlled by GTP proteins, belonging to the Rabs family [6].

Exo were described for the first time in the 1980s as a mechanism that helps to deliver/eliminate transferrin receptors during the maturation of erythrocytes [7, 8]. Therefore, this role in the elimination of intracellular material helps to keep the cell homeostasis. For instance, the elimination of aggregated intracellular proteins, such as TDP-43 throughout Exo by neurons, promotes cell clearance and probably decreases the gravity of clinical signs of neurodegenerative disease amyotrophic lateral sclerosis (ALS) [9].

Almost a decade after the first description of the Exo, some groups showed the evidence that Exo could be associated with intercellular communications during the immune response [10, 11]. Since Exo are considered a mini reflection of the original cells, Exo secreted by tumor cells can be both a rich source of tumor antigens [12–14] and bring suppressive molecules to hinder the immune response and enhance the tumor progression [15–17], while those delivered Exo delivered by antigen-presenting cells bring proteins directly involved in the induction of T cell response, such as the molecules of the major histocompatibility complex (MHC) class I and II and costimulatory molecules [10, 11, 18, 19].

Exo are also found in bodily fluids such as saliva, urine, and serum, leading several authors to show the feasibility of using them as a "liquid biopsy" for cancer diagnosis [20–22].

20.2 Tumor Cell-Derived Exosomes

The role of tumor cell-derived Exo in carcinogenesis is controversial since they can both contribute to an antitumor immune response and to evade this response enhancing angiogenesis and metastasis [23].

Dissemination of tumor cells to secondary sites depends on the formation of pre-metastatic niches, and tumor Exo contribute for this process inducing higher vascular permeability inside the primary tumor and in the secondary site as well [24, 25], facilitating the cell migration and colonization of this new site. It was observed that the integrity of endothelial barrier is broken by interference microRNAs, such as miR-105 found in tumor Exo, since treatment with anti-mi-R105 decreases the tumor volume in animals bearing xenogeneic tumor [25].

In fact, miRNAs seem to have a significant contribution to the role of Exo in promoting tumorigenesis, since those derived from metastatic breast cancer cells express more miRNA than those secreted by nonmetastatic cells [26]. Pre-miRNAs, such as Dirce, are found in Exo and are able to induce proliferation of tumor cells both *in vitro* and *in vivo*. Such a proliferative response can be blocked by antibodies (anti-Dirce) that reduce the tumor size in murine models *in vivo* [26]. The transcriptome of non-transformed MCF-10A cells is also modified by exposition to Exo, inhibiting the expression of tumor suppressor gene PTEN and converting them into tumor cells. In addition, motility and invasive growth are enhanced by Exo-derived metalloproteinases that directly modulate the extracellular matrix [15].

Tumor stromal cells also deliver Exo and are able to transfer miRNA (miR-21, -143 and -378e) to T47D breast cancer cells that gain an invasive feature, increasing the formation of mammospheres and the expression of SNAIL (zinc-finger transcriptional repressor) while reducing the expression of E-cadherin [27]. This phenotype is associated with the epithelial-mesenchymal transition (EMT), a process related to tumor progression. In addition, there is an increased expression of markers associated with cancer stem cells (CSCs) such as oct3/4, Nanog e SOX2 [27]. Maus et al. [28] have demonstrated, for the first time, the presence of extracellular vesicles in afferent lymphatic channels of patients with metastatic melanoma, suggesting their role in the formation of pre-metastatic niches.

Tumor Exo bring tumor-associated antigens as demonstrated in vesicles derived from colorectal cancer cells (CRC) as well as in plasma of metastatic CEA^+ CRC patients [29]. Presence of circulating tumor Exo may have negative consequences since they could be recognized by antitumor therapeutic antibodies. For instance, Exo carrying Her-2 can be recognized by trastuzumab, a humanized anti-Her-2 monoclonal antibody used in the clinic. Such a reaction seems to cause sequestration of antibodies, hindering their antitumor effect [30]. Depletion of tumor Exo can be an alternative to keep specific antitumor antibodies working as observed by depletion of $CD20^+$ Exo that increases the cytotoxic activity of anti-CD20 antibodies against $CD20^+$ lymphoma B cells *in vitro* [31].

Another suppressive mechanism of tumor exosomes is their ability to induce apoptosis of immune cells. Concerning this, it was observed that Fas-ligand (Fas-L) loaded Exo secreted by melanoma cells induce apoptosis of T lymphocytes [32]. In addition to Fas-L, tumor Exo also carry TNF-related apoptosis-inducing ligand (TRAIL), being able to induce apoptosis of autologous $CD8^+$ lymphocytes [29]. Galectin 9 expressed by Exo obtained from plasma of patients with EBV-associated nasopharyngeal carcinoma is able to induce apoptosis of EBV-specific $CD4^+$ T cells by binding with TIM-3 receptor [33].

Subversion of protective response by tumor Exo can affect several defense cells. For instance, dendritic cells (DCs) treated with hypoxia-induced melanoma-derived extracellular vesicles show a reduced expression of markers CD83 and CD86, as well as reduced production of cytokines and chemokines involved in the Th-1 profile [28]. They also hinder the differentiation of DC precursor cells or drive this differentiation into TGF-β producing myeloid-derived suppressor cells (MDSC) [34]. Under the influence of tumor Exo, monocytes increase the expression of the programmed cell death ligand-1 (PD-L1), a potent regulatory molecule [35]. This increased PD-L1 expression on monocytes is due to the transference of Y RNA hY4 (a type of non-codificated miRNA) through the Exo, as happens with plasma vesicles isolated from patients with chronic lymphocytic leukemia (CLL), which happens in a TLR-7-dependent fashion [35]. The suppressive role of tumor Exo can favor the differentiation of both MDSC and regulatory T lymphocytes to keep the control on Th-1 lymphocytes and NK, probably due to the loading of TGF-β by nanovesicles [17, 36–38].

In opposition to this suppressive role, tumor Exo represent a rich source of tumor antigens, being able to trigger an antitumor response. It happens because Exo are incorporated by DC more efficiently than irradiated tumor cells, apoptotic bodies, or tumor cell lysates [13]. Even in patients bearing weakly immunogenic tumors, Exo isolated from the ascitic fluid were shown to carry relevant tumor antigens such as Her2/neu, Mart1, and Hsc70. DC sensitized with said Exo induced the generation of tumor-specific T lymphocytes, increase the production of IFN-γ, and enhance the antitumor cytotoxicity [14].

Besides tumor antigens, chaperones such as HSP70 and HSP90 can also be found in those Exo isolated from ascitic fluid of patients with T cell lymphoma [39]. Immunization of animals with these Exo triggers the generation of tumor-specific T lymphocytes following further challenge with live tumor cells, with a significant proliferation of $CD4^+$ and $CD8^+$ lymphocytes, high levels of IFN-γ production, and enhanced resistance to tumor growth.

In another study it was observed that DCs treated with Exo isolated from patients with glioma, expressing MAGE-1 and HSP70, presented with increase on CD86 and HLA-DR, showing higher effectiveness to induce tumor-specific $CD8^+$ lymphocytes than those sensitized with tumor lysate. In addition, resulting lymphocytes showed higher toxicity than their counterparts [40]. In a murine model, vaccination with DC pulsed with Exo from WEHI3B myeloid leukemia cells improves the survival of tumor-bearing animals, in comparison with the treatment with DC pulsed with WEHI3B lysate [41]. Authors demonstrated that Exo is a rich source of tumor antigens with long-term storage in MHC class II compartment. These Exo induce strong trogocytosis (a kind of intercellular transfer of cell surface proteins and membrane patches) with T lymphocytes that can be the reason for the high proliferation of WEHI3B-specific CD4+ T cells. In agreement with this view, it was reported that co-delivery of tumor derived Exo and

α-galactosylceramide to DC is better than the tumor lysate to induce the proliferation of tumor-specific T cells, against glioblastoma [42].

DCs loaded with Exo of murine leukemia cells have significant prophylactic effect protecting 87% of animals against the development of leukemia [43]. These DCs showed therapeutic effect, delaying the tumor development in 100% of animals.

Clinical studies show that Exo isolated from ascites of cancer patients seem to be useful for inducing antitumor lymphocyte responsiveness. For instance, Exo obtained from ascites of colorectal cancer patients express CEA (carcinoembryonic antigen) and their administration together with GM-CSF (granulocyte-macrophage colony-stimulating factor) induced a delayed hypersensitivity to the Exo. Challenge of CTL infiltrating the delayed-type hypersensitivity (DTH) region with tumor cells showed the presence of specific anti-CEA lymphocytes [12]. This ability to transfer immunogenicity was also observed in Exo obtained from ascites of patients with weakly immunogenic cancers in which tumor markers Her2-neu, Mart1, and Hsc70 were identified [14].

Increasing the immunogenicity of tumor cells is one of the goals for using their Exo for active immunotherapy. According to this, Exo of heat-treated ascites of gastric cancer patients show to be enriched for HSP70 and HSP60, being more effective than non-heated material to increase the expression of CD40, CD80, CD86, and MHC class II on DC [44]. This change is followed by increased functional effectiveness to induce lymphocyte proliferation in mixed lymphocyte reaction as well as the generation of tumor-specific CTL *in vitro*.

These data indicate that feasibility of using tumor-derived Exo seems to be dependent on the expression of danger signals provided by heat shock proteins, while those expressing regulatory signals (e.g., PD-L1 and CTLA-4) or bringing interference micro-RNA are rather associated with the facilitation of tumor growth. Therefore, blocking these regulatory signals and/or enhancing the expression of DAMPS (HSPs, HMGB-1, calreticulin) on tumor Exo, as well as select phenotypically immunogenic vesicles, may be rationale strategies to allow their use to achieve active antitumor immunity. Another strategy proposed by some authors is to use Exo obtained from antigen-presenting cells, as a tool for transferring selected immunogenic signals.

20.3 Exosomes Secreted by Dendritic Cells

Dendritic cells (DCs) are the main antigen-presenting cells, with the singular ability to activate *naïve* T lymphocytes [45]. DC classically present exogenous peptides linked to MHC class II molecules, while endogenously generated peptides are loaded on MHC class I molecules [46]. In addition, these cells are able to cross-present exogenous peptides in association with MHC class I molecules [47, 48].

Such a functional feature and the expression of costimulatory signals (including CD80, CD86, CD40, and ICAM-1) [46] are reflected in their Exo (DC-Exo), making them potential immunomodulatory nanovesicles. In fact, it was observed in P815 murine models of mastocytoma and TS/A spontaneous mouse mammary adenocarcinoma that treatment of tumor-bearing mice with DC-Exo loaded with tumor antigen peptides was able to induce tumor regression by direct activation of cytotoxic T lymphocytes [11]. Although Exo are able to directly activate T lymphocytes, this *in vivo* activity seems to be rather dependent on the incorporation by host DC [49, 50] and is more effective to stimulate primed lymphocytes than *naïve* T cells [51]. In addition, DC sensitization by antigen-loaded Exo seems to be more efficient than their exposition to soluble antigens, since the former induces higher levels of antigen-specific T lymphocyte hybridomas [52].

In another study it was demonstrated that Exo obtained from DC pulsed in vitro with a lysate of glioblastoma cells enriched with chaperones can be incorporated by syngeneic DC to induce a significant *in vitro* and *in vivo* antitumor responsiveness, featured by increased CTL activity, increased systemic production of IL-12 and IFN-γ, and enhanced survival of tumor-bearing mice [53].

DC-Exo are able to activate other effector cells such as natural killer (NKs) cells. For instance, it was observed that they carry the surface ligand for NKG2D, providing the activation of NK cells, as well as their proliferation in an IL-15Ra-dependent fashion, leading to tumor regression [54].

DC-Exo can also be incorporated into different tumor cell lines (SK-BR-3, U87, and K562), altering their phenotype. This Exo incorporation seems to be dependent on the tetraspanin CD9, expressed in tumor cells [55]. Furthermore, challenge with this new phenotype of tumor cell was able to induce IFN-γ production by previously sensitized T lymphocytes [56]. Interestingly, tetraspanins (e.g., CD9, CD63, and CD81) are a constitutive label of Exo [57] that can be relevant for their adhesion on the target cells, since they are involved in cell adhesion and cell stimulation as well as in functional signaling [58, 59].

Bioactive DNA, mRNA, and miRNA inside Exo [34, 60] contribute to their immunomodulatory property. For instance, it was observed that miRNA isolated from DC-Exo suppresses target mRNA of acceptor DCs, indicating that the luminal contents of these nanovesicles can also be transferred to target cells with posttranscriptional implications on their activity [61].

Immature and mature DCs can bind Exo on their surface following their internalization into endocytic vesicles [62]; however mature DCs retain more Exo on their surface [19]. Internalized Exo can be processed and antigens are presented via self MHC [19, 62, 63]. The interaction of Exo with cell membranes can occur through proteins such as integrins and tetraspanins [55, 64–66]. Extracellular cleavage of Exo surface proteins by proteases originate soluble ligands, which can bind to receptors on target cells [64]. Another Exo interaction fashion is cross-dressing, where proteins of Exo surface are transferred to the membrane of target cells, as happens with the MHC/tumor peptide complex [19, 67].

The expression of some molecules on the surface of Exo seems to reinforce their functional role in the immune response. In this aspect, LFA-1 integrins (CD11a/CD18) can work as receptors for these nanovesicles, since LFA-1 on murine $CD8^+$ DCs interact with ICAM-1 on Exo, promoting their uptake. These Exo-loaded DCs further increase the expression of activation marker CD69 by lymphocytes [68]. In addition, ICAM-1 expressed by DC's Exo interact with LFA-1 on activated T lymphocytes, facilitating the transference of Exo MHC class II to the lymphocytes [69]. The interaction of C-type lectin with mannose-rich C-type lectin receptor is also involved in the incorporation of Exo by DCs, favoring the development of antitumor immune response in a murine model [70].

As previously described in preclinical studies, tumor antigen-loaded DCs show high potential to induce both *in vitro* and *in vivo* antitumor response [11, 56, 71]. In the first clinical trial involving patients with stage III/IV melanoma, it was observed the feasibility and safety of Exo of autologous DC pulsed with MAGE-3 peptide [72]. Two out of 15 patients have the disease stabilized, one of them for 24 months. Two other patients showed partial or minor responses. Increased NK activity was observed in 7 out of 13 patients, including that one who experienced partial clinical response to the treatment. Despite this effect on NK cells, no generation of specific anti-MAGE cytotoxic lymphocytes was observed.

A second clinical study was developed with patients with non-small cell lung cancer (NSCLC) treated with Exo of autologous DC pulsed with MAGE peptides [73]. In this study six out nine vaccinated patients have the disease stabilized with no evidence of toxic effects. Their antitumor specific responsiveness was checked by DTH for MAGE peptides, and three out of nine patients showed the positive response.

In these two studies, DCs were pulsed with tumor peptides alone with no additional activation signal. DCs stimulated with tumor peptides and IFN-γ provide highly immunogenic Exo able to directly induce the generation of effector T lymphocytes *in vitro* and *in vivo* in the experimental model [74]. In a third clinical trial with NSCLC patients, the administration of Exo obtained from autologous DCs pulsed with tumor peptide and IFN-γ was well tolerated by 82% of patients with no signals of toxicity and overall median

survival of 15 months [75]. After the administration of four doses, a longer progression-free survival correlates with increased NK activity that showed to be dependent on NKp30 that links to BAG6 expressed by Exo.

20.4 Diagnostic Application of Exo

Although the presence of some oncoproteins can sometimes hinder the therapeutic usage of Exo, this feature enables their use for diagnostic purposes, since their bilipid membrane preserves their rich proteic and genetic content from degradation by extracellular enzymes. Exo in biological fluids can be isolated to be used as a noninvasive liquid biopsy. Melo et al. [22] elegantly demonstrated that Exo isolated from serum of patients with pancreatic cancer precursor lesions (PCPL) and pancreatic ductal adenocarcinoma (PDAC) have a rich expression of the proteoglycan glypcan-1 (GPC1) on their surface. Identification of $GPC1^+$ Exo showed 100% of specificity and sensibility for both pathological conditions, being superior to the gold standard identification of carbohydrate antigen 19-9 (CA19-9) for diagnosis of PDAC that showed 63–80% of specificity/sensibility for adenocarcinoma. Using a genetically engineered murine model for PCDA, the authors showed that $GPC1^+$ Exo can be identified within 16 days, much earlier than the tumor identification by magnetic resonance (only visible of the fifth week) or by histopathological analysis. Another interesting point is that $GPC1^+$ Exo are also loaded with $KRAS^{G12D}$, a gene frequently mutated in PCDA patients, reinforcing the proposal for using plasmatic Exo for diagnosis in the early phase of the disease.

Exo isolated from serum or plasma of patients with colorectal cancer also showed to be useful for diagnosis as reported by Hon et al. [76], since they are loaded with RNA (mRNA and long noncoding RNAs, lncRNAs), miRNA, and proteins associated with early and late phases of tumorigenesis, tumor proliferation and progression, increase of vascular permeability, remodeling of extravascular matrix, drug resistance, shorter disease-free survival, and poor prognosis.

In addition, Exo obtained from urine, saliva, and serum of patients with different kinds of cancer (such as bladder, breast, lung, melanoma, and prostate) can also be used for identification of biomarker [20]. Therefore this is a field that deserves new investigations in order to standardize and simplify the methodology for isolation and characterization of Exo for diagnostic purposes.

20.5 New Perspectives of Using Exo for Therapy

An attractive feature of Exo is the feasibility of using them as a biotechnological tool for drug delivery, since their biological nature favors their circulation and permanence in the blood, reducing the natural clearance by phagocytic cells observed when a synthetic nanomaterial is used as drug carriers [77, 78]. In addition, they show low toxicity according to previously reported trials [12, 72, 73, 75]. Depending on their original source and the administration route, Exo have an intrinsical ability of homing to target cells [77, 79]. For instance, Exo of B lymphocytes are fivefold more efficient to adhere to follicular dendritic cells (FDCs) than immune cells [80]. It was also observed that regions of lymphoid organs where DCs are surrounded by B lymphocytes show several Exo expressing MHC class II. Since FDC express, but are not able to synthesize MHC class II, the finding of these molecules on the cell surface could be explained by incorporation of Exo from B lymphocytes. The potential of migration of Exo for sentinel lymph nodes [81], associated with metastasis and growth of tumor cells, can also be explored for loading them with antitumor or immunostimulating agents, in order to avoid the development of pre-metastatic niches.

Exo can be loaded with lipophilic and hydrophilic drugs both during their biogenesis and after their purification [77]. Then, Exo of different cell lines were loaded with anti-inflammatory agent curcumin, enhancing the *in vivo* effect of

this drug in order to decrease the development of glioblastoma [82].

Antitumor agent paclitaxel was incorporated to macrophage-derived Exo (Exo-PTX) to achieve a stable and adequately disperse product that showed higher *in vitro* toxicity for drug-resistant cell lines than pure paclitaxel [83]. In vivo, this Exo-PTX was tested in the murine model of pulmonary metastasis of Lewis lung carcinoma and showed considerable ability to inhibit the growth of cells in the lungs. Other authors have shown that both Exo derived from iDC and tumor cells loaded with doxorubicin (DOX) accumulate in the tumor site when injected in mice bearing ovarian cancer. These Exo nanocarriers were more effective than the higher doses of pure DOX [84, 85]. In addition, cardiotoxicity usually associated with the administration of DOX is reduced by its incorporation into Exo [84, 86].

20.6 Concluding Remarks

Since Exo bring a variety of antigens, receptors, and nucleic acids of the cells that originate them, it can be considered that they may reflex both the stimulatory and suppressive properties of those cells. Exo secreted by tumor cells show immunoregulatory properties rather than ability to stimulate an antitumor immune response, unless the original cells are previously submitted to stressing conditions to induce the expression of danger signals. This suppressive role limits the use of tumor-derived Exo for therapeutic purposes but the variety of surface markers they bring points out them as reliable liquid biopsies for early diagnosis of cancer.

On the other hand, taking DC as the main APC for triggering an antitumor immunoresponse, the expression of co-stimulatory signals and processed tumor-associated antigens by DC-derived Exo can make them a useful source of immunostimulatory signals, helping to overcome the immunosuppressive status induced by cancer cells, deserving more studies to support their clinical use.

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Photodynamic Therapy and Antitumor Immune Response

21

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21.1 Introduction

Despite high investment in the field of cancer research, the overall results have been somewhat discouraging and have only produced marginal improvements in some types of cancer [1–4]. New-generation cancer drugs are now being tailored according to the patient and tumor genetic signatures and designed to exploit biochemical characteristics associated with tumors (such as ligands, receptors, and signaling pathways). But these approaches come with certain limitations, such as high cost, and more importantly, they are not applicable to a broad range of cancer patients and thus have limitations in comparison with older cheaper chemotherapeutic drugs [5]. Moreover, there are other difficulties, which arise due to the fact that the tumor often develops drug resistance and is often only detected at an advanced stage [6–12]. To complicate and worsen the situation further, some tumors appear to acclimatize and adapt to these initially active tailored drugs. Any time a specific pathway is blocked, the tumor tends to overcome this obstacle to its survival by developing an alternative pathway to continue its growth. Regardless of advances in cancer treatment, the conventional treatment package including surgery + radiation therapy + chemotherapy remains the most prevalent option for oncologists. In this chapter, we will discuss in detail an alternative antitumor technique called photodynamic therapy (PDT) and its ability to stimulate antitumor immune responses.

21.2 Photodynamic Therapy

There have been many preclinical and clinical studies carried out worldwide, showing that PDT has been proven to be a promising modality for the treatment of cancer and other malignancies [13–16]. PDT is now a clinically approved modality for the treatment and management of both nonmalignant and neoplastic diseases. It has the potential to overcome many of the shortcomings and problems associated with conventional cancer treatments. In photodynamic therapy a PS is the administered either systemically, locally, or topically to a patient bearing a lesion (mostly cancer), followed after some time by the illumination of the lesion with visible light of appropriate wavelength. In the presence of oxygen, the excited PS generates cytotoxic reactive oxygen species (ROS) and therefore leads to cell death [17–21] .

Since the lifetime of the ROS such as singlet oxygen is very short, approximately 10–320 ns, it has very limited cellular diffusion (10–55 nm), therefore PDT is highly localized [22], and the photodynamic damage only occurs in the vicinity of the PS molecular location. The PDT effect on the tumor occurs by three interrelated mechanisms: (1) killing of tumor cells directly; (2) tumor vasculature damage; and (3) induction of a strong inflammatory reaction that can lead to development of systemic immunity. The interaction between these three mechanisms and the tumor mass depends on factors such as the type and dose of the PS, the time frame of the PS administration (drug-light interval), the light characteristics (wavelength, total energy exposure or light dose, fluence rate, etc.), and the oxygen concentration in the tumor (Fig. 21.1).

PDT has numerous advantages over other cancer treatment options presently in use. In addition to its selectivity and the possibility of repeated or multiple application, it is considered inexpensive (in comparison with some recent targeted agents) and has tolerable side effects. Moreover, tumors are rarely resistant to PDT [23, 24]. Several types of economical PS compounds are commercially available, and some are already approved to be used on patients. Most of the PS classes in common use are based on porphyrin or chlorin-type backbones or their derivatives. With the newer PS classes, problems such as prolonged skin photosensitization have been virtually eliminated [25]. In addition, these compounds absorb in the far-red region of the visible spectrum, optimal for deep tissue penetration. The list of benefits can be extended to include the absence of the adverse effects produced by radiation therapy and chemotherapy, lack of any significant change in tissue temperature during illumination, preservation of the connective tissue structures (collagen) at the site of PDT application, minimal induction of

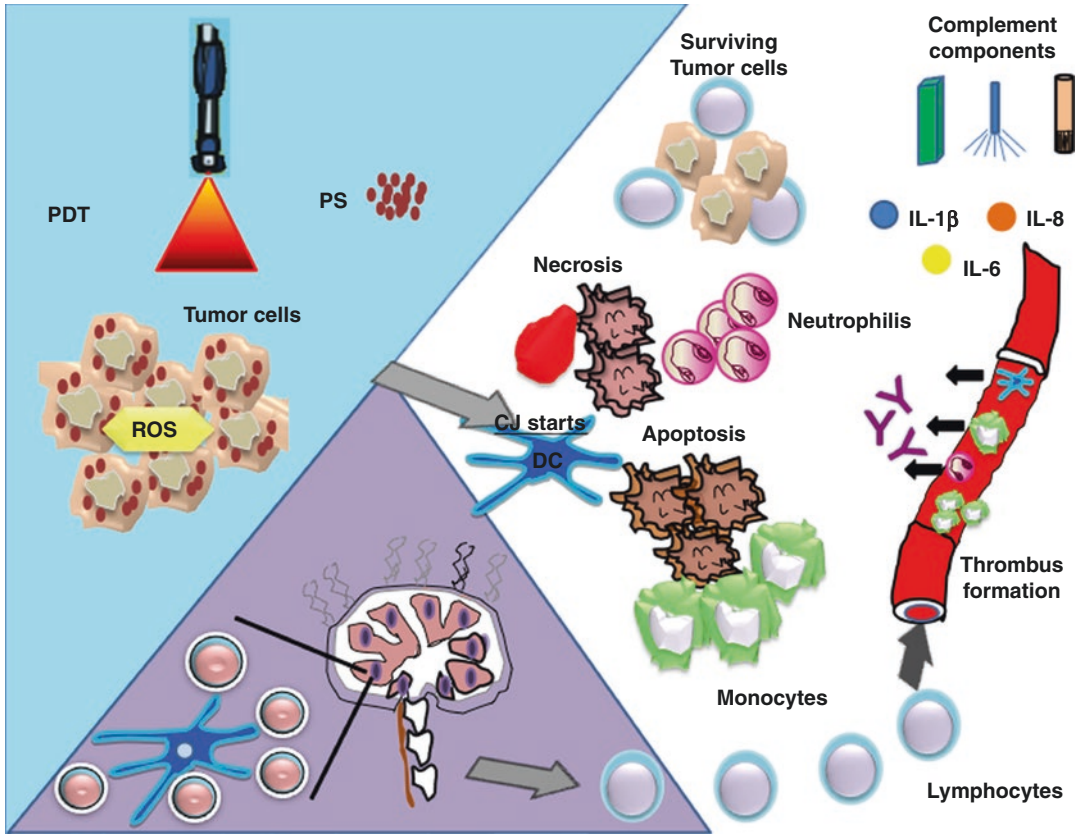

Fig. 21.1 PDT-induced antitumor effects. In tumors, cells loaded with PS absorb light and generate ROS species, which leads to predominantly apoptotic and necrotic cell death. Tumor cell death is accompanied with activation of the complement cascade, pro-inflammatory cytokine activation, rapid accumulation of neutrophils, followed by DCs and macrophages. Dying tumor cells and their debris are phagocytosed by phagocytic cells and DCs, which then migrate to the local lymph nodes and differentiate into antigen-presenting cells. Tumor antigen presentation is then followed by clonal expansion of tumor-specific lymphocytes that home to tumor sites and eliminate residual tumor cells

fibrosis compared to radiation therapy, and an improved cosmetic outcome. Therefore, PDT is a very promising treatment modality that needs further translational and clinical studies.

Studies have shown several and interconnected biological and physiological effects that occur during in vivo PDT. These effects depend on various factors such as the PS concentration, the location of PS in the organism/tumor site, and the dosage and rate of the applied irradiation. PDT effects include direct cell killing, occlusion of the tumor-associated vasculature, and modulation of the immune system, and sometimes all of these effects can be observed occurring simultaneously in a tumor model. At the cellular level, both necrosis and apoptosis have been observed to occur after PDT [14, 26–29]. It is a known fact that direct damage of the tumor cells and the nearby vasculature initiates several cell signaling cascades. Besides this, damage to endothelial cells leads to formation of thrombosis and consequently leads to occlusion of the tumor vasculature. In all these cases, the released fragments from the damaged cells and cytokines trigger a range of inflammatory mediators, which in turn activate the body's defense mechanism, i.e., the innate immune response, which can also affect adaptive immunity. Thus, we can say that PDT generates a distinct systemic effect as well as working in sync with the body's natural

defense mechanisms. The overall success of PDT lies in the fact that it employs the body's "natural pathways" of defense. PDT has been clinically applied to the treatment of early stage pulmonary, gastric, and esophageal carcinoma and has been examined for application to other diseases such as retinal diseases [30, 31] or cardiovascular disorders [32, 33].

21.3 DAMPs (Damage-Associated Molecular Patterns) and Tumor Ablative Therapies

The immunogenicity of cancer cells is an emerging determinant of anticancer immunotherapy [34]. One of the most attractive features of PDT is that besides destroying the tumor itself, it can also trigger an acute inflammatory reaction, thus activating the body's immune system against the cancer cells as discussed above (Fig. 21.2). Thus, induction of a strong inflammatory reaction is a vital part of the antitumor effect of PDT. The local effect of PDT is localized edema and a strong acute inflammation reaction [35, 36]. PDT ends up generating an acute chemical insult within the tumor tissue which is recognized by the body as a type of localized trauma. After this trauma, there occurs a protective mechanism to reestablish tissue integrity and restore homeostasis at the damaged site. This includes removal of damaged cells, and then promoting the healing process at the affected area, in order to reinstate normal homeostasis. This elicited inflammation is initially nonspe-

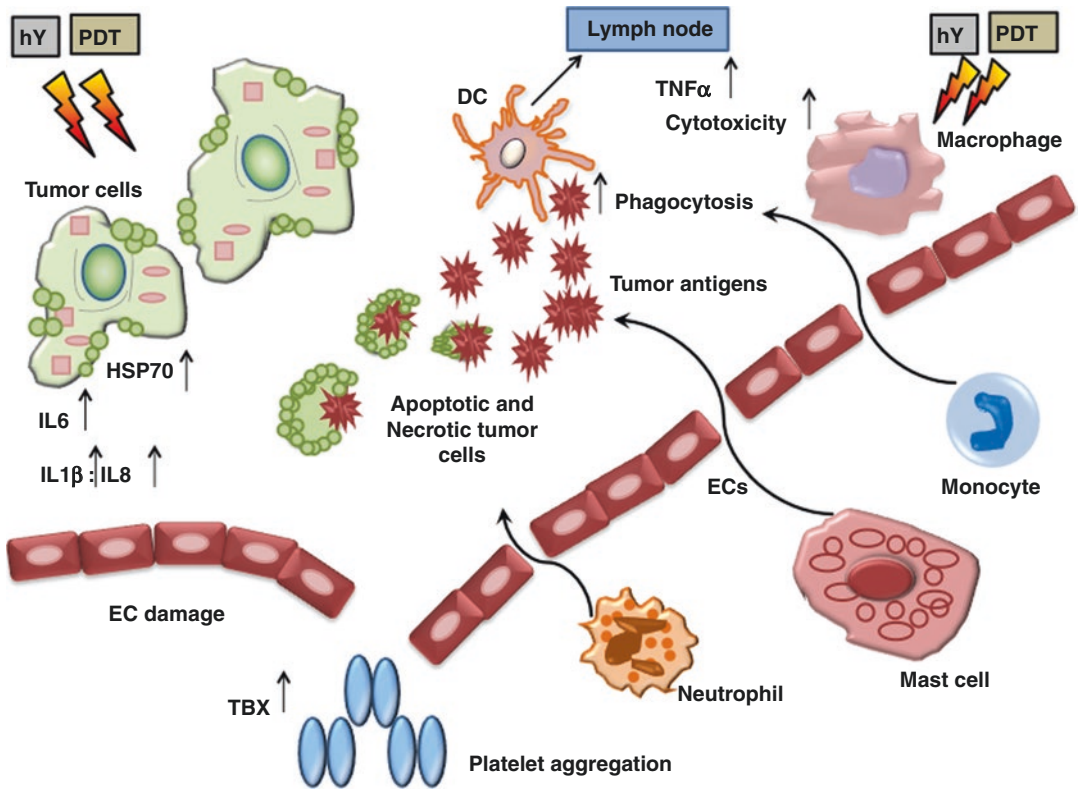

Fig. 21.2 PDT-induced inflammation. Damaging the endothelial cells (*ECs*) activates a cascade of events leading to local inflammation, vessel dilation, and platelet aggregation. Much of these effects are caused by the release of thromboxane (*TBX*), cytokines (such as interleukins IL1β, IL6, IL8, tumor necrosis factor-α), and infiltration of immune system cells (necrotic and apoptotic cells provide antigens to the DCs that migrate to lymph nodes)

cific for the tumor antigens and is orchestrated by the innate immune system [37].

PDT generates rapid and prolific "danger" signals, called damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs), at the site of treatment, which are detected by the innate immune system [38–42]. The pattern of recognition receptors is responsible for detecting the PDT-caused localized insult perceived as "altered self" [37]. This response has probably developed over evolution to protect the host against pathogen invasion at sites of tissue damage. At the onset of inflammation, the tumor vasculature undergoes significant changes and becomes adhesive for inflammatory cells and permeable/leaky for blood proteins [37]. Numerous inflammatory cells, first neutrophils followed by mast cells, monocytes, and macrophages, infiltrate the PDT illumination site [43]. At this stage, the primary function of these cells is to "neutralize" the DAMPs/CDAMPs by eliminating cellular debris, compromised tissue components, etc. [37]. The vascular occlusion, observed after PDT illumination, effectively "walls off" the damaged area, until the damaged cells are removed by phagocytosis, thus preventing further spreading of the tissue damage [37]. Studies have shown that depletion of these inflammatory cells or inhibiting their activity diminishes the therapeutic effect of PDT [44–47]. Moreover, it has been shown that interleukins IL-1β and IL-6 are among the most critical cytokines in this process. Furthermore blocking the function of various adhesion molecules can render PDT ineffective [48, 49]. On the other hand, blocking the anti-inflammatory cytokines, IL-10 and TGF-β, can remarkably improve the outcome of PDT [37, 50].

In recent years a large volume of data has emerged on the effect of in situ tumor destruction (radiotherapy, chemical and biological ablation, PDT, cryoablation, high-temperature ablation (radiofrequency, microwave, laser, and ultrasound), and electrical-based techniques) on the inflammatory and immune components resulting in systemic antitumor immune responses. It is clear that in situ tumor ablation can allow release of tumor antigens, antigen cross-presentation, and the release of DAMPS, thus making the tumor act as its own cellular vaccine [51]. It is now clear that cancer cells can succumb to some anticancer therapies by undergoing a particular form of cell death that is characterized by an increased immunogenic potential, owing to the production of DAMPs. The release of DAMPs and other immunostimulatory factors by the cells gives rise to an immunogenic cell death (ICD) favoring the establishment of a productive interface with the immune system. ICD results in the elicitation of tumor-targeted immune responses associated with the elimination of residual, treatment-resistant cancer cells, as well as with the establishment of long-term immunological memory. Although ICD has been characterized with increased precision since its discovery, several questions remain to be addressed [52].

21.4 PDT and Adaptive Immunity Recognizing Specific Antigens

As discussed earlier, the long-term efficiency of the PDT treatment strongly depends on the initiation of antitumor immunity; and this response is reduced in immunocompromised mice [44, 53]. Moreover this reduced efficacy can be restored by transfer of bone marrow or T-cells, from immunocompetent mice. In this process, recognition of the major histocompatibility complex class I (MHC-I) is critical for activation of $CD8^+$ T-cells; thus tumors that lack MHC-I expression are generally resistant to cell-mediated antitumor immune reactions [54, 55]. In a case in point, patients with vulvar intraepithelial neoplasia (VIN) who lacked high expression of MHC I molecules did not respond as well to PDT treatment, as did patients expressing high levels of MHC-I [56, 57]. Moreover, patients who responded well to PDT treatment had increased $CD8^+$ T-cell infiltration into the treatment site as compared to nonresponders.

Research has shown that PDT treatment of cancer involves both innate and adaptive immune response by stimulating the release or expression of different pro-inflammatory mediators [35, 36,

49]. As a result, a powerful acute inflammatory response is launched causing accumulation of extensive numbers of neutrophils and other inflammatory cells at the PDT-treated site that can attack the cancer cells [36, 43]. The fact is that this initial reaction is not only a powerful tool to elicit direct antitumor effects [58–60], but as importantly, it stimulates the cells to release secondary inflammatory mediators (including the cytokines IL-1β, TNF-α, IL-6, and IL-10 and prostaglandins, histamines, leukotrienes, etc.) [61]. The one area that needed to be further explored was to study the local treatment effects on eliciting systemic immunological response, in particular, establishing the link between PDT-mediated immunity and tumor antigen recognition. Our laboratory was one of the first to recognize this effect. The authors designed a study in which a pair of equally lethal BALB/c colon adenocarcinomas were used: firstly, CT26 wild-type tumors (CT26WT), i.e., antigen negative, and, secondly, CT26.CL25 transduced with lacZ gene, thus expressing the tumor antigen β-galactosidase (β-gal). The idea was to study if PDT treatment would elicit a systemic antigen and epitope-specific antitumor immune response in otherwise identical cancer cells [62]. In this study, both used cell lines were equally lethal, and the level of β-gal expression in CT26.CL25 cells was low enough to allow the tumor to grow without triggering any clinically significant immune response (often seen in cancer patients). The PDT application could therefore generate significant differences in the therapeutic outcome and the observed elicitation of immune response.

The outcome was that PDT induced a local response in all β-gal antigen-negative CT26WT tumors, with clear reduction in size, but this lasted only until day 18 (Fig. 21.3) after that local regrowth occurred. The net result was that the growth was only stalled for 8–10 days. In the case of CT26.CL25 tumors, however, the difference was dramatic (Fig. 21.4); tumor reduction was not only complete after day 20, but most importantly, 100% of these β-gal antigen-positive tumors stayed in remission during the complete trial period of 90 days [62]. During the study, the PDT-induced immune response leading to elevated levels of released IFN-γ and TNF-α cytokines was also observed. Our study also showed that PDT can induce a very strong antigen-specific immune response, capable of generating memory immunity which allows mice to reject a rechallenge with the same antigen-positive cells. The induced immune response was potent enough to cause regression of a distant well-established antigen-positive tumor outside the treatment area (on the opposite flank) [62] (Fig. 21.5). The presence of activated antigen-specific and epitope-specific effector CTLs was also confirmed. During the study, it was found that regression of distant and untreated tumors took place in 70% of the treated mice.

For the first time it was demonstrated that tumor cells may escape PDT-induced immunosurveillance due to loss of the tumor antigen. In clinical settings, it is known that some tumors escape from immune recognition and resist elimination; only now, we realized that this is occurs due to tumor antigen loss. We also demonstrated that PDT-induced antitumor effects are abrogated when there is no functional adaptive immune response as in athymic nude mice (Fig. 21.4). Clearly, effective vascular PDT treatment can not only destroy a local tumor but also induce systemic strong antigen-specific antitumor immune response. In addition, this immunity is so potent that it is able to induce regression and destruction of distant, antigen-positive tumors outside the irradiation field. The treatment also proved to be effective in inducing long-term immune memory effect, imparting a resistance to rechallenge. Our study was successful in proving that the observed tumor-destructive effect was mediated by tumor antigen-specific cytotoxic T-cells, induced after PDT, which are capable of recognizing the immuno-dominant epitope of the β-gal antigen.

To examine antigen-specific PDT-induced antitumor immune response in a more clinically relevant tumor model, the authors designed a different study, where a naturally occurring cancer antigen, namely, P1A, a mouse homologue of the human MAGE-type antigen, was employed [63]. We decided to use this specific cancer-testis antigen, since it is not only well-established, but more importantly, it is mostly expressed in testis and cancers and only at very low levels in other tissues [64–67]; P1A antigen-positive mouse

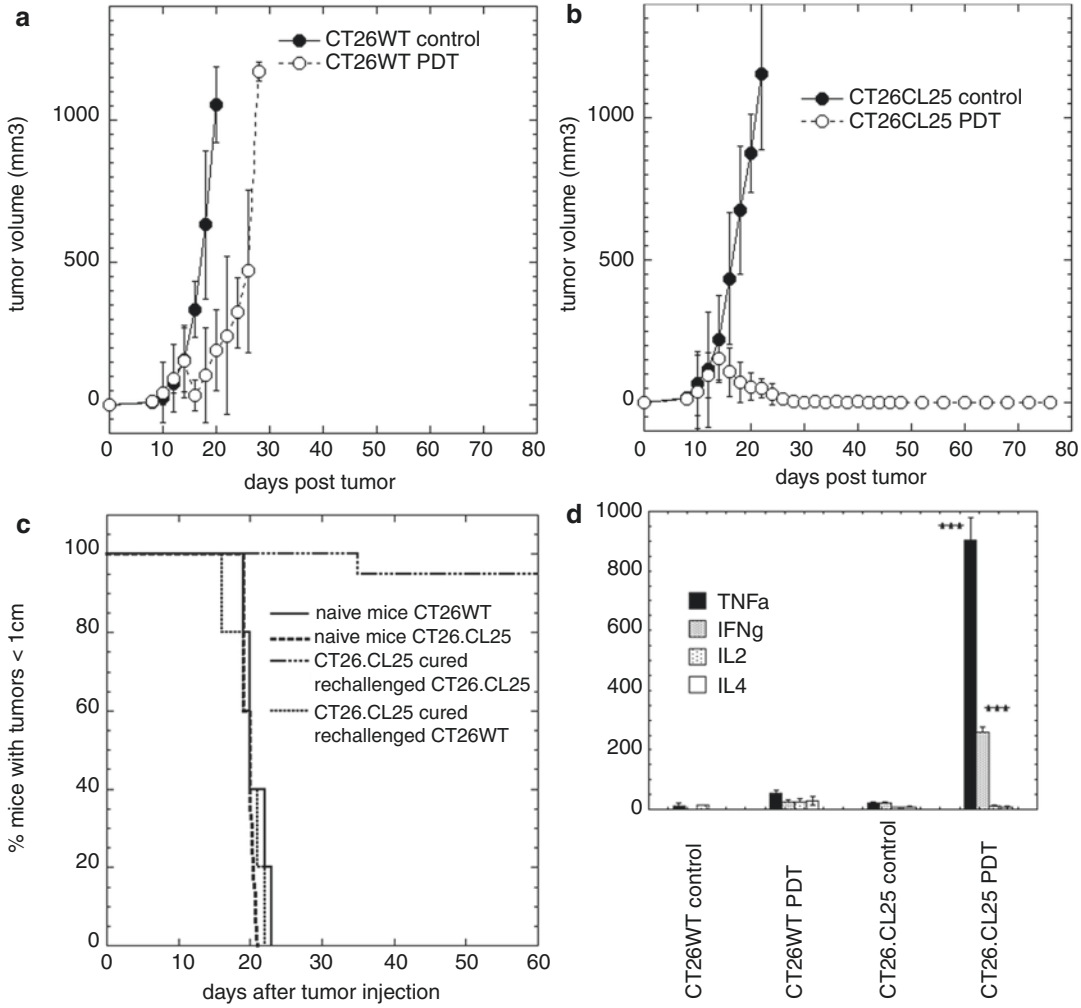

Fig. 21.3 In vivo PDT of tumor (one leg model). (**a**) Mean tumor volumes of CT26WT tumors and (**b**) CT26.CL25 tumors; means of 10–15 tumors. (**c**) Kaplan-Meier survival curves of % of mice cured from CT26.CL25 tumors and rechallenged either with CT26.CL25 or CT26WT tumor cells. (**d**) Mean level of cytokines TNF-α, INF-γ, IL-2, and IL-4; measured 5 days after PDT in CT26.CL25 and CT26WT tumor-bearing mice and control mice (Used with permission from Ref. [62])

mastocytoma P815 wild-type (parental) and P1A antigen-negative P1.204 (P815 derived) cell lines were compared.

Murine methylcholanthrene-induced mastocytoma P815 cancer cells are known to generate very interesting immunologic response patterns. The significance of P815 antigen arises from the fact that it shares many characteristics identified in TAA genes in human, such as those belonging to melanoma MAGE family and other tumors [68, 69]; these antigens are not expressed in most mature tissues with the exception of testis and placenta [70]. It is known that P815 can elicit CTL response against at least four distinct antigens: AB, C, D, and E [70–79]. It appears that the main CTL response against P815 tumor is geared toward AB and E antigens [73]. Also, it has been shown that T-cells isolated from DBA/2 mice implanted with P815 tumors primarily recognize either antigen AB or C-D-E, but not both [79]. Moreover, the two epitopes of the P815AB, P815A, and P815B are recognized by two different CTLs. Another gene codes for P815E and different CTLs recognize this antigen. On the other

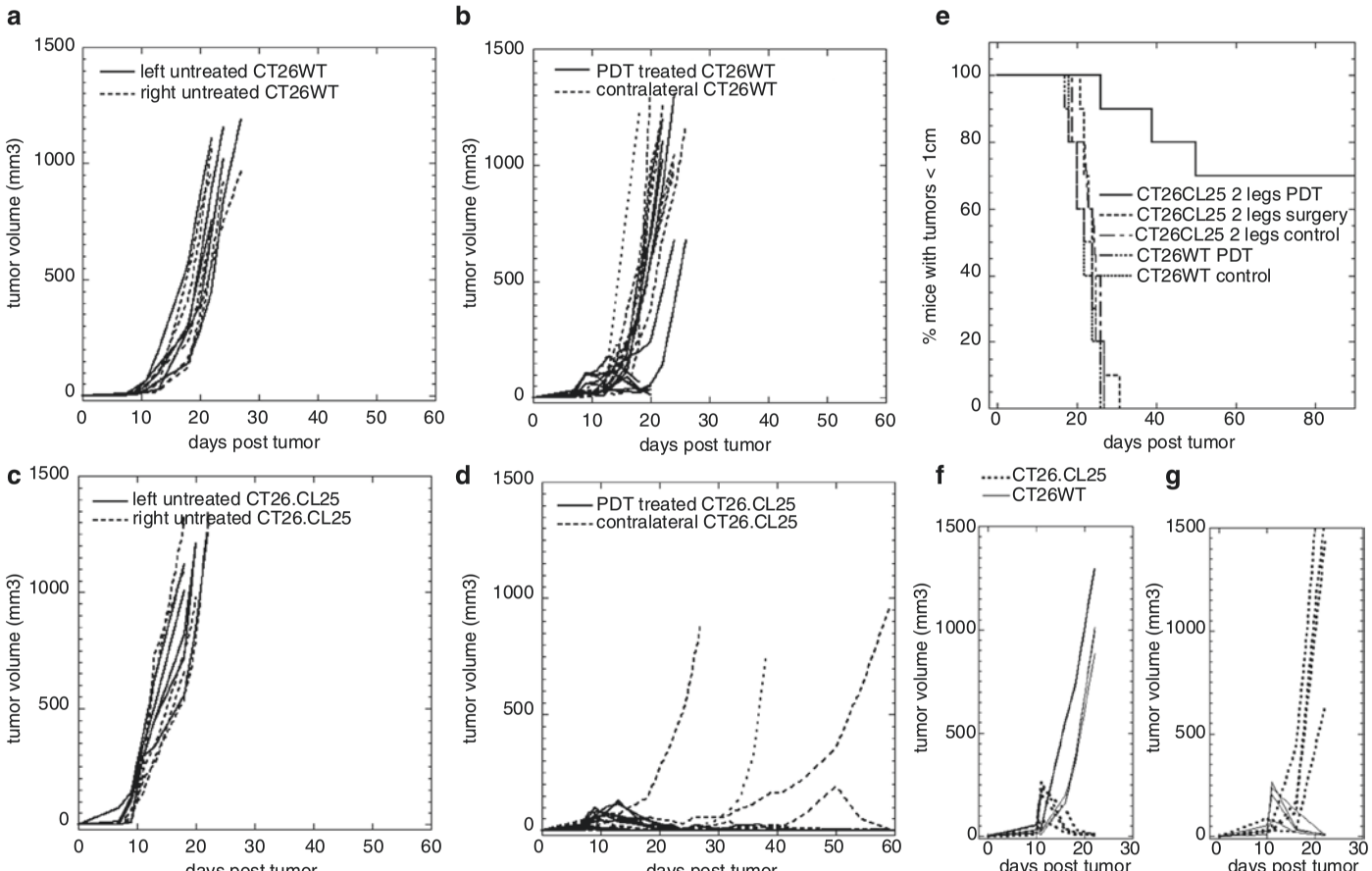

Fig. 21.4 In vivo PDT of tumors (two-leg model). Time courses of individual tumor volumes with two similar or mismatched bilateral tumors on the right and left legs. (**a**) Bilateral CT26WT tumors; right leg PDT treated. (**b**) Bilateral CT26WT tumors, untreated. (**c**) Bilateral CT26.CL25 tumors; right leg PDT treated. (**d**) Bilateral CT26.CL25 tumors, untreated. (**e**) Kaplan-Meier survival curves of % mice with tumor volumes smaller than 1 cm, in five groups: three groups with two similar bilateral CT26.CL25 tumors (one group untreated, one group with right leg tumor and PDT treated, and one group with right leg tumor surgically removed); two groups with two bilateral CT26WT tumors (one group untreated, one group with right leg tumor PDT treated). (**f**) Mismatched CT26.CL25 and CT26WT tumors; CT26WT treated with PDT. (**g**) Mismatched CT26.CL25 and CT26WT tumors; CT26.CL25 treated with PDT (Adapted from Mroz et al. open access [62])

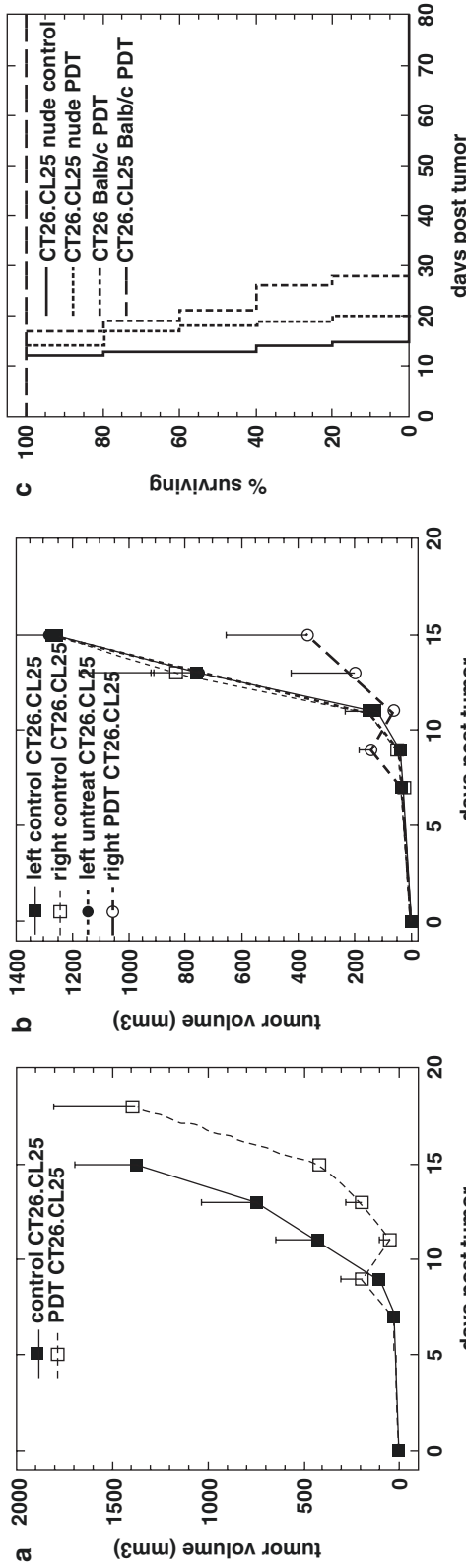

Fig. 21.5 (**a**) Tumor volumes of CT26.CL25 tumors PDT treated and untreated in BALB/c Nu/Nu immunocompromised mice. (**b**) Tumor volumes in bilateral CT26.CL25 tumors PDT treated and untreated in BALB/c Nu/Nu immunocompromised mice. (**c**) Kaplan-Meier survival curves of % surviving BALB/c and BALB/c Nu/Nu mice with either CT26.CL25 or CT26WT tumors, PDT treated. Non-treated BALB/c Nu/Nu mice with CT26.CL25 tumor is used as control (From Mroz et al. [62]; open access)

hand, the P815-derived P1.204 cell line is an immune system escape variant [80]; it has lost the P815AB antigen and only retains the P815E antigen.

During in vivo experiments performed by the authors, the majority of mice with P815 tumors demonstrated tumor regression after PDT irradiation and no recurrence during the trial period of 90 days. In stark contrast, mice with P1.204 tumors did not respond with tumor regression but rather with progression. The difference in response between the two tumor types was hypothesized to be due to differential triggering of immune response. To confirm the PDT-generated long-term immune system "activation" in this clinically relevant tumor model, we rechallenged the cured mice with the same tumor from which they were originally cured. Only mice cured for P1A antigen-positive P815 tumors rejected the rechallenge with P815, while all the naïve mice injected with either tumor cell type grew tumors. The implication of the finding is that P1A antigen-positive P815 tumors, after PDT treatment, develop strong and robust enough immune response that prevents tumor growth upon challenge with a tumorigenic dose of cells [80].

> In the ex vivo study, the extent of induction of an antitumor immune response, as a result of PDT treatment of P1A expressing P815 tumors, and whether the antigen activated T-cells before and/or after PDT, was investigated. Cytokines secreted from $CD4^+$ and $CD8^+$ T-cells were measured upon stimulation. Our results showed that PDT of P1A antigen-positive tumors led to marked increase in IL-2 and TNF-α levels. Moreover, we were able to identify a population of $CD8^+$ T-cells that were able to recognize the known epitope (LPYLGWLVF) of the P1A antigen using a pentamer approach and flow cytometry. In addition, when nude mice (lacking an adaptive immune system) bearing the P1A antigen-positive P815 tumors were treated with PDT, the antitumor effectiveness of PDT was curtailed to nil. Interestingly, the survival of these mice could be significantly prolonged by adoptive transfer of activated lymph node cells isolated from PDT-treated immunocompetent mice bearing the P815 tumor.

The initial escape of P815 tumors from immunosurveillance (and accordingly lack of response) has been documented to be due to antigenic loss [22, 38, 39]. It has been shown [74] that there are three different escape mechanisms employed by P1A tumors, presenting the peptide epitope LPYLGWLVF (expressed in different tumor models). In P815 tumors, all progressions occurred due to antigenic loss, while in J558 tumors (another P1A-positive tumor), all progressions took place due to antigenic drift (antigen mutation) [38], whereas all progressing methA tumors (a third P1A-positive tumor) developed resistance to CTLs.

Green fluorescent protein (GFP) is used as an optical reporter to noninvasively image the progression of mouse tumors (using whole-body fluorescence imaging) and, in addition, may act as a foreign (jellyfish) antigen. We asked whether GFP-expressing tumors could be used to monitor the response of tumor-bearing mice to PDT and whether the tumor response differed when a non-immunogenic tumor cell line was transduced with GFP. RIF-1 or RIF-1 EGFP (stably transduced with a retroviral vector) cells were injected in the leg of C3H/HeN mice and both cells and tumors grew equally well. We used PDT with benzoporphyrin derivative and a short drug-light interval. There were complete cures and 100% mouse survival of RIF-1 EGFP while RIF-1 wild-type tumors all recurred. Cured mice were resistant to rechallenge with RIF-1 EGFP cells and a rechallenge with wild-type RIF-1 cells grew significantly slower. There was also slower RIF-1 EGFP rechallenge growth but no rejection when RIF-1 EGFP tumors were surgically removed. There was a low rate of PDT cure of tumors when RIF-1 cells were transduced with an empty retroviral vector. The presence of antibodies against EGFP in mouse serum suggests EGFP can act as a foreign antigen and PDT can then stimulate a long-term memory immune response [81].

21.5 Cancer and Immunosuppression

Cancer often develops as a complication of severe immunosuppression. Tumor cells proliferate in an immunosuppressive microenvironment, which can be an obstacle in the immunotherapy of cancer. Cancers take advantage of the immune regu-

latory mechanism of the host that prevents autoimmunity, resulting in evasion of immunosurveillance and resistance to immune destruction. Regulatory T-cells, myeloid suppressor cells, inhibitory cytokines, and immune checkpoint receptors are the major components of the immunosuppression mechanisms in cancer progression [82]. Advances in the understanding of tumor immunology are opening up a new range of therapeutic targets, including overcoming immunosuppressive factors in the tumor microenvironment [83]. Manipulating immune responses may thus provide an exciting new option for cancer immunotherapy [84].

21.5.1 Regulatory T-Cells

$CD4^+$ regulatory T-cells (Tregs) are a highly immunosuppressive subset of $CD4^+$ T-cells that protect the host from developing autoimmune diseases and allergies, whereas in malignancies, they promote tumor progression by suppressing antitumor immunity. The elucidation of factors influencing Treg homeostasis and function has important implications for anticancer therapies. Thus, the manipulation of Tregs for up- or down-regulation of their suppressive function is a new therapeutic strategy for treating cancer and autoimmune diseases [85]. Treg depletion augments antitumor immune responses in animal models. Additionally, increased numbers of Tregs and, in particular, decreased ratios of CD8(+) T-cells to Tregs among tumor-infiltrating lymphocytes are correlated with poor prognosis in various types of human cancers. Thus, implementation of a strategy restricting Treg-mediated immune suppression may expand the therapeutic spectrum of cancer immunotherapy, especially in patients with a lower number of neoantigens [86].

21.5.2 Myeloid Suppressor Cells

Tumor-associated myeloid cells comprise a heterogeneous population acting systemically (myeloid-derived suppressor cells/MDSCs) and/or locally in the tumor microenvironment (MDSCs and tumor-associated macrophages/TAMs). Both populations promote cancer cell proliferation and survival, angiogenesis, and lymphangiogenesis and elicit immunosuppression through different pathways, including the expression of immunosuppressive cytokines and checkpoint inhibitors. Several studies have demonstrated that myeloid cells can express different functional programs in response to different microenvironmental signals, a property defined as functional plasticity. Myeloid suppressor cells can on one hand support tumor growth and, on the other, limit autoimmune responses, indicating that their therapeutic reprogramming can generate opportunities in relieving immunosuppression in the tumor microenvironment or reinstating tolerance in autoimmune conditions [87].

Development of metastasis is determined by both the accretion of essential changes in cancerous cells and by their communication with different stromal elements in the tumor microenvironment. Specifically, the inflammatory response and emergence of immune regulatory cells, such as myeloid-derived suppressor cells (M2-activated macrophages, tolerogenic dendritic cells, neutrophils, myeloid-derived suppressor cells (MDSCs)) and lymphoid-derived regulatory cells (regulatory T, B, and NK cells) to the tumor site have all been reported to support tumor growth, in addition to tumor invasion and metastasis. Although the potential role for myeloid regulatory cells in tumor invasion and development of the pre-metastatic niche has been suggested, the concept still requires further supportive experimental and clinical evidence, as well as data related to specific factors and mechanisms responsible for myeloid regulatory cell functioning at malignant sites [88]. Different approaches are currently being explored to target MDSC with the aim to enhance immune-based therapies [89].

21.5.3 Immature Dendritic Cells

Dendritic cells (DCs) comprise a heterogeneous population of cells that play a key role in initiating, directing, and regulating adaptive immune responses, including those critically involved in tumor immunosurveillance. The efficiency of

anticancer therapy exploiting dendritic cells depends upon the maturation status of the DCs and how it changes following their interaction with cancer cells. In a study, using mouse xenograft models of human tumors, it was shown that fast-growing "angiogenic" tumors were infiltrated by a more immature DC population than comparable dormant nonvascular tumors. Since immature DCs actively promote angiogenesis and tumor growth, strategies to promote DC maturation or methods for DC ablation suppresses this response. It was thus concluded that angiogenesis could be dependent on the presence of immature DCs. Thus, cancer immunotherapies that promote DC maturation may act by both augmenting the host immune response to the tumor and by suppressing tumor angiogenesis [90].

DCs are the sentinel antigen-presenting cells of the immune system, such that their productive interface with the dying cancer cells is crucial for proper communication of the "nonself" status of cancer cells to the adaptive immune system. The efficiency and the ultimate success of this communication depends upon the maturation status of the DCs and their interaction with cancer cells. Immature DCs facilitate tolerance toward cancer cells, while fully mature DCs that secrete the correct combinations of cytokines can strongly promote anticancer immunity [91].

21.5.4 Indoleamine 2,3-Dioxygenase

Indoleamine 2,3-dioxygenase (IDO) is an inducible enzyme that catalyzes the rate-limiting first step in tryptophan catabolism. This enzyme is overexpressed in response to IFN gamma in a variety of different malignancies. IDO causes immunosuppression through breakdown of tryptophan in the tumor microenvironment and the tumor-draining lymph nodes. The depletion of tryptophan and production of toxic catabolites renders effector T-cells inactive and dendritic cells immunosuppressive. Thus, the IDO pathway is an important mechanism for tumor-related immunosuppression, and blocking it could improve cancer immunotherapy outcomes. Preclinical data suggest that IDO inhibition can delay tumor growth, enhance dendritic cell vaccines, and synergize with chemotherapy through immune-mediated mechanisms [92]. IDO is an immunosuppressive enzyme, which mediates tumor immune escape in various cancers including hepatocellular carcinoma (HCC). Therefore, IDO inhibitors as adjuvant therapeutic agents may have clinical implications in HCC. This review proposes future prospects of IDO not only as a therapeutic target but also as a prognostic marker for HCC [93].

21.6 PDT and Immunostimulant Combinations

Treatment with PDT alone is often non-curative due to tumor-induced immune cell dysfunction and immune suppression. Motivated by this fact PDT can be combined with immunostimulants and other strategies designed overcome the tumor-induced immune suppressive mechanisms described above, in order to enhance antitumor immunity. There have been many studies reporting good results using this approach.

A study was performed in an animal model of metastatic cancer, to compare PDT alone with PDT combined with low-dose cyclophosphamide (CY). Low-dose CY is a treatment that has been suggested to deplete regulatory T-cells (T-regs) and augment the immune response to some tumors. We used J774 tumors (a highly metastatic reticulum cell sarcoma line) and PDT with benzoporphyrin derivative monoacid ring A, verteporfin for injection, and a short (15 min) drug-light interval. CY (50 or 150 mg/kg i.p.) was injected 48 h before light delivery. PDT alone led to tumor regressions and a survival advantage but no permanent cures were obtained. BPD-PDT in combination with low-dose CY (but not high-dose CY) led to 70% permanent cures. Low-dose CY alone gave no permanent cures but

did provide a survival advantage and was shown to reduce CD4+FoxP3+ T-regs in lymph nodes, whereas high-dose CY reduced other lymphocyte classes as well. Cured animals were rechallenged with J774 cells, and the tumors were rejected in 71% of mice. Cured mice had tumor-specific T-cells in spleens as determined by a (51)Cr release assay (Fig. 21.6) [94].

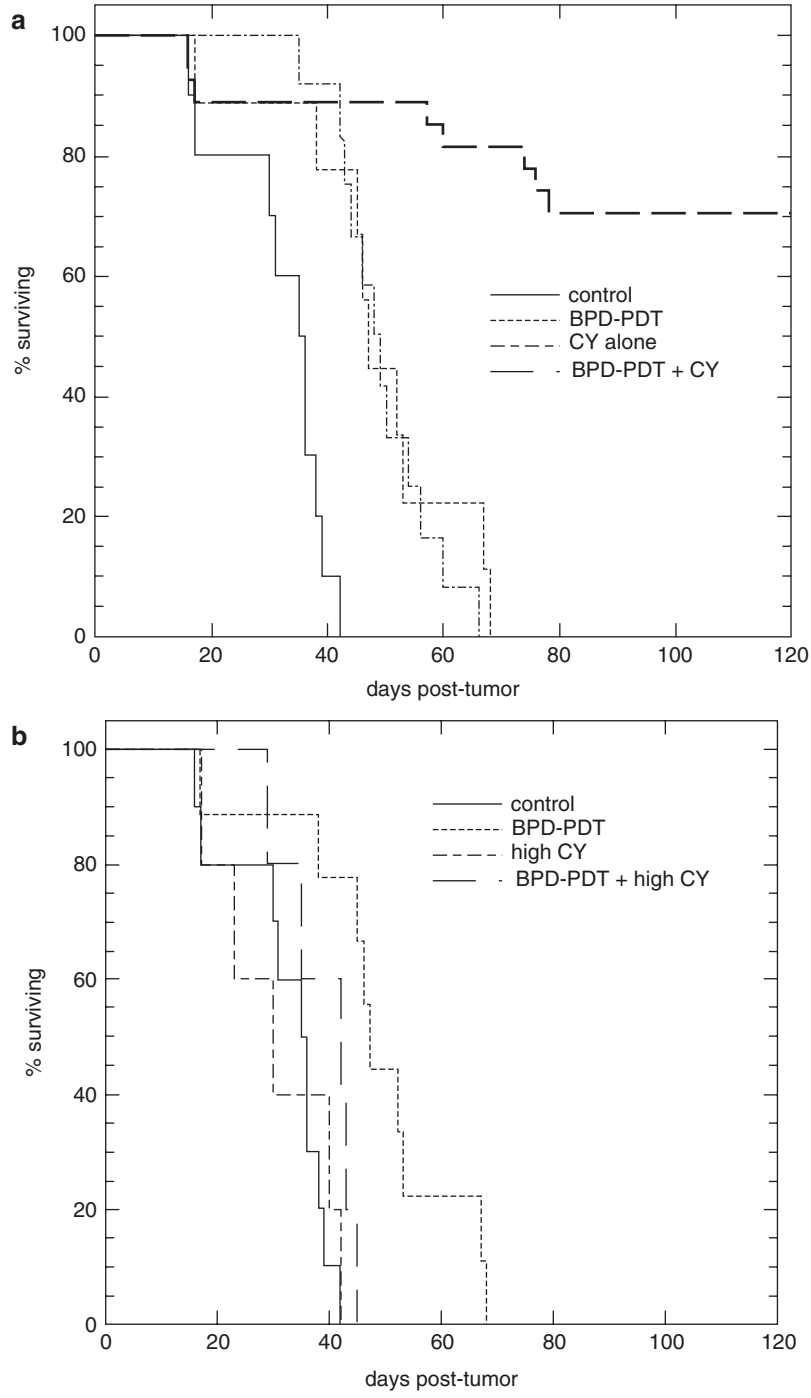

Fig. 21.6 Kaplan-Meier survival curves of mice treated with PDT combined with low-dose CY. (**a**) Plots represent no tumor treatment (*as control*), only PDT, low-dose CY, and low-dose CY + PDT. (**b**) Plots represent no tumor treatment (*as control*), only PDT, high-dose CY, and high-dose CY + PDT. Mice were killed in cases when the primary tumor diameter reached 1.5 cm or body weight dropped >15%

Our lab also investigated PDT mediated by verteporfin and 690 nm light delivered 15 min later, in combination with an immunomodulation approach using CpG oligodeoxynucleotide for the treatment of 4T1 metastatic breast cancer in a BALB/c immunocompetent mouse model. In vitro, CpG primed immature dendritic cells (DC) via toll-like receptor 9 to phagocytose PDT killed tumor cells leading to DC maturation and activation. Peritumoral injection of CpG after PDT in mice gave improved local tumor control and a survival advantage compared to either treatment alone ($p < 0.05$). CpG may be a valuable dendritic cell targeted immunoadjuvant to combine with PDT [95].

In another study, we investigated whether the combination of PDT with low-dose CY could foster immunity against wild-type CT26 tumors expressing self-antigen (gp70) [96]. We had previously shown that CT26 wild-type tumors did not produce a long-term memory immune response when treated with PDT alone [62]. Administration of CY before PDT led to depletion of Treg and potentiated PDT-mediated immunity, leading to long-term survival. However the development of memory immunity (resistance to rechallenge) was only uncovered by a second round of Treg depletion using a second administration of low-dose CY [96].

It was recently reported that PDT can induce strong antitumour immunity toward tumor cells expressing the tumor-associated antigen P1A. Using four different mouse tumor models, we showed that antitumor immune response could be further improved when PDT is combined with a clinically approved epigenetic reversal agent that induces expression of an epigenetically silenced P1A antigen. Taken together these findings showed that PDT leads to strong specific antitumor immune responses and that epigenetic modification of tumor antigens levels may be a novel approach to further enhance the effectiveness of PDT providing a strong rationale for clinical development of this therapeutic approach [97].

The purpose of one of the studies was to determine if local PDT followed by intratumoral injection of naïve dendritic cells (IT-DC) could induce systemic antitumor immunity that could inhibit the growth of untreated tumors. It was concluded that PDT plus IT-DC administered to one tumor site led to tumor regression at distant sites, including multiple lung metastases. PDT + IT-DC induced potent systemic antitumor immunity in mice and should be evaluated in the treatment of human cancer [98].

21.7 PDT and Checkpoint Inhibitors

In recent years the introduction of checkpoint inhibitors has revolutionized the clinical treatment of many forms of advanced cancer [99]. Checkpoint inhibitors are particularly useful for potentiating T-cell-mediated immune attack against tumors. Ipilimumab (Yervoy), a monoclonal antibody targeting CTLA-4 receptor, is approved for the treatment of melanoma. Normally the CTLA-4 receptor antagonizes T-cell-mediated immunity; ipilimumab blocks this receptor leading to increased tumor killing by cytotoxic T-cells [100]. Another new anticancer drug is pembrolizumab (Keytruda), a monoclonal antibody, which targets the programmed cell death 1 (PD-1) receptor. Pembrolizumab is approved for the use against melanoma [101]. PD-1 is expressed on the surface of T-cells and B-cells and negatively regulates immune response. Inhibiting PD-1 prevents its cognate ligand PD-L1 (which is expressed on tumor cells) from binding to PD-1 and thereby killing the attacking T-cells. There are now other checkpoint inhibitors that target PD-1 or its cognate ligand PDL-1, such as nivolumab (Opdivo), atezolizumab, avelumab, and durvalumab.

There have recently been several papers that have explored the combination of PDT with checkpoint inhibitors in experimental animal tumor models. A study by Kleinovink et al. [102] studied PDT mediated by Bremachlorin and 660 nm light with a 6-h drug light interval on day 8 after MC38 tumors were implanted in C57BL/6 mice. PDT was combined with anti-CTLA4 antibody injected three times on days 7, 10, and 14 after tumor inoculation. The combination had an

improved effect on double-tumor-bearing mice (only one tumor treated with PDT). Muchowicz et al. [103] tested the combination of BPD-PDT (15-min drug light interval) with anti-PDL-1 antibody injected every second day, in six doses, starting from 1 day before PDT in BALB/c mice with orthotopic 4T1 tumors. The combination led to 50% cures in this difficult model. A study by Gao et al. [104] looked at a combination of PDT using an integrin αvβ6-targeted phthalocyanine with an anti-PD-1 antibody in a 4T1 tumor model. The combination gave improved antitumor immunity and suppressed lung metastases metastasis.

The laboratory of Wenbin Lin at the University of Chicago has published a series of papers describing the combination of various nanotechnology-based PDT agents and checkpoint inhibitors in mice. One study [105] investigated the combination of nanoscale coordination polymer (NCP) core-shell nanoparticles loaded with oxaliplatin in the core and the PS pyropheophorbide attached to the shell, with anti PD-L1 antibody against CT26 tumors in BALB/c mice. They showed regression of both PDT treated primary tumors and nonirradiated distant tumors. Another study [106] used core-shell nanoparticles with zinc pyrophosphate and a lipid-conjugated pyropheophorbide PS in combination with anti PDL-1 antibody to produce antitumor immunity against 4T1 tumors. A third paper [107] reported PDT using a chlorin-based metal-organic framework (MOF) that also contained the indoleamine 2,3-dioxygenase (IDO) inhibitor (4-amino-N-(3-chloro-4-fluorophenyl)-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide) encapsulated in the channels of the MOF nanoparticles. PDT with this nanovehicle caused effective tumor regression of both primary, treated tumors and distant, untreated tumors in two syngeneic mouse models of colorectal cancer.

Xu and coworkers [108] constructed upconversion nanoparticles (UCNPs) loaded with the PS chlorin e6 and imiquimod (R837), a toll-like-receptor-7 agonist. PDT using NIR light excited the UCNP-Ce6-R837 nanoparticles when combined with anti-CTLA-4 antibody resulted in strong antitumor immune response to inhibit the growth of untreated distant tumors and produce memory immunity.

It should be noted that two very recent papers [109, 110] have reported that the response to checkpoint inhibitors has been shown to depend on the precise composition of the intestinal microbiome in both experimental models and also in patients. Apparently some bacteria in the gut encourage the development of antitumor immunity, while other bacterial species inhibit this response [111].

21.8 Concluding Remarks and Clinical Applications

There have been few reports as yet of antitumor immunity in patients treated with PDT. Abdel-Hady et al. [69] reported that high-risk HPV-infected premalignant genital lesions showed a poor response to ALA-PDT when the patients showed loss of HLA class I in the lesion, and when there was high CD8 infiltration in the lesion after PDT, the response was likely to be better. Kabingu et al. [112] reported that patients with cutaneous basal cell carcinomas (BCC) treated with ALA-PDT were more likely to have peripheral blood leukocytes that recognized Hip1, a transmembrane protein, which is overexpressed in BCC and can function as a tumor antigen, compared to patients that underwent surgery. Superficial lesions appeared to be especially susceptible to increased systemic antitumor immunity. Thong et al. showed [101] using Fotolon (a chlorin-based PS) in a single angiosarcoma patient that high fluence rate PDT showed success in local control, but only for up to 1 year. After recurrence, the tumor was treated again with low fluence rate PDT, but this time the treatment achieved tumor eradication, and spontaneous remission of non-treated distant lesions was observed, showing that an antitumor immune response had been activated.

Nevertheless, it is clear that antitumor systemic immunity after clinical PDT remains the exception rather than the rule. The reasons for

this variability are many and diverse. The PDT parameters such as choice of PS, doses of both PS and light, fluence rate, and drug-light interval are all important in optimizing the immune response. The expression of the appropriate type and amount of antigens and neoantigens within the tumor is of critical importance. Another possible reason for this failure is the weakness of the immune system in older people as well as in patients with advanced tumor stages. Stage 4 cancer patients can often suffer from severe immunosuppression. Identifying and overcoming the immunosuppressive mechanisms that allow the tumor to grow in the first place provides a wealth of opportunities for combination treatments. These may include coadministration of various immunostimulatory adjuvants, strategies that involve dendritic cells, depletion of regulatory T-cells, and epigenetic reversal agents. In particular, the recent growth in popularity of checkpoint inhibitors, many of which are already approved for use in cancer patients, urgently suggests these agents should be clinically tested in patients who are receiving PDT. Future research will be able to test and optimize many of these PDT-based combinations.

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Reprogramming of Tumor Microenvironment in Therapy

22

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Contents

22.1 Introduction

Cancer is an abnormal variant of tissue in which proliferating and dying cells coexist in a low-pH and oxygen-deficient environment. It is created by the unique metabolism of cancer cells and abnormal vascularity. Hypoxia with the presence of danger signals from dying cells induces an inflammatory reaction similar to the one present in damaged tissue. "Repairing" of the altered tumor tissue includes mechanisms of wound healing such as neovascularization, removal of cellular debris, transformation of the environment, and immunosuppression [1].

Autonomic cancer cells develop specific relationships ("dialogs") with normal cells. They create a new microenvironment, a specific ecological niche that allows the growth of cancer cells [2]. Paradoxically, the cancer environment also consists of normal cells. The behavior of cancer cells therefore is determined by not only accumulated mutations and mutational profiles but also "social" interactions with other cells [3]. At each stage of tumor formation, cancer cells coinhabit with different cell types [4]. The main interactions between cancer cells and microenvironment cells are cell-cell-like [5].

The cancer microenvironment is a dynamic structure that changes over time. Important structural and functional elements of the tumor microenvironment include cancer-associated fibroblasts, myofibroblasts, immune system cells,

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blood and lymph vessels, and extracellular matrix (ECM) [3, 5–7]. Immune cells are recruited by tumor cells. In a pro-inflammatory environment (inflammation is an important hallmark of cancer [2]), cancer cells secrete signals that trigger specific reprogramming of normal cells recruited to the tumor [8, 9]. This reprogramming leads to the appearance of a cell phenotype that promotes tumor growth [2, 10]. Modified immune reaction cells form a novel-specific microenvironment, which is both proangiogenic and immunosuppressive [11]. This environment is created inter alia by the emerging blood vessels and immunosuppressive properties of cancer and inflammatory cells. Such a *milieu* shields cancer cells from immune surveillance [12, 13].

The formation of a network of tumor blood vessels influences the progression of cancer [14, 15]. The structure of tumor blood vessels is defective and functionally impaired [16–18]. In the initial stage of tumor growth, oxygen and nutrients are delivered through the vessels. In the further progression, deliveries are insufficient and hypoxia regions occur. Hypoxia is a factor that induces the formation of new blood vessels (angiogenesis) by the activation of hypoxia-inducible factor (HIF), which increases the secretion of vascular endothelial growth factor (VEGF) by cancer cells. VEGF and released growth factors induce defective vessel formation. In hypoxic conditions, cancer cells produce lactates, as well as many cytokines that affect the tumor microenvironment [19]. Hypoxia also affects the cytotoxic properties of immune cells, including T-lymphocytes and dendritic cells (DCs). It inhibits their proliferation, and it also transforms the macrophage phenotype into immunosuppressive, pro-tumor one [17].

The tumor microenvironment is dynamic and undergoes constant changes. Using the appropriate treatment, tumor microenvironment may be reprogrammed into an antiangiogenic and immunomodulatory one, in other words, an environment that inhibits the growth of tumors. The purpose of our article is to draw attention to the role of cells of microenvironment in tumor progression, as well as to the possibility of taking advantage of reprogramming tumor microenvironment cells for therapeutic purposes.

22.2 Recruitment of Inflammatory Cells by Cancer Cells

Mutations of certain genes in cancer cells (including *RET*, *RAS*, *Myc*, and *p53*) trigger transcription of genes encoding chemotactic factors, for instance, CC and CXC subgroup of chemokines, the main chemoattractants of inflammatory reaction [8].

Cancer cells release tumor-derived factors (TDFs) that alter hematopoiesis and promote the expansion of myeloid cells. The increased myelopoiesis causes the accumulation of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). Reprogrammed cancer myeloid cells block the function of T-lymphocytes and stimulate many processes associated with tumor progression. Among TDFs are CC chemokine ligand 2 (CCL2) (MCP-1) and CCL5 (RANTES) chemokines that recruit and activate macrophages. These chemokines are produced by tumor cells, fibroblasts, endothelial cells, and TAMs themselves. Other chemokines involved in the recruitment of monocytes include CCL3, CCL4, CCL8, and CCL22 [20]. In addition, CCL20 recruits dendritic cells (DCs); CCL22 is a chemoattractant of regulatory T-lymphocytes (T_{regs}), and CXCL1, CXCL5, CXCL6, and CXCL8 mobilize polymorphonuclear (PMN) leukocytes [21, 22]. In addition to chemokines, cytokines and growth factors are involved in the recruitment of inflammatory cells. For example, TAMs are mobilized by (besides CCL2, CCL5, CCL7, CXCL8, and CXCL12 chemokines) VEGF and platelet-derived growth factor (PDGF) cytokines as well as M-CSF [23]. The CSF-1 cytokine, produced by monocytes, macrophages, and other cells, recruits macrophages to the tumor. In contrast, endothelial monocyte-activating polypeptide II (EMAP II) is a pro-inflammatory cytokine that recruits macrophages to necrotic and apoptotic areas of the tumor to remove dead cells [20].

Mobilization and recruitment processes involve also damage-associated molecular pattern (DAMP) molecules, especially high mobility group box 1 (HMGB1) protein. HMGB1 is passively released from necrotic cancer cells, whereas actively from immune cells. HMGB1 stimulates neutrophils and monocytes to release pro-inflammatory cytokines [13]. HMGB1 is also a proan-

giogenic factor [24, 25]. Under hypoxia conditions, HIF-1α accumulates in cancer cells and induces HMGB1 translocation and secretion. This results in the production of IL-10 and the activation of alternative M2 macrophages [19]. Cancer cells under hypoxic conditions also produce large amounts of lactates that affect the inhibition of immune responses. Low-pH and high-lactate levels reduce the activation of pro-inflammatory macrophages by inhibiting NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity, which in turn affects the activation of T-cells and NK cells [19]. HIF-1 and HIF-2 genetic programs shift oxidative phosphorylation to glycolysis in cancer cells, which results in a change in the concentration of intermediate metabolites, including glucose and amino acids. These changes in the microenvironment not only recruit macrophages but also switch their phenotype into promoting tumor growth [19].

Recruited immune cells undergo a specific reprogramming (polarization) in response to factors secreted by tumor cells (cytokines, growth factors, and chemokines) [10]. This polarization, in fact, consists in the appearance of a specific phenotype in the cells of the inflammatory reaction—broadly speaking—the phenotype that promotes the growth of tumors. The main populations of inflammatory cells promoting tumor growth are tumor-associated macrophages (TAMs), Tie2-expressing monocytes (TEMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and T-cells [7]. The population of macrophages is one of the better-studied cell subsets in which the pro-cancer phenotype appears. The appearance of tumor-promoting phenotype among macrophages is possible due to the extraordinary plasticity of these cells [26].

22.3 The Role of TAM Macrophages in the Tumor Microenvironment

Macrophages are a highly heterogeneous cell population [27]. In tumors, three populations of macrophages may be distinguished: tissue-resident macrophages (arising from yolk sac progenitors), monocyte-derived TAMs, and undifferentiated monocytic-like cells called MDSC [1, 19].

Cancer cells under hypoxic conditions secrete a number of growth factors and chemokines, including CCL2, M-CSF, and VEGF, which recruit monocytes circulating in the bloodstream. In hypoxic conditions, in the presence of factors secreted by microenvironment cells, recruited cells undergo a specific reprogramming. Monocytes differentiate into tumor-specific TAMs [28, 29]. TAMs are one of the most abundant and crucial nonneoplastic cell types in tumor microenvironment [7]. TAMs may constitute up to 50% of tumor mass [23]. The removal of TAMs inhibits the growth of tumors in mice, indicating the involvement of TAMs in tumor progression [19]. TAMs are involved in all stages of tumorigenesis: angiogenesis, immunosuppression, matrix remodeling, invasiveness, and metastasis (Fig. 22.1) [28, 29]. The phenotype of TAMs is similar to that of M2 macrophages [7, 21, 29, 30]. The formation of the M2 phenotype depends on Th2 cells, which are the source of IL-4 and IL-13, and also tumor cells, cancer-specific fibroblasts (CAFs), and T_{reg} lymphocytes, which produce transforming growth factor-β (TGF-β) and IL-10. CAFs secrete CC chemokine ligand 2 (CCL2) that recruits macrophages to the tumor. In addition, they release stromal cell-derived factor 1 (SDF-1)/CXCL12, which is a macrophages chemoattractant and affects their polarization toward M2 phenotype [7]. Tumor-infiltrating macrophages produce autocrine factors CXCL12, IL-10, and migration inhibitory factor (MIF) that affect TAM self-polarization. The components of the extracellular matrix, including biglycan and hyaluronan, are also important factors in the polarization of TAMs [29].

TAMs play different roles in tumor environment [7, 9, 31]. They directly affect the growth of cancer cells by promoting the process of angiogenesis and resistance of cancer cells to chemotherapy and indirectly by inducing dysfunctions of the immune system [4, 7]. TAMs synthesize EGF, which stimulates the growth of cancer cells. They release proangiogenic factors (VEGF, PDGF and TGF-β) and several FGF (fibroblast growth factor) family factors. TAMs stimulate

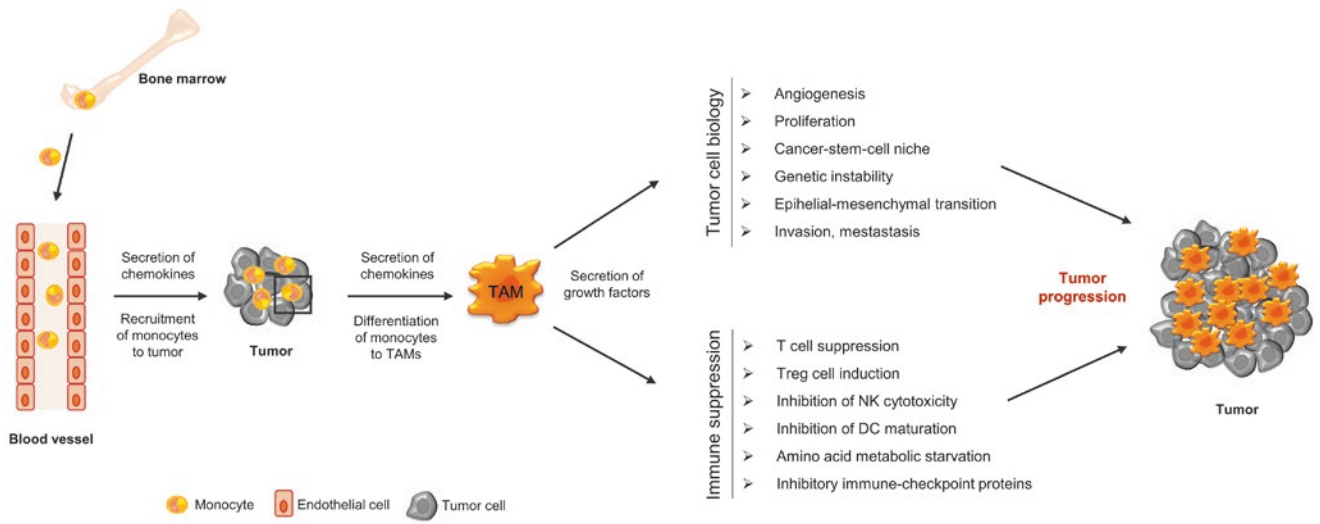

Fig. 22.1 The role of TAMs in cancer progression. Cancer cells secrete a number of cytokines and chemokines that affect the tumor microenvironment. Signals of the inflammatory reaction recruit immune system cells, including monocytes. Recruited monocytes undergo specific reprogramming in the presence of factors secreted by tumor cells. Monocytes are transformed into macrophages specific for tumors (TAMs). TAMs are involved in tumor progression: angiogenesis, immunosuppression, matrix remodeling, invasiveness, and metastasis (Data from Tariq et al. [28] and Mantovani et al. [9])

immunosuppression (IL-10) [11, 29]. By secreting CCL17 and CCL22 chemokines, TAMs recruit T-lymphocytes (T_{reg} and Th2) and inhibit $CD4^+$ and $CD8^+$ cell effector functions [7, 32]. TAMs secrete a CCL8 chemokine that recruits "naïve" T-lymphocytes. In tumor environment, these lymphocytes become anergic. TAMs also inhibit NK cell cytotoxicity by secretion of TGF-β [7].

TAMs accumulate in hypoxic areas [30, 31]. Macrophages are recruited into the hypoxic areas of the tumor, by tumor cells that secrete chemoattractants, i.e., VEGF, endoglin, and CCL2 [33]. The hypoxic environment increases the expression of M2 pro-tumor genes of TAMs [34]. Under such conditions, TAMs induce transcription factors HIF-1α, VEGF, and CXCL12 (and its receptor CXCR4), which modulate TAM migration into avascular regions [10, 28]. HIF-1α controls the expression of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1). At low concentrations of IFN-γ, transcription factor HIF-2α induces the expression of Arg1, inhibits the synthesis of NO, and favors the formation of Th2 phenotype. Under high IFN-γ concentration, HIF-1α dominates. The latter stimulates induction of iNOS, which metabolizes arginine to NO and leads to the appearance of Th1 phenotype [10]. The accumulation of TAMs in hypoxia regions correlates with angiogenesis and the invasive phenotype [35].

TAMs release immunosuppressive cytokines (TGF-β and IL-10) and synthesize the immunosuppressive arginase 1 enzyme [1, 30, 36]. These cytokines and arginase exert considerable effect on the growth of cancer cells. TGF-β stimulates M1 to M2 polarization of macrophages and inhibits the cytolytic activity of NK cells, as well as migration and activity of dendritic cells. TGF-β stimulates the differentiation of $CD4^+$ T-cells to Th2 and blocks the activity of $CD8^+$ T-cells by inhibiting the activity of granzyme A and B, as well as of IFN-γ. TGF-β also promotes the activity of T_{reg} lymphocytes [11, 20, 36]. In addition, TAMs induce T_{reg} lymphocytes via prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) as well as chemotactic factors CCL17, CCL18, and CCL22 [29]. TAMs inhibit the activation of $CD8^+$ lymphocytes mainly through several mechanisms: removal of metabolites important for T-cell proliferation, inhibition of T-cell function through the production of anti-inflammatory cytokines, and activation of T-cell checkpoint blockade by blocking inhibitory receptors [4].

TAMs are programmed to release proangiogenic factors and enzymes involved in the formation of blood vasculature [11, 29, 36]. Proangiogenic agents include, among others, VEGF, PDGF, TGF-β, and FGF, whereas enzymes modifying extracellular matrix (ECM) are matrix metalloproteinase (MMP)-2, MMP-7, MMP-9, MMP-12, and "plasmin system." MMP-9 metalloproteinase releases proangiogenic factors sequestered by extracellular matrix proteins [11, 30]. TAMs also participate in the formation of vascular junctions [37] and play a major role in the creation of the so-called angiogenic switch [20, 38, 39]. As a result of this switch, tumors shift from avascular type of growth to vascular one (and become dependent on the formation of own blood vascular supply). TAMs, which synthesize VEGF-C and VEGF-D, also participate in the formation of lymphatic vessels [11].

22.4 Polarization of the Microenvironmental Cell Phenotype

Macrophages possess a dual nature (thus, they have been called "a double-edged sword") (Fig. 22.2): under certain conditions, they are cytotoxic and eliminate cancer cells (e.g., M1 macrophages), while under others, they stimulate tumor growth being proangiogenic and immunosuppressive (e.g., TAMs (M2)) [26, 28, 30, 40, 41].

Polarization of macrophages depends on environmental context of various signals secreted by both cancer and other tumor *milieu* cells [1, 9, 42]. The signals may be divided into immune signals (e.g., IL-4, IL-13, IL-10, TFN-α, CCL2, periostin (POSTN), CSF-1), tumor cell death signals (e.g., fragments of nucleic acids, ATP,

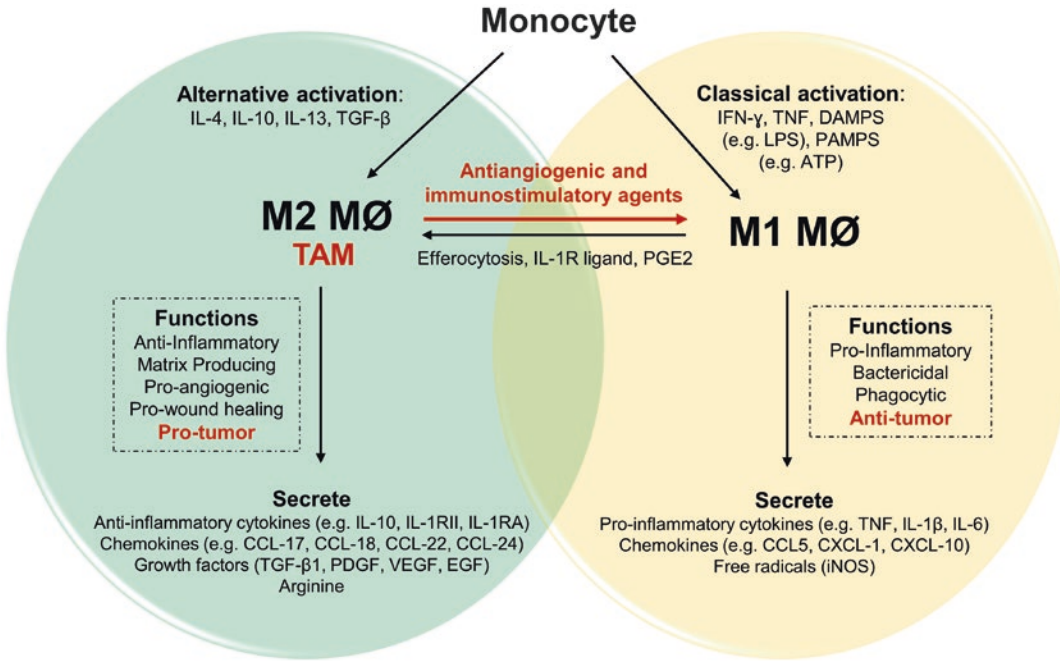

Fig. 22.2 The polarization of M1 and M2 macrophages. Monocytes may be activated at the classical (M1) or at an alternative way (M2). The M1 phenotype is a pro-inflammatory phenotype. Cells with this phenotype have the ability to phagocytosis. The M2 phenotype can "turn off" the inflammatory response and promote the emergence of new blood vessels (acc. to Hesketh et al. [41], changed). The phenotype of tumor-associated TAM macrophages is similar to M2. The combination of antiangiogenic factors with immunostimulating agents converts the phenotype of TAMs from proangiogenic and immunosuppressive M2 to antiangiogenic and immunostimulatory M1: tumor-suppressive phenotype

HMGB, calreticulin), and tumor metabolism signals (e.g., lactate). Signals of the surrounding environment determine the polarization of TAMs [1]. Depending on certain signals' domination, macrophages present either M1 or M2 phenotype. The domination of IFN-γ results in the appearance of M1 phenotype. On the other hand, IL-4/IL-13 and TGF-β in tumor microenvironment induce M2 phenotype (TAM) in macrophages [28, 43]. In a hypoxic environment, macrophages display the M2 phenotype. In contrast, M1 macrophages are present in well-oxygenated areas [19]. The process of angiogenesis and normalization of tumor blood vessels is associated with dynamic changes in TAM phenotype [33, 44, 45]. When M2 macrophages participate in the formation of abnormal, dysfunctional blood vessels, M1 are involved in the process of normalization of irregular tumor vascular network [33, 39, 44–46].

Interactions between innate (among others by macrophages) and adaptive (including T-lymphocytes) immune system are essential in preventing cancer progression [4]. M2 macrophages display immunosuppressive properties and affect lymphocyte infiltration. They produce chemokines, including CCL-17 and CCL-22, which recruit regulatory T-cells (T_{regs}) and Th2 cells and inhibit Th1-mediated response [32]. They also inhibit the activation of $CD8^+$ lymphocytes, whereas M1 macrophages increase the recruitment and activation of $CD8^+$ and NK cells [7, 47]. M1 macrophages induce tumor infiltration by T-lymphocytes and increase their ability to kill cancer cells [47]. M1 macrophages affect NK cells by cell-to-cell as well as through soluble interactions. This leads to the activation of NK cell cytotoxicity [48].

Dual (bipolar) phenotypes are exhibited also by other cells of the immune system. Depending

on circumstances, such cells display a phenotype that either inhibits tumor growth or stimulates it [13]. The presence of TGF-β, a strong immunosuppressant and proangiogenic factor, in tumor *milieu* results in tumor-associated neutrophils (TANs) becoming cells that stimulate tumor growth (type II) [49]. *Milieu* lacking TGF-β causes neutrophils to participate in the elimination of cancer cells (type I) [50]. Dual nature is also shown by NKT cells [51], dendritic cells [52], mast cells [53], T_{reg} cells [54], and NK cells [55, 56].

22.5 Reversion of Tumor Microenvironment

Reprogramming of the microenvironmental cell phenotype, including macrophages, has a therapeutic meaning [4, 43]. There are at least three main therapeutic approaches to modify TAMs: (1) reduction of the presence of TAMs, (2) prevention of the accumulation of TAM, and (3) induction of functional TAM reprogramming toward the anticancer phenotype [4, 28–30, 57]. Trabectedin is one of the anticancer factors that affect the survival of TAMs. It is an agent cytotoxic for mononuclear phagocytes. It activates caspase 8, which is essential in monocyte apoptosis [4, 7, 9, 57]. In addition, anti-204 immunotoxin directed against scavenger receptor A (204) overexpressed on the surface of TAMs is a promising target. After administration, it eliminates TAMs and inhibits tumor progression in mice bearing peritoneal ovarian cancer [7]. Another promising strategy is the inhibition of TAM accumulation by targeting factors that affect the differentiation of monocytes into tumor-suppressive M1 or tumor-promoting M2. Among these factors are tumor-derived chemokines CCL2 and CSF-1 [4, 9]. Bindarit, a CCL2 inhibitor, significantly restrains the recruitment of M2 and the growth of tumors in human melanoma. In contrast, CSFR-1 inhibitors restrain M2 infiltration and improve the effectiveness of chemotherapy with an increased response of $CD8^+$ cytotoxic lymphocytes [4, 7, 27, 30, 57].

However, the main goal of the new therapeutic strategies is targeting the tumor-promoting functions of TAMs rather than TAMs per se [1]. Due to the plasticity of macrophages, through the manipulation of environmental factors, the polarization of macrophage phenotype from M2 to M1 may be affected [4, 28, 57]. For example, by administering IL-12 or polyI:C, a conversion of tumor-promoting toward tumor-inhibiting macrophages was observed [7]. Reprogramming of macrophages toward M1 may also result in the normalization of tumor blood vessels. It improves the drug delivery process by increasing tumor blood supply [6, 33, 39]. Rolny et al. [44] observed that histidine-rich glycoproteins through downregulation of PlGF repolarizes M2 macrophages toward M1, which leads to the stimulation of antitumor response and vessel normalization. Similar results of TAM repolarization have been observed in our experiment where endoglin-based DNA vaccine in combination with interleukin 12 (IL-12) was used [46]. Endoglin (ENG) is overexpressed not only on the surface of activated vascular endothelial cells but also on some cancer cells (among others, B16-F10) [58–61]. Endoglin plays important roles in vascular remodeling [62] and blood vessel maturation during angiogenesis [63]. ENG-based DNA vaccine inhibits angiogenesis [60]. IL-12 gene therapy, in turn, acts as immunostimulant [64–66]. The combination of endoglin-based DNA vaccine with interleukin 12 repolarizes TAM phenotype from M2-like (protumor) into M1-like (antitumor), which affects the structure of tumor blood vessels (improves tumor vessel maturation and perfusion and reduces hypoxia), enhances tumor immune cell infiltration ($CD4^+$, $CD8^+$ lymphocytes, and NK cells), improves the effect of chemotherapy, and leads to tumor growth regression [46]. After administration of 5,6-dimethylxanthenone-4-acetic acid (DMXAA), we also observed a change in TAM phenotype. 5,6-Dimethylxanthenone-4-acetic acid ((DMXAA) also known as ASA404 or vadimezan) is a xanthene, which induces apoptosis in tumor vascular endothelium cells, that results in necrosis appearance at tumor core. DMXAA, besides destroying existing ves-

sels, stimulates the immune response in mice. The stimulation is carried out by reprogramming proangiogenic and immunosuppressive M2 macrophages toward cytotoxic M1 phenotype [67, 68]. Our studies have shown that DMXAA increases the levels of M1 macrophages in tumors and inhibits the tumor growth [69].

22.6 Instead of Conclusion

In general, the tumor microenvironment determines two main processes: the formation of new blood vessels (angiogenesis) and the escape of tumor cells from the immune surveillance (immunosuppression). During progression, cancer cells recruit and reprogram normal cells. Reprogrammed cells become involved in all stages of cancerogenesis. TAMs, which are the main cells of the immune system involved in tumor progression, display remarkable plasticity. TAM phenotype is similar to M2 macrophage phenotype. New therapeutic strategies take advantage of the possibility of TAM reversion from the proangiogenic and immunosuppressive phenotype of M2 to the antiangiogenic and immunostimulatory M1 that inhibits tumor growth. We believe that this therapeutic approach deserves attention and requires closer scrutiny.

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Stanisław Ryszard Szala was born on May 16, 1942, in Kremenets in Volhynia. He spent his childhood in Gorzów Wielkopolski. He studied at the University of Lodz, Faculty of Biology and Earth Sciences. He worked at the Institute of Oncology in Gliwice for 43 years, where he held the positions of head of the Department of Molecular Biology and the scientific director . A member of Polish Academy of Arts and Sciences and scientific councils of numerous institutes, scientific foundations, and periodicals. Author of books and over a hundred scientific papers on genes, gene therapy, and tumor blood vessels, published in world scientific journals: Nature, PNAS, Gene Ther., and PLOS One. He held numerous internships abroad—over 5 years in Paris, Copenhagen, Edinburgh, Bethesda, and Philadelphia. Winner of numerous scientific award in the country and abroad, including the award of Polish Academy of Science, Polish Academy of Arts and Sciences, European Association for Cancer Research, and the American Association for Advancement of Science. He was awarded the Gold Cross of Merit and the Knight's Cross of the Order of Polonia Restituta. He also devoted himself to the education of young researchers whom he used to call his children. Promoter of 21 Ph.D.s, last in June 2018, and supervisor and reviewer of over 70 M.A. theses. A contemporary Renaissance man: violist, painter, and poetry and philosophy lover. He loved classical music, especially Bach's fugues and the Goldberg Variations. He painted a series of watercolors, which were exhibited in Gorzów Wielkopolski in September 2018. Professor Szala passed away on November 9, 2018.

23 Immunotherapies Targeting a Tumor-Associated Antigen 5T4 Oncofetal Glycoprotein

Peter L. Stern

Contents

23.1 Introduction

Historically, a starting place for developing any immunotherapy was the identification of a suitable tumor-associated target antigen. Such targets need to show selective expression in tumors compared to normal tissues. Neoantigens are generated as a result of specific mutations (e.g., *p53*) or translocations (e.g., *BCR-ABL*) or oncogenic viruses (e.g., HPV 16 E6 and E7) associ-

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ated with mechanisms of carcinogenesis as well as the frequent genomic instability that occurs in tumor evolution. In addition, re-expression of embryonic products by tumor cells (oncofetal antigens; e.g., CEA) or aberrant overexpression of adult molecules can also be useful immune targets where there is no immune tolerance. TAAs which are characteristic of a range of different tumor types provide for wide usage of any developed therapy although the idiotypic antigens of tumors can also be targeted in a personalized medicine approach. This chapter will focus on the identification of an oncofetal antigen, 5T4, and its use as a target for multiple immunotherapeutic strategies in human cancer.

23.1.1 5T4 Trophoblast Glycoprotein Is an Oncofetal Antigen

The 5T4 oncofetal glycoprotein was identified by searching for shared surface molecules of human trophoblast and cancer cells with the rationale that they may function to allow survival of the fetus as a semi-allograft in the mother or a tumor in its host. It was hypothesized that such functions would be likely to include those concerned with growth, invasion, or altered immunosurveillance in the host.

Purified glycoproteins from human syncytiotrophoblast microvillous plasma membranes were used as an immunogen to raise monoclonal antibodies (mAbs) which were screened for binding to trophoblast and different tumor cell lines but not normal human peripheral blood mononuclear cells [1]. Subsequently, immunohistochemistry established that the specific mAb (mAb-h5T4) detected expression by many different types of carcinoma but only low levels in some normal tissue epithelia [2, 3]. Further biochemical and genomic studies established the molecules as approximately 72 kDa heavily *N*-glycosylated proteins encoded on the long arm of chromosome 6 at q14–15 [4–6]. Importantly, there was a useful expression profile in many different primary and metastatic cancers characterized by high tumor levels, but in some cases, there was an additional stromal expression. The cancers characterized include cervical [3], cervical precancer [7], colorectal [8–10], gastric [11, 12], ovarian [13], oral [14], prostate [15], lung [16, 17], renal tumors [18], and some others [19]. For colorectal, gastric, and ovarian cancers, there was evidence of tumor expression levels correlating with poorer clinical outcome. Studies in non-small-cell lung carcinoma have shown that among heterogeneously positive tumor cells, 5T4-expressing subpopulations are markedly enriched for tumor-initiating cells [16]. Such cells reflect the sustained properties of normal tissue renewal and are exploited by the cancer to maximize survival and proliferation [20]. Importantly, the presence of 5T4 tumor-initiating cells is associated with poorer clinical outcome possibly derivative from their ability to avoid treatment-induced toxicity and correlated with their increased clonogenicity [16]. 5T4 expression has recently been shown to correlate with the risk of relapse in pre-B acute lymphoblastic leukemia (ALL) patients [21]. The high-risk cytogenetic category patients showed significantly higher 5T4 transcript levels than the low-risk or "other" groups. Flow cytometric analysis determined that bone marrow from relapse patients have a significantly higher percentage of 5T4-positive leukemic blasts than healthy donors. Several reports based on xenotransplantation of ALL in NOD/SCID mice have led to the hypothesis that ALL may be maintained from a rare subpopulation of leukemia-initiating cells (LICs) [20]. It is possible that 5T4 might be a marker of such LICs and correlate with relative resistance to chemotherapy including through increased ability to migrate to extramedullary sites providing for disease relapse following treatment.

Isolation of the human gene coding for the 5T4 protein showed that it was a member of the leucine-rich repeat (LRR)-containing family of proteins [22] (Fig. 23.1). The latter motif is associated with protein-protein interactions of a functionally diverse set of molecules [23]. The extracellular part of the 5T4 molecule has ~3.5 LRRs in two domains separated by a short hydrophilic sequence with each domain having N- and C-terminal LRR flanking region motifs; there is a transmembrane domain and a short cytoplasmic sequence. Overexpression of the 5T4 gene in different cell types provided the first indications of

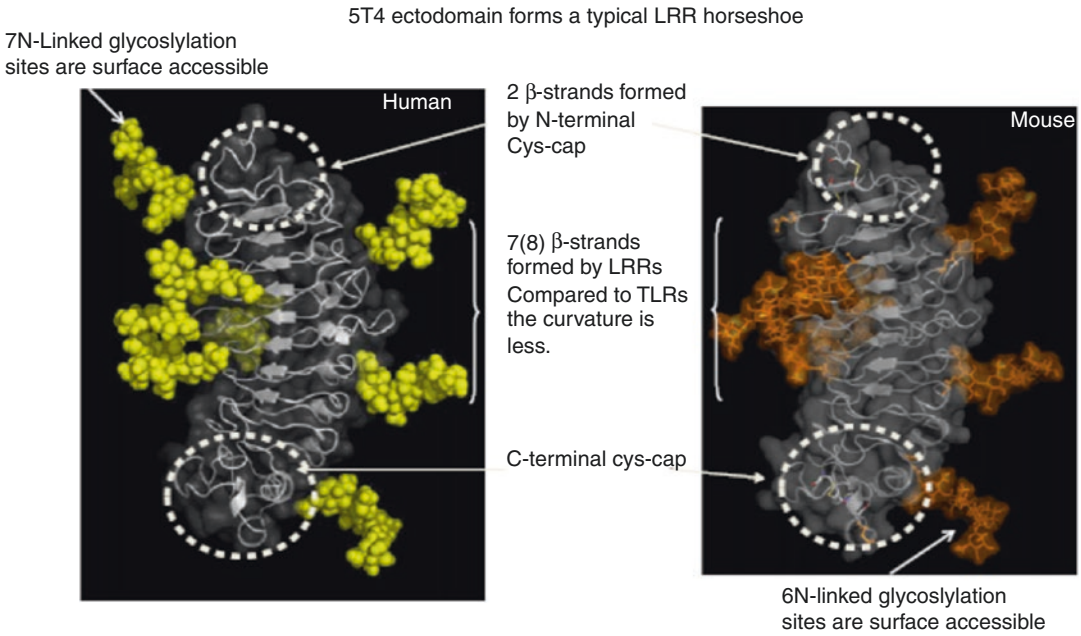

Fig. 23.1 Structure of 5T4 molecules. Human and mouse 5T4 analyzed by a homology modeling approach using the variable lymphocyte receptor A29 (PDB entry 2o6q) and energy minimized to produce RAW structures (Courtesy of Alex Weber and Andriy Kubarenko, DKFZ, Germany)

functionality relevant to cancer spread. Constitutive expression of human 5T4 cDNA in murine fibroblasts showed 5T4 to be found on the tips of microvilli and induced a more spindle-shaped morphology, disruption of cell contacts, and a reduction in adherence [24]. Similar changes occurred when h5T4 was overexpressed in normal murine epithelial cells where there was also clear evidence of E-cadherin downregulation, increased motility, and cytoskeletal disruption dependent on the intracellular part of 5T4 [25]. Furthermore, a yeast two-hybrid screen using the 5T4 cytoplasmic domain as a probe identified a PDZ domain-containing interactor, TIP2/GPIC, which is known to mediate links to the actin cytoskeleton [26]. The isolation of the murine 5T4 gene confirmed its evolutionary conservation and provided additional tools for evaluating 5T4-targeted immunotherapies [27, 28].

These expression patterns and mechanistic studies supported the use of 5T4 as a suitable target for several different types of immunotherapy. More recently, further insights into the function of 5T4 in modulating cancer spread have been established.

23.2 5T4 and Epithelial Mesenchymal Transition (EMT)

EMT occurs during embryonic development and is important for the metastatic spread of epithelial tumors [29]. The 5T4 oncofetal antigen is an early marker of differentiation of mouse and human embryonic stem (ES) cell [30–32]. This process is also an EMT-like event characterized by the differentiation of ES cells in monolayer culture associated with an E- to N-cadherin switch, upregulation of E-cadherin repressor molecules (Snail and Slug proteins), and increased matrix metalloproteinase (MMP-2 and MMP-9) activity and motility [33, 34]. Interestingly, undifferentiated E-cadherin KO ES cells constitutively express surface 5T4, while abrogation of E-cadherin-mediated cell-cell contact in undifferentiated ES cells using neutralizing antibodies results in increased motility, altered actin cytoskeleton arrangement, and a mesenchymal phenotype with cell surface expression of 5T4 molecules [33, 34]. These data and

our previous observations showing 5T4 overexpression in epithelial cells associated with downregulation of E-cadherin [25] suggest that the latter functions to prevent cell surface localization of 5T4 possibly by stabilizing cortical actin cytoskeletal organization.

23.3 5T4 Modulation of Chemokine and Wnt Signaling Pathways

To further investigate additional changes on early ES differentiation, a comparative microarray analysis of undifferentiated (5T4 –) and early differentiating (5T4 +) murine ES cells was performed. One particular transcriptional change identified was the downregulation of transcripts for the dipeptidyl peptidase IV, CD26, which codes for a cell surface protease that cleaves the chemokine CXCL12 [35]. CXCL12 binds to the widely expressed cell surface seven transmembrane domain G-protein-coupled receptor CXCR4 [36] and to the recently identified receptor CXCR7/RDC1 [37]. Subsequently, 5T4 molecules were shown to be required for functional expression of CXCR4 at the cell surface in some embryonic and tumor cells [17, 21, 38]. Both CXCL12 expression and CXCR4 expression have been associated with tumorigenesis in many cancers including breast, ovarian, renal, prostate, and neuroblastoma [36, 39, 40]. These CXCR4-expressing tumors preferentially spread to tissues that highly express CXCL12, including the lungs, liver, lymph nodes, and bone marrow. The observation that some mAbs against m5T4 can inhibit CXCL12 chemotaxis of differentiating ES cells and mouse embryo fibroblasts (MEF) suggests a 5T4 contribution at the cell surface facilitating the biological response to CXCL12 through CXCR4. It is apparent that 5T4 is not a simple chaperone providing for trafficking of the receptor to the cell surface since CXCR4 surface expression depends on microtubules, whereas 5T4 does not [38]. Further, FRET studies do not support a direct interaction between the molecules, while preliminary proteomic analysis following cross-linking of 5T4 molecules indicates many cytoskeleton-associated interactions [41] (Vaghjani and Stern, unpublished). This regulation of CXCR4 surface expression by 5T4 molecules provides a novel means to control responses to the chemokine CXCL12, for example, during embryogenesis, but can also be selected to advantage the spread of a 5T4-positive tumor from its primary site. Interestingly, the absence of 5T4 expression is associated with CXCR7 expression (the other CXCL12 receptor) in embryonic cells and some human tumors [17]. This receptor has a higher affinity for its ligand and activates a different signaling pathway involving transactivation of the EGR receptor with stimulation of proliferative or anti-apoptotic rather than chemotactic pathways [17]. A functional scenario could include that at the periphery of a tumor, surface 5T4 expression favors a chemotactic response to a CXCL12 chemokine gradient and spread toward local vasculature, whereas at the center, 5T4-negative parts may respond through proliferation to the same, albeit weaker, stimulus.

Using B-ALL cell lines (Sup 5T4 + and Sup 5T4 –) derived from Sup-B15 (BCP-ALL), 5T4 expression was shown to correlate with a more immature ALL phenotype, CXCR4/CXCL12 chemotaxis, increased invasion, and adhesion in vitro. Significantly, following intraperitoneal challenge of immunocompromised mice while both Sup and Sup5T4 cells most often migrated to and expanded within the gonadal fat tissue, Sup5T4 cells had a much greater propensity to spread to the omentum and ovaries [21]. In addition, patient-derived BCP-ALL 5T4-positive cells show preferential ability to overcome a NOD-*scid* IL2R γ^{null} mouse xenograft barrier, migrate in vitro on a CXCL12 gradient, preferentially localize to bone marrow in vivo, and display ability to reconstitute the original clonal composition on limited dilution engraftment in xenografts [42]. It is possible that 5T4 might be a marker of putative LICs and correlate with relative resistance to chemotherapy including through increased ability to migrate to extramedullary sites providing for disease relapse following treatment.

Cellular regulation through Wnt protein signaling is an important factor in development and normal tissue homeostasis, but aberrant signaling can lead to disease including cancer [43]. We

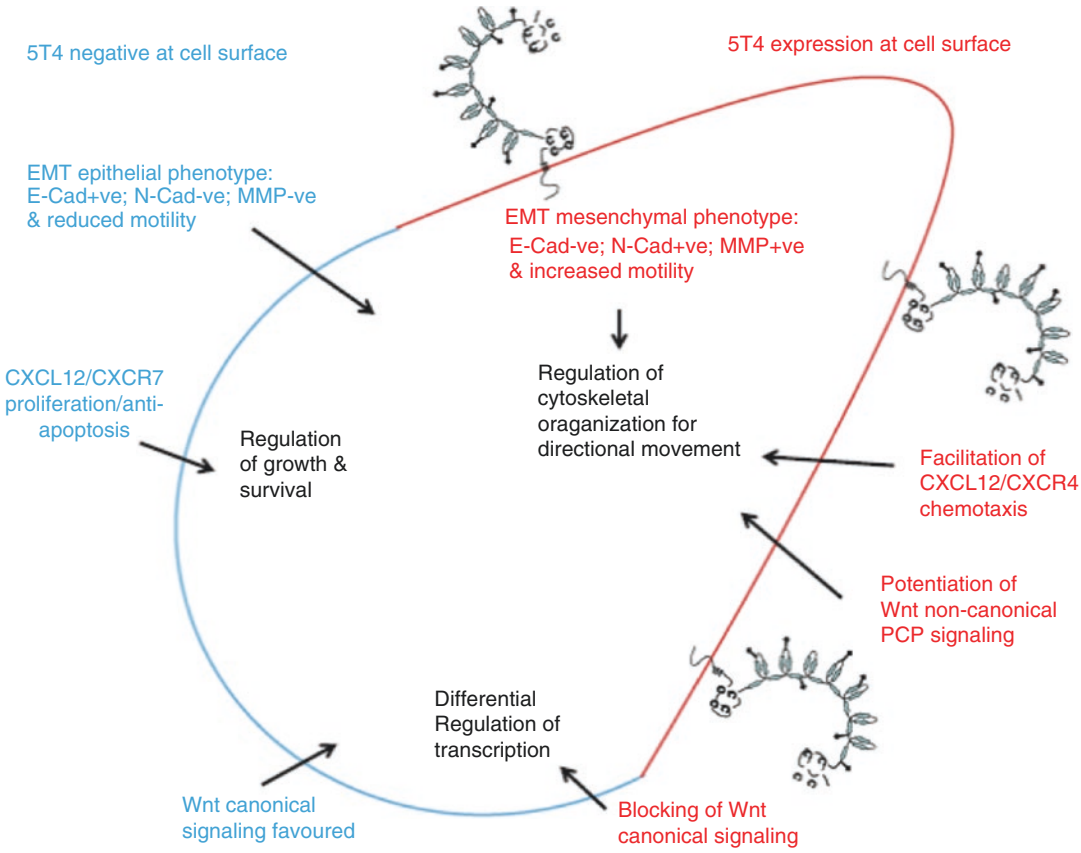

Fig. 23.2 5T4 functional influences on tumor spread. Integrated 5T4 regulation of both the chemokine and Wnt pathways acts to promote cancer spread as well as functional migration in development and cancer

have recently shown that 5T4 inhibits Wnt/β--catenin canonically while concomitantly activating the noncanonical Wnt signaling pathway associated with increased motility [44]. 5T4 interferes with canonical signaling by binding to the Wnt coreceptor LRP6 which then blocks Wnt-induced LRP6 internalization that is required for activation of the Wnt-β-catenin pathway. A 1.8 Å resolution of an 5T4 extracellular domain crystal has confirmed the structural basis of this 5T4 inhibition [45]. At the same time, 5T4 enhances the β-catenin-independent Wnt signaling through promoting a noncanonical function of Dickkopf-1 influencing the actin and microtubular skeleton [44, 46].

It is likely that the integrated 5T4 regulation of both the chemokine and Wnt pathways acts to promote cancer spread as well as functional migration in development (Fig. 23.2).

23.4 Vaccines

When recently assessed by the National Cancer Institute priority ranking methodology for TAAs as vaccine targets based on predetermined and preweighted criteria [47], 5T4 was found to rank 9/75 which is above NY-ESO-1, CEA, gp100, PSA, and p53 [19]. Its favorable properties include a good tumor/normal tissue expression profile, an association with tumor-initiating subpopulations, and its several functional attributes that enhance metastasis. Viral vector-based immunotherapy aims to overcome the relative poor immunogenicity of TAAs by presenting the antigens in a foreign viral vector with the principal goal of generating effector T-cells able to kill 5T4-positive tumors. Lack of high-avidity T-cell receptors (TCRs) in the T-cell repertoire and specific or nonspecific T regulatory cells may be

major limiting factors for vaccine immunogenicity and effectiveness. The highly attenuated and modified vaccinia virus Ankara (MVA) strain was an early choice for the viral vector to express either human or mouse 5T4 and evaluation of immunogenicity and antitumor activity in preclinical studies.

23.4.1 Preclinical Studies

Immunization of mice with MVA-h5T4 and MVA-m5T4 constructs induced antibody responses to human and mouse 5T4, respectively. Mice vaccinated with MVA-h5T4 were protected when challenged with syngeneic tumor line transfectants expressing h5T4. In active treatment studies, inoculation with MVA-h5T4 was able to treat established CT26-h5T4 lung tumor and to a lesser extent B16.h5T4 subcutaneous tumors [48]. In this xenogeneic-TAA model, it was shown that the likely component of protection was antibody with induction dependent on the $CD4^+$ T-cells [49]. Vaccination of mice with MVA-m5T4, a perhaps more relevant model for human cancers, was able to control the growth of autologous B16 cells expressing m5T4 in a tumor protection scenario. Furthermore, mice vaccinated with MVA-m5T4 showed no signs of autoimmune toxicity [48].

Further studies investigated the human T-cell repertoire. Human $CD8^+$ T-cells recognizing HLA-restricted 5T4 peptides have been identified by methods using monocyte-derived dendritic cells (DC) to stimulate peripheral blood lymphocytes from healthy individuals in the absence of $CD4^+$ T-cells [50, 51]. These data are consistent with the influence of Tregs on limiting immune responses to TAA [52]. Subsequently, it was shown that the generation of $CD4^+$ cells recognizing 5T4 peptides also required initial depletion of T regulatory cells. Interestingly, $CD4^+$ T-cells spontaneously recognizing a 5T4 epitope restricted by HLA-DR were identified in tumor-infiltrating lymphocytes from a regressing renal cell carcinoma (RCC) lung metastasis. These cells produced both interferon gamma (IFN-γ) and IL-10 suggesting that such h5T4-specific $CD4^+$ T-cells boosted or induced by vaccination could act to modulate both cell- or antibody-mediated antitumor response either positively or negatively depending on the differentiation status of the T-cell [53].

23.4.2 Early-Phase Clinical Trials of MVA-h5T4 (TroVax)

The preclinical data supported the development of TroVax for tumor immunotherapy. A succession of phase I or II clinical trials in colorectal cancer, prostate cancer, and RCC patients (including with chemotherapy or cytokine treatments) established the optimal dose and route of vaccination as well as safety, tolerability, and vaccine immunogenicity (serology, lymphocyte proliferation, and ELISPOT assays). Two or three TroVax immunizations were needed to generate somewhat transient 5T4-specific cellular immunity, and this was independent of the vector-specific response leading to a protocol of multiple booster vaccinations. In several trials, there was evidence of association of 5T4 immune responses with better clinical outcome albeit in relatively small study sizes (summarized in Table 23.1). For example, in a clinical trial of TroVax in patients undergoing surgical resection of colorectal cancer liver metastases, 17 of 19 colorectal cancer patients showed 5T4 expression in the liver metastases or surrounding stroma, and 18 mounted a 5T4-specific cellular and/or humoral response. In patients who received at least four vaccinations and potentially curative surgery ($n = 15$), those with above median 5T4-specific proliferative responses or T-cell infiltration into the resected tumor showed significantly longer survival compared with those with below median responses [56]. Further investigations assessed the levels of systemic T regulatory cells, plasma cytokine levels, phenotype of tumor-infiltrating lymphocytes including T regulatory cells (Tregs), and tumor HLA class I loss of expression. More than half of the patients showed phenotypes consistent with relative immune suppression and/or escape, highlighting the complexity of positive

Table 23.1 TroVax: early clinical studies of immunogenicity and clinical response

Indication trial (patients)	Patient treatment regime	% 5T4-specific immune response (IR)				Immune and clinical responses (patients with IR measures)	Reference
		Antibody	Proliferation	ELISPOT	Total		
Metastatic colorectal phase 1 (22)	Post chemotherapy	82	88	100	94	Antibody vs. TTP/survival (17)	Harrop et al. [49]
Metastatic colorectal phase II (19)	First line + 5FU/LV/ irinotecan	83	83	92	100	None (12)	Harrop et al. [54]
Metastatic colorectal phase II (17)	First line + 5FU/LV/ oxaliplatin	91	91	91	100	ELISPOT vs. tumor response (11)	Harrop et al. [54]
Metastatic colorectal phase II (20)	Adjuvant to liver metastasis surgery	100	88	53	100	Proliferation vs. survival (17)	Elkord et al. [56]
Prostate-hormone refractory phase 11 (27)	Second line ± GM-CSF	100	Nt	36	100	ELISPOT vs. PFS (24)	Amato et al. [55]
Metastatic renal cell carcinoma phase II (11)	First and second line + IFN-α	100	Nt	36	100	None (11)	Amato et al. [57]
Metastatic renal cell carcinoma phase II (28)	First and second line ± IFN-α	91	Nt	30	91	Antibody vs. survival (23)	Amato et al. [58]
Metastatic renal cell carcinoma phase II (25)	Second-line low-dose Il-2	90	Nt	30	90	ELISPOT vs. survival (20)	Amato et al. [57]
Metastatic renal cell carcinoma phase II (28)	Second-line high-dose IL-2	100	Nt	36	100	Antibody vs. survival (19)	Kaufman et al. [59]

TroVax clinical development overview
nt not tested

and negative factors challenging any simple correlation with clinical outcome [60].

23.4.3 TroVax Phase III Clinical Trial in RCC

Building on the several phase II studies in RCC (Table 2 in Ref. [61]), a phase III trial in RCC patients was designed to determine if the addition of TroVax to available standard of care (SOC) therapy could improve survival for patients with metastatic RCC. This international multicenter trial randomized 733 patients who received seven or eight injections of TroVax ($n = 365$) or placebo ($n = 368$) along with either interferon-α (IFN-α), IL-2, or sunitinib as first-line treatment [62]. The primary end point was overall survival, and progress-free survival, objective response rate, and safety were secondary measures. When the survival data was censored, there was a median follow-up of 12.9 months. While TroVax was safe and well tolerated in all these patients, it failed to meet its primary end point, as there was no significant difference in survival for the TroVax- and placebo-treated groups. However, in

the subset of patients with a good prognosis (Motzer grade 0) receiving IL-2, there was a significantly improved survival with TroVax compared to the placebo group. No other SOC subset, albeit less mature, showed evidence of a TroVax benefit. Analysis of a selected group of 50 TroVax-vaccinated patients with the highest increase in 5T4 antibody responses showed a favorable survival compared to placebo patients, while a similar group with the highest increase in MVA antibody did not.

5T4 antibody response was quantified after the third and fourth vaccinations, and an immune response surrogate (IRS) was constructed and then used to evaluate survival benefit in 590 patients from the phase III study. A high antibody response was associated with longer survival within the TroVax-treated group. The IRS was derivative from a linear combination of pretreatment 5T4 antibody levels, hemoglobin, and hematocrit and was able to predict patient benefit in the phase III study. Importantly, the IRS was associated with antibody response and survival in independent data sets from other TroVax trials [63, 64]. Further statistical modeling identified several baseline clinical factors associated with inflammatory anemia (CRP, hemoglobin, hematocrit, IL-6, ferritin, platelets), which demonstrated a significant relationship with tumor burden and survival. From these prognostic factors, the mean corpuscular hemoglobin concentration (MCHC) was shown to be the best predictor of treatment benefit and was positively associated with tumor shrinkage in different clinical studies of TroVax in vaccinated patients. These results support a view that patients with a relatively small tumor burden and high MCHC would be most likely to benefit from TroVax vaccination [65]. However, our studies in colorectal cancer patients with liver metastasis highlighted a multiplicity of immune regulatory factors that can negatively influence the outcome of patients even with effective immunogenicity of the vaccine [53, 60].

TroVax has now been tested in over 500 patients in ten different clinical trials, and in most patients, antibody responses are induced, whereas cellular T-cell responses are less frequently detected (reviewed by Kim et al. [61]). A desired goal of vaccination is the generation of 5T4 effector $CD8^+$ T-cells although the most frequently used T-cell assay was proliferation which probably reflects a CD4 response. Only relatively rarely have high-frequency $CD8^+$ T-cell responses been definitively demonstrated by ELISPOT. The available evidence from the TroVax clinical studies has suggested that the use of the same vaccine for priming and multiple boosting does not limit the 5T4 immune response as a result of anti-vector responses. However, preclinical studies of different prime/heterologous boost vaccine combinations (replication-defective adenovirus (rAd) and retrovirally transduced DC lines expressing h5T4) have shown that the order of immunization can influence the overall therapeutic efficacy by the generation of different 5T4-specific cellular immune responses in tumor-bearing mice [66]. In particular, a role for Tregs in limiting the therapeutic value of vaccination was demonstrated. The use of the complete 5T4 coding sequence in the vaccine construct could provide epitopes able to stimulate both regulatory and effector T-cell responses.

23.4.4 Insights from the 5T4 KO Mouse

A recent study exploited the 5T4 knockout (KO) mice to analyze the mechanisms by which endogenous expression of 5T4 influences autologous T-cell immunity and tolerance [67]. While the 5T4 KO mice show no obvious changes in T-cell, B-cell, and/or myeloid populations, 5T4 is expressed in murine thymus and thus might influence the repertoire and/or induction of specific Tregs cells leading to the control of natural or vaccine-induced immunity [68]. Mouse 5T4-specific T-cell epitopes were identified using the 5T4 KO mouse, and wild-type (WT) responses were evaluated as a model to refine and improve immunogenicity. Studying the immune response (INF-γ ELISPOT) of 5T4 KO mice to rAdm5T4 vaccination identified only two dominant H2b-restricted epitopes for which the WT

mouse response was either significantly reduced (only low-avidity CD8) or absent (CD4). Other data suggest the possibility that in the absence of WT 5T4-specific $CD4^+$ T helper cells, there is an alternative differentiation process generating 5T4-specific Tregs. While a single rAdm5T4 vaccination of 5T4 KO mice provides protection against B16m5T4 tumor challenge, there is no effect in WT mice. Treatment of WT mice with folate receptor 4 (FR4) antibody to deplete Tregs [69], after Adm5T4 vaccination, alters the balance of effectors and provides a modest protection against autologous B16m5T4 challenge. These data are consistent with the efficacy of 5T4 and some other TAA vaccines being limited by the combination of TAA-specific Tregs, as well as the deletion and/or alternative differentiation of $CD4^+$ and/or $CD8^+$ T-cells [67]. An alternative to vaccination is the adoptive transfer of tumor-specific lymphocytes. To test the potency of this approach in the m5T4 model, primed 5T4 KO splenocytes were adoptively transferred to naïve WT recipient animals but failed to protect against B16m5T4 tumor challenge. Attempts to in vivo modulate Tregs using FR4 mAb were unsuccessful in achieving major protection against tumor challenge despite the clear evidence of survival of adoptively transferred T-cells. Protocols for clinical adoptive cell therapy now incorporate preconditioning which results in a reduction of suppressor cells and conditions which favor homeostatic expansion [52, 70, 71]. However, a clinical study investigating the adoptive transfer of CD25-depleted (includes Tregs) peripheral blood mononuclear cells in cyclophosphamide/fludarabine preconditioned RCC patients showed that this treatment resulted in only a short period of in vivo Tregs depletion [72].

23.4.5 Improving Vaccine Regimens

The challenge for optimizing 5T4 (and other TAA) vaccine immunogenicity requires a means to stimulate appropriate effector T-cell responses and not concomitantly immunomodulatory cells which may always limit the therapeutic effect. We are exploring the use of 5T4-specific CD8 epitopes engineered into an ImmunoBody DNA as this approach [73] can potentially improve vaccine immunogenicity by favoring generation of high-avidity $CD8^+$ T-cells capable of functioning in an autologous tumor-bearing animals.

Successful licensing of treatment following clinical trials evaluating blockade of the immune checkpoints like CTLA-4 and PD1 is currently driving immuno-oncology [74, 75]. However, the benefits of increased survival are still only seen in a subset of patients. Indeed, in these terms, a study of the Pfizer CTLA-4 antibody, tremelimumab, in 18 patients with metastatic gastric and esophageal adenocarcinomas as a second-line treatment also gave encouraging results [76]. Four patients had stable disease with clinical benefit, and one patient achieved a partial response after eight cycles (25.4 months) and remained well at 32.7 months. Interestingly, de novo proliferative responses to 5T4 (8 of 18 patients) and carcinoembryonic antigen (5 of 13) were detected. Indeed, patients with a posttreatment carcinoembryonic antigen proliferative response had a median survival of 17.1 months compared with 4.7 months for nonresponders. Such in vitro evidence of enhanced proliferative responses to relevant TAAs suggests that combining CTLA-4 blockade with specific vaccination may provide additional benefit [76].

A recent study of a prime boost regime based on simian adenovirus (ChAdOx1) and MVA expressing h5T4 shows that it was able to protect against B16-h5T4 challenge in mice but only delay tumor growth in a therapeutic setting [77]. However, the ChAdOx1/MVA h5T4 vaccination in combination with immune checkpoint inhibition by anti-PD-1 antibody was able to therapeutically delay growth and improve survival [77]. To be effective, cancer vaccines will most likely need to stimulate polyclonal antitumor-specific immune responses as well as avoid stimulating immune suppressive factors. Combinatorial approaches that aim to remove or reduce existing immune suppressive factors can stimulate more effective antitumor activity [78].

Current trial designs for evaluation of TroVax are utilizing biomarker information to target patients most likely to benefit from cancer vaccination [79,

80]. This approach is being implemented in investigator-led studies in prostate cancer (VANCE; NCT02390063), mesothelioma (SKOPOS; NCT01569919), ovarian cancer (TRIOC; NCT01556841), and colorectal cancer (TaCTiCC). In the phase I/II TaCTiCC trial of advanced colorectal cancer patients, TroVax plus low-dose cyclophosphamide (delivered prior to vaccination) led to robust 5T4 immune responses that were associated with improved progression-free and overall survival. Low-dose cyclophosphamide alone also produced strong immune responses that were associated with prolonged remission [81].

23.5 5T4 Antibody-Targeted Superantigen Therapy

Bacterial superantigens such as staphylococcal enterotoxin A (SEA) can activate T-cells by linking the latter through binding to a particular family of V-beta chain containing TCRs to MHC class II molecules on antigen-presenting cells. With an antibody-superantigen fusion protein, large amounts of cytotoxic and cytokine-producing T-cells can be targeted by the antibody specificity for a TAA for in vivo tumor treatment [82, 83]. Challenges in developing safe and efficacious therapy for cancer depend on selection of a suitable TAA, overcoming the toxicity associated with MHC class II binding, and any preexisting immunity to the bacterial protein [84].

23.5.1 Preclinical Studies

A first-generation 5T4 mAb-derived Fab-SEA fusion (ABR-214936) incorporated a point mutation in the SEA sequence reducing the affinity for binding to MHC class II molecules and optimized for bacterial production [85]. This agent (ABR-214936) maintained 5T4-specific superantigen antibody-dependent cellular cytotoxicity (SADCC), while toxicity for MHC class II-expressing cells was reduced by 1000-folds in vitro (SDCC); therapeutic efficacy was demonstrated in murine xenograft tumor models [86]. Recently, a humanized 5T4 scFv fused to streptococcal pyrogenic exotoxin C, mutated at the high-affinity MHC II binding site, has also been successfully evaluated in xenograft models and might provide an alternative strategy for tumor targeting of superantigens [87].

23.5.2 Early-Phase Clinical Studies

In a phase I study of ABR-214936 in non-small-cell lung carcinoma (NSCLC) patients, a maximum tolerated dose (MTD), given intravenously over 4 days, as a function of the preexisting anti-SEA antibody was determined [88]. In phase II studies of ABR-214936 in RCC patients, the treatment cycle was repeated after 1 month, and survival was significantly prolonged compared to that of expected. Patients receiving higher drug exposure had greater disease control and lived almost twice as long as expected, whereas low drug exposure patients survived as expected (Fig. 23.3); sustained IL-2 production at day 2 appeared to be a biomarker for the clinical effect [89].

The high degree of disease control and the prolonged survival suggested this treatment could be effective and led to the development of an improved variant (ANYARA or naptumomab estafenatox or ABR-217620). This version has 90% homology to ABR-214936, incorporating a hybrid SEA/E-120 superantigen sequence with additional point mutations reducing MHC class II binding and antigenicity [90, 91]. Preclinical evaluation showed reduced binding to preformed anti-superantigen antibodies, lower toxicity, higher affinity for 5T4, and improved tumor cell killing. Phase I clinical studies showed that ANYARA was well tolerated both as monotherapy and in combination with docetaxel, and there was a good correlation of the preclinical studies with the MTD [92]. Evidence of immunological and antitumor activity included a dose-dependent induction of IL-2 and INF-γ (biomarkers for T-cell activation), selective expansion of ANYARA reactive T-cells, infiltration of T-cells into the tumor, and selective retention of ANYARA in tumor tissue as demonstrated using PET. ABR-217620 selectively engages with

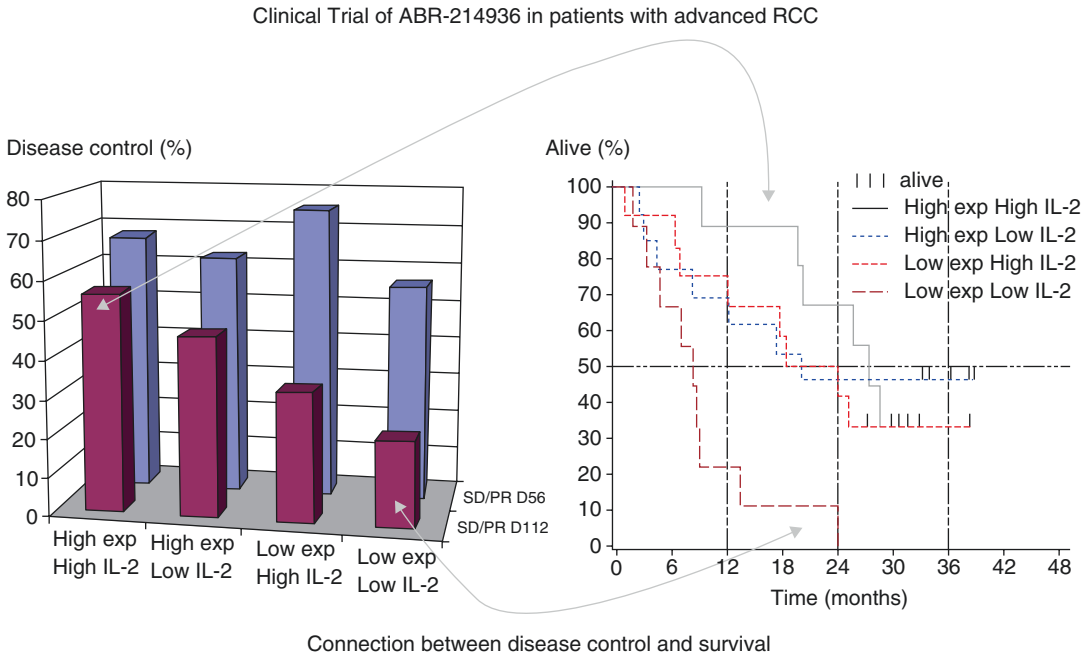

Fig. 23.3 5T4 antibody-directed superantigen therapy. Clinical trial of 5T4 antibody-directed superantigen therapy in patients with advanced RCC. This shows that patients with high IL-2 after the second infusion and high exposure are more likely to have disease control at day 112 and the longest survival (Adapted with permission from *British Journal of Cancer*: Shaw et al. [89])

TRBV7-9 and exploits TCR-peptide-MHC affinity mimicry in mediating T-cell cytotoxicity [93].

23.5.3 A Phase II/III Clinical Trial in RCC

A multinational (50 sites in Europe: United Kingdom, Russia, Ukraine, Bulgaria, Romania), randomized phase II/III study of ANYARA in combination with IFN-α vs. IFN-α alone in 513 advanced RCC patients has been conducted. The safety profile was good, and in line with previous observations, the most common adverse events associated with ANYARA treatment were grade 1–2 fever, nausea, and vomiting. No new and unexpected safety concerns were identified in the study. Unfortunately, the primary end point—to show a survival advantage in the intention to treat population—was not reached. Unexpectedly, and in contrast to previous studies conducted in other countries, a majority of the patients showed high levels of preformed antibodies against the superantigen component of ANYARA. A subgroup analysis, excluding patients with high levels of preformed antibodies, resulted in a trend for survival benefit with ANYARA treatment. This was consistent with the results of the previous version of ABR214936 in RCC patients [89]. Interestingly, high baseline levels of IL-6 were associated with a poorer outcome in this study, and this was also seen in trials of RCC patients treated with TroVax [65] or pazopanib [94]. In a hypothesis-generating analysis of approximately 25% of patients with low/normal levels of baseline IL-6 and low anti-superantigen antibody levels, a statistically significant treatment advantage for overall survival was seen (p = 0.02, HR = 0.59). In North America and Western Europe, this subgroup accounts for 40–50% of the total number of advanced RCC patients [95]. Patients with low baseline IL-6 and normal anti-SEA/E-120 may respond well to ABR-217620 by T-cell activation and expansion paving the way for antitumor effects [96]. Future development strategies for optimizing use of ANYARA are

likely to include combination use with other treatment modalities such as a tyrosine kinase inhibitor in the favorable RCC subgroup.

23.6 Other 5T4 Antibody-Targeted Therapies

This section will consider therapies using 5T4 antibody for the delivery of toxins and inhibition of function in cancer spread and in the context of chimeric antigen receptors expressed in T-cells using retroviruses.

23.6.1 Antibody-Drug Conjugates (ADC)

ADCs chemically combine the specificity of the antibody with a cytotoxic drug. The challenge is to produce an efficacious and safe agent, and this demands optimizing the properties of a suitable TAA-specific antibody in combination with the linkage chemistry and the payload characteristics. The original mAb 5T4 (clone H8) was shown to internalize into cells and utilized to target the calicheamicin toxin. The latter is a potent cytotoxic drug which causes double-strand DNA breaks. The conjugation methodology used stable chemical linkers between antibody and drug which restricted the release of calicheamicin to cells that internalize the ADC. The efficacy of the anti-5T4 conjugates was demonstrated in several tumor models including an orthotopic model for 5T4-positive lung cancer [97]. This efficacy derives, at least in part, from the targeting of tumor-initiating cells (TICs) in (NSCLC) xenografts, and the abundance of these 5T4-positive TICs is correlated with worse clinical outcome for the patients [16]. Consistent with other mechanistic studies [33, 34], co-expression of 5T4 and factors involved in the epithelial-to-mesenchymal transition was observed in undifferentiated but not in differentiated lung tumor cells.

These observations support the possibility that the anti-5T4 ADC might cause complete regression of tumors through targeting 5T4-expressing TICs, even where there is considerable heterogeneity in expression of 5T4 within the tumor. To test this, the efficacy of an anti-5T4 ADC on the growth of two patient-derived xenograft (PDX) lines with heterogeneous and different levels of 5T4 expression predominantly at the lung tumor-stroma interface was assessed. These tumors were treated with anti-5T4 ADC, anti-CD33 ADC, or vehicle; the anti-CD33 ADC served as a negative control because these PDX lines do not express CD33. In both cases, treatment with anti-5T4 ADC caused tumor regression, and no regrowth was observed even 3 months after the last dose; in contrast, treatment with anti-CD33 ADC or vehicle did not inhibit tumor growth. Treatment with calicheamicin (not conjugated to an antibody) did not show any significant impact on tumor growth. In contrast to the efficacy observed with anti-5T4 ADC, treatment of both PDXs with cisplatin at the maximum tolerable dose regressed tumors only transiently, and the tumors regrew after treatment was completed. These results highlight the superior long-term efficacy of an ADC that targets TICs as compared with a conventional chemotherapeutic. Thus, despite heterogeneous expression of 5T4 in NSCLC patient-derived xenografts, treatment with an anti-5T4 antibody-drug conjugate resulted in complete and sustained tumor regression. Thus, the aggressive growth of heterogeneous solid tumors can be blocked by therapeutic agents that target a subpopulation of cells near the top of the cellular hierarchy [16].

A further development of this approach has used a different 5T4 humanized mAb (A1) linked by sulfhydryl-based conjugation to deliver a tubulin inhibitor, monomethyl auristatin F (MMAF) via a maleimidocaproyl linker [98]. This conjugate (A1mcMMAF) showed potent in vivo activity in a variety of tumor models, with induction of long-term regression after the last dose. Evidence of the selective accumulation of the 5T4 (but not control) conjugates with release of the payload and consequent mitotic arrest in the tumor tissue was demonstrated. Depending on the particular tumor, 3–10 mg/kg doses given three times every 4 days were sufficient to produce a complete pathogenic response; this was independent of the degree of heterogeneity in 5T4 expression. This effect was shown to be con-

sistent with the targeting of TICs within the tumors.

Outcome in childhood acute lymphoblastic leukemia is prognosticated on levels of minimal residual disease after remission induction therapy [99]. Higher minimal residual disease levels are associated with inferior results even with intensification of therapy and suggest identification and targeting of minimal residual disease cells as a therapeutic strategy [100]. It has been shown that there is high expression of 5T4 in subclonal populations of patient-derived xenografts from patients with high post induction minimal residual disease levels [42]. Treatment with A1mcMMAF significantly improved survival without overt toxicity in mice engrafted with a 5T4-positive acute lymphoblastic leukemia cell line (Fig. 23.4). Mice engrafted with 5T4-positive patient-derived xenograft cells, were treated with combination chemotherapy or dexamethasone alone and then given A1mcMMAF in the minimal residual disease setting. While dexametha-

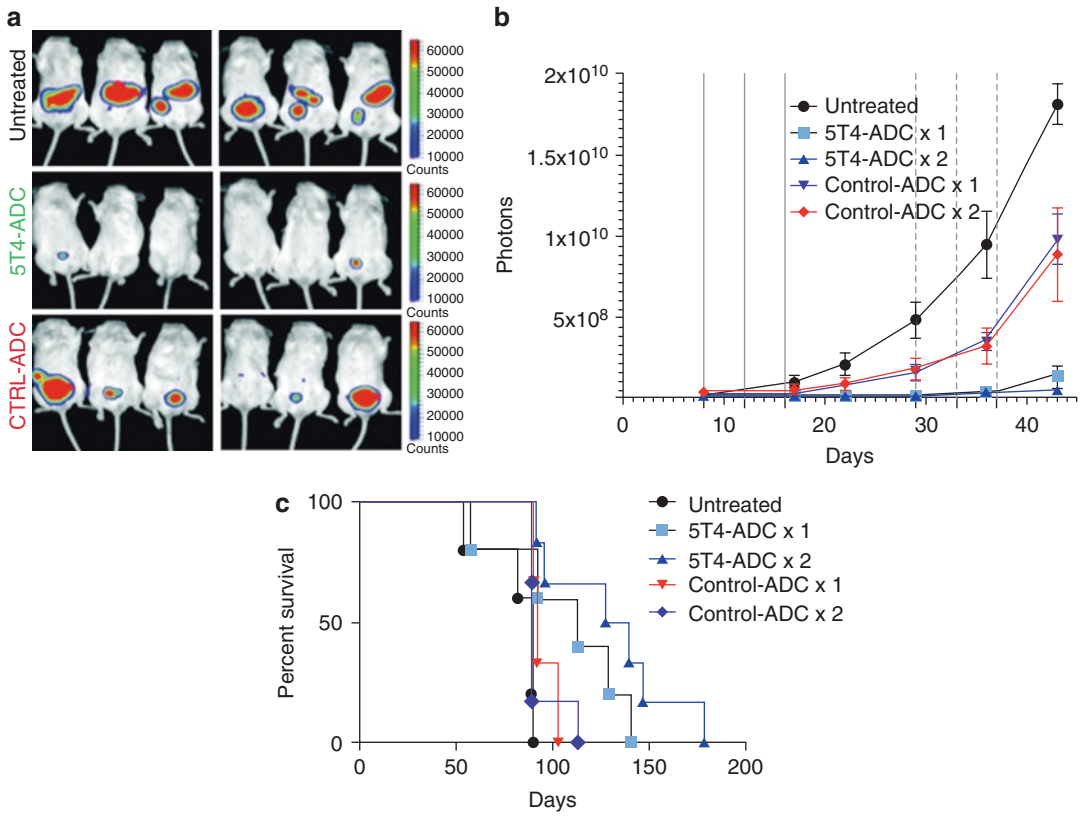

Fig. 23.4 A1mcMMAF monotherapy of Sup5T4 cells in vivo. Animals were challenged with Sup5T4 cells ip at day 0 and received either no treatment (black circles/line) or one (light blue squares/line) or two (dark blue triangles/line) cycles of A1mcMMAF or one (red triangles/line) or two (purple diamonds/line) cycles of control-ADC treatment starting after 1 week. (**a**) IVIS images of tumor growth at day 43. (**b**) Growth of tumors was quantified using log radiance (photons/sec/cm^2/sr) = photons. A1mcMMAF shows significant growth control: ANOVA-Tukey: untreated vs. one cycle or two cycles of A1mcMMAF; $p < 0.0001$; control-ADC one or two cycles vs. A1mcMMAF one or two cycles, respectively: $p < 0.05$ and $p < 0.01$. (**c**) Kaplan-Meier plots show that only A1mcMMAF (one or two cycles) but not the control-ADC treatments influences the overall survival. Log-rank Mantel-Cox shows significant affects compared to untreated animals of one and two cycles of A1mcMMAF, respectively ($p = 0.04$, HZR: 6.3 (1.08–36.52), and $p = 0.002$, HZR: 24.14 (3.36–173.4)) and no significant differences of control-ADC treatments. Dotted vertical lines represent timing of doses of ADC therapy (Reproduced from McGinn et al. [42], Haematologica 2017 Jun; 102(6):1075–1084; Haematologica Journal website http://www.haematologica.org)

sone or A1mcMMAF alone improved outcomes, the sequential administration of dexamethasone and A1mcMMAF significantly improved survival over either monotherapy [42]. These data show specifically targeting minimal residual disease cells improved outcomes and support further investigation of A1mcMMAF in high-risk B-cell precursor acute lymphoblastic leukemia patients identified by 5T4 expression at diagnosis.

The A1 antibody is cross-reactive with cynomolgus monkey 5T4, and this species was used to explore any potential toxicity and the pharmacokinetics of the conjugate and its payload as a first step for translation into clinical treatments. The A1mcMMAF exhibited no overt toxicity at doses up to 10 mg/kg/cycle × 2 and displayed a half-life of 5 days. Importantly, after treatment with the A1mcMMAF, the cys-mcMMAF concentrations remained very low in the plasma of monkeys; cys-mcMMAF was shown to accumulate in the tumor tissue in mouse studies. These observations suggest that the A1mcMMAF provides sufficient targeted payload to the tumor tissue with limited nonspecific exposure of the cytotoxic agent [101]. A first in human trial of A1mcMMAF showed tolerable toxicity in patients with solid tumors [102].

23.6.2 Direct 5T4 Antibody Effects

We have shown, as for mouse embryonic cells [38], that some mAbs to 5T4 can block 5T4-positive SupB15 leukemic cells [21] and PDX blasts CXCR4/CXCL12 chemotaxis in vitro [42]. In the latter case, one can speculate that this capacity is reflected in the enrichment of 5T4-positive blasts in mouse bone marrow in vivo [42]. Notably, in vivo antibody treatment is able to prevent the spread of 5T4-positive Sup-B15 B-ALL cells in the xenograft model [21] (Fig. 23.5). This may be of clinical relevance when considering ways to increase the exposure of leukemia cells to cytotoxic drugs. A CXCR4 inhibitor, AMD3100, has been used as a means to mobilize leukemic blasts from the bone marrow systemically to increase the relative bioavailability of chemotherapy [103]. A limitation of such therapy is that CXCR4 is a chemokine receptor widely expressed by many cell lineages. Since normal tissue levels of 5T4 are low, if its influence on chemotaxis could be specifically targeted, it might allow a disruption of CXCR4 function more specifically to malignant hematopoietic cells. In the context of BCP-ALL, the use of 5T4 as a relapse risk prognostic and potential

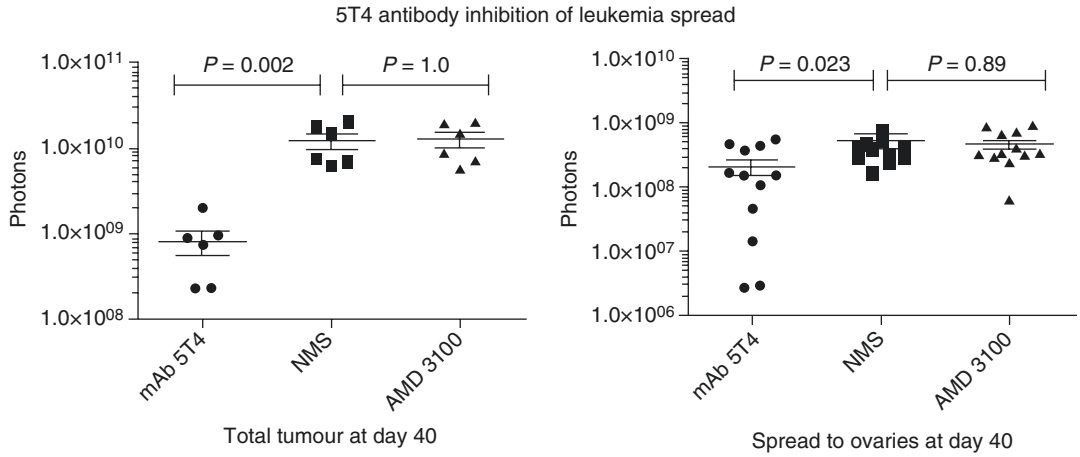

Fig. 23.5 5T4 antibody inhibition of leukemia spread. One hundred μg mAb 5T4 but not normal mouse serum (NMS) (both given at day 1 and then every other day for 10 days) or AMD3100 (plerixafor at 1.25 mg/kg, given daily for 10 days) blocks spread of intravenous Sup5T4 leukemia (5 × 10e6). Significant reduction in total tumor load and for spread to the ovaries at day 40 for mAb5T4 compared to either NMS- or AMD3100-treated animals (Mann-Whitney)

therapeutic target and insight into its mechanistic involvement of tumor spread and relapse are the focus of ongoing research.

23.6.3 5T4 Chimeric Antigen Receptors

There are a plethora of reports documenting dramatic tumor responses in conditioned patients receiving adoptive transfer of ex vivo expanded TILs [70, 74]. The precise specificity and differentiation status of the TILs is largely unknown but when successful presumably favors an antitumor effector rather than T regulatory cell bias. Genetic modification of T-cells to express chimeric antigen receptors (CARs) can produce effector populations with defined antigen specificities that function independently of the natural TCR. First-generation CARs typically expressed immunoglobulin-derived single-chain variable fragment (scFv) as the antigen recognition motif fused to either TCR CD3 ζ or Fc receptor of IgG (FcεRIγ) signaling domain for T-cell activation [104]. Recently, CAR variants incorporating costimulatory elements such as CD28 or 4-1BB or inducible IL-12 production to promote the survival and local expansion of the CAR T-cells in the patient's tumor have been developed. Early clinical testing of modified T-cells expressing such CARs targeted CD19 (leukemia/lymphoma), PSMA (prostate), and CEA (colorectal and breast cancer) [104–106]. Recently, clinical proof of concept using CAR T-cell directed at CD19, based on its expression at the cell surface in many leukemia and lymphomas, has now been delivered. The elimination of normal B-cells has been deemed a tolerable and manageable side effect with three different B-cell malignancies, diffuse large B-cell lymphoma, chronic lymphocytic leukemia, and ALL, all showing high rates of complete response in spite of differences in disease histology, CAR construct, and production [107].

A high-affinity scFv specific for h5T4 [108] was used to construct a first-generation CAR. This CAR, in contrast to CEA- and CD19-specific CARs, showed enhanced specific cytokine release and cytotoxicity in vitro only when possessing an extracellular spacer region [109]. This might reflect the relative accessibility of the target antigen epitopes. In a proof of concept study, 5T4 CAR-modified T-cells from RCC patients were shown to kill 5T4-expressing RCC cell lines [18]. The in vivo activity and use in combination with vaccination were also tested in an animal model [110]. Human 5T4-specific engineered murine T-cells demonstrated antigen-specific, non-MHC-restricted cytolysis of h5T4-positive mouse B16 and CT26 tumor cells in vitro by cytotoxicity assay and antitumor activity in vivo using a Winn assay. In subcutaneous B16h5T4 melanoma challenge, early local but not systemic intravenous administration of the h5T4-specific CAR T-cells significantly increased mouse survival. This improvement was further enhanced when combined with immunization with rAd-h5T4 vaccine, followed by post-CAR T-cell treatment with bone marrow-derived dendritic cells (BMDC) in the active therapy model. An autologous tumor model would provide a more realistic platform for assessing such bystander effects and for safety testing. Therefore, scFv from mouse antibodies to 5T4 [38] have been used to construct CARs with modified murine T-cells, and they were able to kill m5T4-expressing tumor cells in vitro [111]. The next step will compare m5T4-specific natural T-cells (generated in the 5T4 KO mouse; [67]) and gene-modified T-cells in therapy of an autologous m5T4B16 tumor in WT and 5T4 KO mice. Overall, 5T4 CAR T-cells are powerful means to bypass a number of mechanisms which allow tumors to escape T-cell killing [60] and can be readily scaled up for clinical use. The 5T4 expression by TIC/LICs with CAR T-cell targeting may ensure more complete responses, for example, in B-ALL and might be used in combination with other specific CAR T-cells or immunotherapies modulating local immune suppression [19, 78].

23.7 Concluding Remarks

The functional biology of 5T4 molecules is consistent with a role in the directional movement of cells. These processes are highly regulated in normal developing and adult tissues. 5T4 expression by cancer cells contributes to their spread and allows for immune targeting of 5T4. Several different 5T4-specific immunotherapies have been evaluated in late-phase clinical trials, and the data suggest certain subgroups of patients can get clinical benefit from the treatments. Further clinical studies are needed to focus the use of 5T4-specific immunotherapies in the management of particular cancers. Metastatic cancer continues to be very difficult to cure in most cases as is clear from the relatively low response rates to most conventional chemo and/or radiation treatments. The heterogeneity of tumors likewise poses immense hurdles for individualized treatment strategies based on blocking particular signaling pathways. To most immunologists, immunotherapy is the most rational and potentially efficacious approach to the treatment of such disseminated and heterogeneous targets. It is clear that the immune system can be vital in controlling the tumors but in some circumstances can also promote their development. Understanding how to control this balance is the key to the effective use of immunotherapy, and this will involve both systemic and local tumor microenvironment factors. It is imperative that oncologists begin to consider how their conventional treatment strategies influence the immune system since it may be controlling otherwise "unseen" cancer or be required for optimal disease resolution.

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Aging and Cancer Prognosis

24

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Contents

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24.1 Introduction

Aging is a natural and biological procedure that definitely occurs in all living organisms and could induce a progressive loss of function or decrease the capacity of tissues for regeneration [1, 2]. Aging could increase the risk of many chronic diseases with different pathways such as mutation accumulation, wear and tear, and antagonistic pleiotropy [3]. It is considered that aging is one of the potential risk factors for cancer developments; majority of cancer diagnoses are in individuals over 65 years old [4]. In this regard, cancer is an aging-related disease [5–7], and a better understanding of the aging process could clarify the reason for increased cancer incidence in advanced ages. Typical features of the age pattern for cancer incidence rate include a peak in early childhood, low rate in youth, and increase in elderly [3, 8–10].

Advances in the clinical and experimental research of aging and cancer have shown insight into the molecular and cellular pathways of these processes. The antagonistic pleiotropy hypothesis indicates that the genes which induce aging could survive in the evolutionary selection, because they could induce some useful and valuable effects during the reproductive period [11, 12]. Briefly, many of these biological procedures during aging could perform a pro-survival function during earlier periods of life. In this regard, cellular senescence, as an example of this paradigm, was demonstrated to be an essential process during embryonic development [13]. Also, this process is a robust tumor suppressor mechanism which could play as a preventer agent of cellular damages and oncogenic mutations [14, 15]. This process is the same as the one which could induce a multiple age-related pathologies [16], including cancer [17].

Recently, researchers have shown that the inflammation that is commonly induced by exogenous pathogens, DNA impairments, UV radiation, and physical trauma could modulate some multiple biological processes, including cancer and aging-related pathologies [18–21]. On the other hand, it is clear that during the aging process, the function of the immune system declines, and other tissues deteriorate [22]. One of the theories, which shows the cause of decreasing function of the immune system during aging, is age-related thymic involution. This is a progressive shrinking process of the thymus and is related to the natural decline of the immune system over time [23]. It has been suggested that immune surveillance is a key factor in avoiding cancer progression; therefore, immunosenescence is an important key factor that could link tumorigenesis and aging [24].

24.2 Aging and Cancer Demography

Cancer diagnoses vary during an individual's life span and depend on many factors. It has been estimated that the cumulative risk for cancer increases by age 70 years old and then decreases slightly [25]. The lifetime risk of ever being diagnosed with cancer in the total US population is about 41% [25]. However, most of older individuals remain without any diagnoses of cancer in their life span. Also, it should be noted that cancer became a rare condition after 90 years old [26]. It has been estimated that more than half of all cancers occurred in people more than 65 years old in 2009, and by considering the growing number of older adults, it's predicted that the numbers would increase to 70% until 2030 [27]. Therefore, this topic shows the necessity to focus on opportunities for primary prevention rather than relying on treatments.

In the midlife, health becomes a valuable condition establishing the foundation for longevity later in life. In the period of midlife, people are confronted with many risk factors for a variety of diseases including cancer. Tobacco use, lack of physical activity, poor nutrition, infection, etc. are considered to be among these risk factors [28, 29]. Although many of these factors are changeable, others including genetic and aging of the cells are unchangeable throughout life. In addition, some preventable conditions and disorders such as diabetes and obesity, which could increase during midlife, are correlated with elevated cancer risk or decreased malignancy

survival. The incidence of chronic conditions such as obesity, lower physical activity, and diabetes has increased in the recent decades. Hence, it could be estimated that prior generations which include adults who are currently aged from 45 to 64 years old are expected to live longer than their descendents who seem to be experiencing higher rates of these chronic conditions [30, 31]. Therefore, the prevention or management of chronic conditions and the promotion of general health during midlife are promising strategies to prevent or delay cancer incidence at older ages.

24.3 General Content of Cellular Aging

Cellular aging and the age-related physiological changes are fascinating subjects to investigate. Aging is commonly characterized by a developing accumulation of cell and tissue destructions, resulting in reduced organ function and increased susceptibility to disease and age-related disorders [32]. Aging could affect all macromolecular components at the cellular level. For example, the yellow-brown granular pigment lipofuscin that contributed to brown atrophy of tissue, in the elderly individuals, was one of the first to be reported; this process consists of complexes of oxidized lipids covalently linked to proteins [33]. On the other hand, nonenzymatic biological side reactions such as glycation, as a part of the free radical hypothesis of aging, have been suggested to interpret the main mechanism of aging in elderly animals [34, 35].

Recently, researchers have been focused on the decline in proteostasis process and protein quality control. These impairments lead to an increase in number of the abnormal proteins in aged individuals [36]. The ubiquitin-proteasome, chaperone, and autophagy systems are the intracellular proteostasis mechanisms that are typically acting in the normal cells. It has been suggested that aging could induce changes in all of these pathways. Chaperons could recognize the initial protein misfolding; this process requires ATP, which might be limited in older ages [37, 38]. Therefore, repairment of misfolded or damaged proteins might be decreased by aging, and subsequently, the number of abnormal proteins in cells increases. In addition, both proteasomal and autophagy functions could be affected by aging; decline in these pathways leads to both intracellular and extracellular abnormal accumulation [39]. Aging is also correlated with epigenetic modifications including changes in histone and DNA methylation patterns, which result in the progressive and profound modification of transcriptional profiles of coding and noncoding RNA [40, 41]. Several lines of experimental evidence have been indicating that such large-scale changes are related to the inflammatory status and are in response to environmental stimuli and nutrient availability [42]. In addition, a decrease in the proliferative capacity in senescent cells is correlated with the general loss of histones and with an imbalance between activating and repressive histone alterations [43, 44]. Also, aging could affect DNA methylation patterns, and the methylation status of some specific regions (termed clock CpGs) could correctly predict cellular age [45]. Interestingly, studies have revealed that more than 30% of chromatin, including the formation of large-scale domains of H3K4me3 and H3K27me3 over lamina-related domains, as well as significant losses of H3K27me3 outside these domains, is dramatically reorganized and linked to the transcriptional downregulation of lamin B1 in senescence. These processes could be a key trigger of global and local chromatin alterations that could affect gene expression, aging, and cancer [40, 46, 47]. Overall, age is correlated with global DNA hypo-methylation and local hypermethylation in some particular regions. These conditions in combination with histone alteration are linked to inflammation, aging, and oxidative stress, which could affect the activation or the repression of specific transcriptional programs, including those involved in the expression of cytokines, oncogenes, and tumor suppressor genes. Therefore, these conditions could make tissues prone to chronic inflammatory diseases associated with age and cancer [48]. In general, both endogenous and exogenous sources of DNA damage could be accompanied with genotoxicity

[49]. Furthermore, alternation in all macromolecule such as membrane lipids, proteins, and DNA and the underlying implications could influence the organ functions at both cellular and tissue levels, which is the primary hallmark of aging [50–52].

For half a century, one of the most potent hypotheses indicated as an increment factor for survival and reducing age-associated changes was to restrict the caloric intake. Animal studies showed that decreasing the caloric intake by 20–40% could increase the life span for about 20–50% without any increment of survival in mice [53]. However, in primates, investigations have shown no significant increase in the lifetime with lower cholesterol intake, better insulin sensitivity, etc. [54, 55]. On the other hand, molecular studies have suggested that telomere shortening as a mechanism of aging could increase the vulnerability of aging cells to DNA damage and dysregulation [56–58]. The decreased telomere sequence, which is called "replicative senescence," as well as other replicative dysregulation, might result in an unsatisfactory replacement of damaged or dead cells from their respective precursor cell populations. Many of these resting precursor cells begin to differentiate along adipocyte-like pathways, rather than into other tissue types [59]. Subpopulations of adipocytes, hepatocytes, fibroblasts, and other cells might enter the senescence period with aging and develop the senescence-associated secretory phenotype (SASP) [60]. SASP cells have a potential to release the inflammatory cytokines, growth factors, proteases, and other damaging factors that could change the activity of other localized normal cells [61]. Researchers have been focused on the damaging effects of SASP to develop a chemical which has an ability to kill and eliminate senescent cells to decrease the age-related diseases (Fig. 24.1) [62]. In this regard, eliminating these cells has improved the cardiac and vascular function in mice [62]. Therefore, senescent cell removal might increase

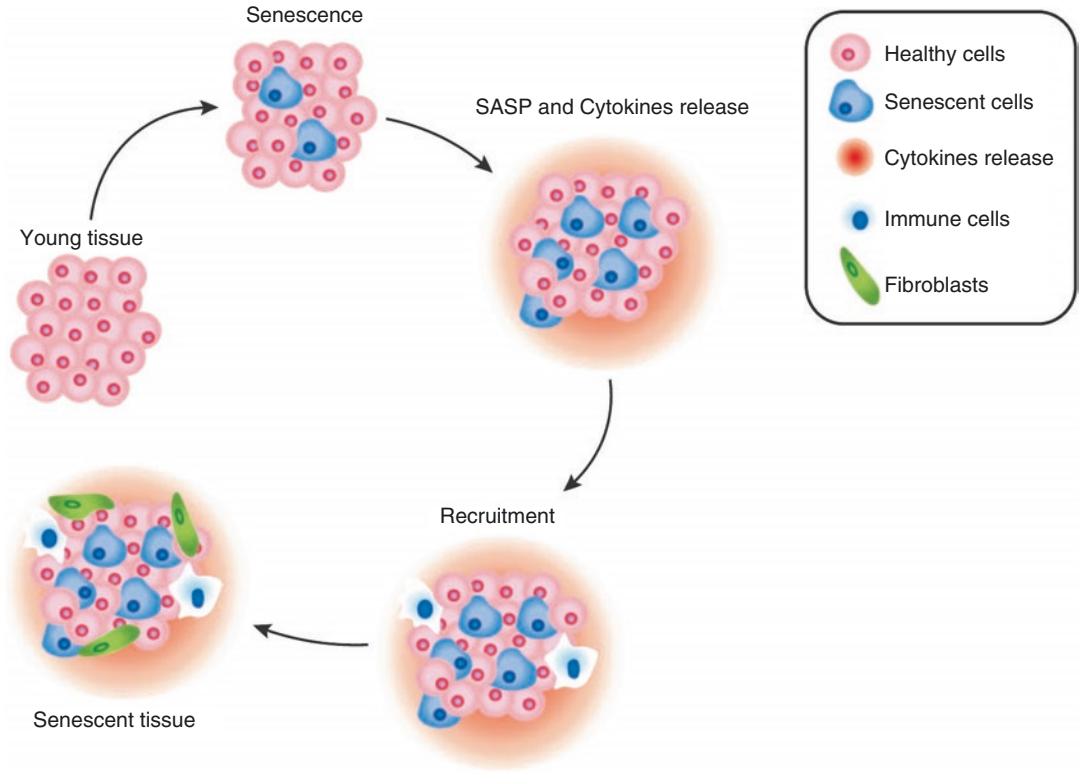

Fig. 24.1 This schematic briefly shows the senescent procedure in a normal tissue which could lead to an increase in the risk of malignancies

the life span and life expectancy [63]. It has been demonstrated that in the senescent cells, the nucleus is defined by senescence-associated heterochromatin foci (SAHF) and DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) [64]. In addition, senescence could affect tumor suppression, cell development, and wound healing and plays as an important pathological agent for age-associated diseases. In this regard, experimental studies showed that eliminating the senescent cells in mice could result in greater resistance against the age-related disorders [65].

Also, other hypotheses have shown that DNA damage could activate the p53 gene. Activation of this gene results in many molecular pathways, which could affect the cell function and viability. For those cells that have a rapid turnover, an activated p53 gene could stop the normal cell growth and turn it to the apoptosis state. Also, this process leads to loss of function of peroxisome proliferator-activated receptor gamma coactivators alpha and beta (PGC1-alpha and PGC1-beta) and might result in mitochondrial dysfunction and subsequently increase the level of free radicals with loss of antioxidant defenses [66].

Interestingly, recent articles have focused on the mammalian target of rapamycin (mTOR) pathway, which modulates nutrient delivery and is considered to play an essential role in the ability of caloric limitation to increase life span. Rapamycin has been determined to provide longevity in mice [67]. Therefore, although senescent cell removal and preventing senesce could influence the duration of life span, aging is a natural biological process leading to increase of the age-related diseases; hence, struggling with this condition remains a novel topic to discuss.

24.4 Clinical Aspects of Aging, Age-Related Disease, and Immunity

It has been well recognized that aging could induce functional decline in multiple organs which does not occur in young, normal, and healthy individuals [68]. For example, the renal function could decrease while aging [69]; this reduction has been proven to be a useful biological marker of aging in the clinical studies. However, these changes could not be accompanied with renal complication in the absence of any other disorders or exposure to a nephrotoxic agent. In addition, it has been observed that bone marrow is affected by aging through a decrease in marrow stem cells and their proliferative potential [70, 71]. Also, studies show that there are significant age-related changes in the immune system functions [72, 73]. However, these changes either do not influence health of the aged individuals or are associated with minimal clinical consequences in the absence of any other diseases. Aging is not a disease, but these physiological changes could make individuals prone to a variety of disorders. In this regard, studies indicated that aging-related changes could induce the following factors due to change in immune system responses: increased reactivation of tuberculosis [74, 75], or herpes zoster [76], and less responding capability to vaccination against diseases such as influenza [77, 78]. This decrement of immune responses might also be correlated with malignant conditions in elderly individuals [79].

Clinical studies suggest a significant inverse relationship between cardiovascular, respiratory, nervous, endocrine, gastrointestinal, and genitourinary system functions and age in elderly individuals in comparison to younger patients [80, 81]. The immune system like any other organ might be affected by aging. The immune system acts as a defensive factor against infection and also a detector and removal agent for malignant cells. By aging, the immune system responses inappropriately against various conditions, and this process could cause increased susceptibility to infections, cancer, and incidence of autoimmune disease.

Age-related immune dysfunction is an interesting topic to discuss, and there is limited documents investigating the effect of aging on the immune system and its consequences [82]. Although many experimental studies have assessed this association at a basic level, few clinical studies evaluated the effect of aging on immune system changes. The prevalence of cancer and mortality notably increases in individuals

more than 65 years old and reduces by the age 85–90. Overall, there are two causes of immunodeficiency, that is, primary and secondary. The most important primary causes of immunodeficiency are correlated with antibody deficiency, aging, and immuno-senescence. Secondary causes of immunodeficiency include malnutrition, malignancy treatment or immune-suppressive drugs, immunomodulatory agents (such as infliximab), drug-induced hypogammaglobulinemia, metabolic conditions, and infections. It has been indicated that both innate and adaptive immune systems are involved in the first barrier against malignant cells. Individuals with no sufficient response of the immune system are highly at risk of malignancies; this condition is observed in immune-deficient patients which indicates the role of the immune system in defending against malignant cells [83]. On the other hand, exposure to the carcinogenic agents and accumulation of mutation load could increase the risk of cancer in the elderly [84].

It has been suggested that immuno-senescence is characterized by reduction in the number of naive T-cells in peripheral blood and lymph nodes [85–87]. Although the number of memory T-cell increases by aging, the functional integrity of T-cells including CD4+ and CD8+ cells decreases in elderly individuals. This condition might be the reason of reduced immune response to cancer antigens that are expressed by malignant cells [88, 89]. It seems that antigen presentation by dendritic cells (DCs) remains unchanged during the aging process; this subject caused researchers to focus more on T-cells in immuno-senescence [90]. Also, many documents have shown that the activity of innate immune system could increase the level of pro-inflammatory cytokines and subsequently induce inflammation, which associates with an adverse effect on health in the elderly [91]. These hypotheses have been proven by many clinical trials showing decrease in immunity responses to vaccination in older individuals [92]. However, the most important factors involved in immuno-senescence and the underlying causes of age-associated changes remain mostly unclear [93].

24.5 Hypothesis of Increase in Cancer Risk by Aging

There are many types of theories that have been evaluated to show the increased risk of cancer is correlated with aging. Right after genetic factors, one of the most critical risk factors is exposures to carcinogenic agents. Carcinogenic exposures seem to affect similarly across human and other mammals. This hypothesis was evaluated in preclinical experiments on rodents and by an observational study on occupational exposures in humans [9, 94, 95]. These studies showed that skin administration of the regular benzpyrene significantly increases the prevalence of malignant epithelial tumors. This increment was related to the duration of exposure; however, it was not related to the age onset of exposures. On the other hand, the study by Doll et al. [94] showed that the occupational exposures could increase the incidence of cancer in humans. Overall, it could be concluded that the risk of exposures and accumulator dose of carcinogenic factors in the body could be increased by aging [3, 96].

Another hypothesis for increasing the risk of cancer in the elderly is the increment in vulnerability of individuals to cancer, and aging-related procedures might be a powerful reason for this hypothesis [3, 96–100]. In this regard, animal studies demonstrated that tissues obtained from elderly mice are more susceptible to be transformed by carcinogenic factors rather than tissues taken from younger subjects [101]. There have been many hypotheses investigated, showing how aging could increase individual's vulnerability to cancer. Some papers [3, 97] suggested that aging could decrease threshold of an organism to cancer due to several pathways including disturbance in hormonal balance, an increase in the number of loci of chronic proliferation, and the decline in the immune system by aging. The exact mechanism in which immuno-senescence leads to increased incidence of malignancies is still unclear and contradictory [102, 103]. Krtolica et al. proposed that the accumulation of senescent cells in the stroma, while aging, dis-

rupts the local tissue integrity with factors secreted by these cells [10]. This may—in authors' opinion—create a pro-oncogenic tissue microenvironment. Overall, the increment in cancer risk by aging cannot have a single cause, and it is assumed to be a multifactorial process; therefore, decreasing immunity and accumulation in carcinogenic factor exposures could increase the mutation load and also escalate individual's vulnerability to cancer [3, 96, 98]. Recently, there are many researches that indicate aging or aging-related pathologies could produce a change in the immune system with a low-grade inflammation, which is triggered by various damage-associated molecular patterns (DAMPs) and autophagy-related immune changes. These changes in immunity are considered to create pro-tumorigenic conditions that make aged organisms become more vulnerable to oncogenic insults [22]. Also, it should be noted that the abovementioned concepts not only could increase age-associated cancer risk but also might increase age-related diseases.

24.6 An Epitome of Aging, Immunity, and Cancer

As mentioned above and in Chap. 23, Vol 1 entitled "Immuno-senescence, Oxidative Stress, and Cancers," it has been indicated that aging is related with a low-grade of chronic sterile inflammation, which could be accompanied with all aging-associated diseases [18, 91, 104]. Results obtained from some epidemiological analysis demonstrate a direct relation between elderly and high levels of inflammatory factors including IL-6 and C-reactive protein (CRP). This is the biological theme of the elderly, and it is considered as the outcome of exposure to various internal and external factors throughout life and in turn a driving factor in multiple age-related pathologies [23, 105, 106].

Generally, inflammation is a sophisticated biological reply to detrimental provocations including pathogen invasion, physical trauma, or irradiation and also is considered as an eliminator factor for harmful agents and then plays a role in restoring the tissues and homeostasis [107, 108]. Chronic inflammation, as a low-grade permanent process, could lead to tissue remodeling or dysfunction, while acute inflammation is considered as a beneficial process for promoting the tissue repairment [109]. It has been suggested that chronic inflammation could lead to induction or distribution of multiple pathological procedures, including degenerative disease that follows with aging and cancer [64, 110, 111]. It has been indicated that immune cells, mainly macrophages, and nonimmune cells such as epithelial and fibroblast cells are considered as the inflammatory responses which accompany aging [112]. However, there have been several methods that stop inflammation in aging. Two sources have been suggested in the pathophysiology of chronic low-grade inflammation (inflammaging); one source is the increased frequency of cellular aging and inflammatory factors especially IL-6, and another one is contributed to innate immune system responses which result from various pro-inflammatory factors. DAMPs, DNA fragments or DNA culprits, and various microbial elements that might be debris of macromolecules are those known agents which are involved in the pathophysiology of this chronic low-grade inflammation [113]. The immune response involved in the aging inflammation initiates with activation of innate immune receptors which is the result of the accumulation of DAMPs [114, 115]. Toll-like receptors (TLR) are the kind of transmembrane receptors that are typically considered as innate sensors and are commonly activated by these components [115]. Activation of TLRs could subsequently result in activation of the pro-inflammatory transcription factors including NF-κB and activator protein 1 (AP-1); upregulation of various inflammatory cytokines including TNF-α, IL-1β, and IL-12; and activation of type I IFN immune response via myeloid differentiation primary response 88 (MYD88). Other intracellular receptors such as NOD-like receptors (NLRs) establish a fundamental component of the inflammasome complex. The inflammasome is a multi-protein cytoplasmic complex that uses

a signaling core for inflammatory responses. NRL family senses the DAMPs and leads to activation of caspase-1 (inflammasome complex) and subsequently results in secretion of mature pro-inflammatory cytokines such as IL-1β and IL-18 [116, 117].

Chronic inflammation is correlated tightly to the growth of age-associated disorders such as cancer. It has been shown that the chronic low-grade inflammation could increase tumorigenesis, which is related to myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogenic group of myeloid lineage-derived cells that could play as an immune-suppressive factor. Past studies showed that MDSCs could accumulate in melanoma lesions and lymphoid organs in a mouse model of melanoma. Their accumulation was correlated to reduce the representation of T-cell receptor ζ chain and decreased antitumoral immune activity [118]. Studies have determined that breast cancer is a well-known example of the robust linkage between pro-inflammatory malignancies. Also, they showed the connection between IL-6, as a major component of aging inflammation, and cancer development and progression [119]. Interestingly, it was suggested that IL-6 could be used as a prognostic factor in breast cancer, and high concentration of IL-6 correlates with poor prognosis in breast cancer [120]. On the other hand, studies revealed a raised IL-6 mRNA in aggressive breast ductal cell carcinoma in comparison to a healthy tissue [121]. In a different in vitro model, IL-6 stimulated non-stem cancer cells of breast and prostate cancer cell lines to gain cancer stem cell properties [122]. Breast cancer is one example that can serve to highlight the possible contribution of inflammaging to cancer propagation. Also, there are many topics that are in line with each other on various types of cancers such as prostate [122]; thus, it could be concluded that these hypotheses could highlight the possible connection of aging inflammation and cancer developments.

Recently, Hanahan et al. demonstrated the new hallmarks of cancer; they showed the association between cancer and immune responses [123]. It has been demonstrated that aging could induce transformations in the immune system such as sensitivity to infections, autoimmunity, decrease in vaccination response, and cancer development [124, 125]. From the perspective of immunity, aging is described by thymic involution, decreased in T-cell diversity, reduced naive T-cell population, increased in memory T-cells, and decreased cytotoxic activity of natural killer cells (NK) and macrophage age-related changes [24, 126–129]. Therefore, age-induced changes in T-cells, macrophage, neutrophils, and NK cells could significantly affect the changing tumoral microenvironment and provide compromising immune surveillance (Table 24.1). In an interesting study, the effect of the aging immune system on cell fate in mouse model of squamous cell carcinoma (SCC) has been studied. They showed that induction of mutation in a growth gene of keratinocyte could result in the rapid cell growth and hyperplastic reaction in younger mice with no creation of malignant cells; however, in older mice, in addition to high speed of growth, they observed evidence of dysplastic changes, which half of them converted to SCC. Also, they demonstrated a shift toward the pro-tumorigenic Th2 inflammatory response, increased expression of the immune checkpoint activator PD-L1, and increased SA-β-Gal staining in the dermis which probably represents senescent immune cells in older mice [130].

24.7 Aging and Immunity as Prognostic Factors in Cancer

Numerous studies have indicated that age is a critical risk factor for cancer prognosis and development, and it is clear that an elderly patient diagnosed with cancer has a higher risk for recurrence and lower survival rate. The neoplastic prevalence and mortality of malignant conditions are directly correlated with aging. As mentioned previously, in patients older than 65 years old, nearly more than 50% of all types of malignant neoplasia will appear [131]. However, it should be noted that this condition does not apply to all cancers.

Table 24.1 Effect of aging on immune system: consequent increased tumorigenesis due to the decrease of immune responses to malignant conditions and increased tumorigenesis and cancer

Affected items	Outcome	Involvement function	Pathological results	Final outcome
Inflammation	Increase low-grade chronic inflammation	↑ Cytokines ↑ DAMPs ↑ TLR activation NLRs	Increased tumorigenesis	Increase risk of cancer
Thymic involution	Decreased T-cell diversity	Decreased in naive T-cells	Decreased immune responses to malignant condition	
Natural killer cells	Decreased in cytotoxicity	Impairment toxicity	Decreased immune responses to malignant condition	
Macrophages	Decrease in antigen presentation, phagocytosis, ROS production, and cytokine production	Controversies for phagocytosis Impaired recruitment ↓ Response to stimuli factors ↑ Response to IL-4/IL13 Products released	Decreased immune responses to malignant condition	
Neutrophils	Impairment of neutrophil function	Intact adhesion, Controversies for migration and chemotaxis ↓ Phagocytosis Intracellular killing ↓ and ↑ various products released ↓ Apoptosis	Decreased immune responses to malignant condition	

Many clinical and experimental studies have shown a poor prognosis in aged patients who suffer from cancer. For example, a study run by Høst et al. on 31,594 females with breast cancer reported the poor outcome among elderly individuals [132]. However, they also indicated that this condition might be due to the lower efficacy of treatments in elderly patient because they have more organ disability and lower tolerance to treatments used in younger patients. On the other hand, other studies demonstrated that in aged individuals, the cancer might become more aggressive with high rates of metastasis. For example, Faruk et al. showed that the rate of metastatic pancreatic carcinoma was significantly higher in elderly individuals and also the overall survival of aged patients with metastatic cancer was significantly lower in comparison to younger individuals suffering from metastatic cancer [133]. In line with recently mentioned studies, Balch et al. showed that the incidence of melanoma increased among younger population; however, the mortality rate was significantly higher in older patients [134]. However, it is not well recognized how aging affects cancer prognosis. One of the most important reasons is using less invasive treatments in older patients due to their organ dysfunction during their aging process. Recent manuscripts showed that immune senescence could be a recently identified reason for poor prognosis of cancer in elderly.

As mentioned before in this chapter, the most critical aspects describing the aging process are the inevitable loss of renewal capacity and involution of tissues and organs. Also, in aged individuals, the immune system does not have an efficient response against malignancies, and this condition is tightly associated with induction of more aggressive cancers along with poor prognosis. It is clear that the immune system acts as a preventing factor by activating and inducing an efficient immune response against tumors and

malignant conditions. Therefore, aging could increase the risk of cancer development by affecting the immune system [135]. For example, the immune stimulation of T-cells by dendritic cells is essential for their efficient activation, and this is changed by aging within the co-receptors including B7.1, B7.2, OX40, CD27, CD30, and CD40. Aging leads to weakening of the T-cell responses. One clinical study showed that NK cells might play a critical and prognostic role in metastatic colorectal cancer; they also observed that these cells are able to eliminate metastasis. On the other hand, they showed that in addition to tumor stage, infiltration of CD8+ and CD57+ cells in the tumor margin is an independent prognostic factor in these individuals [136]. Also, Walsh et al. suggested that preoperative neutrophils to lymphocyte ratio could be a promising predictor for colorectal cancer prognosis [137]. The unique features of immuno-senescence prevent an efficient immune response against malignancies and contribute to the overall decrease threshold to malignant conditions with aging [138].

24.8 Cancer Treatment Approaches Based on Aging and Immunity

Researchers have evaluated many mechanisms to increase longevity and life expectancy. The results of the most parts of these efforts demonstrated that longevity is tightly related to reduction of the risk factors for infections, autoimmunity, or cancer. It has been well documented that good nutrition and exercise could significantly relate to longevity. In this regard, some vitamin or mineral supplements including vitamins A, D, E, B6, B12, folate, and C, selenium, zinc, copper, and iron are necessary for normal immune system function, and lack of these components could lead to decrease in the immune system's function. Also, it has been suggested that immune responses increase in elderly if the adequate amount of these components is received [139–141]. In addition, past studies showed that chronic stress is the most powerful agent that could affect the immune system and its responses. It has been suggested that chronic stress is associated with accelerated immunosenescence; therefore, stress management therapies might reverse some features of immuno-senescence [142–144].

Cytokine therapies as a novel treatment could affect the immune system aging. In this regard, experimental studies have revealed that recombinant interlukin-7 (IL-7) could increase thymic output of T-cells or T-cell function (CD4+, CD8+, central memory CD8+, and T-cell receptor excision circle or TRECs) in a mouse model of thymic atrophy [112]. Interestingly, a phase I study of recombinant human IL-7 on 16 individuals with refractory cancer showed the increment of naive and central memory cells [145]. On the other hand, animal studies have demonstrated that mTOR inhibitors could extend the longevity and partially reverse aging effects on immune cells. Studies suggested that using mTOR inhibitors could increase the life span in 9–14 percent of mice and this range is sex dependent [146, 147]. In contrast, long treatment of mTOR inhibitors could not affect the life span in marmosets [148]. Human studies of mTOR inhibitors have revealed that this agent might counter some measure of immuno-senescence. After using the mTOR inhibitors, researchers observed the high response to influenza vaccine in 20% of individuals with more than 65 years old [149].

Recently, it has been shown that the immune system could play a critical role in tumorigenesis, and immunotherapy could be used as an effective anticancer treatment. In this regard, FDA approved that IL-2 could be administrated as a treatment for renal cancer and melanoma, and this treatment became the first immunotherapeutic agent for achieving durable cancer response [122]. On the other hand, a phase I clinical trial of anti-CD28 antibody showed that using this treatment leads to destructive cytokine storm response, causing intensive care unit (ICU) admission of those individuals who underwent this treatment [123]. However, immunotherapy is now one of the fundamental treatments in oncology that is owing to advances in immunological researches.

Overall, immunotherapy methods in this field include chimeric antigen receptor (CAR) T-cell-based therapy, T-cell transfer, and immune checkpoint inhibitors (ICI) [125–127]. In clinic, these treatments are the most common immunotherapeutic strategies. Although cancer occurs mainly in the elderly, most of the experimental studies evaluated the effect of immunotherapies on young rodents. Past studies on the effectiveness of combination therapy with anti-CD40 and IL-2 in a mouse model showed that younger mice achieved good response with metastatic tumor regression; however, aged mice suffered from severe macrophage-mediated cytokine storm and died within 2 days [128]. These data suggest that the therapeutic and toxic effects of immunotherapy are based on age. Finally, a new meta-analysis of randomized controlled trials reviews the effectiveness of ICI among younger and older cancer individuals [129]. When a cutoff point was set in the range of 65–70 years old, both younger and older patients presented similar improvement in overall survival and disease-free survival. However, in a subgroup of patients older than 75 years, no significant effect of anti PD-1was observed. This further indicates the possible impact of immuno-senescence on anticancer treatment.

24.9 Conclusion

Aging overlaps with decrease in immune system's function and responses, thus resulting in increased vulnerability to cancer in elderly individuals. Age-induced changes in T-cells, macrophages, neutrophils, natural killer cells, and autophagy could significantly affect the response of the immune system to cancerous cells. On the other hand, over-release of pro-inflammatory cytokines could lead to low-grade inflammation and subsequently age-related disease. It could be concluded that age can be considered as a prognostic factor in origination of malignancies. According to previous studies on cancer treatment, based on aging and immunity, enhancement of the immune system would lead to significant decline in incidence, morbidity, and mortality of cancer. However, supplementary research is required to demonstrate age-related changes in immuno-senescence in the aspect of cancer.

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Biomarkers for Immune Checkpoint Inhibitors

25

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25.1 Introduction

Perhaps the history of immunotherapy goes back to 1891 when William Coley tried to cure cancer patients with the injection of a vaccine that contained killed *Streptococcus pyogenes* and *Serratia marcescens* (Coley's toxins). This was due to his observation of a patient with a recurrent sarcoma, which spontaneously regressed after an episode of erysipelas [1]. Obviously, the outcomes of such treatment were inconsistent. However, his strategy was such a miracle in that era. In 1909, Paul Ehrlich and then, in 1957, Burnet and Thomas described the "immune surveillance" hypothesis. This theory suggested that the immune system constantly screens all cells for having malignant transformations [2]. With a variety of clinical and experimental evidence against this theory, in 2002, Dunn and Schreiber described the "cancer immunoediting" hypothesis, which has implied both tumor-suppressing and tumor-promoting functions of the immune system [2]. Cancer immunoediting consists of three phases: the first one is the elimination, in which neoplastic cells will be destroyed by the immune system. The next phase is equilibrium, characterized by the presence of some survived resistant cancer cells. This happens when new mutations give rise to the resistance of such cells to the immune system, a process described by authors as "Darwinian selection." The last phase is escape, as transformed cells begin to insanely outgrow and make an immunodeficient microenvironment, that ends in apparent clinical manifestations of the disease [2].

25.2 Overview of Immune Checkpoint Inhibitors: Mechanism of Action

Among various therapeutic strategies that are based on cancer immunotherapy (including cancer vaccines and chimeric antigen receptor T-cells or CAR T-cells), immune checkpoint inhibitors (ICIs, also known as immune checkpoint blockade or ICB) are one of the most important and effective ones. In 2011, the Food and Drug Administration (FDA) approved ipilimumab as the first ICI for the treatment of patients with metastatic melanoma [3]. Ipilimumab is an anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) antibody that, in comparison with glycoprotein 100 (gp100) therapy, showed an increase in overall survival (OS) of aforementioned patients [3].

Up to now, there are six more ICIs approved by the FDA, including pembrolizumab, nivolumab, and cemiplimab-rwlc (anti-programmed cell death protein 1 or anti-PD-1) and atezolizumab, avelumab, and durvalumab (anti-programmed death-ligand 1 or anti-PD-L1).

25.2.1 Central Tolerance

The development of T-cells occurs primarily in the thymus [4]. During this process, double-positive precursors (DP cells, which are $CD4^+$ and $CD8^+$) undergo positive and negative selection. The first one will remove all immature DP cells except those which bind to peptide-MHC

complexes (expressed on cortical thymic epithelial cells) with intermediate avidity. Thymocytes with too high or too low avidity will be eliminated by apoptosis or neglect, respectively. This process results in single-positive (either $CD4^+$ and $CD8^-$ or $CD4^-$ and $CD8^-$) precursors. During the negative selection, thymocytes that interact with self-peptide-MHC complexes (expressed by medullary thymic epithelial cells) with too high or too low avidity will be eliminated. The negative selection is the cornerstone of central self-tolerance [5].

25.2.2 Peripheral Tolerance

Peripheral tolerance is where the importance of CTLA-4 and PD-1 comes into action. T-cells with low avidity or sometimes with high avidity for self-antigens can escape the negative selection [6]. In peripheral tolerance, tolerogenic DCs present self-antigens in the peripheral lymphoid tissues. Tolerogenic DCs lack the stimulating signals needed for the T-cell activation and, instead, induce expression of CTLA-4 and PD-1 in those. CTLA-4 is the mainstay of inducing anergy (functional unresponsiveness) in self-reacting T-cells, and its effects are maintained by the PD-1 [7]. Therefore, CTLA-4 and PD-1 are necessary for the induction of peripheral self-tolerance, but neoplastic cells can also use their ability to escape from the immune system, as discussed later.

25.2.3 CTLA-4 Receptor

For an effective response to antigens, in addition to attachment of T-cell receptors (TCRs) to the MHC complexes, T-cells require a variety of stimulating signals, which are initiated by interactions between T-cell and antigen-presenting cell (APC) receptors. One of such attachments is between CD28 on T-cells and B7-1 (CD80) or B7-2 (CD86) on APCs. CTLA-4 is a homolog for CD28, with a higher affinity for binding to B7. The attachment of CTLA-4 on the T-cell surface with B7 will impede the induction of stimulatory signals required for T-cell activation [8, 9].

25.2.4 PD-1 Receptor

PD-1 is another inhibitory receptor expressed on T-cells. PD-1 is mainly expressed during the chronic and endured stimulation of activating receptors (mainly TCR and CD28) by antigens, which usually happens during chronic infections or cancers [8]. After interaction with its ligands (PD-L1 and PD-L2), it makes its inhibitory effects mainly via suppression of production of cytokines involved in the differentiation and survival of T-cells (including TNF-α, IFN-γ, IL-2, and Bcl-xL) [10].

25.2.5 Immune Escape Mechanism

As mentioned earlier, under normal circumstances, all neoplastic cells get eliminated by the immune system (mainly cytotoxic T-cells). This happens because of specific neoantigens that cancer cells produce and express on their surface with MHC complex I [11]. Immune surveillance has the most important role in eliminating cancer cells but also can contribute to the emergence of some immune-resistant neoplastic cells via the "Darwinian selection," as described earlier. In the immune equilibrium phase, neoplastic cells either might be under the control of the immune system or might eventually harbor enabling mutations and enter the immune escape phase [12]. Such mutations might make neoplastic cells capable to stop expressing neoantigens (by mutations in MHC class I and its signaling pathways or by expression of modified weaker antigens) [12]. Besides, neoplastic cells may induce an immunosuppressive state in the tumor microenvironment (TME). This happens by the production of immunosuppressive cytokines (IL-10, transforming growth factor-β, indoleamine 2,3-dioxygenase, etc.) and molecules (mainly PD-L1) and recruitment of suppressor immune

cells (regulatory T-cells and myeloid-derived suppressor cells or MDSCs) [12, 13].

25.3 The Essential Need for Biomarkers

ICIs interfere with two of the most important mechanisms of peripheral tolerance, as discussed earlier. Therefore, immune-related adverse effects (irAEs) are the concerning side effects of this therapy. Also, despite promising results in some groups of patients, not all of them respond to these agents. In fact, in some groups, ICIs have not had any difference with other conventional chemotherapeutic regiments (which will be discussed later). Hence, reliable biomarkers can help clinicians choose which patients to be enrolled in the ICB therapy programs. Also, appropriate biomarkers can guide how to choose the first-line drug and when to administer it and anticipate whether a patient needs combined ICB therapy or not. The following parts of this chapter will focus on the numerous biomarkers developed for ICB therapies.

25.4 Demographic Characteristics

Before we discuss molecular and invasive approaches known as ICI biomarkers, it is worth to mention that some simple demographic information can also act as biomarkers.

25.4.1 Sex

Most original studies have not reported sex as an important factor in response to ICI therapy. A study has developed a model for predicting the response to anti-PD-1 therapy in patients with advanced melanoma. It has shown that the response to therapy has been better in men [14, 15]. Besides, a meta-analysis has shown that OS and PFS have been more favorable in male melanoma patients [15].

25.4.2 Age

In the mentioned model developed by Nosrati and colleagues, age is another predictive biomarker; patients younger than 65 years have not responded to therapy [14]. However, most studies and clinical trials have not considered age as a predictive biomarker.

25.4.3 Tumor Size

It has been reported that in melanoma patients who received pembrolizumab, reactivation of $CD8^+$ T-cells after the initiation of therapy has been associated with physical tumor burden. Furthermore, higher values of the reactivation rate divided by tumor size have been correlated with more favorable OS and ORR [16]. However, almost none of the large validated clinical trials reported the tumor size as a predictive biomarker for ICI therapy.

25.5 PD-L1 Expression

Due to the mechanism of action of anti-PD-1 and anti-PD-L1 antibodies, measurement of the amount of PD-L1 expression in tumoral tissue biopsies seems a logical approach for predicting the response to ICIs. This measurement is done by immunohistochemistry. Many trials have reported the expression of PD-L1 in their patients, but the results are quite paradoxical. Here, we review the results of some of the more recent and important studies.

In a phase 1 study that evaluated the effect of nivolumab on five types of cancers (advanced melanoma, non-small-cell lung cancer (NSCLC), castration-resistant prostate cancer, renal cell carcinoma, and colorectal cancer), 9 of 25 are PD-L1 positive, and interestingly, none of 17 PD-L1 negative patients had an objective response (OR) to the therapy. They have considered 5% expression in IHC as the threshold for considering PD-L1 expression positive [17]. In

patients with advanced NSCLC, the efficacy of nivolumab (in terms of progression-free survival (PFS), overall survival (OS), and objective response rate or ORR) had been enhanced in groups with higher expression of PD-L1 (with thresholds of 1%, 5%, and 10%) [18]. Similar correlations have been reported for patients with NSCLC, albeit with a 50% threshold [19]. In patients with metastatic melanoma to the brain that received combined nivolumab and ipilimumab, the rate of clinical benefit (defined as the percentage of patients with stable disease for at least 6 months or complete response or partial response) was higher in those with PD-L1 expression of at least 5% or more [20].

However, there is evidence against the importance of PD-L1 expression as a biomarker; a study that evaluated short and long effects of nivolumab on NSCLC (CheckMate 227) has concluded that in patients with high tumor mutational burden (threshold of at least ten mutations per megabase), progression-free survival and overall survival have not been different between high and low PD-L1 expression groups (as ≥1% or <1%, respectively) [21, 22]. Of note, in these studies [21, 22], the method for measuring PD-L1 only contains tumor cells (and not immune cells). Another phase 3 trial (KEYNOTE-522), which aimed to evaluate pembrolizumab for early triple-negative breast cancer, has concluded that pathological complete response in the pembrolizumab group has been achieved irrespective of PD-L1 expression status (on both immune cells and tumor cells, reported with the method known as combined positive score) [23]. Treatment with nivolumab has been effective for metastatic urothelial carcinoma, without any difference between PD-L1 expression-based groups (with thresholds of 1% and 5% for PD-L1 expression on the tumor cells) [24]. In another trial of nivolumab and ipilimumab for advanced melanoma, PD-L1 expression is reported as a poor biomarker for the efficacy of therapy [25]. Several other trials have observed similar results in advanced RCC and NSCLC [26, 27].

There are debates about the method of measuring PD-L1. Older studies have reported PD-L1 expression on either immune cells (IC) or tumor cells (TC), but more recent studies have reported it only for IC. The difference can be huge. For example, Massard and colleagues have concluded that with reporting the PD-L1 status based on its presence on IC or TC, independently, there is no significant difference between positive and negative PD-L1 groups in response to durvalumab for urothelial bladder cancer (UBC). However, with measuring the PD-L1 on either IC or TC (with 25% as the threshold), the ORR has been different between the two groups [28]. A similar conclusion is made by Chow et al., with the administration of pembrolizumab for patients with recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) [29]. Moreover, different studies use different IHC kits, elicit different measurement methods, and establish different thresholds. Besides, it seems that the distribution of PD-L1 is not the same throughout the tumoral tissues [30]. There is also evidence of the dynamic changes in the expression of PD-L1, which means that it is controlled by many signaling pathways and microRNAs that might be propitious targets for cancer immunotherapy [31]. It is also worth to mention that significant outcomes in PD-L1-positive groups do not necessarily mean that other groups will not benefit from ICB therapy. Finally, to date, FDA has approved the measurement of the PD-L1 in patients with NSCLC, gastric or gastroesophageal junction adenocarcinoma, cervical cancer, urothelial carcinoma, triple-negative breast cancer, esophageal squamous cell carcinoma, and HNSCC as a companion diagnostic test for the treatment with pembrolizumab and atezolizumab (available at https://www.fda.gov/medical-devices/vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-vitro-and-imaging-tools).

25.6 Tumor-Infiltrating Lymphocytes (TIL)

As discussed earlier, one of the escape mechanisms of neoplastic cells from the immune system is the induction of apoptosis and/or anergy in the infiltrated immune cells. The higher number

of immune cells in the TME has shown to be associated with a more favorable prognosis in some cancers, including CRC, advanced ovarian cancer, melanoma, NSCLC, and breast cancer [32–36]. Hence, it seems reasonable that a high infiltration rate of IC, and especially $CD8^+$ T-cells, inside the tumoral tissue would result in more favorable outcomes of ICB therapy. Emens and colleagues have shown that there is a better (yet nonsignificant) ORR and PFS among patients with metastatic triple-negative breast cancer who were treated with atezolizumab and had higher baseline IC infiltration [37]. A phase 2 study of ipilimumab in advanced melanoma has shown that the increase in TIL from baseline is associated with the clinical activity (detailed definition is provided in the article) [38]. Loi and colleagues administered pembrolizumab and trastuzumab for patients with trastuzumab-resistant HER-2-positive breast cancer. They found that TIL had been higher in patients with objective responses and those with controlled disease [39].

As for the PD-L1, reports of TIL measurement may also vary due to IHC techniques, the heterogenic rate of infiltration throughout the tumoral tissue, and the timing of the biopsy.

25.7 TIL Molecular Characteristics

A few studies have further investigated the distribution of different subclasses of TIL and the diversity in their receptors. In a study on 46 patients with metastatic melanoma, $CD8^+$ T-cells in the responding group had more clonal and tumor antigen-specific TCR β chains. Besides, after treatment with an anti-PD-1 antibody, the number of such cells in the responding group had a ten times expansion, compared with the progressive disease group [40]. In 20 patients with metastatic melanoma who were treated with an anti-PD-1 antibody, PFS and PR were significantly associated with the level of expression of CTLA-4 on $CD8^+$ T-cells. Furthermore, such cells also had the highest amounts of PD-1 [41]. Hamid and colleagues observed a better clinical activity in patients with higher baseline expression of FoxP3 (traditionally known as the marker of naïve and regulatory T-cells) in the nuclei of mononuclear cells [38].

25.8 Tumor Mutational Burden

Along with PD-L1 expression level, measurement of tumor neoantigens, and its underlying etiology, mutational burden (TMB) is another more accepted method as a predictive biomarker. Mutation in the genomic content of cells is one of the cornerstone events in the development of neoplasms. Triggers and mechanisms of DNA damage are discussed in detail elsewhere [42]. The neoantigens then express with the MHC class I on the surface of neoplastic cells. It is postulated that as the amount of expressed neoantigens increases, the ability of the immune system and especially cytotoxic T-cells in detecting these non-self-antigens and killing such cells will be enhanced too [43]. There are growing pieces of evidence that the outcome of cancer immunotherapies is also dependent on the TMB [44]. For the assessment of TMB, whole exome sequencing (WES) has been the conventional method. However, because of the extended need for time, required comparison with normal tissue genome, and high cost, it has been replaced with a novel method named next-generation sequencing (NGS) [42]. Comprehensive genomic profiling (CGP) is based on the NGS method and measures the number of indel mutations and somatic coding base mutations (determinants of TMB), as well as copy number alterations and microsatellite instabilities. Chalmers and colleagues have shown that compared with WES, CGP has acceptable validity and reliability [42]. They also have reported that TMB is higher in melanoma and NSCLC, two common targets for immunotherapies. This high TMB is probably because these two neoplasms are mainly caused by environmental mutagens (e.g., cigarette smoke, radon, and ultraviolet radiation). Based on these findings, they have further suggested that the other types of cancers with high TMB (defined as 20> mutations per megabase), such as skin squamous cell carcinoma, diffuse large B cell lym-

phoma, and other types of lung cancers, might be good targets for immunotherapy [42]. This study has also identified some genes which are associated with higher TMB [42]. Here, we will mention a small number of numerous trials that have tried to evaluate the effects of TMB on the outcomes of ICI therapy.

Hellmann et al. reported TMB as a predictive biomarker in patients with advanced NSCLC, with the evidence that TMB-positive group had longer PFS with ipilimumab and nivolumab, compared with chemotherapy [21]. In patients with advanced NSCLC, high TMB (measured by WES with cutoffs of 100 and 243 somatic missense mutations) has been associated with longer PFS and higher RR in nivolumab arm, compared with the chemotherapy [45]. High TMB (16 mutations per megabase, measured by NGS) has been associated with longer OS in patients with locally advanced and metastatic urothelial carcinoma who were treated with atezolizumab [46]. In patients with CRC, those with mismatch repair deficiencies (analyzed by microsatellite instability analysis system, Promega) had a better response to anti-PD-1 therapy [47]. The findings of the previous study have also reported for some other solid cancers [48]. In two cohorts of patients with advanced NSCLC who were treated with pembrolizumab ($n = 16$ and 18), higher TMB has been associated with higher ORR and PFS and durable clinical benefit (DCB, defined as a partial or stable response for more than 6 months) [44]. In patients with early resectable NSCLC, pathological response (defined as less than 10% viable cancer cells) to nivolumab has been associated with TMB [49].

Neoantigen intratumoral heterogeneity (ITH) is another predictive biomarker candidate for ICI therapy. It has been shown that in patients with lung adenocarcinoma, high TMB and low ITH, together, are associated with a longer survival period, regardless of the type of therapy [50]. This study also has analyzed the information of a previous study [44] and has concluded that DCB is higher in patients with high TMB and low (less than 1%) ITH, compared with high TMB alone [50].

However, there are limitations to the measurement of TMB as a predictive biomarker. A great number of such mutations seem to be specific for individuals [51]. As for the PD-L1 expression, until now, there is no accepted cutoff for grouping the number of mutations as high or low.

25.9 Mutations in the Specific Genes

A study tried to find the underlying etiologies of relapse during the treatment with pembrolizumab in four melanoma patients. Patients 1 and 2 had mutations in Janus kinase 1 and 2 (jak1 and jak2) encoding genes, respectively. The third patient had a mutation in the beta-2-microglobulin subunit (β2M) of MHC class I, which results in the absence of MHC class I on the cellular surface. The authors could not find any prominent gene alteration in the fourth patient [52]. It is suggested that mutations in *JAK1* and *JAK2*, which are parts of the interferon receptors, make neoplastic cells resistant to antiproliferative effects of IFN-γ [52].

A retrospective analysis of two sets of patients with NSCLC (treated with pembrolizumab and either pembrolizumab or nivolumab, respectively) showed that mutations in *TP53* and *KRAS* were associated with longer PFS. This is probably because of higher TMB, increased infiltration of cytotoxic T-cells, and enhanced IFN-γ-associated signaling [53].

Another study that aimed to assess the impact of mutations in DNA damage response and repair genes (DDRs) found that harboring DDR correlates with the favorable ORR, PFS, and OS in patients with metastatic urothelial carcinoma who were treated with nivolumab or atezolizumab. Common altered DDR genes in this study included *ATM*, *POLE*, and *BRCA2*. This correlation has been stronger for deleterious DDRs (defined as all loss of function mutations) [54].

Many other studies have analyzed the effects of various genomic mutations on the outcome of the ICI therapy. However, most of them are retro-

spective, which warrants the need for clinical trials to establish robust predictive biomarkers based on genomic analyses.

25.10 Heterogeneity in the *HLA* Genes and Expression of MHC

HLA genes, which encode the MHC classes I and II, are assumed as the most polymorphic genes of humans. MHC class I is composed of heavy and light chains (α chain and β_2 microglobulin, respectively). HLA class I consists of three genes: *HLA-A*, *HLA-B*, and *HLA-C*, which encode the heavy chain of the MHC class I [55]. Because of the importance of MHC complexes in the immune system (discussed earlier), several studies have tried to find an association between variations in the *HLA* genes and the outcome of ICI therapy.

A study of two cohorts of patients with different cancers (mainly melanoma and NSCLC) who received anti-CTLA-4 or anti-PD-1/PD-L1 antibodies revealed that homozygosity in HLA class I genes was associated with reduced survival in both cohorts [56]. Interestingly, in multivariate analysis that included TMB, the combined association of TMB and HLA heterozygosity with enhanced survival has been greater than that of the TMB alone, although the TMB had not been significantly different between heterozygous and homozygous patients [56]. They further realized that the homozygosity is mostly caused by *HLA-B* and *HLA-C*, probably because of their more expression on cells and APCs, respectively, and the ability of HLA-B to present more different peptides. Besides, the clonality of TCR has been higher in patients with heterozygous HLA class I genes [56]. In a subgroup of melanoma patients who received anti-CTLA-4 antibodies, B44 and B62 supertypes have been associated with improved and decreased survival, respectively. The authors have suggested that this might be due to the presentation of specific antigens (e.g., MAGEA3) by the B44 supertype, which is associated with favorable outcomes of anti-CTLA-4 therapy [56].

Another study of patients with advanced melanoma who were treated with either nivolumab and ipilimumab or ipilimumab monotherapy (CheckMate 069) showed that absence of the expression of MHC class I on $SOX10^+$ cells (defined as the absence on more than 50% of cells) was associated with poor OS in the ipilimumab, but not combination therapy arm [57]. This study also evaluated patients of another trial (CheckMate 064) for the expression of MHC class II on neoplastic melanoma cells and concluded that the presence of MHC class II (defined as the expression on more than 50% of cells) is associated with improved ORR in patients who first received nivolumab and then ipilimumab [57]. Similarly, analysis of the expression of the MHC class II on $SOX10^+$ cells of melanoma patients who were treated with anti-PD-1 or PD-L1 antibodies showed that the presence of HLA-DR (with 5% cutoff) on $SOX10^+$ cells is associated with ORR, PFS, and OS. Notably, such association was not observed in patients who were treated with ipilimumab before participating in this cohort [58]. The association between MHC class II and ORR was confirmed in another set of patients with melanoma who were treated with anti-PD-1 antibodies [58].

25.11 Expression of Immune-Related Genes

After the recognition of cancer cell antigens with TCRs, numerous cytokines and ligands are required for the effective activation and function of T-cells. Among these cytokines, TNF-α, IFN-γ, and IL-12 are of greatest importance [59]. In patients with NSCLC and melanoma, who received nivolumab and pembrolizumab, respectively, PFS has been longer in those with higher *IFNG* (IFN-γ gene) expression [60]. Also, OS has been longer in melanoma patients with higher *IFNG* expression [60]. Another study has evalu-

ated the immune-related gene expression in melanoma patients who were treated with ipilimumab. They measured the expression of more than 170 genes and concluded that higher baseline and posttreatment expression of immune-related genes (including IFN-γ, granzyme B, perforin 1, and MHC class II) are associated with more favorable clinical outcome and longer survival [61]. In contrast, Forde and colleagues found no difference in *IFNG* expression between responsive and nonresponsive groups, as for changes in other immune-related genes (for *CTLA-4*, *HLA*, *JAK1*, *JAK2*, etc.) [49].

25.12 Blood Biomarkers

Blood biomarkers have been accepted and validated as a predictive tool for response to the conventional chemotherapeutic regiments. The advantages of blood biomarkers include ease of obtaining, minor invasiveness (compared with the biopsy), and the possibility of repeated sampling.

25.12.1 Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is an indicator of anaerobic cellular metabolism, which usually happens in tumors with an accelerated rate of growth, as tumor vasculature cannot provide the oxygen for all cells, and generally, higher values of LDH are correlated with worse prognosis [62]. In a phase 3 trial that investigated the effects of combined nivolumab and ipilimumab on patients with advanced renal cell carcinoma, baseline LDH values of more than 1.5 times the upper normal of limit were an indicator of poor OS [63]. In two cohorts of patients with advanced cutaneous melanoma, higher values of LDH have been associated with shortened overall survival. This study then proposes an LDH value of more than two times of upper limit of normal as a cut-off for selecting patients for ipilimumab therapy. However, certain limitations of the study hinder this value as being a predictive biomarker [64]. Another retrospective study of patients with unresectable stage III or IV melanoma who were treated with pembrolizumab revealed that baseline LDH value of more than 2.5 times the upper limit of normal had the strongest association with a poor OS [65]. On the other hand, Tawbi and colleagues showed that in a trial of patients with metastatic melanoma to the brain, the rate of the clinical benefit (as defined before) of combined nivolumab and ipilimumab therapy had been higher in patients with LDH more than upper limit of the normal values [20]. As it is apparent, studies that have investigated the effects of LDH on the ICI therapy outcome are largely done on the patients with melanoma, which implies its limited usage as a predictive biomarker in other cancers.

25.12.2 Peripheral Cell Count

Another studied variable as a potential predictive biomarker is blood cell count. Neutrophil to lymphocyte ratio (NLR) is known as a marker of inflammation [63], and high NLR is associated with a grim prognosis in a variety of cancers [66]. Similar findings have reported for the outcome of ICI therapy. In patients with metastatic RCC (mRCC) who received anti-PD-1/PD-L1 therapies, higher NLR was correlated significantly with a shorter OS, PFS, and less ORR [67]. In another trial of nivolumab for mRCC, a baseline NLR value of more than 4.2 has been associated with an increased risk of progression. This study also reports that there has been a relation between baseline eosinophil count more than 100 μL^{-1} and decreased risk of disease progression [68]. Motzer and colleagues also have shown that NLR value of more than 2.9 has been associated with decreased OS [63]. Finally, a meta-analysis of NLR values in different types of neoplasms (melanoma, NSCLC, RCC, and urothelial carcinoma) treated with ICIs has concluded that higher NLR values have been correlated with poor OS and PFS in all of the studied cancers [66]. There is

also evidence that elevated absolute and relative eosinophil count, relative lymphocyte count, and decreased absolute monocyte count are associated with improved survival in melanoma patients treated with ICIs [65, 69].

25.12.3 Other Blood Biomarkers

An analysis of blood samples of patients with melanoma showed that they had high circulating extracellular vesicles containing PD-L1. The amount of PD-L1 significantly differed with those of healthy individuals. Furthermore, responders to the pembrolizumab had a significantly lower baseline and higher increment in the circulating PD-L1 values [70]. In HLA-A*0201-positive patients with metastatic melanoma who received ipilimumab, high serum baseline levels of CXCL11 and, to a lesser extent, soluble MHC class I polypeptide-related chain A (sMICA) were indicators of poor OS [71]. In a retrospective study on nine cohorts of ipilimumab-treated patients with melanoma, high baseline soluble CD25 (soluble IL-2 receptor-α) and LDH have been indicators of poor OS [72].

25.13 The Importance of Gut Microbiota

Regarding the increased focus on the role of gut microbiota in different aspects of human health, some studies have tried to find a possible relation between the composition of gut microbiota and response to ICIs. Treatment of metastatic melanoma with ipilimumab has resulted in better OS and longer PFS in those who have *Faecalibacterium* and other Firmicutes in their gut microbiota, compared with *Bacteroides* [73]. Similar studies on patients with melanoma treated with anti-PD-1 antibodies have shown better OR in those with gut microbiota composed of *Bifidobacterium*, *Collinsella*, *Enterococcus* [74], and *Faecalibacterium* [75]. Transplantation of these microbiotas to mice has resulted in similar results.

25.14 Other Possible Biomarkers

With the recognition of ICIs as potent antineoplastic agents in recent years, countless studies have tried to examine myriad clinical variables as a potential biomarker. For example, several studies have tried to disclose the role of other specific gene mutations (including TGF-β and β-catenin) [76–79] and spatial characteristics of TIL [40] in the outcome of ICI therapy. Along with CTLA-4 and PD-1, there are other inhibitory molecules on immune cells. Some examples are lymphocyte activation gene 3 (LAG3), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) [80]. It is rational to think these inhibitory molecules might be the reason for resistance to ICI therapy in some patients and are recognized as good targets for the future immune checkpoint inhibitor agents [80]. Indoleamine-pyrrole 2,3-dioxygenase (IDO) is a long known immunosuppressant enzyme which usually secret from IFN-γ-activated macrophages [81]. Following the observations that higher amounts of IFN-γ are correlated with favorable outcome, a "T-cell-inflamed gene expression profile (GEP)" consists of 18 INF-γ-responsive gene set (including *LAG3*, *IDO1*, and *TIGIT*) developed to predict the outcome of patients treated with pembrolizumab. Validation of this GEP in patients with melanoma, gastric cancer, and HNSCC has shown that increased expression of selected genes is correlated with better PFS and OS [82]. A trial of PD-L1-positive patients with more than 20 types of solid tumors has shown that the higher T-cell-inflamed GEP (along with higher TMB and PD-L1 expression) is correlated with better ORR and PFS [83]. A study of nivolumab for patients with melanoma has reported that the serum levels of IL-6, IL-10, and IFN-γ were higher in the responding group [84]. Inducible T-cell co-stimulator (ICOS or CD278) is a stimulatory molecule which expresses on T-cells and acts as a stimulator of T-cells and sometimes suppressor of regulatory T-cells; both are against the outgrow of neo-

plasms, which makes it a good target for cancer immunotherapy [85, 86].

25.15 Combination of Different Biomarkers

TME has a dynamic nature, and there are complex interactions between different cells, cytokines, receptors, and signaling pathways, which may vary in different times and different places of TME. For example, it has shown that the infiltration of lymphocyte to the TME correlates with the expression of PD-L1, PD-1, and genetic mutations [53, 87, 88]. The expression of PD-L1 itself is influenced by several other cytokines, including PI3K, MAPK, several miRNAs [31], and IFN-γ [60]. The association between the expression of different genes might be complicatedly interlaced [89]. Regarding the TMB, ITH should also be considered, as tumors with high TMB and low ITH (an indicator of clonal mutations) seem to respond better to therapy, than those with high TMB and ITH, as mentioned earlier [50, 90]. It is also noteworthy that a high TMB in the absence of its presenting agent (MHC) will not necessarily change anything [56]. Hence, in selecting patients for ICB therapy, it is superior to look out for several different biomarkers. Table 25.1 provides a summary of more conventional biomarkers discussed in this chapter.

Table 25.1 Summary of more conventional biomarkers reviewed throughout the chapter

Type of biomarker	Type of cancer	Association with the clinical outcome	Sampling type	Diagnostic test	Reference(s)
PD-L1 expression	Many, including eight types approved by the FDA	Favorable and in some studies none	Tumor biopsy	IHC	[17–27]
TIL	Many	Favorable	Tumor biopsy	Immunostaining	[32–39]
TIL characteristics					
Clonality of TCR	Metastatic melanoma	Favorable	Tumor biopsy	Immunostaining	[40]
CTLA-4 on CTC					[41]
FoxP3 expression					[38]
TMB	Many	Favorable	Tumor biopsy, blood sample (ctDNA)	WES, NGS	[21, 42, 44–49]
ITH	NSCLC, lung adenocarcinoma	Unfavorable	Tumor biopsy	WES, NGS	[50]
Specific gene mutations					
JAK1, *JAK2*, and *β2M*	Melanoma	Unfavorable	Tumor biopsy	Different	[52]
TP53 and *KRAS*	NSCLC	Favorable			[53]
DDR genes	Metastatic UC	Favorable			[54]
HLA heterogeneity	NSCLC, melanoma	Favorable	Tumor biopsy	Different	[56]
Blood LDH level	Advanced RCC, advanced melanoma	Unfavorable	Blood sample	Conventional lab kits	[63–65]
	Melanoma with brain metastasis	Favorable			[20]

(continued)

Table 25.1 (continued)

Type of biomarker	Type of cancer	Association with the clinical outcome	Sampling type	Diagnostic test	Reference(s)
Peripheral blood count	Many		Blood sample	Conventional lab kits	[65–69]
NLR Eosinophil count Lymphocyte count Monocyte count		UnfavorableFavorable Favorable Unfavorable			
Gut microbiota	Mainly melanoma	Different	Stool or oral samples	NGS, PCR	[73–75]

IHC immunohistochemistry, *TIL* tumor-infiltrating lymphocyte, *TMB* tumor mutational burden, *ctDNA* circulating tumor DNA, *WES* whole exome sequencing; *NGS* next-generation sequencing, *ITH* intratumoral heterogeneity, *NSCLC* non-small-cell lung cancer, *JAK* Janus kinase, *β2M* beta-2-microglobulin, *DDR* DNA damage response and repair, *UC* urothelial carcinoma, *LDH* lactate dehydrogenase, *RCC* renal cell carcinoma, *NLR* neutrophil to lymphocyte ratio, *PCR* polymerase chain reaction

25.16 Conclusion

Throughout this chapter, we reviewed some of the more accepted predictive biomarkers for the ICB therapy outcome. One should remember that these variables can be present in one patient together and can mislead the prediction of physicians if they do not consider them as an interwoven network. Despite remarkable outcomes and durable responses in some patients, the overall ICI rate of success is not high yet. Prospective trails should try to design combination models based on different biomarkers and validate them for different agents and different neoplasms. Until now, among the enormous studied biomarkers, PD-L1 expression, TMB and ITH measurement, and analysis of specific genomic mutations have yielded acceptable predictive values and should be considered for further investigations and combinations.

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Cancer Nanomedicine: Special Focus on Cancer Immunotherapy

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Abbreviations

ABC	ATP-binding cassette
ABCG2	ATP-binding cassette subfamily G member 2
AIF	Apoptosis-inducing factor
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
AnxA2	Annexin A2
Ape1	Apurinic endonuclease 1
Bcl-2	B-Cell lymphoma-2
BCL2L14	Bcl-2-like protein 14
BCL-XL	B-Cell lymphoma-extra large
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CASP2	Caspase-2
CD90	Cluster of Differentiation 90
CDK6	Cyclin-dependent kinase 6
CI	Combination index
CMIIT	Center for Molecular Imaging Innovation and Translation
CNTs	Carbon nanotube
CP	Coordination polymer
CRC	Colorectal cancer
CSCs	Cancer stem cells
CT	Computed tomography
CTAB	Cetyltrimethylammonium bromide
DAMPs	Damage-associated molecular patterns
DCE-MRI	Dynamic contrast-enhanced magnetic resonance imaging
DCs	Dendritic cells
DiMI	Diagnostics in Molecular Imaging
DWI-MRI	Diffusion-weighted imaging–magnetic resonance imaging
EMIL	European Molecular Imaging Laboratories
EP	Ependymoma
EpCAM	Epithelial cell adhesion molecule
EPNs	Enoxaparin sodium–PLGA hybrid nanoparticles
EPR	Enhanced permeability and retention
Fe-bLf	Iron-saturated bovine lactoferrin
gal-C-Dextran	Galactosylated cationic dextran
GMP	Gemcitabine monophosphate
HA	Hyaluronic acid
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
IGF	Insulin-like growth factor
IL2	Interleukin-2
LNA-Aps	Locked nucleic acid aptamers
LNPs	Lipid nanoparticles
MALDI	Matrix-assisted laser desorption/ionization
MB	Medulloblastoma
MBA	Methylenebisacrylamide
MCL1	Myeloid cell leukemia sequence 1
MCM	Mobil crystalline materials
MDR	Multidrug resistance

MRSI	Magnetic resonance spectroscopic imaging
MTC	Medullary thyroid cancer
NanoHH1	Nanoparticle-encapsulated hedgehog pathway inhibitor HPI-1
Nanolipogels	Nanoscale liposomal polymeric gels
NCPs	Nanoscale coordination polymers
NCs	Nanocarriers/nanocapsules
NIR	Near-infrared
NK	Natural killer
Nm	Nanometers
NPC	Nasopharyngeal cancer
NSCLC	Non-small-cell lung cancer
ODN	Oligodeoxynucleotide
OV	Oncolytic virus
PAMAM	Poly(amido amine)
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PET	Positron emission tomography
PHA	PEG-histidine-modified alginate
PLGA-PEG	Poly(D,L-lactide-co-glycolide)–polyethylene glycol
PLK1	Polo-like kinase 1
PTCL	Peripheral T-cell lymphomas
REV3	Reversionless 3
REV3L	REV3-like
RNS	Reactive nitrogen
ROS	Reactive oxygen
SBA-15	Santa Barbara Amorphous type material
shRNA	Short hairpin RNA
SODs	Superoxide dismutases
SP	Side population
SPECT	Single-photon emission computed tomography
STAT3	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAMs	Tumor-associated macrophages
TGF-β	Transforming growth factor-beta
Th	T-Helper
THBS1	Thrombospondin 1
TLRs	Toll-like receptors
TLS	Translesion DNA synthesis
Tregs	Regulatory T-cells
XIAP	X-Linked inhibitor of apoptosis

26.1 Introduction

According to the last update on this context, Fouad and Aaneihave suggested seven hallmarks of cancer: (1) selective growth and proliferative advantage, (2) altered stress response favoring overall survival, (3) vascularization, (4) invasion and metastasis, (5) metabolic rewiring, (6) an abetting microenvironment, and (7) immune modulation. Despite many efforts, cancer has remained one of the main causes of death in humans with not very effective therapeutic options. Surgery, chemotherapy, and radiotherapy are considered the gold standard options available for cancer patients. Chemotherapy alone, or in combination with radiation therapy, is usually used to increase the success rate of surgery. Unfortunately, not all tumors are surgically accessible. Additionally, despite widespread use of chemotherapy drugs, the nonselective nature of most of these agents could severely damage critical organs of the body due to indiscrimination between normal and cancerous proliferating cells, which causes different primary side effects. In other words, chemotherapy agents more accurately fall into the antiproliferative agents category, rather than anticancer agents [1–4]. Thus, it is not surprising that cancer patients always suffer from systemic toxicity of traditional cancer chemotherapy. Considering the fact that cancerous cells may be resistant to chemotherapeutic agents and cell division inhibitors and the nonselective nature of most of those agents, specific targeting of hallmarks of cancer could be a reasonable treatment option. Thereby, selective targeting of cancer cells has become an attractive treatment strategy for modern cancer therapy.

Once a healthy cell transforms into a cancer cell, it may be recognized by immune cells, which could be followed by induction of other immune cells to mount responses in greater scales. However, owing to several reasons, such as impairment of effective immune cell responses or evasion of tumor cells, immune system could inhibit the cancer evolution not all the time, and therefore, tumor/cancer will arise [5–7]. To overcome current cancer treatment plans' pitfalls, dif-

ferent strategies have been proposed. For example, it seems that identification of the mutated components and then selectively targeting these mutations via designed small molecules as mimetic or agonist could be a promising strategy to eradicate cancer cells [8]. Moreover, since tumor cells may escape from antitumor T-cell response through two primary mechanisms, that is, cancer immunoediting and impairment of antitumor immune responses, manipulation of the immune system in order to downregulate immune tolerance against cancer cells has shown promising results [9]. This strategy which is called as cancer immunotherapy has been well known as a potential treatment option of cancer. Although, a large number of immunotherapy approaches have been introduced so far, various clinical challenges remained to be addressed, and some of cancer patients still do not respond well to immunomodulatory compounds. The efficient delivery system could significantly enhance the effectiveness of cancer immunotherapy, which seems achievable through employment of different nanoparticles. Additionally, because of the significant greater chance of survival and successful treatment when cancer is diagnosed in early stage, early detection of a tumor may be as important as treatment. Nanoparticles are synthetic particles available in a wide range of sizes, which could be combined with drugs or other therapeutic agents to be used in the treatment of incurable disorders, such as cancer. Moreover, advances in nanotechnology have caused the emergence of novel approaches for cancer detection at very early stages, which was not possible with the traditional diagnostic methods. Overall, there is increasing evidence to support the fact that engineered nanoparticles have the potential to revolutionize the diagnosis and treatment of multiple types of human disease in all fields as well as cancer medicine [10, 11].

Nanotechnology in medicine, also known as nanomedicine, involves applications of nanoparticles as well as employment of manufactured nano-robots to make repairs at cellular level. Nanomedicine has offered several new possibilities to overcome different treatment obstacles through alternative drug delivery, improvement of treatment efficacy, and minimizing detrimental side effects to normal tissues [12]. As an example, specific targeting of tumor cells and their discrimination from nonmalignant surrounding cells are well-known advantages of nanotechnology in cancer treatment which will be associated with significantly reduced side effects [12]. Moreover, because of the possibility to control the size, shape, and surface properties of nanoparticles, benefits of nanoparticles for biological applications would be significantly higher than conventional treatments [13]. For example, the properties of nanoparticles (e.g., solubility) can be engineered via changing their shapes and chemical compositions. As it was mentioned, nanotechnology not only could be employed in the treatment of cancer but also provides unique capabilities and enables innovative diagnosis. Currently, there are different diagnostic tests for cancer, including laboratory tests (e.g., blood, urine), imaging tests (e.g., X-ray, PET/CT, MRI, ultrasound), nuclear medicine scans (e.g., bone scans), endoscopy, and genetic tests, which should be confirmed by biopsy and pathology. Employing nanomedicine in each aforementioned diagnostic areas has provided great opportunities in more sensitive and specific diagnosis of cancer [14–16]. As Chen et al. [14] have discussed, nanoparticles can be used as probes in in vivo imaging, biosensing, and immunostaining. This technology offers high sensitivity, appropriate size for long-lasting circulation and penetrating in many biological barriers, and multiple targeting ligands.

In this chapter, at first, different aspects of the immune system during carcinogenesis process will be reviewed. Moreover, some of the well-studied immunotherapy approaches will be briefly discussed. Application of nanotechnology in diagnosis and treatment of cancer will be discussed following an overview on immune responses and current related therapeutic approaches. Some of the most critical challenges related to anticancer nanomedicine development will be pointed out at the end of chapter, and traditional immunotherapy approaches will be compared with new nanotechnology-based immunotherapies.

26.2 Overview of the Immune System and Cancer

Immune systems can distinguish between self and non-self most of the time. Hence, it not only protects the host against pathogens or infectious agents including viruses, bacteria, fungi, and other parasites but also specifically identifies and then eliminates abnormal cells to prevent the development of many cancers. Indeed, the immune system can mount cytotoxic immune responses against tumors and thereby acts toward the eradication of cancer cells. However, it does not always happen flawlessly, and cancer cells employ different mechanisms to escape from the immune system in a reactive fashion to be protected from this immune attack [7].

Coordination between two distinct cellular compartments, referred to as the innate and adaptive system, could significantly prevent tumor development. Innate immune system consists of various immune cells, including dendritic cells (DCs), monocytes, macrophages, natural killer (NK) cells, and granulocytes (neutrophils, basophils and eosinophils, and mast cells). These cells are able to cause activation of the adaptive arm through specific signals. DCs also act as a bridge between the innate and adaptive immune systems, and cytokines secreted by activated DCs influence both innate and adaptive immune responses [17]. T and B lymphocytes are the major cellular components of the adaptive immune response which are involved in cell-mediated and humoral immunities, respectively. Cross talk between those two arms may be necessary for polarization of sustained antigen-specific immunity.

Premalignant or malignant cell death might stimulate antitumor response or immune surveillance. Calling damage-associated molecular patterns (DAMPs) as the results of radiation not only causes direct cytotoxic effects but also initiates immune responses against tumor [18, 19]. It has been described that inflammation is a major player in cancer evolution, maybe thanks to the successive changes occurring at the tumor site [20]. However, failure of DAMPs to elicit an effective antitumor response may trigger chronic inflammation and thereby promote the development or progression of tumors [21]. Stress-associated DAMPs trigger innate immune system activation and make a bridge toward adaptive immunity. Although adaptive immunity is able to restrain cancer cells to be grown in an extended time [22], it was suggested that in the absence of adaptive immunity, cells in innate immunity arms (e.g., NK cell) act as important effectors during cancer immunoediting [23]. In addition to radiation, conventional chemotherapeutic agents also stimulate the immune system through different signaling pathways, such as increased extracellular ATP concentrations [24], recruitment and differentiation of APCs [25], and induction of cytokine expression [26].

There are several pieces of evidence to support the critical role of immune system in prevention of cancer. Recent findings have unequivocally documented that immune system, which was previously thought to act as a barrier against tumorigenesis, facilitates cellular transformation, as well. It seems that antitumor effector and suppressor cells contributed in tumor growth prevention and tumorigenesis, respectively. This phenomenon could be confirmed by an incidence of increased cancer cells in immunocompromised patients [27]. Moreover, a large number of immunosuppressive drugs have been found to be associated with multiple tumor types, such as lymphoma and skin cancer [28, 29]. Interactions between tumor-infiltrating immune cells and tumor cells could either interfere with tumor progression or actively promote tumor growth. Some effector cells provide protection against different pathogens but not against tumor cell development. For example, T-helper (Th) 22 cells were found to be associated with different types of cancer, such as hepatocellular carcinoma and colorectal cancer [30, 31]. Moreover, there are some other cells referred as regulatory T-cells (Tregs) that are highly immunosuppressive and play central roles in prevention of autoimmunity process [32]. These cells have an opposite role in cancer progression and may promote local tumor growth. Shedding light on cancer cell interaction with innate and adaptive immune system may enable us to

develop novel, effective, and safe therapeutic options by manipulating the immune system at molecular level in human cancers.

26.2.1 Immune Cells and Mediators in Tumors

Tumor microenvironment is a highly heterogeneous mix of cellular and noncellular components including various immune cell types from both innate and adaptive systems such as effector T-cells (CD8+ and CD4+ T-cells), Tregs, macrophages, DCs, NK cells, and NKT cells. Their percentages and phenotypes markedly vary among different types of tumor and even among patients with the same tumor type. As it was previously mentioned, both innate and adaptive immunities are essential to exert effective antitumor responses. Among the innate immune cells which are involved in fighting against cancer cells, NK [33] and NKT cells [34] play important roles in the immune surveillance of cancer and are able to lyse and directly kill the tumor cells. NK cells are specialized to eliminate virus-infected as well as malignantly transformed cells. Those frontline soldiers of the innate immune system act through different strategies, such as releasing perforin and granzymes, expression of the death receptor ligands TRAIL and FasL, and secretion of cytokines and chemokines [35]. Moreover, NK cell activity could recruit other immune cells to the tumor site. Despite their potent and powerful cytotoxic activity, their dysfunctional deficiency in cancer patients highlights the fact that their activity may be eluded by the tumor microenvironment [36]. Those findings have resulted in employing NK cells for cancer immunotherapy [35]. Similar to NK cells, NKT cells are usually considered as an interface between innate and adaptive immune systems and are critical modulatory cells in shaping adaptive immune responses. NKT cells (especially type I) directly and indirectly fight with cancer cells via their cytolytic activity and activation of additional immune cells, respectively. However, these cells (especially type II) also may effectively suppress the early tumor-specific immunity, and therefore, these cells could be considered as a double-edged sword in cancer evolution [37].

DCs are the most potent antigen-presenting cells which cause initiation of antitumor immunity by unleashing a T-cell response. Infiltration of maturated and active DCs into the tumors confers an increase in recruitment of tumor-specific effector T-cells. However, DC maturation within the tumor site makes them unable to induce sufficient immunity [38]. To elicit enough tumor-specific effector T-cell responses, a concerted effort has been initiated to use DC-based immunotherapies as a weapon against cancer [39]. Tumor-associated macrophages (TAMs) have been found to be largely present in the tumor microenvironment, and they are major players of the cancer-related inflammation [40]. TAMs seem to be directly involved in tumor progression and growth and may be indispensable for angiogenesis, invasion, and metastasis [41] as high TAM content was found to be associated with poor cancer prognosis [42]. Owing to the ability of TAMs to promote the development and migration of tumor, selective targeting of these cells has attracted considerable interest and may be proved to be beneficial in the treatment of cancer [40, 43]. Neutrophils are other players belonging to innate immune system that could have contribution in tumor initiation, tumor growth, and metastasis cascade. Several mechanisms have been suggested that show neutrophils promote tumorigenesis (reviewed in [44]). Oxidants produced by neutrophils, such as reactive oxygen (ROS) and reactive nitrogen (RNS) species as well as proteases, could result in epithelial damage and subsequent tumor-promoting inflammation. Stimulation of proliferation through IL-1 receptor antagonist in addition to impairing CD8+ T-cells-mediated antitumor immune responses accelerates tumor growth, as well. Moreover, they are involved in several steps of metastasis through stimulation of cancer cells to migrate.

Regarding the role of adaptive immunity, T-cell responses are relatively more tumoricidal compared to most humoral responses, and they have important roles in establishing antitumor immunity [45]. Induction of optimal systemic

antitumor immunity involves priming of both CD4+ and CD8+ T-cells (effector T-cells) which can finally lead to tumor regression.

Although activity of one effector cell group, CD8+ or CD4+ T-cells, is adequate for tumor eradication, higher antitumor effect has been shown to be exerted when those cells work together [46]. Naïve CD4+/CD8+ T-cells can differentiate among different functionally distinct tumor suppressor or tumor promoter subsets. The latter group could suppress the activity of tumor-specific T effector cells; for example, cytotoxic CD8+ and CD4+ Th1 T-cells function as the major antitumor immune effector cells through production of cytokine IFN-γ, a critical cytokine involved in tumor suppression. However, a subpopulation of CD4+ T-cells, which abrogates the attack of effector cells against self-somatic cells, acts as a promoter of tumor growth through inhibition of the effector T-cells. Traditionally, research in cancer immunity has focused almost exclusively on Th1/Th2 cell balance. Identification of different other subsets of Th cells including Th17, Th9, and Th22 has shed light on the significant roles of T-cells in control of tumor evolution during the past decades. Among those cells, Th1 is the most studied type of T-cells which is critically important for induction of in vivo antitumor cellular immunity [47]. Conversely, Th2 inhibits Th1 differentiation and interferes with antitumor CTL activity, and therefore, their activity would be associated with tumor progression. Those humoral-mediated cells can inhibit cell apoptosis via IL-4 and IL-10 secretion, as well [48]. The cells, a relatively novel subset of CD4+ T-cells, have recently attracted more attention due to its ability to enable CD8+ and CD4+ and can activate the adaptive antitumor immunity as well favoring DC survival [49–51]. However, there is an evidence suggesting that Th9 cells can function as a promoter of cell proliferation and migration, as well [52]. In spite of a huge number of conducted studies on the role of Th17 in cancer, there are several contradictory results indicating that it may function as a double-edged sword in cancer pathogenesis [53]. There are mounting evidences suggesting that Th22, as a recently identified subset of human CD4+ T-cells, may be involved in the development of tumors, and therefore, tumor-infiltrating Th22 cells could be suitable therapeutic targets in cancer patients [31, 54–56]. In contrast to effector T-cells with antitumor immunity, such as Th1, Tregs interfere with the eradication of tumors. Tregs, which are composed of a diverse and heterogeneous subset cells (e.g., Th3, Tr1, iTr35), suppress tumor-primed T-cell activity. It was demonstrated that Tregs infiltration was significantly associated with poor prognosis in multiple tumors [32, 57, 58] and therefore depletion of Tregs has demonstrated to result in augmentation of antitumor immune responses and immunotherapy [59].

26.2.2 Tumor Immune Surveillance and Cancer Immunoediting

Cancer immune surveillance is a hypothesis which has been postulated by Burnet and Thomas in more than half a century ago [60, 61]. As it was discussed, the immune system can specifically identify and eliminate tumor cells through recognition of expressed antigens that are not found in normal cells and/or molecules induced by cellular stress. According to the cancer immune surveillance hypothesis, adaptive immunity was responsible for hindering tumor growth in immunocompetent hosts. However, Stutman [62] demonstrated that there is no difference in cancer susceptibility among the immunocompetent mice and nude mice with substantial but not total immunodeficiency, which has led to widely abandon immune surveillance theory. However, a more comprehensive hypothesis was still required to explain those observations. In the early twenty-first century, it was revealed that this surveillance function could be extended to a more comprehensive one, known as cancer immunoediting which was describing novel aspects of the immune system–tumor interactions. Immune system not only protects the host against cancer development but also shapes tumor immunogenicity (composed of three phases which is elimination, equilibrium, and escape) which is the basis of this new hypothesis [63, 64]. It is implementing the dual

host-protective and tumor-promoting actions of immunity on developing tumors. During the elimination phase, which refers to cancer immune surveillance theory, both arms of immunity work together to detect the presence of a developing tumor cells and eradicate them before clinical appearance. However, some tumor cells may survive from the elimination phase and enter the equilibrium phase in which the immune system does not cause eradication of cancerous cells while holding the tumor in a state of functional dormancy. In the third phase, some tumor cells that have acquired resistance to elimination may circumvent immune recognition and escape from immune destruction, followed by progressively growing and visible tumors. Exhaustion of immune system as a result of the emergence of tumor cell variants may be responsible for bypassing elimination phase.

26.2.3 Tumor Immune Evasion

Complicated cross talk between immune system and cancer cells can either inhibit or enhance tumor growth which is now classified as a hallmark of cancer, and tumors could learn how to avoid immune-mediated elimination by employing various mechanisms to evade immune surveillance. Some of those mechanisms include decreasing or shedding the expression of tumor-associated antigen (TAA), impaired expression of MHC class I, downregulation of co-stimulatory pathway (e.g., CD28), aberrant expression of co-inhibitory molecules (e.g., CTLA-4), downregulation of adhesion molecules, expression of the apoptosis-inducing protein (e.g., Fas ligand), recruitment of immunosuppressive cells (e.g., Tregs), and secretion of immunosuppressive factors (e.g., transforming growth factor-beta [TGF-β] and IL-10) [5–7].

The immunogenicity of a tumor is significantly dependent on its antigenicity. Most tumor cells express antigens which can be recognized by the host and have the potential to elicit tumor-specific immune responses [65]. These antigens could be mainly encoded by either germline or somatic cancer, genetic mutations, and oncogenic viruses (e.g., human papillomavirus [HPV]) [66]. However, to avoid immune-mediated elimination, cancer cells may lose their dominant antigens or harbor defects through underexpression of MHC class I and other components of the antigen-processing machinery. In addition to the loss of antigenicity expression, tumor cells may fail to function as effective APCs due to the lack of positive co-stimulatory ligands or even presence of inhibitory ligands. As it was previously mentioned, most tumors lack the expression of positive co-stimulatory molecules that cause abortive proficient T-cell activation, and thereby, the situations would be suitable for tumor cells to enter into the escape phase.

Tumor cells often show a decrease in cell–cell adhesiveness which seems to be a critical phase in the invasion and metastasis of human cancers [67]. It is now well accepted that cell adhesion molecules that function as tumor suppressors are able to suppress cancer cell growth, but not necessarily migration [68]. Fas activation through ligand–receptor interaction triggers apoptosis in cells. Expression of FasL during T-cell activation is indispensable for maintaining the homeostasis and the proper functioning of the immune system. However, increased FasL levels in some tumors such as melanoma [69], lung cancer [70], pancreatic cancer [71], and breast cancer [72] were found to induce effector T lymphocytes to die. Effector T-cell death might accelerate T-cell activation-induced cell death and also leads the cancer cells to escape from immune recognition and interference. In addition to FasL, increased expression of some other members of the TNF family as well as TRAIL may contribute in inducing antitumor effector cell death [73].

Recruitment and expansion of immunosuppressive cell populations by tumors are another well-discussed strategy to escape immune surveillance [74]. Moreover, reprogramming of normal antitumor immune cells into the tumor-promoting cells plays critical role in expansion of tumors. Tregs are a good example of those cells that facilitate tumor immune escape through inhibition of antitumor immune responses and therefore induction of immunosuppression. Moreover, impairment of antitumor

immunity may be mediated by tumor-derived immunosuppressive soluble factors including galectin-1, TGFβ, and IL-10 which cooperate in advanced stages of cancer to limit the antitumor activity of immune system [75].

26.2.4 Current Immunotherapies

Owing to the limited efficiency and emergence of several serious side effects in conventional therapies of cancer, novel therapies are urgently needed with more desirable outcomes and less side effects. It is worth to note that considering the components of both innate and adaptive immune system is required for the design and development of effective immunotherapy approaches. Fortunately, during the recent decades, advances in cancer immunology and revealing the role of the immune system in cancer initiation, progression, and invasion have provided new therapeutic options. Generally speaking, cancer immunotherapy harnesses the immune system to eradicate tumor cells and prevent future relapse. Several forms of immunotherapy have been explored to boost or restore the ability of the immune system to detect and eliminate tumor cells. These approaches act through overcoming the mechanisms by which tumors evade from immune cells which are exercising their antitumor activities. Some of the well-described options include cell-based therapies (cancer vaccines and adoptive cell therapy) checkpoint inhibition, cytokine therapy, therapeutic administration of monoclonal antibodies, and oncolytic virus immunotherapy (reviewed in [9]).

26.2.5 Cancer Vaccines

Cancer vaccines could be categorized as biological response modifiers, which either stimulate or restore the impaired immune responses against tumors. Cancer vaccines could be divided into two broad types, that is, prophylactic and therapeutic vaccines. The prophylactic vaccines are used as a predictive treatment in high-risk normal individuals such as those who are infected by HPV or hepatitis B virus. Therapeutic vaccines, which are a form of immunotherapy, are intended to treat existing cancer by boosting anticancer immunity and can be used through major approaches including autologous patient-derived immune cell vaccines, engineered viruses to express tumor antigen transgenes, protein/peptide-based cancer vaccines, DNA/RNA vaccines, and allogeneic whole tumor cell vaccines [76, 77].

26.2.6 Adoptive Cell Therapy (ACT)

Adoptive cell therapy (ACT) is another approach which can be used in harnessing the immune system for cancer therapy. It is a highly personalized cancer therapy that refers to the ex vivo expansion of autologous or allogeneic immune cells and then reinfusion of the cells back into the patient. Using this approach, isolated tumor-infiltrating lymphocytes from patients will be reinfused back into the patient after ex vivo expansion, with the goal of recognizing, targeting, and destroying tumor cells [78]. ACT was found to be a promising strategy to induce regression of established tumors in a number of malignancies, including metastatic melanoma [79], leukemia [80], and prostate cancer [81]. Over the past decade, many efforts have been made for engineering immune cells before reinfusion to the patient with the aim of revolutionizing adoptive cell immunotherapy. This technology has opened up a whole new avenue of research in cancer immunotherapy. Engineered T lymphocytes have been used to express chimeric antigen receptors (CARs) allowing the T-cells to recognize antigens on targeted tumor cells. Although this approach has been shown to be successful in treatment of various hematologic malignancies [82, 83], there are some multicenter clinical trials using CAR T-cells targeting expressed TAA, such as EGFR and HER2, to investigate the antitumor effects of engineered T lymphocytes on solid tumors [84]. In addition to the CAR T-cells, owing to the great potential of NK cells in mounting immune system against the tumor cells,

adoptive transfer of allogeneic CAR-modified NK cell and NKT cells which have been ex vivo expanded has emerged as another novel strategy of cancer immunotherapy. NK cells expressing CARs have demonstrated to have significantly improved specificity and efficiency in detection and elimination of tumor cells through recognition of surface antigens overexpressed on cancer cells. Those engineered cells seem to be able to recognize cancer cells and can be used as a magic bullet against not only hematologic cancers but also solid tumors [83]. Although CAR T-cell therapy strategy offers several advantages over other immunotherapy approaches, due to lack of enough CAR NK clinical studies, it is still waiting to receive regulatory approval.

26.2.7 Checkpoint Inhibition

As it was previously discussed, cancer cells employ checkpoints for T-cell exhaustion and thereby protect themselves from the immune system attack. Targeting immune checkpoints with CTLA-4- or PD-1-blocking antibodies has held a lot of promises among cancer treatment strategies. So far, various drugs have been introduced to restore antitumor immunity especially in various types of solid tumors in either single targeting manner including PD-1 inhibitors (pembrolizumab, nivolumab), PD-L1 inhibitors (atezolizumab, avelumab, durvalumab), and CTLA-4 inhibitor (ipilimumab) or dual targeting as well as PD-1- and CTLA-4-blocking agents [85, 86]. In spite of remarkable results obtained by checkpoint inhibition therapy, development of autoimmunity in genetically susceptible patients is a serious concern which has remained to be addressed in this approach [87].

26.2.8 Cytokine Therapy

Two cytokines could achieved FDA approval as single agent for cancer treatment, so far: high-dose bolus IL-2 for metastatic melanoma and renal cell carcinoma and IFN-α for adjuvant therapy of stage III melanoma [88]. Treatment of cancer using IL-2 and IFN-α cytokines has been designed to mainly target adaptive immunity (e.g., activation of T-cells) and innate immune cells (e.g., promotion of DCs and macrophages). However, owing to the observed different side effects as well as flu-like disease in subsequent studies, using IFN-α has been shown to be used in limited cancer treatment programs.

26.2.9 Monoclonal Antibody

Monoclonal antibody-based treatment of cancer was found as a promising therapeutic option for both solid tumors and hematologic malignancies. Various drugs which belong to this class of new agents have been approved for the treatment of human cancer (e.g., trastuzumab, rituximab, cetuximab, alemtuzumab) [89]. Although they are safer than conventional cancer chemotherapeutic agents, some side effects have been reported which are majorly related to the targeted antigens and intravenous route of administration [90]. Targeting of tumor-associated macrophages (TAMs) as a critical player in modulating the local microenvironment in order to facilitate tumor growth and metastasis is another suggested approach for cancer immunotherapy [91]. It has been accepted that TAMs are correlated with increased tumor angiogenesis, metastasis, and poor prognosis of most of the human cancers (reviewed in [91]). Therapeutic advantages of targeting TAM have been confirmed in several clinical trials using different agents to target TAMs, including carlumab, alemtuzumab, and tremelimumab (reviewed in [92]).

26.2.10 Oncolytic Virus Immunotherapy

Oncolytic virus (OV) immunotherapy is a novel form of cancer therapy which utilizes native or genetically modified viruses with the capability to selectively replicate and spread within the tumor cells without affecting the surrounding healthy tissues [93]. OVs are believed to promote antitumor responses mainly through their direct oncolytic activity as well as induction of systemic antitumor immunity. Generally, employed

viruses as vectors for OV immunotherapy could be classified into nonpathogenic viruses that naturally replicate preferentially in cancer tissue (e.g., paramyxovirus, picornavirus) and genetically modified viruses that become nonpathogenic before administration (e.g., herpes simplex virus, measles virus, vaccinia virus) [94].

26.3 Application of Nanotechnology in Cancer

Nanotechnology is a relatively novel and rapidly growing field, which provides new molecular contrast agents enabling earlier diagnosis and imaging and selectively targeting tumor cells. Recent advancements in managing various types of cancers are thanks to implication of nanomaterials in different aspects of diagnosis and treatment. In this part, the most important and frequent applications of nanomaterials will be discussed in different fields of cancer control with final debate on immunotherapy enhanced by nanoparticles.

26.3.1 Nanodiagnostics

The role of nanomaterials in diagnostic area of medicine especially early diagnosis, screening, or follow-up of cancer patients could be classified in three major spectrums including diagnostic and screening biosensors and various medical imaging technologies especially magnetic resonance imaging (MRI). One of the fascinating promises of nanomaterials is the possibility of detection of tumor site and specific targeting of treatment in the identified malignant area. Herein, aforementioned diagnostic fields which sometimes have led to theranostic applications of nanomaterials will be described in details.

26.3.2 Nanomaterials in Medical Imaging

Application of nanomedicine in imaging-based diagnosis in modern imaging can be divided into main tow categories, that is, traditional imaging and modern molecular imaging. MRI is the most frequent conventional imaging technique in which using nanoparticles had increased its sensitivity and specificity, especially in cancer diagnosis. Modern molecular imaging which is sometimes called as nanoflare is a novel category of bio-imaging field therein nanomaterials would be conjugated with a molecule complementary to a molecular change typical of a specific cancer cell population. Interaction of two complementary molecules which are attached to nanoparticles will cause a chemical reaction and emit a signal indicating the presence of a particular change in living cells. However, nanotechnology-enhanced conventional imaging systems may sometimes have overlap with molecular imaging especially in early-stage diagnosis of the disease. In the following section, the details of each nano-imaging category will be described with specific focus on recent advancements.

26.3.2.1 Nanotechnology in Traditional Imaging

General Principles

MRI, computed tomography (CT), and positron emission tomography (PET) are the most common advanced imaging techniques which are frequently used in cancer diagnosis. In all of those techniques, characteristics of contrast agents have pivotal roles in the identification of abnormalities within target organ. Contrast agent or contrast medium as its name calls it is a material or substance used to increase the contrast and visibility of internal body organs through absorbing or changing the external electromagnetism or ultrasound. The most important advances mediated by nanotechnology in traditional imaging field have been made in developing novel contrast agents with enhanced contrasting capability. Some of the major challenges of using nanoparticle-based contrast agents are their recognition by the immune system and removing them from circulation. An ideal contrast agent should be maintained within the circulation till the imaging process will be fulfilled and then rapidly degraded and cleared from the human body without precipitation or interaction with each elements of clearance route [95, 96]. In this regard,

size and surface modifications of nanoparticles have substantial effects on their interactions with every part of the body. For example, hydrophobic, more surface-charged particles such as iron oxide, quantum dots, silica, and larger nanoparticles have more chances to be detected by the immune system and be opsonized for degradation [97]. However, the ideal size of nanoparticle should be adjusted to be larger than 5 mm as in much smaller size they are more prone to be quickly eliminated from circulation through kidney filtration system [98]. However, coating of nanoparticle with polymers like polyvinyl alcohol (PVA) or PEG can conceal their surface charge (except using small molecules as well as thiol-containing molecules), and inevitably results in larger particle [99, 100].

Nanoparticle-Mediated Targeting in Traditional Imaging

Another fantastic application of nanotechnology in imaging is specific targeting of cancerous tissue and determining its precise margins and extension by nanoparticle-based contrast agents. There are two main types of targeting, that is, active and passive. Passive targeting uses special characteristics of cancer cells such as enhanced permeability and retention (EPR) which leads to accumulation of macromolecules within the cell or specific trend of some molecules as well as Feridex, a contrast agent, to be entered into the liver or spleen cells [101, 102]. In active targeting approach, ligand of specific markers expressed on cancer cell is conjugated to a nanoparticle which will be used as contrast agent. The interaction between receptor and ligand leads to internalization of nanoparticle, and therefore, emission of a signal would be indicating the presence of tumor tissue and its exact margins, as well [103, 104].

Nanotechnology in MRI

As it was previously referred, the most frequently performed studies on the application of nanotechnology in imaging were reported in MRI field. MRI is still one of the most potent noninvasive imaging technologies which has excellent sensitivity and specificity in detection of soft tissue tumors. MRI images are basically obtained by the interaction between external magnetic field of instrument and protons present in the water of soft tissue. Type of contrast agents used in MRI and the possibility of their accumulation within the target cells will provide further details with a higher resolution [105]. Gadolinium (III) ion is one of the most frequent contrast agents used in MRI clinics due to its large paramagnetic and unpaired electrons which help to get more resolution in taken images [106]. Conjugation of gadolinium with specific ligand molecules (i.e., chelates) not only changes it to a nontoxic contrast agent but also makes it a suitable choice for active targeting imaging. It was frequently reported that addition of various types of nanomaterials to gadolinium including polymers, carbon nanotubes, and liposome had a significant effect on gadolinium accumulation within target cells and therefore increased the overall resolution and contrast of images taken by MRI [107, 108]. Of note, among all nanoformulated contrast agents, only gadolinium-based nanoparticles could receive FDA approval. Gadolinium oxide (GO) nanoparticles are another chemical form of gadolinium which has various types of surface chemical groups including hydroxyl, carboxyl, and epoxides. Those surface chemical groups provide a promising situation for conjugation and loading of chemotherapeutic drugs to start the treatment of cancer in real-time diagnosis. In more advanced simultaneous mode of cancer diagnosis and therapy called as multimodal theranostic delivery system, one chemotherapeutic and multiple diagnostic agents are loaded on a nanoparticle-based contrast agent which can be tracked by more than one imaging technology [109]. As an example, application of a nanocomposite that consisted of Si–Ti nanoparticles, gadolinium, and folic acid was demonstrated to be associated with higher contrast and resolution of MRI images [110]. In designing such a nanocomposite, some studies have used a combination of gadolinium and gold (Au) as contrast agent and had shown promising results in both imaging and drug delivery [111, 112].

The other frequently used contrast agent in MRI is superparamagnetic iron oxide nanoparti-

cle (SPION). They are small synthetic polymers of γ-Fe_2O_3, Fe_3O_4, or α-Fe_2O_3, and the two former oxides are the most commonly used SPIONs in medical imaging [113]. Superparamagnetic characteristics of SPIONs are strongly dependent on their size as the highest degree could be seen in particles with a core diameter of nanoparticle ranging 10–20 nm. SPIONs are appropriate to be used in drug delivery as upon an external magnetic field they can pull the therapeutic agent toward its target cells. In addition, owing to the dispersed form of SPIONs in the absence of magnetic field, their activity could be controlled by adding or removing the external magnetic field to significantly reduce the chance of their detection by the immune system in agglomerated form [114]. This feature of SPIONs has made them a less toxic alternative to gadolinium in MRI imaging especially in patients with renal dysfunction. However, it was demonstrated that the shape of SPION nanoparticle has a determinant effect on the toxicity of those particles on cells as larger nanoparticles as well as nanobeads or nanoworm particles are more prone to be toxic than smaller ones such as nanorods and colloidal nanocrystal clusters [115].

26.4 Nanotechnology in Other Imaging Systems

Implication of nanoparticles in other imaging technologies, as well as computed tomography (CT) scanning, has opened a new horizon toward specific diagnosis of tumors within human body cavities including chest, abdomen, pelvis, and cranium. CT scanning is based on X-ray rendering detailed imaging sections from various types of tissues using iodine- or gadolinium-based molecules as contrast agent [116]. Current contrast agents suffer from the necessity for injecting materials into the circulatory system and therefore the possibility of renal toxicity and nonspecific systemic distribution and eventually poor resolution [117].

GNPs maybe are the most type of nanoparticles which have been investigated in many CT imaging studies as contrast agent. Larger sizes of GNPs decrease the probability of extravasation of particles and therefore would have longer half-life due to a decrease in chance of filtering and excretion by urinary system [118]. The other major advantage of using GNPs as contrast agents in CT imaging is that due to their large size, they are able to absorb a lower range of X-rays, decreasing the general dose of radiation a patient should receive meanwhile increasing the resolution. Application of GNPs in CT imaging has been demonstrated to be useful in radiosensitization of choroidal melanoma cells, as well [119]. Another notable advantage of GNPs as contrast agent is their minimal biological toxicity which has been reported in two animal studies [117, 120]. GNPs capped with mannan as stabilizer and reducer have been recently shown to be effective in targeted lymph node CT imaging with a significant resolution [121]. Using other nanomaterials including bismuth sulfide (Bi2S3) and iodinated nanoparticles besides targeted liposomal carriers of traditional contrast agents has revolutionized the sensitivity and specificity of CT imaging technology, as well. Although requiring specialized and precise protocol of synthesis, Bi2S3 nanoparticles have demonstrated to be significantly stronger than conventional contrast agents with long blood circulation half-life [120, 122]. Iodine nanoparticles and nanoformulation have somehow overcome the iodine pitfalls including short half-life and less specific targeting. Encapsulation of iodine within polymers or liposomes provided the opportunity to increase the circulation time and local concentration on the targeted tissues to give stronger resolution [123, 124].

Implication of nanomaterial in PET as a functional imaging system has provided two scopes of advantages in both medical diagnosis and researches. Using radiotracers as well as ^{11}C, ^{13}N, or ^{15}O in PET scan not only can provide functional information about the metabolism within targeted organ especially cancer tissues but also can be a valuable tracking system for assessment of nanoparticle pharmacodynamics and kinetics and their distribution throughout the body. However, to obtain specific and precise results, ideal imaging technologies take advantage of the combina-

tion of structural and functional systems which are known as multimodal imaging systems [125–127]. The same scenario is held for fluorescence imaging in which organic fluorophores and fluorescent proteins are used to demonstrate the molecular actions including uptake and intake of various macromolecules and nanoparticles around the target cell. In this way, implication of nanomaterials in the structure of fluorophores and fluorescent proteins as carrier could enhance obtained signals through increasing the fluorophore skin infiltration and stability [128]. Owing to the reported low toxicity and high possibility of making different surface chemical linking, silica nanoparticles are the most widely investigated nanomaterials used to encapsulate hydrophobic fluorophores through covalent linking using various method of synthesis [129].

26.4.1 Nanotechnology in Molecular Imaging

Modern molecular imaging is aimed to noninvasively provide a detailed description of molecular and intracellular events for more sensitive diagnosis, treatment, and follow-up of various types of human diseases especially cancers at their initial stages as much as possible [130]. Molecular imaging includes a vast medical research and diagnostic area which are fundamentally based on tracking a molecular biomarker that its interaction with subcellular elements and its changes within the cell herald for initiation of a disease or even disease response to treatment. Owing to the potential of molecular imaging in the diagnosis of pre-disease status, some European and American organizations as well as the Center for Molecular Imaging Innovation and Translation (CMIIT), Diagnostics in Molecular Imaging (DiMI), or European Molecular Imaging Laboratories (EMIL) have invested on molecular imaging researches. Single-photon emission computed tomography (SPECT), diffusion-weighted imaging–magnetic resonance imaging (DWI-MRI), dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), magnetic resonance spectroscopic imaging (MRSI), and matrix-assisted laser desorption/ionization (MALDI) as a mass spectrometry technique are the most studied examples of molecular imaging methods in various diseases as well as cancer [131]. Gas bubbles in micron ranges of size have been used as a contrast agent to enhance ultrasound imaging system in the assessment of intracellular process. The other molecular imaging has been specified in using a combination of fluorescent or near-infrared (NIR) probes and light photon detection camera [132–135].

Recent advances in molecular imaging are mostly dependent on molecular probes which are including one specific molecule targeting special marker on cancer cell of interest and a reporter element which helps ligand and target marker interaction be visible. Although the most frequently used reporters were fluorescent markers and radionuclides, nanoparticles may be a better choice owing to their low toxicity and the simultaneous possibility of delivering multiple drugs to an identified defective site which will be discussed in detail in nanocarrier section of the current chapter. One of the other amazing superiority of nanoparticles in molecular imaging is the introduction of a category of liposome-based nanoparticles called as porphysomes. Porphysomes consist of a pyropheophorbide–lipid in which its number per every liposome determines porphyrin packing density. By regulation of packing density, we will be able to determine the capability of porphysomes as a diagnostic tool when the packing density is low or as a theranostic agent with high packing density through local specific changing of light energy into heat within tumor site (photothermal therapy) [131]. For effective implication of porphysomes in various types of human solid tumors with different expression profile, porphysome–liposome structure has been encapsulated in other nanoparticles to modulate the particle size and increase their bioavailability besides providing the opportunity for further surface modifications [136, 137].

Although using nanotechnology could overcome most of the concerns, the following items

are the most important limiting factors in development and extension of molecular imaging in clinic:

1. Emerging novel techniques requires much more research funds to be absolutely approved.
2. Introduced novel methods should be confirmed on animal and human level at large scale to be approved as a reproducible one.
3. Necessity of cooperation between basic researchers and medical professionals to translate the approved methods from bench to the bedside [138, 139].

26.4.1.1 Biosensors and Role of Nanotechnology in Their Developments

The term biosensor is basically used to call a device implicated for detection or measuring of an analyte in a preferably visible manner. Primary biosensors have been developed for major task of early and sensitive diagnosis of diseases including different types of human cancers, while the following generations of biosensors were specifically designed to trace the residual of the disease or to determine the level of response to the treatment schedule. Using biosensors in cancer medicine takes advantages of rapid, relatively cheap, and early detection of malignancy which almost requires no need to be in a specific laboratory with skilled personnel. Moreover, biosensors remove the necessary labors behind test performance and sample preparations and processing problems for every patient. The most important aspect of home-based biosensors may be the quickest response given to a worried patient [140]. The principle of most of the biosensors is an electrochemical reaction which takes place within a miniaturized device as much as possible, and the result of reaction can be recognized through a color change of test band compared to control band or more precisely through digital demonstration. Implication of nanomaterials not only has made the biosensor technology development easier but also helped to design more specific and smaller user-friendly sensors for patients and healthcare professionals [141].

The basic electrochemical reaction that takes place in a biosensor structure is detection of one or a set of specific biomarkers of disease of interest including proteins, microRNA (miRNA), and circulating tumor cells (CTCs). Identification of the CTCs and cell-free DNA of the tumor as a result of core tumor apoptosis in peripheral blood of cancer patients has opened a promising wide window toward early diagnosis and follow-up of various types of cancers which may remove the need for tissue biopsy in future of cancer medicine [142]. Recent biosensor optimization has been focused on molecular reactions at the micro- or nanomolar scales as well as polymerase chain reactions to detect cancer-specific genetic and epigenetic alterations. In general, most of the studied and developed electrochemical biosensors and nanobiosensors especially in cancer diagnostic field have been optimized based on the following transducers: potentiometric, impediometric, amperometric, and voltammetric. Amperometric transducers, as well as glucometer, measure produced electric current proportional to the chemical interaction and amount of analyte present in the sample.

Potentiometric transducers measure present charge potentials on two electrodes in the absence of any current as well as CEA biosensor used in colon cancer screening and diagnosis. These types of biosensors have a special advantage in the detection of minor quantities of analyte (as low as 10^{11} molar) which is impressive in recognizing cancer biomarkers in early stages. Application of potentiometric biosensors in cancer diagnosis has been more highlighted when simultaneous detection of thousand markers has been possible using hybridization-based potentiometric microarray [143, 144]. Other developments in potentiometric biosensors include specific detection of CTCs, and cancer cell microenvironment through chemical and metabolic changes occurs in cancer medium [145, 146].

Impediometric transducers measure the resistance related to the nonconducting nature of various types of molecular markers used to define a specific cancer or disease status [147]. In

amperometric-transducer-based biosensors, produced signal would be enhanced when a conjugation has occurred between a designed ligand and its corresponding target. Using antibody-functionalized gold nanoparticles in the structure of biosensor's probe was associated with detection of annexin II and MUC5AC as biomarkers of lung cancer in the range of 280 ± 8.0 pg/mL [148]. Graphene nanocomposite is another example of using nanotechnology in designing amperometric biosensors which was demonstrated to be effective in the detection of miR-21 as a biomarker of cervical cancer when it has been functionalized with GNP [149].

Emerging voltammetric transducers make the sensitivity of biosensors to be increased up to the detection limit of femtogram (fg)/mL of biomarkers circulating in blood. Implication of zirconia nanoparticles in voltammetric biosensors has demonstrated to accelerate the response time for detection of cancer biomarkers in salivary samples [150]. Although conjugation of GNPs with anti-human epidermal growth factor receptor 2 (HER2) antibody didn't increase the overall biosensor detection capability and performance, it was associated with efficient recognition of cancer cells among the normal cell population [151]. Moreover, voltammetric biosensors not only have been used to identify cancer cells but also have shown to be helpful in gene expression analysis through real-time PCR [152].

26.4.2 Nanotherapy and Nanotoxicity

Despite advancements in the understanding of cancer mechanisms over the last few decades, the therapeutic efficacy of cancer treatments is still undesirable. Unfortunately, current approaches in management of cancer including surgery, chemotherapy, radiotherapy, and sometimes combination of them have demonstrated insufficient efficacy for a large number of cancer patients especially those who have been diagnosed in later stage of the disease. Additionally, because of several side effects of chemotherapy and radiotherapy, such as cumulative toxicities, more effective methods with fewer side effects are demanded to be employed for cancer patients. Almost all the anticancer agents act through a nonspecific targeting paths which are associated with many side effects. The most critical barriers against reaching a high efficacy in the treatment of cancer patients include failing in differentiation between cancerous cells and normal body cells as well as poor drug delivery of those agents into the cancer site. Failing in effective penetration to the core of solid tumors is another limitation of chemotherapeutic agents, which make it critical to use alternative strategies to treat cancer patients in more effective and accurate ways. Thanks to the advances in our understanding of the tumor microenvironment, development of new treatment approaches for cancer has been significantly facilitated during the last decade. Nanoparticles as a promising alternative to conventional chemotherapy are able to accumulate on the tumor via enhanced permeability and retention (EPR) effect followed by releasing their therapeutic payloads. In order to overcome the mentioned limitation of currently available anticancer agents, many efforts have been aimed to engineer the drug in such a way that it can effectively deliver the anticancer agent into cancerous cells [153]. Because of the flexibility in the modification of size, shape, and surface chemistry of nanoparticles, this emerging field has recently attracted widespread attention in novel cancer therapy strategies. Chemical and physical modifications of nanoparticles could affect their accumulation, retention, and penetration in tumors of interest leading to accurately targeting of desired misbehaved cells.

Generally, nanoparticles could target tumor in passive or active fashions which are actually complementary to each other. Passive approach is based on EPR effect, while the active approach relies on molecular recognition of cancer cells. Following facilitating the efficient localization of nanoparticles in the tumor, further enhancing the uptake of cancer drugs into tumors could be mediated by nanoparticles by either ligand–receptor interaction or antibody–antigen recognition [154, 155]. In passive targeting, deposition of nanoparticles within the tumor microenviron-

ment will be facilitated, but not in healthy tissues. Regarding active targeting, the delivery of anticancer agents will be optimized through recognizing various targeting ligands, such as antibodies (e.g., HER2, EGFR), antibody fragments, aptamers, peptides and whole proteins (e.g., transferrin), and different receptor ligands (e.g., folic acid) (reviewed in [155]). Targeting each of those ligands has its own advantages and disadvantages, which made it difficult to announce the optimum targeting strategy. For example, immunogenicity, stability, and their expression on tumor cells are critical factors in choosing the optimum strategy. It was suggested that combining these approaches may lead to a better outcome in treatment of cancer patients [155]. In addition to active and passive targeting, different strategies, such as pH-dependent drug delivery, hyperthermia (thermal therapy or thermotherapy), and combination therapy, are other suggested options to overcome numerous limitations of conventional chemotherapy [153].

Although selectively targeting cancerous cells using nanomaterial-based drug delivery is an optimum strategy to eradicate tumors, some unfavorable outcomes also could occur which are known as toxicity of nanomaterials. During the recent decade, many studies have been published which have been focused on the interconnections between nanotoxicity and drug delivery [156]. One of the most important factors contributing in nanotoxicity is the size of nanomaterial as it was found that small particle size was associated with higher toxic effects [157]. Other critical factors that influence nanomaterial's toxicity include aspect ratio and shape, surface chemistry, surface charge, and prescription dosage. Nonphagocytic cells ingest cationic nanoparticles to a greater extent that may lead to a higher cellular uptake and therefore higher toxic effects [158]. Prescribed dosage is another predictor, which usually is correlated with the nanotoxicity [159].

Having a great insight into the mechanisms of nanotoxicity is required for minimizing the adverse effects associated with drug delivery aided by nanomaterials. Different mechanisms have been proposed for nanotoxicity as the first one is oxidative stress, which could be defined as the disturbance in the balance between the production and elimination of reactive oxygen species (ROS) [160]. Since nanoparticles could induce ROS production, they may lead to impaired physiological function through cellular damage of macromolecules such as proteins, lipids, and DNA, followed by detrimental effects on cells [161]. Inflammation-mediated nanotoxicity and genotoxicity are the other critical toxic paradigms of nanomaterials.

Following revealing the toxic effects of nanoparticles used in medicine, numerous researches have been looking for new strategies to overcome nanotoxicity. Regarding oxidative stress associated with using nanoparticles, several enzymatic and nonenzymatic antioxidant systems have been identified which could efficiently protect the body against produced free radicals [162, 163]. The most important enzymatic antioxidants include superoxide dismutases (SODs) (e.g., CuZn-SOD, Mn-SOD, and EC-SOD), catalase, and several peroxidases catalyze. In the nonenzymatic antioxidant group, small-molecular-weight compounds such as vitamins (vitamins C and E), β-carotene, uric acid, and glutathione have been etensively studied in various studies. Surface modification of nanoparticles is another approach to decrease toxicity of nanoparticles [164, 165]. Owing to its higher toxicity with higher doses of nanoparticles, it was recommended that high experimental doses should be interpreted with caution [159].

26.5 Nanotechnology Against Tumors

26.5.1 Aims and Mechanisms of Action

In spite of recent developments in cancer medicine, there are still many pitfalls and limitations in specific diagnosis and treatment of various types of cancers. According to the concerns described in the previous part of the current chapter, nanotechnology not only could pave the way for specific tumor targeting but also may have a critical role in personalized medicine of cancer

treatment. In this regard, using nanomaterials in structures of cancer-fighting medicine design has got great attention in recent years. Each classification of nanomaterials could be used for a variety of cancer diagnosis and treatment options based on their physical and chemical characteristics which will be described in the following section.

26.5.2 Nanoparticle's Characteristics

Based on International Union of Pure and Applied Chemistry (IUPAC) definition, any particle sized in the range of 1 × 10–9 and 1 × 10–7 m (generally less than 500 nm) and that has $<10^6$ atoms per every particle with any shape is considered as nanoparticle [166]. However, given that novel characteristics have been found in nanoparticles with diameters less than 100 nm, the scale of nanoparticles usually is defined as particles with a dimension less than 100 nm as well as tubes and fibers [167]. Different types of nanoparticles have some general and some specific characteristics which limit their application in a special scope of medicine. The most important general characteristics of all types of nanoparticles are size, high surface-to-volume ratio, and the possibility to adapt their features to be useful in various aspects of medicine. High surface-to-volume ratio is one of the significant features of nanoparticles which in a simple word provides a rich source of atoms at the surface of molecule to be involved in various chemical and physical reactions.

26.5.3 Optical Properties of Nanoparticles

Similar to most of the nanoparticle characteristics, optical properties are related to electronic features of nanomaterials and are described as the interaction of electromagnetic radiation with matter [168]. This interaction is strongly dependent on topographical features and anisotropic shape of nanoparticle, and the produced ray may be reflected, refracted, or absorbed. Reflection of produced electromagnetic ray can be in either scattering or diffuse manner. For the first time, optical properties of nanoparticle have been noted in eminent paper of Michael Faraday in which he described that upon high temperatures, the metal (silver or gold) layer on glass (as a coloring agent) will be degraded and therefore the white light will be emitted accompanying increase in electricity [169, 170].

The optical characteristics of nanoparticles can be described in linear or nonlinear format. By emitting a laser beam containing an electromagnetic field to the significant bulk of atoms at the surface of nanoparticles, electric polarization will be induced which leads to amazing features with nonlinear properties and different frequency among various nanomaterial compounds [171].

26.5.4 Physical Properties of Nanoparticles

Unique physical properties of nanoparticles compared to nanobulked materials had left fantastic improvement footprints in novel medical diagnosis and treatment strategies. One of the major features of nanoparticles is color which is strongly dependent on the interaction between free electrons and oscillating electric fields of a light ray within a nanoparticle called as surface plasmon resonance (SPR). Every nanoparticle has its specific wavelength absorption of light and emits special color based on its dimension, size, and density of particles. As an example, the SPR of gold nanoparticles interacts with the wavelength of 450 nm (blue-green) of the visible light and, in turn, emits the purple color with a wavelength of 700 nm. Any further changes in particle size or shape of nanoparticles can affect the wavelength of absorption and emission, and therefore, the color of solution will be changed. Distinctive visible change in color can be made through binding of the nanoparticle to target molecules and therefore can be an indicator of the probing molecule. This is one of the most applicable features of gold nanoparticles used in nanobiosensors [172–174]. Of note, the degree of nanoparticle distribution within the solution has a significant effect on color (shift toward blue spectrum) as well as particle aggregation and may mimic the coupling of them with the target [175].

The other physical characteristic of the nanoparticle is their melting temperature which is mainly determined by its size. The lower the nanoparticle size, the lower the melting temperature owing to lower needed energy to dissociate and unbound atoms which are known as melting point depression [176]. This is a major point that should be considered in various medical and even nonmedical applications of nanoparticles which will be described in later parts of the current chapter.

One of the most applicable features of nanoparticles is that they tend to be in suspension form. This characteristic enhances the possible detection and bounding of the target by nanoparticle within the interaction solution [177].

26.5.4.1 Chemical Characteristics of Nanoparticles

Chemical features of nanoparticles refer to the detailed structure of nanoparticles especially the type and distribution of electrons on their surface [178]. Given that every category of nanoparticles has its specific chemical properties, we will briefly describe the chemical characteristics of nanoparticles within their general classifications.

26.5.4.2 Metallic and Metal Oxide

One of the most amazing features of metallic nanoparticles, as well as silver and gold NPs, is flexibility in their structure which allows for synthesizing particles in size and shape of interest according to the research, diagnosis, or treatment's demands. Owing to their high thermal and electric conductivity, metallic and metal oxide nanoparticles are good options for cancer cell-targeted hyperthermal therapy and ultrasensitive diagnostic chips, as well [179, 180].

26.5.4.3 Quantum Dots

Quantum dots (QDs) are attractive nanoparticles due to their specific composition which commonly includes different variations of metals (magnetic, semiconductor, etc.). Similar to the most of other nanoparticles, they can be surface modified with additional chemical group to be more efficient and water soluble in bioactive applications [181].

26.5.4.4 Carbon Nanoparticle

Carbon is one of the most plentiful elements on the earth which is frequently found in coal deposits and is the most frequent molecule of the human body following oxygen. Carbon nanoparticles with the high spherical surface area (30–50 m^2/g with the size of 10–45 nm) afford great scope of applications in medical diagnosis and treatment. The possibility of using carbon nanoparticles in a tubelike structure as one cylindrical tube or multiwall nanotubes has made them a powerful carrier for targeted transportation of drugs and imaging agents [182, 183].

26.5.4.5 Polymeric Nanoparticles

Polymeric nanoparticles include mostly of nanospheres and nanocapsules and are polymers of caprolactone, acrylamide, acrylate, DNA, albumin, chitosan, and gelatin [184, 185]. These types of nanoparticle may be the most suitable tools for more efficient and specific targeting of drugs which have been used more frequently in plant-derived drugs [186].

26.5.5 Challenges and Opportunities

Although nanotechnology has offered several attractive properties which enable us to make them a magic bullet against the tumor cells, many challenges still remained to be undertaken. Although a large number of studies have found nanomedicine therapeutics as an effective alternative option for treatment of cancer patients, only a few of them have successfully entered into the clinical trials. This is implying to some reported challenges and limitations which had restricted their application. Similar to the majority of currently available treatments, this new technology faces many challenges. For instance, changing instability, solubility, and pharmacokinetic properties of the carried drugs and also toxic effects of some nanoparticles (e.g., carbon nanotubes and quantum dots) are some of the possible challenges associated with nanotechnology [187]. In a deeper insight, a large number of those challenges and limitations could be converted into opportunities, which even make nanomedicine more practical

than before; for example, moving toward personalized medicine for selection of given nanotherapy could present automation with unprecedented opportunities. To date, different nanomedicines are under review by FDA, and although some of them have been approved and have successfully been brought to market, results from some clinical trials and studies were disappointing [188, 189]. By reviewing the risks and challenges associated with current nanoparticles used in cancer treatment and lessons from past successes and failures, next-generation nanomedicines could be even more efficient and safer. One of the most important areas which can help to prevent failure of nanomedicines in the clinic is preselecting patients who are more likely to respond to nanomedicine-based therapy. Choosing the right nanomedicine for a patient offers much more hopes in this regard and has caused the emergence of personalized cancer nanomedicine which could increase the efficacy and reduce systemic toxicity [190, 191].

26.5.6 Nanoparticle's Interaction with Cancer Cells

One of the most important actions of nanoparticles as antitumor tools is their interaction with immune system cells. The nanoparticle can direct antitumor activity through the various mechanisms of actions including antiangiogenesis and interaction with the immune system. Interaction of nanoparticles with cancer cells mainly happens through antiangiogenesis mechanism which will be described in the next part. Other mechanisms are based on the type of nanoparticles which will be discussed in following sentences, as well.

26.5.7 Antiangiogenesis

Angiogenesis or creation of new blood vessels is one of the main hallmarks of cancer cells to support nutrients for proliferative cancer cells and provide a more suitable situation for their metastasis to other sites of the body [2]. It is a multistep process including secretion of endothelial-specific growth factors and degradation of extracellular matrix (ECM) to make the way open for new endothelial cells as the bricks of new vessel tubes [192]. Vascular endothelial growth factor (VEGF) is the major angiogenesis element which has been shown to be overexpressed in most types of cancer and has a critical role in the proliferation of cancer cells through activation of anti-apoptosis genes as well as B-cell lymphoma-2 (*Bcl-2*) [193]. The other important involved factor is nuclear factor-κB (NF-κB) which plays a pivotal role in control of the expression of *VEGF* gene [194]. Although it has raised much tumor-mediated resistance, targeting either VEGF or its receptor (VEGFR) can limit the growth and proliferation of cancer cells in most of the chemotherapy approaches [195]. However, the most effective chemotherapy strategy is combining more than one drug targeting various angiogenic factors in parallel which would be associated with higher rate of severe adverse effects owing to targeting of other normal cells [196]. In this regard, nanoparticles have demonstrated a powerful role in direct and efficient targeting of multiple angiogenic factors toward only cancer cells [197]. Several types of nanoparticle have been implicated to decrease tumor angiogenesis which include gold nanoparticles (GNPs), silver nanoparticles, chitosan, cerium oxide, silica-based nanoparticles, tetrac, and selenium nanoparticles.

GNPs or AuNPs have demonstrated to have cancer cell toxicity against breast, lung, and cervical cancer cells which was enhanced in a higher dose and longer exposure with the most cellular uptake [198, 199]. GNP's interaction with cancer cells is strongly dependent on nanoparticle surface modification as either cancer cell membrane destruction induced by GNPs (modified with CTAB) or driving apoptosis by citrate GNPs was more significant compared to other modifications as well as PSS and polyethyleneimine (PEI) [199, 200]. It was also shown that with minimum harmful effect on noncancerous cells, citrate-coated GNPs caused human lung cancer cell cycle (A549) to stop and induced apoptosis in them [201, 202].

Among all types of nanoparticles used as angiogenic therapy, GNPs may be the best option due to the reported lower toxicity compared to other nanoparticles and targeted therapy. Mukherjee and his coworkers primarily demonstrated that gold nanoparticles tend to bind proteins with heparin-binding domain which led to prevention of VEGFR2 phosphorylation and suppression of some other types of VEGF proteins as well as VEGF165 [203]. This interaction between GNPs and heparin-binding domain has been demonstrated to be more efficient and enhanced when the diameter of used GNPs is 20 nm and the GNPs have no surface chemical modifications [204]. Interaction of GNPs with heparin-binding domain has been demonstrated to be useful in the inhibition of metastasis process through direct suppression of epithelial–mesenchymal transition (EMT) [203]. The other interesting anticancer approach using GNPs is their conjugation with chemotherapeutic agents which have shown remarkably different success rate compared to treatment plans including the same drugs alone. One of the best examples is quercetin (Qu) in which its conjugation with GNPs has been associated with overexpression of *E-cadherin* and underexpression of *VEGFR-2* and specific introduction of the drug to cancer cells has resulted in angiogenesis and metastasis suppression [205]. One of the amazing features of GNPs is amplification of laser radiation through local heating of nanoparticle for targeted photothermal therapy (PTT) and photodynamic therapy (PDT) of cancer cells [206]. In this regard, plasmonic GNPs have demonstrated higher efficiency in amplification of radiations which is strongly influenced by the shape of nanoparticle [207, 208]. This characteristic of GNPs is also useful in the local treatment of microbial infections owing to the tendency of GNPs to bind bacterial cell wall. Conjugation of microorganism-specific antibiotic with GNPs can lead antibiotic straightforward to the infection site, and then, the present microbe will be killed by absorbing the near-infrared (NIR) light. It is especially helpful in the treatment of antibiotic-resistant microorganism and targeted treatment of infected areas of the body to avoid side effects frequently created by systemic administration of drugs [209]. Nonetheless, the GNP-based PTT or PDT of cancer can be applied to the superficial tumors, and using them in other deep cancer sites needs further studies and approval [210].

26.5.8 Silver NPs (AgNPs)

Another widely used nanoparticle in modern medicine is silver NPs (AgNPs). AgNPs is well known as antimicrobial tools in killing fungal and bacterial infections, as well. Antiangiogenic effects of AgNPs have shown to be stronger than GNPs as it has stopped the whole process in both cell line and animal models through direct inhibition of PI3K/Akt pathway and HIF-1α protein and its target genes including *VEGF-A* and *GLUT1*, as well [211, 212]. Regarding direct interaction between AgNPs and cancer cells, Yilmaz VT et al. have recently demonstrated that a novel AgNP complex (Ag(barb)(PCy3)) caused a significant antiproliferative effect on breast cancer cells compared to chemotherapeutic agents [213]. Satapathy SR et al. have shown that treatment of HCT116 colon cancer cells with AgNPs led to apoptosis of cancer cells which has been demonstrated by increasing p21 and p53 expressions as well as caspase family [214]. Similar dose-dependent effects of AgNPs have been described in prostate cancer (PC-3) cells which were associated with overexpression of apoptotic genes and downregulation of some oncogenes including signal transducer and activator of transcription 3 (STAT3), *Bcl-2*, and *survivin* [215]. Kovács D et al. have demonstrated that anticancer effect of AgNPs is independent of molecular background of osteosarcoma cells which is a great achievement in traditional therapeutic plans of cancer patients in whom p53-defective cells poorly respond to chemotherapy [216]. Administration of gemcitabine in combination with AgNPs also demonstrated significant induction of apoptosis in A2780 ovarian cancer cells, as well [217]. The other great interaction of AgNPs with cancer cells has been described in the specific cytotoxic effect of AgNPs on ovarian cancer stem cells [218].

26.5.9 Chitosan NPs (CNPs)

Chitosan is a linear polysaccharide composed of randomly distributed β-(1 → 4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) which is widely used in agriculture and medicine [180]. Owing to low in vivo toxicity, biodegradability, and biocompatibility, CNPs have seized great attention in various aspects of biomedicine. Interaction of CNPs with cancer cells has been identified through finding downregulation of angiogenesis-inducing gene, *VEGFR2*, in human hepatocellular carcinoma (HCC) (BEL-7402) cells [218]. Almada et al. have demonstrated that hydrophobization modification of CNPs not only enhanced nanoparticle internalization into cervical and breast cancer cells but also its cancer toxicity effects have been significantly increased compared to bare CNPs [219]. Interestingly, it was recently reported that synthesis of aerobic CNPs containing chlorin e6 (Ce6) as photosensitizer and transfection of cancer cells with it caused successful photodynamic therapy (PDT) in the hypoxic microenvironment of the tumor. The underlying mechanism is based on the radiation-induced degradation of the synthetic CNPs and release of Ce6 and generation of toxic $^1O_2$ under the acidic tumor microenvironment [184, 185]. One of the major obstacles against chemotherapy of brain tumors is blood–brain barrier (BBB) which has been shown to be overpassed by CNPs through cerebral microvessel endothelial cells (hCMECs). Successful crossing of CNPs over BBB is a hopeful promise toward targeted treatment and prophylaxis of central nervous system malignancies and infections [179, 186].

26.5.10 Silica NPs (SiNPs)

The nanoparticles which are based on SiO_2 is called as nanosilica or silica nanoparticles and have been widely used in various fields of medicine due to their low toxicity and high in vivo stability [2]. Mesoporous forms of silica nanoparticles include Mobil Crystalline Materials (MCM) and Santa Barbara Amorphous type material (SBA-15) that, due to their special hexagonal array of pores as molecular docking sites, have created great advances in medicine [192]. Mesoporous SiNPs (MSPs) have also seized great attention in PDT of tumor through surface modification with substances as well as hyaluronic acid (HA) and poly-(L-lysine) which have a specific tendency to be bound with CD44 receptor overexpressing on cancer cells [220]. Nanocomposition of MSPs and GNPs demonstrated that it not only has higher affinity to nasopharyngeal cancer (NPC) cells but also could differentiate between normal and precancerous cells [221]. Although antiangiogenic characteristics of SiNPs has not been generally accepted in all reported studies, it has been shown that SiNPs, dependent on their size, caused downregulation of ERK 1/2 pathway through interfering with VEGFR2 phosphorylation and finally suppression of angiogenesis process within human microvascular retinal endothelial cells [222, 223]. It is worth to note that safety of topical ophthalmic and oral administration of SiNPs has been accepted in some separate studies [224].

26.5.11 Selenium NPs (SeNPs)

Surface amino acid modification of SeNPs has demonstrated to be a major factor in their inhibitory activity on cancer cells as lysine modification had revealed the highest level of anticancer activity [225]. It was shown that SeNPs could impair cancer cell metabolism and invasion through several mechanisms including suppression of glycolysis and thereby normal mitochondrial function, microtubule depolymerization, induction of oxidative stress, and downregulation of annexin A2 which may be generated by self-assembly of SeNPs [226, 227]. It was demonstrated that ruthenium modification of selenium nanoparticles (Ru-SeNPs) has an antiangiogenic effect on human umbilical vascular endothelial cells through inhibition of FGFR1, ErK, and AKT and can pass through the cancer cells via clathrin-mediated endocytosis [228, 229]. Treatment of human umbilical vascular endothelial cells with a combination of doxorubicin and SeNPs was shown to have antiangiogenic

effect by suppressing the expression of *VEGF–VEGFR2–ERK/AKT* besides activation of apoptosis cascades [230].

26.5.12 Tetrac NPs

Tetraiodothyroacetic acid (tetrac) is an antagonist of thyroid hormone and integrin αvβ3 which has been shown to act as anticancer agent on human renal cancer cells [231]. Antiproliferative effect of tetrac NPs has been investigated in different cancer cells and animal studies with great and amazing results. Treatment of MDA-MB-231 breast cancer cells with tetrac NPs has led to underexpression of antiapoptotic agents including X-linked inhibitor of apoptosis (XIAP) and myeloid cell leukemia sequence 1 (MCL1) and also most of the RAS-dependent pathway's elements, while the expression of main apoptotic factors, as well as caspase 2 (CASP2) and Bcl-2-like protein 14 (BCL2L14) besides antiangiogenic factors including CBY1 and thrombospondin 1 (THBS1), has been demonstrated to be increased [232]. As it was expected, tetrac NPs have demonstrated antitumor activity against various types of thyroid cancer cells in either cell line and xenograft models through the same mechanism in breast cancer cells [233, 234]. However, Lin et al. have described that anticancer activity of tetrac would be enhanced in a combination of chemotherapeutic agents with different mechanism of action [235]. Antiangiogenic activity of tetrac NPs also has been demonstrated on human retinal endothelial cells mediated by downregulation of VEGF and erythropoietin [236]. Anticancer activity of tetrac NPs has been reported in two OVCAR3 and A2780 ovarian cancer cells via activation of caspase-dependent and caspase-independent (apoptosis-inducing factor, AIF) apoptosis pathways and key DNA repair genes including *ATM* and *PARP-1* [237]. Another interesting and strong antiangiogenic effect of a tetrac NP modification (nano-diamino-tetrac, NDAT) has been shown on xenograft mice model of glioblastoma in which devascularization was associated with significant tumor cells necrosis [238].

26.6 Nanocarriers

Nanocarriers have demonstrated a promising potential in targeted diagnosis and treatment of various types of human disease, in particular in diseases with inflammatory mechanisms. AIDS, hepatitis, tuberculosis, melanoma, cardiovascular diseases, pulmonary infections, brain diseases, inflammatory bowel diseases, diabetes mellitus, allergic diseases, and different types of cancers are the best examples in which nanocarriers have shown hopeful horizons in targeted diagnosis and treatment [239]. General strategies in the implication of nanoparticles in drug delivery include encapsulation of drugs within nanoparticle, creating chemical interaction between one or more drug and nanoparticle, and the combinatory strategy of two former methods [240]. In this section, we will describe the most significant researchers which have been performed in various types of human cancers.

26.6.1 Nanocarriers in Cancer

Nanoparticles can overcome the pitfalls of current cancer treatment plans including radiotherapy, chemotherapy, and immunotherapy. Encapsulation of anticancer agents within nanoparticles not only increases their solubility, bioavailability, and stability but also helps the drug to pass through biological barriers and specifically in targeting cancer cells in order to decrease systemic therapeutic side effects. The most widely used nanocarrier systems are dendrimers, liposomes, polymer micelles, and nanoparticles.

26.6.2 Nanocarriers in Cancer Treatment

Owing to the multidimensional aspects of cancer cell growth, novel trends of cancer treatment have been deviated toward combination therapies. In this regard, simultaneous presentation of drugs especially with different identity and solubility (protein, DNA, or regulatory RNA) as well

as a combination of hydrophobic and hydrophilic compounds is a major challenge which has been substantially addressed in novel combination therapies using nanomaterials [241]. By implicating different nanomaterials, it is possible to deliver various drug combinations to different tumor sites [242]. In this way, nanomaterials as carrier have made significant advances in overcoming chemotherapeutic agent's resistance which will be discussed below.

One of the major obstacles against drug delivery is overexpression of efflux ATP-binding cassette (ABC) transporters as well as P-glycoprotein (P-gp) which causes decrease in intracellular concentration of administered drugs [243]. Although using various P-gp inhibitors like elacridar has led to increased concentrations of chemotherapeutic agents, there are some reports which have used nanocarriers to enhance the efficiency of targeted siRNA and natural drug (e.g., curcumin) delivery, as well [244]. Mesoporous silica nanoparticles (MSNs) were demonstrated to be effective in the combined presentation of doxorubicin and siRNA complementary to P-gp mRNAs in either breast cancer cell line or animal model [245, 246]. Nanoemulsion encapsulation of curcumin and paclitaxel was shown to be successful in P-gp suppression and inducing breast cancer cell toxicity [247, 248]. In the other recent study on using chitosan nanoparticles for co-delivery of gefitinib and chloroquine into EGFR-TKI-resistant cells, it was demonstrated that chitosan nanoparticles caused significant cytotoxicity and suppressed autophagy pathway in human hepatocellular carcinoma cell lines resistant to gefitinib [249]. Another advance in this field is introduction of orally absorbed doxorubicin with lowest cellular efflux which has been encapsulated in enoxaparin sodium–PLGA hybrid nanoparticles (EPNs) [250]. Deng et al. have also used liposome–silica hybrid nanocarrier to co-administer cyclosporine and paclitaxel which has been associated with high oral absorption [251]. Sadekar et al. have demonstrated that using poly(amido amine) or PAMAM dendrimers was not associated with epithelial toxicity and increased oral absorption, as well [252]. Aforementioned studies indicate that the novel nanocarrier-based strategies of drug delivery would be a promising approach in changing the route of administration of chemotherapeutic drugs from parenteral to oral with remarkably lower side effects and will remove the need for patient's hospitalization.

The other approach in the implication of nanocarriers in treatment of cancer is targeting DNA repair system. Activation of DNA repair system is one of the main mechanisms behind chemotherapy resistance especially for alkylating agents as a potent inducer of DNA repair pathways. In this regard, conjugation of chemotherapeutic drugs with nanoparticles may have additional detrimental effects on cancer cells in order to bypass activation of DNA repair systems and directly induce apoptosis. It was demonstrated that treatment of breast cancer cells with combination of doxorubicin and mesoporous silica nanoparticles has led to downregulation of multidrug resistance genes (MDR) and inhibition of p53-dependent DNA repair pathway. Cancer cells killing using MSPs was achieved through induction of autophagic lysosome pathway and tumor necrosis which has been activated by high intracellular oxygen concentration [253, 254]. MSPs were also used to be co-treated with CTAB drug-resistant breast cancer cells and demonstrated enhanced cell cycle arrest and apoptosis in cancer cells [255]. The other silica nanoparticle based including iron (II) acetate and polyoxyethylene (5) nonylphenyl ether was used to further decrease the DNA damage repair response against doxorubicin and arsenic in HCC cells. A significant reduction in PARP-1 expression as a major element of DNA damage response was detected in cancer cells which were associated with apoptosis, as well [256].

Another spectrum of using nanomaterials in targeting DNA repair system to overcome cancer treatment resistance is effective transportation of anti-DNA repair molecule siRNAs through cell membrane [257]. Kievit et al. and Liu have implicated a nanoparticle carrier which was cored with superparamagnetic iron oxide and GNPs, respectively. Nanoparticle carriers were similarly coated with chitosan, polyethylene glycol (PEG), and polyethyleneimine (PEI) to load siRNA

which has been designed to be complementary with apurinic endonuclease 1 (Ape1) transcript in medulloblastoma (MB) and ependymoma (EP) patients. The results were promising that in addition to effectively surpassing against blood–brain barrier and protection of siRNA from lysosomal degradation, Ape1 has been significantly decreased and correspondingly the DNA damage has been shown to be increased following radiation, as well [258, 259]. Self-assembly of lipid nanoparticles (LNPs) is the other category of nanoparticles which has been extensively used to carry siRNAs. LNPs need helper lipids to not be detected by the immune system and thereby facilitate passing them through cell membrane [260]. To be efficiently delivered, each siRNA is encapsulated in an LNP structure which itself should be coated with cholesterol to cover the free spaces among lipids and stabilize the overall structure of LNPs and finally covered with helper lipids and PEG lipids. Several surface modifications have been applied to LNPs to increase bioavailability and specificity such as conjugation with the ligand of target receptor molecule, 2′-*O*-methyl, 2′-fluoro, or phosphorothioate to avoid natural intracellular degradation as much as possible [261]. LNPs have been shown to be successful in treatment of various types of cancers including HCC and adrenocortical and neuroendocrine malignant tumors by effective targeting of *MYC* oncogene and polo-like kinase 1 (*PLK1*) genes and are proceeding in the required steps to be approved by fulfilling their clinical trials [262].

Proliferation of a subset of tumor cells called as cancer stem cells (CSCs) is the other major cause of chemoresistance and metastatic relapse of human cancers. Similar to stem cells, they have the capability to self-renew and differentiate into cancer cells [263]. It is interesting that according to the CSCs hypothesis, CSCs have a special potential to repair DNA damage induced by chemotherapeutic agents through activation of common repair pathways as well as nucleotide excision repair (NER). It could be considered as a possible mechanism behind the role of CSCs in chemoresistance. CSCs have been identified in leukemia and many solid tumors such as prostate, pancreas, ovary, breast, lung, brain, and colon, hepatocellular carcinoma, and oral cancer [264–268]. CSCs have typical surface markers such as epithelial cell adhesion molecule (*EpCAM*), Cluster of Differentiation 90 (CD90), CD44, CD24, CD144, CD117, CD133, and some other CD markers based on the cancer cell type and phenotypic marker as well as side population (SP) which is determined based on Hoechst dye exclusion in flow cytometry [269]. For identification and effective targeting of CSCs, various molecular markers have been reported including the expression of aldehyde dehydrogenase (ALDH) especially ALDH1, ATP-binding cassette (ABC) transporters, B-cell lymphoma-2 (Bcl-2), and B-cell lymphoma-extra large (BCL-XL) [270]. Primary generation of nanocarriers with the aim of targeting CSCs was based on conjugation of nanoparticles with antibodies against specific CSC markers as mentioned above [271]. The other strategy is the conjugation of nanoparticle with ligands of surface marker molecules including hyaluronic acid (HA) with high tendency to bind to CD44 and biotin. Conjugation of HA with a CD44 inhibitor in the form of antibody or siRNA and their intracellular transportation by liposome have shown a dramatic decrease in tumor cell proliferation in breast cancer and head and neck cancer patients [272]. Yang Y et al. have demonstrated that conjugation of magnetic Prussian Blue@Quantum Dot Nanoparticles with HA and bovine serum albumin (BSA) could specifically localize the radiation to the tumor site in a mouse model [273]. One effective cancer treatment strategy is the simultaneous introduction of chemotherapeutic agents and anti-CSC factors to guarantee tumor eradication. In this regard, the role of MSPs in efficient targeting of ATP-binding cassette subfamily G member 2 (ABCG2) by both siRNA and a chemotherapeutic agent as well as cisplatin was shown to be significant in laryngeal cancer cell growth limitation in BALB/c-nu/nu mice in vivo and Hep-2 cell line in vitro models. Of note, it was demonstrated that ABCG2 expression is a necessary factor for self-renewal characteristics of CD133-positive CSCs, and therefore, blocking its expression can indirectly suppress CSCs proliferation and activity [274, 275]. In a study on glioma cell line,

CD133-positive tumor cells were targeted with curcumin which has been conjugated with NanoCurc, as a polymer of N-isopropylacrylamide, vinylpyrrolidone, and acrylic acid-based polymeric nanoparticle. NanoCurc not only has increased bioavailability of curcumin in brain tumor cells but also had demonstrated a remarkable decrease in CSCs population which was due to the suppression of insulin-like growth factor (IGF) and Hedgehog signaling pathways [276]. Similarly, nanoparticle-encapsulated hedgehog pathway inhibitor HPI-1 (NanoHH1) was significantly successful in restriction of the growth and metastasis of CD133-positive hepatocellular carcinoma cells in both cell line and animal models compared to the frequently used drug sorafenib [277]. It was found that breast cancer stem cells with high expression of CD44 and low expression of the CD24 marker are highly prone to be resistant against thermal therapy. In this regard, the implication of carbon nanotubes (CNTs) and polyelectrolyte-coated gold nanorods loaded with salinomycin had surprising effects on enhanced sensitivity of both tumor cells and CSCs to thermal treatment and prevention of cancer recurrence [278]. Zhou et al. have demonstrated that combination of photothermal therapy and radiation therapy using copper-64-labeled copper sulfide nanoparticles not only was associated with lower rate of CSCs proliferation but also exhibited significantly lower formation of secondary tumor metastasis in the lung of mouse model [279].

Prostate stem cell antigen (PSCA) has been detected to be expressed on specific cancer stem cells especially in hormone-independent prostate cancer and indicates the absolute probability of metastasis [280]. Targeting of PSCA with its specific monoclonal antibody conjugated with CNTs in prostate cancer cells was associated with significant increase in cellular uptake of antibody and cancer cell growth suppression beyond more effective real-time tumor imaging [281]. Upregulation of *annexin A2 (AnxA2)* and *SOX2* genes in chemoresistant lung cancer cells (H1650 SP cell line) marked them as candidate lung cancer-specific stem cell marker. Designing a polymer containing short hairpin RNA (shRNA) complementary to *AnxA2* gene transcript conjugated with a cationic liposome and treatment of the H1650 SP cell line and mouse cancer model with this polymer has led to the significant decrease in the corresponding gene and protein expression besides tumor size, growth, and metastasis regression [282].

Various miRNA-based CSC nanotherapy approaches have been trialed in different types of human cancers and demonstrated promising results in their initial clinical investigations. Encapsulation of miRNA-34 which functions as a tumor suppressor with SMARTICLES liposome and its presentation to different types of cancer patients including pancreas, brain, stomach, and prostate could induce tumor apoptosis and regression mainly through induction of CD44 expression in CSCs population [283–286]. The other examples of miRNA and siRNA nanoparticle conjugate have been represented in Table 26.1.

26.6.3 Combinatorial Strategy in Cancer Treatment Using Nanocarriers

As it was previously discussed in brief, many studies have taken advance of using combination therapy considering one or more chemotherapeutic agents targeting different molecular pathways in addition to a nanoformulation structure to specifically target cancer cells and increase the overall efficiency of treatment. This is owing to the heterogeneity pattern of tumor cells with different mutation panels which causes each tumor cell population to respond to a specific classification of chemotherapeutic agents [300]. In this regard, combination index (CI) has been defined to determine the synergistic effects of used drugs that based on the cutoff = 1, the lesser, greater, or similar effect compared to expected results could be extrapolated [301]. Tumor microenvironment (TME) including various populations of cancer cells, normal cells, and blood network cells is the other factor which has frequently been focused

Table 26.1 Regulatory RNA nanocarriers used in targeting CSCs

miRNA	Nanocarrier	Targeting genes	Trialed cancer
miRNA-107	Cationic lipid nanoparticles	*Protein kinase C (PKC), cyclin-dependent kinase 6 (CDK6),* and *HIF1-b*	Head and neck squamous cell carcinoma (HNSCC) cells, HNSCC mouse model [287]
MDR1 siRNA	Lipid nanoparticles	*MDR1*	Colorectal cancer (CRC) [288]
miRNA-34a	Cationic liposomes	*ALDH1, CD44, Notch1*	Genetically engineered and xenograft pancreatic cancer mouse models, medullary thyroid cancer (MTC) cells [289–291]
CD44 siRNA	HA	*CD44*	Lung cancer, melanoma, liver cancer, different types of breast cancer cells, pulmonary adenocarcinoma, and gastric cancer [292–295]
miRNA	Iron-saturated bovine lactoferrin (Fe-bLf) nanocarriers/nanocapsules (NCs)	Survivin, lf receptor genes, and other genes involved in iron metabolism pathway	CRC cells [296]
Locked nucleic acid *aptamers* (*LNA*-aps)	Fe-bLf	*EpCAM* and nucleolin markers	CRC cells, breast cancer mouse model [297, 298]
Aptamer–siRNA	PEGylated cationic liposome	*BRAF*	Melanoma mice model [299]

on combinatorial therapies [302]. It also contains some critical factors such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages, and endothelial cells which play pivotal roles in metastasis and cancer cell proliferation [302]. There are gaps among those endothelial cells within the TME structure, and owing to the enhanced EPR effect, the tumor will be more leaky to provide an efficient situation for tumor cell aggregation [2]. On the other hand, low oxygen pressure within the center of tumor cells makes them produce their ATP and energy from alternative anaerobic glycolysis pathway which finally leads to them making acidic TME due to high generation of lactate. Implication of nanoparticles in the specific presentation of drug to cancer cells has been spiked based on those two aforementioned characteristics of TME which is interestingly stimulated by desired internal and external factors as well as magnetic field, ultrasound, or light [303, 304].

Three general nanocarrier structures have been introduced which have been designed based on the level of co-delivery of drugs including macromolecule, cell, and tissue. In other words, nanocarriers could be implicated to delivery of two hydrophilic drugs, one hydrophilic drug and one hydrophobic drug, two hydrophobic drugs, or one hydrophobic drug along with a nucleic acid like siRNA (Fig. 26.1).

At macromolecule level, reported nanocarriers have been designed to simultaneously deliver a regulatory RNA along with another chemotherapy drug. In this way, self-assembled nanoscale coordination polymers (NCPs) were used to subcutaneously deliver three siRNA against *survivin*, *Bcl-2*, and *P-glycoprotein* genes accompanying cisplatin into the xenograft ovarian cancer model. This combinatory drug delivery model was associated with significant cisplatin-induced cancer cell apoptosis and targeted gene silencing with less cisplatin nephrotoxicity side effects [305]. NCP nanoformulation was also used to deliver miR-655-3p in conjunction with oxaliplatin into the metastatic CRC tissue and demonstrated significant synergistic results in tumor growth and invasion limitation [306]. In the other study, siRNAs complementary to the reversionless 3 (*REV3*) encoding DNA directed polymerase and *REV3-like* (*REV3L*) encoding DNA directed polymerase zeta catalytic subunit were co-loaded with a cisplatin prodrug using a nanoparticle polymer consisting of poly(D,L-lactide-co-glycolide)–polyethylene

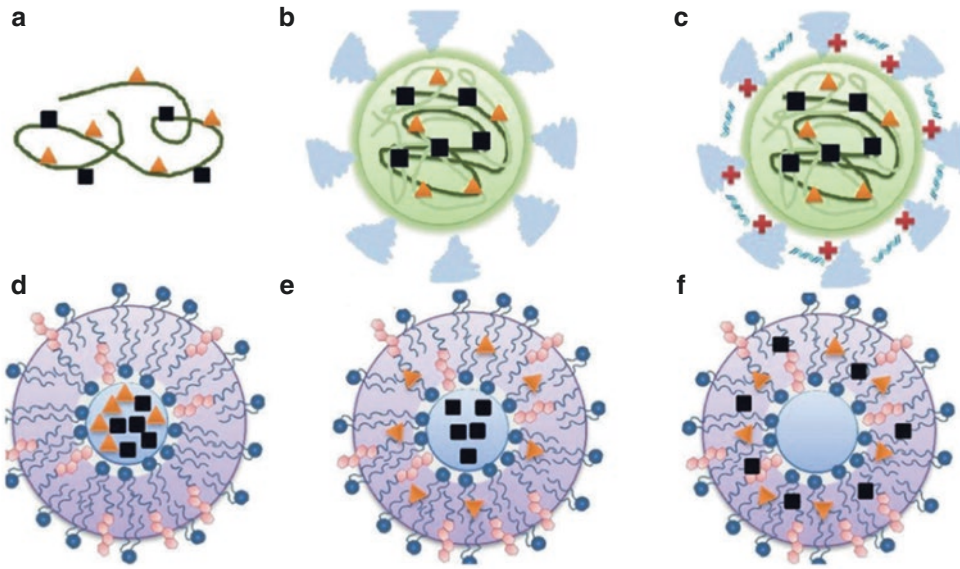

Fig. 26.1 Schematic representation of nanopolymers used in combination therapy of cancers; (**a**) polymer and drug conjugate; (**b**) polymeric carrier encapsulating two hydrophobic drugs, (**c**) polymeric carrier encapsulating one hydrophobic drug with one nucleic acid-based drug, (**d**) nanocarrier liposome encapsulating two hydrophilic drugs, (**e**) nanocarrier liposome encapsulating hydrophilic and hydrophobic drugs together, and (**f**) nanocarrier liposome encapsulating two hydrophilic drugs

glycol (PLGA-PEG) in both in vitro and in vivo models. PLGA is itself a copolymer in which its bio-application has been approved by FDA and could be considered as an ideal carrier for encapsulation of hydrophobic drugs. In spite of biodegradability of PLGA, it is very unstable in body fluids and therefore needs to be added with a more stable polymer as well as PEG to increase its loading capacity, as well [307, 308]. REV3 and REV3L are actively involved in cancer cell proliferation through translesion DNA synthesis (TLS) process, and in spite of chemotherapy-induced DNA damage, their expression has been shown to be directly correlated with cisplatin resistance [309, 310]. Those target genes have been demonstrated to be significantly downregulated in both prostate cancer cells and xenograft mouse model which have made cancer cells significantly sensitive to cisplatin [311].

At the cellular level, one example is metal-drug coordination polymer (CP) which consisted of the fixed ratio of gemcitabine monophosphate (GMP) and cisplatin as anticancer drugs encapsulated in PLGA/PLGA-PEG/PLGA-PEG-MBA (264*N*,*N*′-methylenebisacrylamide (MBA)) nanopolymer. This nanopolymer caused a significant increase in intracellular uptake of cisplatin and GMP drugs and cancer cell death in bladder cancer animal model and could overcome problems that usually occur in co-presentation of two hydrophilic drugs [312]. This ratiometric approach maybe the highest and most novel of combination therapy using nanocarriers which provide the possibility to study the effect of each drug in separate and synergistic models [313]. Solid polymer–lipid hybrid nanoparticles such as myristic acid/PEG100SA/PEG40SA, EPC/DSPE-mPEG2000 and DXR-poly-L-lactide, and/or CPT–poly-L-lactide have shown to be effective in co-delivery of doxorubicin along with mitomycin C using the first polymer and along with camptothecin using the two later polymers in breast cancer cells and xenograft models [314–317].

Maybe the most simple combination therapy implicating nanoparticles has been focused at the

tissue level. In contrast to nanocarriers used at the cellular level, most of the nanostructures used at tissue level were liposomal nanoparticles encapsulating chemotherapeutic drugs. One of the best examples is a nanoscale liposomal polymeric gel (nanolipogels; nLG) which has been used in combination therapy of melanoma mouse model using interleukin-2 (IL2) and a transforming growth factor beta (TGFβ) receptor inhibitor, SB505124. Beside significant decrease in tumor growth and increase in the survival of affected animal model, induction of innate and adaptive immune system activity was the remarkable result obtained by this approach [318]. This study was one of the initial investigations on the application of nanopolymers in augmentation of immunotherapy against tumor growth [319].

26.6.4 Nanocarriers with FDA Approval for Cancer Treatment

There is a list of nanoparticle-based drugs which have been approved by FDA in the treatment of human cancers (Table 26.2). The first nanocarrier approved by FDA was Doxil® which mainly consisted of PEGylated nano-liposome for delivery of doxorubicin to cancer cells [320]. The most important indications of Doxil® were included: metastatic breast cancer, multiple myeloma, recurrent ovarian cancer, and AIDS-related Kaposi's sarcoma [321]. The other FDA-approved liposome-based nanocarrier is DaunoXome® which has been extensively used in the treatment of various types of human cancers as well as breast cancer [322]. Other nanocarrier-based drugs which have been approved in 2000 was Mylotarg® which has been withdrawn from pharmaceutical markets 10 years later due to its toxic side effects and inadequate anticancer potential. One of the most successful nanocarriers which currently used in chemotherapy is Abraxane, as paclitaxel albumin-bound nanoparticles. In contrast to other types of delivery strategies as well as liposome which are still on clinical trial phases, Abraxane has been absolutely approved by FDA and could be an ideal replacement for Taxol in failed combined chemotherapy with 21% response rate [323]. The other fascinating example of nanocarriers which has been shown to be successful in increasing the survival of leukemia patient is Ontak. It is one of the instances of targeted proteinaceous nanoparticle that could enhance the survival rate up to 63% among non-Hodgkin's peripheral T-cell lymphoma (PTCL) patients when added to the first line of conventional chemotherapy such as cyclophosphamide, doxorubicin, vincristine, and prednisone known as CHOP. It has no remarkable

Table 26.2 List of FDA-approved nanoformulated chemotherapeutic drugs

Drug's name	Nanoparticle formulation	Year	Type of cancer used
Doxil®	Liposome + doxorubicin	1995	Breast, Kaposi sarcoma, ovary
DaunoXome®	Liposome + daunorubicin	1996	Kaposi sarcoma, breast, rhabdomyosarcoma [327–329]
Mylotarg®	Gemtuzumab ozogamicin molecules bonded to the monoclonal antibody	2000	Acute myeloid leukemia (AML)
Abraxane®	Paclitaxel albumin-bound nanoparticles	2005	Metastatic breast and ovarian cancer/ non-small-cell lung cancer (NSCLC) [323, 330]
Abraxane®	Paclitaxel albumin-bound nanoparticles	2013	Metastatic breast and ovarian cancer
Ontak®	Diphtheria toxin and IL2 protein fusion mediating by nanoparticle	2008	Non-Hodgkin's peripheral T-cell lymphomas (PTCL)
Marqibo (Spectrum)	Liposomal vincristine (non-PEGylated)	2012	Lymphoma, melanoma, Philadelphia chromosome-negative AML, leukemia, and brain tumor [331]
Onivyde (MM-398) (Merrimack)	Liposomal irinotecan (PEGylated)	2015	Metastatic pancreatic cancer, breast cancer, sarcoma [332]

toxicity owing to the fact that it is not a myelosuppressive chemotherapeutic agent and can be used for the treatment of every hematologic cancer [324].

Some of the nanocarriers have been approved by the European Medicines Agency (EMA) for treatment of cancer which include MEPACT (Millennium) and Myocet (Teva UK). MEPACT consisted of non-PEGylated liposomal mifamurtide which received confirmation to be used in chemotherapy of osteosarcoma after surgery or in high-grade form by 2009 [140]. Myocet was approved by the year 2000, and its nanoformulation is non-PEGylated liposomal doxorubicin. It was initially approved for treatment of metastatic breast cancer, and then, it got confirmation to be considered in chemotherapy of other cancers such as ovarian cancer, soft tissue sarcoma, and lymphoma [325, 326].

26.7 Nanoparticle-Based Immunotherapy for Cancer

Although the initial goal for cancer nanomedicine was to enhance localized delivery of anticancer drugs within tumors, efficiency of drug delivery is low, and demands for powerful nanomedicine approaches are still required [333]. In this way, recent researchers have focused on the manipulation of immune responses to induce more effective antitumor immunity. Thanks to the significant advances in cancer immunotherapy, targeting tumor cells would not be the only approach for cancer treatment using nanotechnology. Nanomedicine and cancer immunotherapy are two emerging fields that have grown in parallel, and combination of these two potent approaches has led to the introduction of nanoparticle-based immunotherapy. Of note, nanomedicine is going to provide multiple new solutions for cancer immunotherapy-associated problems in coming decades. Although ultimate aims of both approaches are mounting, sustained, and specific antitumor responses against the cancer cells, some difference in the efficacy, safety, and cost-effectiveness could be addressed. In fact, multifunctional nanoparticles not only have enabled us to perform targeted delivery into immune cells more effectively than mono- or multiple deliveries of therapeutic agents but also could dramatically reduce adverse outcomes.

As discussed earlier, restoration of impaired immune responses during carcinogenesis could be carried out through several options which are almost based on the alteration of immune responses. However, different immunotherapy strategies have remained elusive due to the insufficient induction of immune responses and associated systemic toxicity. To overcome conventional immunotherapy pitfalls, nanomaterials have been designed to offer many advantages including prolonged half-life of drugs, site-specific targeting, and less toxicity. As it was previously described in details, nanoparticles could be implicated to encapsulate various types of chemotherapeutic agents to be exactly delivered on target organ [334]. In this way, those revolutionary particles could release immunostimulatory agents into the tumor tissues, as well. Those immunostimulatory agents could lead to blockage of inhibitory signals to T-cells resulting in less immune evasion of tumors as well as stimulation of effective immune responses via different co-stimulatory pathways to promote antitumor immunity [335, 336]. Although this is a new field, it holds tremendous potential for personalized therapy of cancer.

There are different pieces of evidence of stimulation and/or suppression of immune responses more efficiently by nanoparticles. As it was previously mentioned, cytokine therapy is one of the oldest immunotherapy approaches used for cancer treatment which is not widely used due to its serious impacts as well as lack of response. One of the major drawbacks of cytokine therapies (e.g., IL-2 therapy) is related to nonspecific lymphocyte activation in circulation as well as the short half-life of some of them in serum, which requires repeated high-dose injections. Conventional cytokine therapy has also been shown to be associated with severe side effects as well as development of autoimmune disease in genetically susceptible individuals. In this regard, engineered nanoparticles could be used for optimal delivery of cytokines as well as IL-2 with the

goal of activation and proliferation of different immune cells (e.g., CD8 T-cells, CD4 T-cell, NK cells) in peripheral blood, and thereby, infiltration of those cells will increase into the tumor environment [337]. The other example of immunotherapy approach is suppression of TGF-β as an anti-inflammatory cytokine which plays important roles in tumorigenesis stimulation of cancer cell proliferation and invasion [338]. Nanoscale liposomal polymeric gels (nanolipogels) have been used to simultaneously deliver IL-2 and TGF-β receptor I inhibitor which resulted in a significantly delayed tumor growth due to the activation of both innate and adaptive immune responses while blocking a key immunosuppressive pathway [318].

Another approach to boost immune responses is cancer vaccine, which may fail to induce significant antitumor responses through conventional ways. Nanotechnology has offered a solution for this problem by presenting antigens/epitopes on nanoparticulate carriers [339]. Nanocarrier-based cancer vaccines can prolong or boost antigen-specific immune responses and subsequently promote antitumor immunity through enhancing uptake of nanoparticle-based vaccines by phagocytes as well as APCs. Co-administration of adjuvants as free drugs conjugated with antigen loaded on nanocarriers could result in a robust antitumor immune response. In this regard, tumor antigens and adjuvants can be co-loaded on the particle core which enables co-delivery of both components to the same DC and then further increases the magnitude of responses against tumor antigens [340, 341]. Since Toll-like receptors (TLRs) especially those with the capacity of Th1 activation (TLR3, TLR7, and TLR9) enhance antitumor immunity, their agonists have been widely investigated as adjuvants for cancer which could be loaded into nanoparticles [342, 343].

Interestingly, there is some evidence implying the effect of nanoparticle vaccine's physical characteristics as well as material on mounting immune responses [344]. For example, Stano et al. [345] have shown that a polymersome with the watery-core structures elicited a different immune response from the solid-core structure nanoparticles. Additionally, particle size, surface characteristics such as hydrophobicity and charge, and conjugating the targeting ligands are other critical factors that can significantly increase uptake and often antitumor efficacy [346].

Checkpoint inhibitors as well as co-stimulatory agents conjugated with nanoparticles have become another interesting topic for the restoration of impaired antitumor responses. To this end, many efforts have been made to develop engineered nanoparticles that can target specific co-inhibitory molecules, such as PD-1 in T-cells as well as tumor cells. It was demonstrated that such nanoparticles when intravenously administered could bind to circulatory T-cells and concentrate immunomodulatory drugs to these cells and load them into the tumor microenvironment [347]. Kosmides et al. [336] have created an immunoswitch nanoparticle with the goal of making delay in tumor growth. Those nanoparticles were able to switch off the immunosuppressive PD-L1 pathway and in turn switch on the co-stimulatory 4-1BB pathway on tumor cells and CD8+ T-cells, respectively. It was demonstrated that polymeric nanoparticles loaded with CTLA-4 siRNA have been able to induce increase and decrease in frequency of antitumor CD8+ T-cells and Tregs, respectively, among tumor-infiltrating lymphocytes (TILs) which were followed by eliciting more effective antitumor immune responses of the TIL cells [348]. Another innovative strategy for nanoparticle-based immunotherapy was using polymeric nanoparticles to target dendritic cells within the lymph node, which was demonstrated to be associated with reduced B16-F10 melanoma cell growth through an increase in the frequency of antigen-specific CD8 T-cells within the tumor [349]. Nanotechnology was also implicated in targeting of TAMS as Huang et al. [350] have developed a multifunctional delivery system which consisted of (1) combination of CpG oligodeoxynucleotide (ODN), anti-IL-10 ODN, and anti-IL-10RA ODN; (2) galactosylated cationic dextran (gal-C-dextran); and (3) the pH-sensitive material PEG–histidine-modified alginate (PHA) to reprogram TAMs in murine tumor model. As a consequence, the production of IL-12, an antitu-

mor cytokine, has been promoted, while the expression levels of IL-10 and IL-10RA have been shown to be reduced.

Using nanoparticles to increase the strength of immune responses against tumors is not limited to modification of only current immunotherapy approaches. It was shown that iron oxide nanoparticles can induce pro-inflammatory antitumor phenotype in pro-tumor macrophages which resulted in destroying cancer cells through releasing cytotoxic molecules (e.g., reactive oxygen species) and induced cancer cell apoptosis [351]. The delivery of nucleic acids such as DNA and short interfering RNA by viral vectors and nonviral nanoparticles is another potential approach for nano-based cancer immunotherapy, especially for drug-resistant lines. In fact, using DNA or RNA interference in conjunction with nanomaterials could regulate the activity of tumor immune cells or induce the expression of specific tumor antigens by APCs [352, 353].

Aside from currently proposed strategies in using nanomedicine for enhancing cancer immunotherapy, some speculations have been released about the potential complementary of CAR T-cells and nanoparticles [354]. It was suggested that conjugation of CAR T-cells which are able to be circulated in the bloodstream for a long time with nanoparticles has led to degradation of the extracellular matrix, disruption of cell–cell interactions, and thermal stimulation of targeted tumor. This nanoparticle-based immunotherapy approach could be efficiently used in CAR T-cell therapy by increasing the chance of tumor accessibility as well as better management of CAR T-cell-related toxicity [354].

One of the major advantage of nanoparticle-based immunotherapy including all described possible approaches is that it could be used to treat all types of human cancers including solid and hematologic malignancies. To the best of our knowledge, except some concerns which still remained about the possible toxicity of nanoparticles especially in long-term administration, no drawback has been reported regarding using nanoparticles in immunotherapy of cancer. However, implication of nanoparticles in immunotherapy has been shown to be decreased in nonspecific cell cytotoxicity frequently observed in conventional immunotherapy. Further investigation is required to confirm the potential of nanomaterials in successful immunotherapy to be moved from bench to bedside.

26.8 Concluding Remarks

Nanomedicine is an emerging science for the treatment of cancer patients more effectively than ever. Mounting evidences suggest that cancer immunotherapies formulated in nanoparticles are capable of bolstering immune responses against cancer much more than when they are administered alone. However, it is relatively new and must mature before its full impacts will be realized. Increasing our understanding of the role of immunomodulatory and immunostimulatory molecules, combined with advancing in designing of multifunctional nanocarrier systems, has enabled us to be one step closer to targeted therapy of cancer. In other words, nanomedicine has not only altered diagnosis of cancer by improving imaging contrast agent but also altered the treatment by enhancing penetrating capability and physicochemical stability, having less toxicity, and also improving therapeutic index for entrapped drugs. With improving our knowledge and experience in this field, it is expected that several challenges and opportunities will appear; nonetheless, it will make a fundamental paradigm shift in treatment and diagnosis of cancer. Taken together, nanomedicine has significantly improved diagnosis and treatment of various types of cancer and made remarkable development in lab-free follow-up of cancer patients. It is also revolutionizing cancer immunotherapy which merits further exploration and investigation due to the significant capacity to boost the magnitude of immune responses as well as reverse immunosuppression in tumor microenvironment. Nevertheless, new findings related to the cancer immunology and other advances in cancer nanomedicine will need to be taken into account in future studies.

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Oncolytic Viruses as Immunotherapeutic Agents

27

Yevhenii Trehub and Andrii Havrilov

Contents

> The most striking sign of leukemia, the excess of leukocytes, disappears, and sometimes the spleen and lymph glands return to their normal size. Yet that the change is not wholly favorable appears from the fact that no case has really recovered… Considering the hopelessness of the ordinary treatment of leukemia, it seems that carefully planned experiments, either with bacterial products or organ extracts, might show a more safe and permanent result.
> —Dock G. (1904) [1].

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27.1 Introduction

Oncolytic viruses are considered as a fundamentally new approach to cancer therapy, which, based on the underlying mechanisms, should be discussed in the context of immunotherapy. Oncolytic viruses (OVs) are viral agents that multiply predominantly

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or exclusively in neoplastic cells and neighboring endothelium, killing them, and do not replicate in cells of normal tissues. Unlike gene therapy, where the virus acts as a gene carrier the product of which is a treatment of a particular disease, the oncolytic virus itself is a means of treatment.

High interest in oncolytic viruses has been observed during the last decade, although the idea of using viruses to fight cancer is not new. Reports of regression of tumors in patients with natural infectious diseases, which now can be retrospectively considered as of viral nature, began to appear since the 1800s [2]. The role of viruses in the treatment of cancer was first mentioned in 1912, when the effect of rabies vaccination on the course of cervical cancer was noted [3]. In 1955, the infection of cervical cancer patients with different adenoidal-pharyngeal-conjunctival virus (APC) serotypes, histological changes in tumor tissue, and the risk of developing a systemic viral disease were investigated deeper and more consciously [4]. In 1949, the effect of viral hepatitis on the course of the Hodgkin lymphoma was investigated, when the volunteer cancer patients were infected with blood plasma or tissue samples of a patient with viral hepatitis. A positive effect was observed in almost half of the cases [5]. In 1952, the infection of patients with various advanced, resistant tumors with the early passage of the West Nile virus (Egypt 101) showed tumor regression in 10% of patients [6]. In 1974 a nonattenuated Mumps virus for the treatment of patients with 18 different types of tumors showed a dramatic effect: a cure or more than 50% regression occurred in 37 of the 90 subjects. At the same time, a killed Mumps virus showed a relatively very weak antitumor effect as a stimulant of immunity in unresponsive melanoma, which indicates the predominant role of the oncolytic but not immunostimulating effect of the virus [7]. These are only a few studies that had been conducted in the field of oncovirotherapy before the 1980s, not to mention the multitude of studies on animals. By the way, Moore in 1949 showed a complete destruction of murine sarcoma 180 on a mouse model under the influence of Russian Far East encephalitis virus under certain conditions [8, 9], which became a milestone in the development of oncovirotherapy. The limiting factor for the widespread use of oncovirotherapy was an inability to restrict the viral process to make it minimally harmful to healthy tissues and limit viral replication to tumor cells alone. Therefore, in the 1970s and 1980s, the research activity around oncolytic viruses was somewhat faded due to certain legal and ethical limitations, but interest in them did not disappear.

At the same time, attempts were being made to reduce the systemic damage of the viruses for the organism. In 1952, Moore notes that the passaging of the virus in a culture of tumor cells increases its tropism 20- to 30-fold to this tumor in vivo [10]. This was the beginning of an era of manipulation of the viruses, although it was still far from real interventions in their structure and genome.

Trying to reduce the harm of viruses a hypotheses of virus competing for the target organ have been put forward: to reduce the harm of a Russian Far East encephalitis virus, it was proposed to simultaneously infect the object with a nonpathogenic neurotropic Newcastle disease virus [11]. This slightly prolonged survival, but the Newcastle disease virus did not show interference with the most oncolytically active at those years Egypt 101 isolate of West Nile virus [2].

Attempts have been made to use viruses that are pathogenic for some animal species to treat tumors of other species. The most successful example was an avian Newcastle Disease Virus. Injected to mice with abdominal cavity carcinoma (Ehrlich ascites carcinoma), it caused a significant tumor response without any manifestations of a viral disease [12]. The very important clue then was the detection of the increase in antitumor immunity after treatment with oncolytic virus—more than 80% of mice cured by the virus did not develop carcinoma after repeated application of this type of cancer cells [13]. This became the basis for understanding that the virus not only causes lysis of the cancer cell but also stimulates anticancer immunity.

However, at that time, the risk associated with an infection of the animal population with a virus that they had never contacted before and had no protection against was underestimated. Such a virus, according to the theory of epidemiology, can adapt, acquire pathogenicity, and increase virulence toward the unprotected species. One of the

viruses used in oncolytic studies was the feline panleukopenia virus, which mutated and acquired the ability to transmit to dogs. It is believed that this virus infected 80% of dogs around the world in the late 1950s as canine parvovirus infection [14, 15].

In the early 1990s, with the advent of DNA recombination technologies and virus-based genetic engineering, oncovirotherapy reached a new stage of development. Now, it has become possible to create recombinant viruses that can only replicate in cells with certain properties—for example, fast-proliferating cells. Martuza's experiment demonstrated the selective activity of the herpes simplex virus with deleted thymidine kinase gene in the malignant glioma tissue [16]. In 1998, the Phase I clinical trial of the G207 virus for patients with brain tumors started in the United States [17], in 2015—the Phase I trial of this virus in children with supratentorial brain tumors [18]. In 2005, H101, a recombinant adenovirus, was approved in China for the treatment of head, neck, and esophageal cancers [19, 20]. In 2015, T-VEC was approved by the FDA for the treatment of melanoma in the United States and in 2016 in Europe and Australia [21–23].

27.2 Model of Oncolytic Virus and Macroorganism Interaction

Immediate realization of the oncolytic potential of the virus occurs, undoubtedly, after its direct interaction with the tumor. This is preceded by the introduction of the virus into the macroorganism—its infection. Depending on the route of administration, which basically can be either intratumoral or systemic, the virus is more or less in contact with the bloodstream, where it is exposed to the primary influence of protective factors that it has to overcome in order to provide the expected effect.

The immune system of the macroorganism was originally considered and indeed is an obstacle to the effective use of oncolytic viruses. Even in the earliest studies in the 1950s, it was observed that active tumor necrosis under the influence of APC virus did not last long due to the eradication of the virus by the host's immune system. In addition, patients who had previously suffered an adenovirus infection showed less response. Viruses which the patient could be contacted with prior to treatment, for example, adenoviruses or poxviruses, are quickly inactivated by the neutralizing antibodies present in the body and demonstrate limited effectiveness. But even in the absence of preimmunization, the viruses rapidly interact with complement and are absorbed by phagocytic cells. Following the injection of vesicular stomatitis virus (VSV) into the systemic circulation, after 2 min, most of the particles become associated with blood cells, and only a small part of them are free in the blood plasma. After 30 min, all the viral particles are already bound to the cells [24]. It turned out that among these cells there are not only ones specialized in virus eliminating but also others which contact with the virus opportunistically. The latter, migrating in the bloodstream, protect the viral particles penetrated into them or adhered on their surface from the immune response and disseminate them into tissues, where the cells migrate to perform their normal functions. Experiments with tumor-antigen-specific T lymphocytes loaded with oncolytic vesicular stomatitis virus and reovirus in vivo showed minimal neutralization of viral particles even at high titers of virus-specific neutralizing antibodies in the animal. In natural conditions, carriers of viruses can be both T lymphocytes and dendritic cells (DCs), which was shown for retrovirus, Newcastle disease virus (NDV), VSV, and reovirus [25–30]. As artificial carriers, different cell lines that can selectively migrate into a tumor or even contact tumor cells are investigated: tumor-antigen-specific T cells, cytokine-induced killer cells, tumor-associated macrophages, mesenchymal stem cells, granulocytes, platelets, and others [31–35]. It is possible to coat the viral particles with polymers, for example, polyethylene glycol or poly-(N-(2-hydroxypropyl) methacrylamide) (pHPMA). This protects the virus from neutralization with antibodies and the T-cell response [36].

In other studies, the best response to OVs in immunosuppressive patients was noted, for example, those with lymphoma or leukemia. Cyclophosphamide was used as an immunosuppressive agent. Many chemotherapeutic agents

are immunosuppressors themselves, so the recent issue is the development of the correct mode of combined chemo-virotherapy, in which the virus would be administrated during a period of slight immunosuppression. In addition, viruses that an individual rarely contacts under normal conditions and against which he does not have neutralizing antibodies (e.g., Seneca Valley virus) still have a theoretical advantage over the common types.

Another obstacle is the permeability of the tumor vessels. The tumor can often have a higher interstitial pressure in comparison with a pressure in the vessels, which makes it difficult to deliver therapeutic agents, including viruses. Chemotherapy, killing tumor cells, somewhat reduces intratumoral interstitial pressure and increases extravasation and intake of substances into the tumor, not affecting directly on vascular permeability [37]. This property should be considered when constructing regimens of combined therapy. Local nitric oxide, bradykinin, nitroglycerin, histamine, local hyperthermia, and low-dose paclitaxel increase vascular permeability and substance leakage into the tumor and enhance oncolytic virus bioavailability [38–40]; systemic angiotensin receptor blockers reduce the collagen deposition inside tumors, which results in the decreasing of intratumoral interstitial pressure [41], VEGF enhances endothelial proliferation and angiogenesis in the tumor, enhancing tumor perfusion by the virus and vascular permeability (see below).

To date, in practical use, only mechanical protection of the virus from immune surveillance and tumor barriers is used so far in a form of direct intratumoral ways of introducing the virus, although this method is sometimes complicated and not always safe for the patient and possible.

27.3 Interaction Between Oncolytic Virus and Tumor

Oncolytic viruses carry with them two mechanisms of antitumor effect: direct cytolysis of tumor cells and enhancement of antitumor immunity. Intracellular replication and accumulation of viral copies in the tumor cell leads to its direct destruction and cell death, resulting in the release of tumor-associated antigens and the provocation of an immune T-cell response [42–45]. In addition, genes of proteins that enhance or modify the immune response and even tumor antigens can be induced into the genome of the virus, which moves the virus to vaccine category.

As stated above, the main task of adapting the virus for use as an oncolytic agent is to make it as affine to tumor cells and associated endothelial cells and minimally pathogenic to normal cells as possible. Some viruses have a natural selectivity in relation to tumor tissue, due to certain features of its altered biology and can be used in a natural, unmodified form. Among such viruses are reovirus, parvovirus, coxsackievirus, and Newcastle disease virus.

The tumor itself with respect to its immunosuppressive microenvironment is an optimal place for the replication of the virus, where it cannot be registered by the immune surveillance in the early stages of the viral process. For example, a number of tumors represent reduced expression of type I IFN and have fewer receptors to it or a disturbed signaling pathway (the pathway that leads to inhibition of cell division and activation of p53). In such conditions, viruses such as VSV, vaccinia, Newcastle disease virus, and mumps virus have an advantage and multiply unhindered [46, 47]. However, the role of type I IFN in the interaction of the tumor with the virus is not completely clear and is probably bivalent, and its formation in the tumor can lead to an increase in tumoristatic or lytic effect (see below).

Knowing the peculiarities and differences of the metabolic or signaling pathways of a cancer cell and the absence of or the altered activity of certain functional proteins in it, it is possible to adapt the virus and make it able to replicate only in conditions of such perverted cell biology. For example, by knocking out viral genes that block the antiviral defense of the host cell, if this defense is absent in the tumor, it is possible to achieve the selective replication of the virus only within the tumor. Among the disturbed metabolic pathways that are potential targets for the virus

selectivity are the defects of the RB/E2F/p16 mechanism, p53, PKR, EGFR, Ras, Wnt, anti-apoptosis, hypoxia conditions, or defects in IFN [48–51]. In general, the mechanism of the virus selectivity can be associated with its penetration into the cell, for example, if the cancer cell expresses unique receptors to which the virus is affine (EGF receptor, Her2-neu, folate receptor, prostate-specific membrane antigen and CD20, and nuclear transcription factors PSA, hTERT, COX-2, and osteocalcin are believed to be potential targets for modified viruses [36, 52]), with a disturbed synthesis of IFN in the tumor (Newcastle disease virus, see below), or with disturbed protective antiviral signaling pathways of the tumor cell (as in T-VEC; see below) [53].

27.3.1 Model of Tumor Destruction Under the Virus Influence

A model of the destruction of tumor formation under the influence of infection with OV is very controversial and, for sure, varies for different tumors and viruses. However, with sufficient confidence, it could be argued that this destruction is multimodal and is mediated by the cooperative impact of several factors. A good model of the complex effect of OV on tumor death is proposed by Mahoney D. on the example of vesiculovirus [54]:

Infection of the tumor cell ultimately leads to its lysis via specific pathways and ultrastructural disorders (immunogenic cell death; see mechanism below) and infection of a number of surrounding tumor cells. At this time, intratumoral resident dendritic cells react to a viral infection (by detecting DAMPs and PAMPs, described below) and activate innate immune response, recruiting NK cells, macrophages, and neutrophils. It is interesting to note that some viruses (in particular, vesiculovirus) can increase the release of type 3 IFN by intratumoral immunocytes, with subsequent increase in the number of NK cell receptors on the tumor cells, making them more vulnerable [55]. Recruited innate immunity cells destroy both infected and noninfected tumor cells. Dendritic cells then absorb tumor and viral antigens, migrate to regional lymph nodes, and present antigens to T lymphocytes, which means activation of an adaptive immune response. Activated antigen-specific T lymphocytes migrate into the tumor and continue destroying its cells. For some viruses, tropism was shown to the endothelium of vessels that supply the tumor (a presumable association with an excess of VEGF). Infection of endothelial cells attracts neutrophils and develops vasculitis and thrombus formation in the vessels of the tumor that leads to ischemic necrosis of the tumor tissue.

27.3.2 Immunogenic Cell Death

Oncolytic viruses, as well as some chemotherapeutic agents and radiotherapy, trigger a specific type of cell destruction. It does not fit completely into any of the classic ways of cell death (necrosis, apoptosis, and autophagy). Until recently, the death of tumor cells due to the effect of any therapeutic agents was considered in the context of nonimmune cell death or arrest of the cell cycle. Immunogenic cell death (ICD) of a tumor cell, or immunogenic apoptosis, is a complex response of a tumor cell to injurious effects, resulting in both apoptosis-like death and activation of a specific immune response to tumor antigens. ICD has been shown for anthracyclines, oxaliplatin, bortezomib, radiotherapy, photodynamic therapy, and viral agents [56–61].

The process of ICD starts when the agent affects certain structures of the cellular matrix and requires a contribution of reactive oxygen species (ROS). ROS cause a stress of the endoplasmic reticulum (ER), but at least, just the presence of ER stress and ROS inside the cell simultaneously is required for ICD initiation. In other words, an ability to induce a ROS-based/ROS-associated ER stress is the determining feature for an ICD inducer. Depending on the way of activation of ER stress, all inducers are divided into two types. Type 1 affects intracellular structures other than ER, triggering its stress indirectly through such targets as cytoplasmic proteins, membrane proteins and channels, and proteins of

Table 27.1 Immunogenic cell death inducers [56, 62–64]

Inducer	Cellular target
Type I inducers	
Anthracyclines	DNA or proteins of DNA replication machinery
Oxaliplatin	DNA synthesis
Bortezomib	ERAD, 26S proteasome, CIP2A
UVC irradiation	DNA
Cyclophosphamide (frequent low-dose administration) [63]	DNA
7A7 (EGFR-specific antibody)	Cell surface receptor (EGFR)
Cardiac glycosides (if combined with chemotherapeutic agents) [62]	Na^+/K^+-ATPase
Vorinostat (HDAC inhibitor)	Nucleus (chromatin structure)
Shikonin	Tumor-specific pyruvate kinase-M2 protein
Wogonin	Mitochondria
Type II inducers	
Hypericin-based photodynamic therapy	Endoplasmic reticulum
Oncolytic viruses	Endoplasmic reticulum

EGFR epidermal growth factor receptor, *UVC* ultraviolet C, *ERAD* endoplasmic-reticulum-associated protein degradation, *HDAC* histone deacetylase, *CIP2A* cancerous inhibitor of protein phosphatase 2A

the DNA replication system. This type mainly includes chemotherapeutic agents and UV radiation. Type 2 agents trigger ER stress impacting directly the ER and disrupt its operation. This type mainly refers to oncolytic viruses [56, 58, 59] (Table 27.1).

ER stress is a state of ER in which it either undergoes synthetic overload and therefore cannot cope with an excessive needs of folding of proteins (physiological stress) or synthesizes pathological proteins that cannot be folded into a tertiary structure properly (pathological stress). Disturbances of protein glycosylation or folding into a soluble form, the presence of mutant proteins, and some viral infections lead to ER stress. Eukaryotic cells have developed a protective mechanism against ER stress—the unfolded protein response (UPR) [65]. UPR is a complex of transmembrane proteins of ER whose domains protrude in both the ER lumen and the cytoplasm of the cell: inositol-requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor (ATF)-6 [66]. These proteins are associated with chaperone glucose-regulated protein 78 (GRP78) in the ER lumen, which detects non-folded or misfolded proteins in ER and releases IRE1, PERK, and ATF-6; they undergo activation by homodimerization and autophosphorylation (but ATF-6 migrates to the Golgi where it is activated by the proteases) [66–68]. Activated PERK inhibits protein synthesis by phosphorylation of eIF-2α (i.e., protein shutoff response); eIF-2α triggers an expression of ATF4 which in turn upregulates expression of CHOP that inhibits a gene encoding anti-apoptotic BCL-2 while enhancing expression of pro-apoptotic BIM. Activated IRE1 triggers an expression of protein degradation enzymes (ERAD). ATF-6 triggers an expression of chaperone genes that refold the misfolded proteins [57]. If an activity of the UPR complex is not sufficient to eliminate ER stress, the described adaptation phase is replaced by an alarm phase and further, through a triggering of signaling pathways such as Fas-associated death domain protein (FADD)/caspase-8-dependent cell death, leads to a cell death [69], which can proceed both via caspase-dependent (apoptosis) and caspase-independent pathway (necrosis) [57] (Fig. 27.1).

Immunogenicity of a cell death is determined by a release of signals into an extracellular environment that indicate a nonphysiological nature of the occurring apoptosis—danger-associated molecular patterns (DAMPs), also called alarmins. DAMPs are intracellular molecules that do not normally come out from the cell but when it is stressed, traumatized, or dying are released into surrounding tissues to be detected by receptors of immune cells. Not all DAMPs are pro-inflammatory—some serve as immunosuppressors to downregulate autoimmune reactions in response to a cell death, thereby providing mechanisms for tolerogenic cell death. Among the latter DAMPs are phosphatidylserine (PS), annexin A1 (ANXA1), death domain 1α (DD1α), and B-cell CLL/lymphoma 2 (BCL2). Main immunogenic DAMPs are adenosine triphosphate (ATP), high-mobility

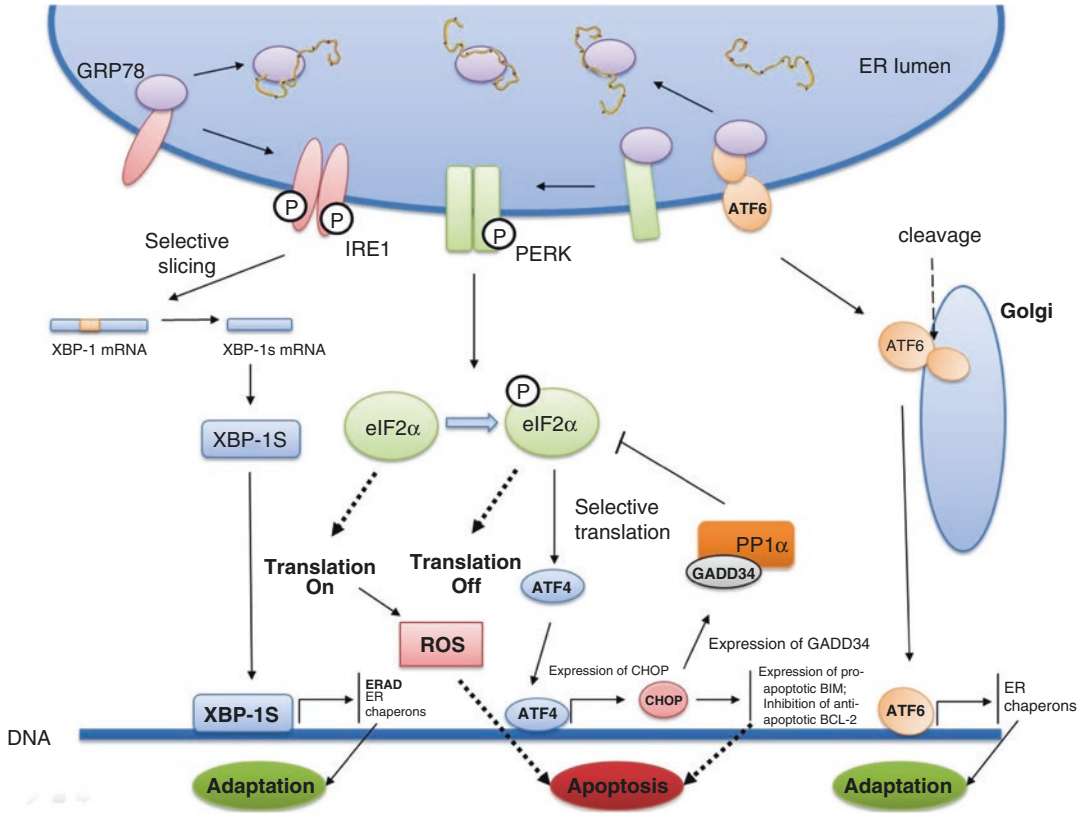

Fig. 27.1 Unfolded protein response. IRE1, PERK, and ATF6 are ER transmembrane proteins that have their domains both in the ER lumen and cytoplasm. GRP78 in normal conditions binds ER luminal parts of IRE1, PERK, and ATF6, attenuating their activity. Accumulation of unfolded or misfolded proteins in the ER lumen leads to GRP78 dissociation and migration into the lumen. Consequently, released IRE1 and PERK are activated through homodimerization and autophosphorylation; ATF6 migrates to Golgi where it undergoes selective proteolysis and subsequent translocation to the nucleus. ATF6 being a transcription factor modulates the expression of genes encoding ER chaperones, which enhance protein folding in ER, and ERAD proteins, which provide degradation of unfolded proteins. Activated IRE1a provides the selective excision of the intron fragment from XBP-1 mRNA (selective splicing). Spliced XBP-1 mRNA translates protein with transcription factor properties that regulates transcription of ERAD pathway proteins and ER chaperons in conjunction with ATF6. Activated PERK phosphorylates eIF2α, which in turn inhibits overall protein translation but enhances translation of ATF4. ATF4 acts as a transcription factor for CHOP, which in turn augments expression of GADD34 and pro-apoptotic BIM but decreases anti-apoptotic BCL-2. GADD34 is a downregulator of the phosphorylated eIF2α activity. Accumulation of ROS due to enhanced protein synthesis along with the expression of pro-apoptotic genes leads to apoptosis [70–73]. *IRE1* inositol-requiring protein 1, *PERK* PKR-like endoplasmic reticulum kinase, *ATF6* activating transcription factor-6, *ATF4* activating transcription factor-4, *GRP78* chaperone glucose–regulated protein 78, *ER* endoplasmic reticulum, *ERAD* ER-associated protein degradation, *XBP-1* X-box binding protein 1, *eIF2α* eukaryotic translation initiation factor 2, *CHOP* C/EBP homologous protein, *GADD34* growth arrest and DNA-damage-inducible 34, *BIM* Bcl-2-like protein 11, *BCL-2* B-cell lymphoma 2 protein, *ROS* reactive oxygen species

group box 1 (HMGB1), heat shock proteins (HSP70, HSP90), and calreticulin (CRT) [59–61]. Their releasing mechanisms, as well as target receptors on immune cells, are presented in Table 27.2.

ER stress, which precedes ICD, is accompanied by an appearance on the surface of the cell membrane of proteins serving as an immunogenic "eat-me" signal for antigen-presenting cells, primarily dendritic cells (DCs). Any ICD, regardless of the inducer, is accompanied by an appearance of calreticulin on the membrane and a release of the immunomodulating molecules such as adenosine triphosphate (ATP) and high-mobility group box

Table 27.2 Main DAMPs occurring in ICD and their brief descriptions

DAMP	Mechanism of release	Immunocytes' receptors	Related mechanisms of cell death
ATP	Actively or passively released	P2Y2 and P2X7	ICD, apoptosis/ secondary necrosis and necrosis
Calreticulin	Mostly surface exposed; sometimes passively released	CD91 (LRP1)	ICD
Heat shock proteins (HSP70, HSP90)	Surface exposure, active secretion, or passive release	CD91 (LRP1), TLR2, TLR4, SREC-1, and FEEL-1	ICD, apoptosis/ secondary necrosis, necrosis
High-mobility group box 1	Mostly passively released; sometimes actively released	TLR2, TLR4, RAGE, and TIM3	ICD, secondary necrosis and necrosis

DAMP danger-associated molecular pattern, *ICD* immunogenic cell death, *ATP* adenosine triphosphate, *LRP1* low-density lipoprotein receptor-related protein 1, *TLR* Toll-like receptor, *SREC-1* scavenger receptor expressed by endothelial cells 1, *FEEL-1* fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1, *RAGE* receptor for advanced glycation end products, *TIM3* T-cell immunoglobulin and mucin-domain containing-3

1 (HMGB1) into an extracellular space [60, 74]. Calreticulin (CRT) is an ER-chaperone protein; its migration from the ER to the surface of the cell membrane is a sign of the onset of apoptosis even before its morphological features appear. Translocation of CRT to the surface of the cell membrane is initiated by an activation of caspase-8. The latter leads to an activation of BAX/BAK and cleavage of their substrate Bap31. This is considered necessary for the beginning of migration of CRT [75]. The translocation of CRT is due to its binding to the ERp57 protein, and an CRT/ERp57 complex migrates to the surface [56, 69]. Various proteins of UPR, apoptosis (BAX/BAK/caspase-8), cytosolic Ca2+ play a role in calreticulin transportation to the cell surface. On the membrane, CRT is deposited on low-density lipoprotein receptor-related protein 1 (LRP1) [76, 77]. It is CRT that is considered to be the main signal that causes the immunogenicity of cell death. A blockade of CRT or depletion of CRT with small interfering RNAs (siRNAs) neutralizes the immunogenicity of cell death [76]. Part of CRT is also secreted into an extracellular space, acting as a pro-inflammatory agent and a modulator for DCs: after the impact of CRT, DCs release IL-6, IL-8, and TNF-alpha [78], and the antigen-presentation mechanism is changed—the MHC II pathway is inhibited, and MHC I is activated and, accordingly, a cross-presentation is, with the activation of CD8-T lymphocytes.

HSP90 is another DAMP released during ICD that also migrates to the cell surface and is exposed associated with LRP1. Both surface-exposed CRT and HSP90 interact with specific receptors on the membrane of the immune cell (for example, LRP1 of the DC), which becomes an immunogenic "eat-me" signal for the latter [79–81].

ATP, being a "find-me" signal, binds to P2Y2 receptors of DCs, making them migrate to the apoptosis region. In addition, ATP binds to P2X7 receptors of DCs that activate the NALP3-inflammasome complex, which acts as a trigger for caspase-1 in monocytes [56, 80]. Caspase-1 serves as a protease of pro-IL-1β protein; thus, its activation increases expression of IL-1β by a DC. IL-1β acts as a pro-inflammatory agent; it, together with presentation of tumor antigens, activates the CD8+ T cells and triggers an antitumor adaptive immune response [82, 83].

HMGB 1 is a nuclear protein that is passively released both in necrosis and in the late phase of apoptosis and is an agonist of Toll-like receptor (TLR)-4 of DCs [56]. Its interaction with the receptor stimulates maturation of the DCs and release of pro-inflammatory cytokines. Additionally, HMGB 1 induces multiplication of the IFN-producing Th1 cells clone [84]. The activity of HMGB 1 depends on its redox state. Reduced HMGB 1 behaves as a chemoattractant for leukocytes, disulfide-bond possessing HMGB1—as an inducer of pro-inflammatory cytokines release, and oxidized state is inactive [85]. Moreover, HMGB 1 inhibits immunosup-

pressive Treg cells of the tumor microenvironment [57].

Along with the release of immunogenic DAMPs during ICD, the cell loses tolerogenic "don't eat me" signals. Among such signals is CD47. Moreover, a decrease in the level of CD47 is considered necessary for CRT to manifest its immunogenic properties as an "eat me" signal [58, 86–88].

A picture of ICD caused by a number of OVs is similar to the ICD resulting from other inducers: coxsackievirus B3 [89], measles virus [90], and CD40-ligand expressing adenovirus [91] lead to cell death, which is accompanied by the release of the main described DAMPs—calreticulin, ATP, and HMGB1. However, processes occurring on the ultrastructural level during the OV-mediated ICD is not identical to that caused by other agents. OV takes control of the protein synthesis machinery and mechanisms of cell death, so its course may differ from the described. For example, OV can regulate the cell death apparatus in a way that allows its activation only after all cell energetic resources (ATP) have been depleted [50]. For Newcastle disease virus, it has been shown that it can trigger both caspase-mediated (apoptosis) and caspase-independent (necrosis) death. Also, for this virus, no exposure of HSP70/90 and ATP by the dying cell was observed during ICD. Concerning ATP, this is probably due to its expenditure on viral replication [92].

DCs consume tumor-associated antigens (both endogenous and neoantigens, as well as viral antigens) and present them to the cells of the adaptive immune response in lymph nodes, which in the presence of the immunogenic (but not tolerogenic) DAMPs leads to liberation of pro-inflammatory cytokines (e.g., IL-6/IL-12/IL-1β) [93, 94] by DCs and activation of T cells: polarization of CD4+ lymphocytes into the Th1 and Th17 cells for type-I antibody-dependent antitumor immune reactions (DC-released IFN-γ polarizes CD4+ and also acts as a cytostatic agent for tumor cells) and activation of CD8+ cytotoxic lymphocytes (CTL) by the aid of Th1 cells (cytotoxic lymphocytes cause direct toxic effects on tumor cells mediated through IFN-γ, FasL-CD95 interaction, and perforin-granzyme action) [59, 61, 74, 95–97]. Different OVs presumably can differently activate different components of the adaptive immune response: for example, preferential activation of Th1 was shown for reovirus-mediated oncolysis, while VSV promotes mostly Th17 cells [98]. During the adaptive immune response, a pool of memory T cells is formed, which provide prospective long-term antitumor immunity, mainly maintained by CD8+ T cells.

An obstacle to an effective immune response to the ICD of a tumor cell is the fact that tumor-associated antigens (TAAs) of solid tumors in fact are often self- or close-to-self-antigens. T lymphocytes carrying high-affinity T-cell receptors (TCRs) to these antigens normally undergo negative selection in the thymus and lymph nodes to prevent autoimmunity [99, 100]. Cells with low-affinity TCRs may elude negative selection, but their activity is usually insufficient to trigger a full-fledged immune response due to the immunosuppressive microenvironment in the tumor [101, 102]. ICD decreases the degree of this immunosuppression and increases activity of the low-affinity clone of T lymphocytes for a while, but this pool is quickly suppressed by mechanisms of peripheral tolerogenicity after the fading of ICD, and immunological memory hardly develops. This is especially relevant for chemotherapy regimens, because they have a limited duration of administration due to the development of adverse effects (e.g., severe lymphopenia, which diminishes the antitumor immunity) [99]. From this perspective, OVs seem to be an effective solution as an inductor of ICD—they replicate in a tumor causing ICD for as long, as they still are able to infect other tumor cells; such prolonged ICD stimulates the activity of low-affinity T cells for a long time [59]. But if mutant antigens are present on the tumor, T lymphocytes carrying TCRs to them are not subjected to central (negative selection) and peripheral tolerogenesis, and therefore will be more active in the immune response and memory formation [103].

Another significant potentially positive difference of OVs from other inducers of ICD is that an infected cell, in addition to DAMPs, releases pathogen-associated molecular patterns (PAMPs), which indeed are structural molecules and the products of the vital activity of the virus (like in the infection of normal non-tumorous tissues). Such additional stimulation may enhance the

activity of immunocytes and increase the efficiency of cross-priming of TAAs and, therefore, the immune response to the tumor [57].

Some OVs, in particular Newcastle disease virus, trigger type I IFN response in tumor tissue additionally to ICD [104]. The effect is achieved both by the direct influence of IFN-α and IFN-β on the tumor cell followed by an activation of the antiproliferative effect by p53 induction [46], mediation of the stimulated CD8+ T lymphocytes and macrophages, and release of pro-inflammatory cytokines. The early phase of type I IFN response is the detection of PAMPs by monocytes and DCs via pattern recognition receptors (PRRs). This signal leads to the initiation of IFN-β and then IFN-α expression by these cells. The late phase is the interaction of the released IFN-α and IFN-β with the surface chain of the type I IFN receptor (IFNAR) and start of the synthetic phase of the IFN response, i.e., the signaling pathway resulting in activation of the expression of a wide variety of interferon-stimulated genes (ISGs) that affect the life cycle of the virus at its various stages [105]. It is not yet clear which of the IFN response links are most effective and are of primary importance in the infection of tumor tissue, taking into account the immunosuppressive microenvironment and the disturbed apoptotic and inflammatory signaling pathways of neoplastic cells. IFN response in the tumor may presumably develop after a sufficiently massive infection of the tissue followed by an increase in pro-inflammatory properties of the microenvironment as far as leukocytes infiltration of the tumor occurs (Fig. 27.2). This mechanism requires further study.

27.4 Oncolytic Viruses of Current Interest

27.4.1 Artificially Modified Viruses

Modified oncolytic viruses are mainly normally pathogenic human viruses, which has been induced with specific modifications in their cell invasion or antiviral defense block apparatus, and therefore, they lose their pathogenicity in normal tissues but manifest it in neoplastic cells with defective defense or demonstrate their selectivity to cells with specific membrane receptors. Among the most studied of such viruses are HSV, adenoviruses, and vaccinia, and the most common modifications are blockades of genes attenuating antiviral protection in host cells, changes in proteins responsible for invasion into the cell, and insertions of immunomodulatory protein genes (e.g., GM-CSF) (Table 27.3).

27.4.1.1 Oncolytic Herpesviruses

Talimogene laherparepvec (T-VEC) is the first drug of the OVs group that has proven to be effective in the Phase III clinical trials and is approved for use in Europe [110] and the United States [21, 111, 112].

The virus is constructed on the basis of HSV-1 with mutations in two genes: deletion of *α47* and *γ34.5*, with the insertion of human granulocyte-monocyte colony-stimulating factor (GM-CSF) gene into the locus of *γ34.5* gene [23]. *γ34.5* is responsible for the virus's ability to inactivate the protein synthesis block (protein shutoff) response to the viral invasion of the host cell and thus maintains its replication in the infected cell. Deletion of this gene makes the virus unable to reproduce in a normal cell. But in the neoplastic cell, where the mechanism of the protein shutoff is frequently disrupted, the mutant Δ*γ34.5* virus can still replicate [113]. The *α47* gene serves as an inhibitor of the transporter associated with antigen presentation (TAP) protein. This transporter is involved in the mechanism of antigen presentation and particularly MHC class I expression on the cell surface. Its inhibition makes infected cells invisible for CD8+ CTL [114, 115]. Switching off the *α47* gene enhances expression of Ag/MHC I complexes on tumor cells and antitumor immune response. In addition, inactivation of *α47* enhances expression of a neighboring *US11* gene that additionally increases viral replication in cells [113, 116]. Expression of GM-CSF further enhances maturation of DCs and, consequently, the immune response. In the murine bilateral flank tumor model, a GM-CSF-expressing virus showed an oncolytic effect

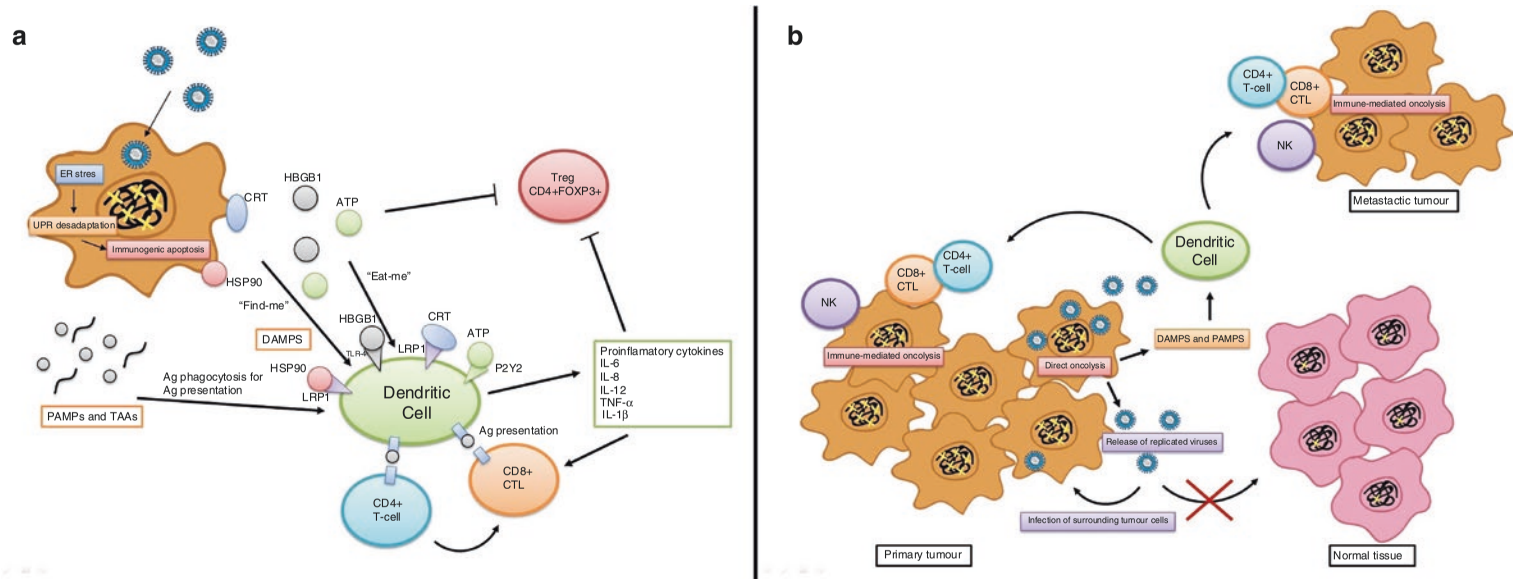

Fig. 27.2 Immunogenic cell death. Intratumoral or systemic injection of OV leads to selective infection of tumor cells due to the dysregulation of their functional pathways (e.g., antiviral defense machinery) or presence of specific receptors or immunosuppressive media (see in the text). Normal tissues are not susceptible to OVs which are either normally nonpathogenic for humans or have genetic modifications providing such selectivity. Infection of cancer cells with an OV causes the consequent response, including ER stress, disadaptation of unfolded protein response pathways, and activation of apoptosis machinery through a caspase-8-dependent cell death pathway. Immunogenic apoptosis is accomplished by the release of DAMPs: surface-exposed CRT and HSP90 which act as "eat me" signals for DCs and extracellular ATP and HMGB1—"find me" signals; TAAs that are processed by DCs for antigen presentation; and PAMPs—viral proteins and nucleic acid that enhance recruitment of immunocytes and are also used for antigen presentation. Replicated viruses released during the cell death invade surrounding cancer cells. Activated DC releases pro-inflammatory cytokines that reduce immunosuppressive properties of tumor microenvironment by Treg attenuation and enhance innate immune response by additional recruiting of NK. Maturated DCs then migrate to regional lymph nodes which present tumor and viral antigens to CTL and Th cells, accordingly initiating adaptive immune response. Tumor (and virus)-specific lymphocytes then infiltrate the primary tumor, as well as distant metastatic tumors that were not exposed to the OV, causing immune-mediated oncolysis [54, 56, 57, 59, 97, 106, 107]. *ER* endoplasmic reticulum, *UPR* unfolded protein response, *DAMPs* danger-associated molecular patterns, *PAMPs* pathogen-associated molecular patterns, *TAAs* tumor-associated antigens, *CRT* calreticulin, *HSP90* heat shock protein 90, *HMGB1* high-mobility group box 1, *ATP* adenosine triphosphate, *LRP1* low-density lipoprotein receptor-related protein 1, *TLR-4* Toll-like receptor-4, *CTL* cytotoxic T lymphocyte, *Th* T-helper, *NK* natural killer, *Treg* regulatory T lymphocyte

Table 27.3 General properties of current OVs under development [108, 109]

Virus family	Virus species	Genome	Mechanism of invasion	Virus strain (name), genetic modification	Current development status
Herpesviridae	HSV-1	dsDNA	Membrane receptors—Glycoprotein D for epithelial cells; HVEM, nectin-1, and nectin-2 for neurons	Talimogene laherparepvec (T-VEC) (Δγ34.5/Δα47/GM-CSF (+))	Approved by FDA for stage IIIB-IVM1a melanoma
Adenoviridae	Adenovirus	dsDNA	Membrane receptors—CAR; HSPG and low-density lipoprotein receptors for hepatocytes	H101 (ΔE1B55K/ΔE3)	Approved by Chinese state Food and Drug Administration for advanced head and neck cancer
				ICOVIR-5 (E1AΔ24/E2F1 (+)/RGD-4C (+) into the fiber knot)	Phase I trial for melanoma
				CG0070 (ΔE3/GM-CSF (+))	Phase II trial for bladder cancer
				OBP-301 (hTERT promoter (+))	Phase I/II trial for hepatocellular carcinoma; phase I for esophageal carcinoma
Reoviridae	Reovirus	dsRNA	Membrane receptors—Sialic acid, JAM-1	Reolysin (non-modified)	Phase III trial for advanced/metastatic head and neck cancer
Paramyxoviridae	NDV	ssRNA	Plasma membrane fusion	NDV (non-modified)	Phase I/II trial for glioblastoma, sarcoma, and neuroblastoma
				NDV oncolysate-pulsed DCs (VOL-DCs) (vaccine)	Received advanced therapeutic medicinal product status
	Measles virus	ssRNA	Membrane receptors—CD46	MV-NIS (sodium/iodine transporter (+))	Phase I/II trial for recurrent ovarian cancer
Picornaviridae	Coxsackievirus	ssRNA	Membrane receptors—CAR, ICAM-1, DAF	Cavatak (non-modified)	Phase I and II trial for melanoma
	Poliovirus	ssRNA	Membrane receptors—CD155	PVS-RIPO (ΔIRES/IRES from human rhinovirus type 2 (+))	Phase I trial for glioblastoma
Poxviridae	Vaccinia	dsDNA	Plasma membrane fusion	JX-594 (ΔTK/GM-CSF (+))	Phase III trial for hepatocellular carcinoma
Rhabdoviridae	VSV	ssRNA	Membrane receptors—LDLR	VSV-hIFNb (IFN-β (+))	Phase I trial for different solid tumors; phase I trial for lymphomas and leukemia
				GL-ONC1 (ΔF14.5L/ΔJ2R/ΔA56R/Renilla luciferase (+)/GFP (+/, β-galactosidase (+))	Phase I/II trial for ovarian, fallopian tube cancer, peritoneal carcinomatosis

Table 27.3 (continued)

Virus family	Virus species	Genome	Mechanism of invasion	Virus strain (name), genetic modification	Current development status
	Maraba virus	ssRNA	Membrane receptors	MG1-MA3 (MageA3 (+))	Phase I/II trial for advanced/metastatic solid tumors
Parvoviridae	Parvovirus	ssDNA	Membrane receptors—Sialic acid, erythrocyte P receptor	ParvOryx (non-modified)	Phase I trial for glioma

OVs oncolytic viruses, *Δ* deletion, *(+)* insertion, *FDA* Food and Drug Administration, *HSV-1* herpes simplex virus-1, *NDV* Newcastle disease virus, *VSV* vesicular stomatitis virus, *HVEM* herpesvirus entry mediator, *CAR* coxsackievirus and adenovirus receptor, *HSPG* heparan sulfate proteoglycan, *JAM-1* junctional adhesion molecule 1, *ICAM-1* intercellular adhesion molecule 1, *DAF* decay-accelerating factor, *LDLR* low-density lipoprotein receptor, *IRES* internal ribosome entry site, *GFP* green fluorescent protein, *MageA3* melanoma-associated antigen 3

both at the site of intratumoral administration and in a distant homologues tumor, whereas the virus without the GM-CSF gene acted only in the primary-injected tumor site [44] (Fig. 27.3). Thus, acomplex theoretical model of the T-VEC virus action can be represented by the following:

At the site of intratumoral injection of the virus, it invades mainly cancerous cells that express an excess of receptors to which the virus has a natural tropism (such as HVEM, nectin-1, and nectin-2) but also normal cells. In normal cells, its replication does not occur since the mechanism of protein synthesis shutoff response is turned on, which cannot be blocked by the virus due to the absence of the *γ34.5* gene. In the tumor cell, the protein shutoff mechanism does not work, so the virus freely replicates in it. During replication, some viral antigens interact with TAP in the Golgi, since the viral protein that normally prevents this event is absent in the virus due to the deletion of *α47*; then, these viral antigens bind with MHC I, and this complex migrates to the cell surface. It promotes virus-specific CD8+ CTL formation, which triggers mechanisms of immune-mediated cell death and attract immunocytes, releasing IFN-gamma. Expression of GM-CSF additionally recruits DCs and macrophages into the tumor and triggers their maturation. Mature antigen-presenting cells then present tumor antigens to CD8+ T cells in lymph nodes; this process stimulates the formation of a tumor-specific clone of CTLs. Lysis of a cancer cell due to the replication of the virus inside it is an achievement of cytoreduction itself. Released from lysed cells, DAMPs, PAMPs and tumor-associated antigens on a background of the immune-activated microenvironment stimulate DCs to trigger an adaptive immune response. Activated antitumor immunity attacks both the primary tumor in which the virus was injected and metastatic foci [110] (see ICD mechanism above).

In Europe, indications for T-VEC is an unresectable melanoma in adults, which is regionally or distantly metastatic (stage IIIB, IIIC, and IVM1a), with no bone, brain, lung, or other visceral diseases [111]. In preclinical studies, T-VEC showed efficacy also in other types of neoplasm, but melanoma was initially chosen for the clinical trial because of the availability of superficial foci for intratumoral virus administration and the known activity of the immune system in this type of cancer.

T-VEC is administrated intratumorally in a maximum dose of 4 ml with a titer of 10^6–10^8 plaque forming units (pfu)/ml diluted in phosphate-buffered saline. The injected dose depends on the size of the tumor: 0.1 ml is used for the tumor smaller than 0.5 cm in the largest dimension; size 0.5–1.5 cm, up to 0.5 ml; 1.5–2.5 cm, up to 1 ml; 2.5–5 cm, up to 2 ml; and lesions more than 4 cm, up to 4 ml. The first injection for the seronegative for HSV-1 patient

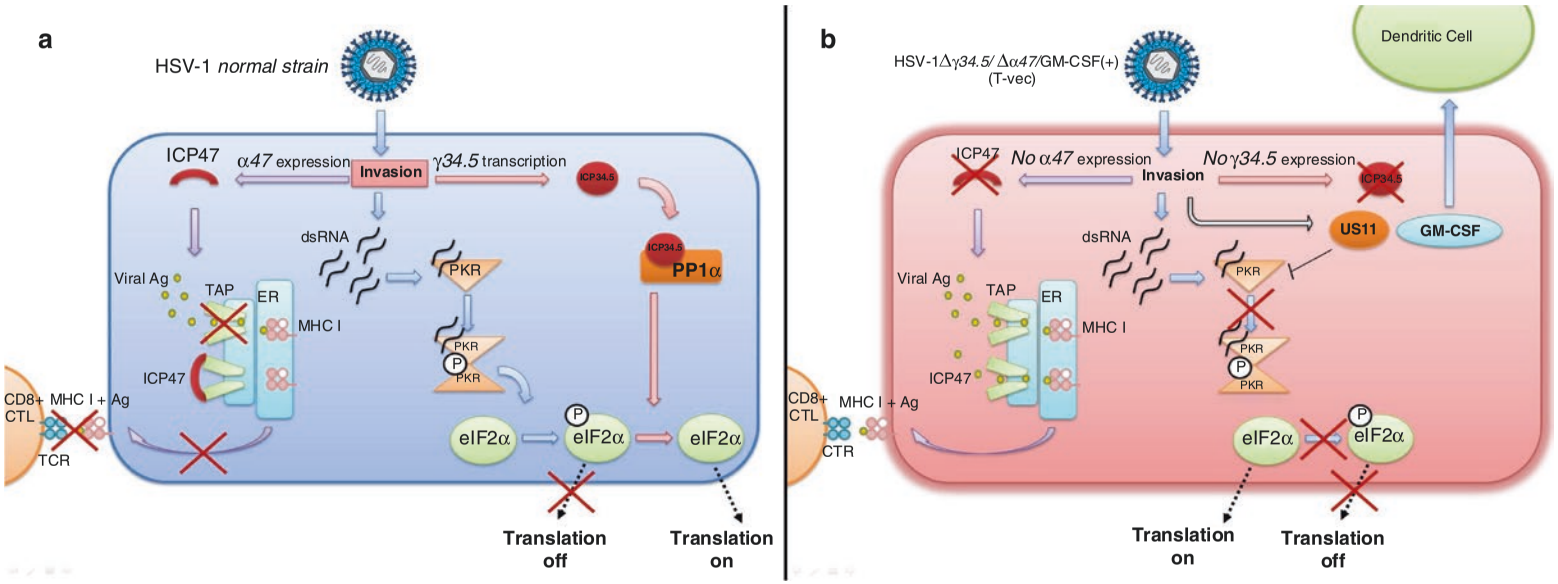

Fig. 27.3 Talimogene laherparepvec (T-VEC) tumor selectivity. (**a**) Following the infection of a normal cell with a wild (normal) HSV-1 virus, a translation of viral proteins starts. Viral dsRNA binds host cell's PKR, being a strong stimulus for its activation. PKR undergoes homodimerization and autophosphorylation. Activated in this way, PKR phosphorylates eIF2α, which causes global protein translation off (protein shutoff response for viral invasion). HSV-1 protein ICP 34.5, a product of *γ34.5* gene, binds PP1α, which redirects its activity in the way of dephosphorylation of eIF2α, accordingly blocking host cell's shutoff response. A product of viral *α47* gene ICP 47 blocks TAP that prevent viral antigen translocation into the Golgi and consequent binding to MHC class I. This inhibits surface exposure of MHC I/Ag complexes and hides the infected cell from CD8+ CTL response. Deletion of these viral genes leads to full-fledged response on viral infection in normal cells. (**b**) Infection of a cancer cell with Δγ34.5/Δα47/GM-CSF (+) modified T-VEC proceeds to the following scenario. PKR in cancer cells is basically attenuated, so it cannot provide eIF2α phosphorylation and protein shutoff. ICP 34.5 does not express in T-VEC (due to *γ34.5* deletion), but it is not necessary in cancer cells as the shutoff response is already blocked. Enhanced expression of the viral *US11* gene (a gene neighboring to *γ34.5*) causes additional inhibition of PKR. The absence of ICP 47 leads to a normal presentation of MHC I/Ag complexes on the cell surface, causing virus-specific CTL-mediated oncolysis in addition to viral oncolysis (ICD). Expression of inserted GM-CSF provides enhanced DCs recruitment to the place of infection and their maturation [113–115, 117–119]. *HSV-1* herpes simplex virus-1, *ICP 47* infected cell protein 47, *ICP 34.5* infected cell protein 34.5, *PKR* protein kinase R, *eIF2α* eukaryotic translation initiation factor 2, *TAP* transporter associated with antigen presentation, *MHC I* major histocompatibility complex class I, *PP1α* protein phosphatase 1α, *CTL* cytotoxic T lymphocyte, *TCR* T-cell receptor, *GM-CSF* granulocyte-macrophage colony-stimulating factor

should be done with a titer of 10^6 pfu/ml solution; the drug is first injected into the largest available tumor and then into others in order of decreasing size until a full one-time dose of 4 ml is applied. The second dose is given after 3 weeks, using a concentration of 10^8 pfu/ml; injections are started with new tumors that have appeared since the previous visit and then the other tumor, starting from the largest, till the full single 4 ml dose is reached. Subsequent visits are conducted at 2-week intervals, with the same regime of injection of the virus. For superficial tumors, the needle is inserted into the central part of the tumor, and the dose is injected into all portions of the tumor, changing the direction of the needle but not removing it, if possible. Each needle removal, as well as injections into different foci, must be accompanied by a needle change. For deeply located formations when it is impossible to insert a needle under visual or palpatory control, ultrasound guidance is recommended. The needle should be removed slowly, during up to 15–30 s, in order to avoid leakage of the drug through the injection site [106, 111, 112].

In the Phase III clinical trial, OPTiM T-VEC showed its efficiency compared with the intratumoral administration of GM-CSF. Durable response rates (which means continuous response of ≥6 months beginning within the first 12 months of therapy), complete responses, and overall survival for patients with IIIB-IVM1a stage melanoma were significantly higher in an arm of talimogene laherparepvec than in GM-CSF. The average overall survival totaled 41.1 months in the T-VEC arm and 21.5 in the GM-CSF one (HR (95% CI) 0.57 (0.40–0.80)). Importantly, not only tumors that had undergone injections responded to the treatment, but also distant tumors did. A total of 64% of injected lesions, 34% of uninjected non-visceral lesions, and 15% of uninjected visceral lesions decreased in size by ≥50% [21]. It means that the theoretical model of the mechanism of action of the virus is confirmed by its practical application.

Adverse effects (AEs) of talimogene laherparepvec are comparatively rare, and it is overall safe for clinical use. Among the most common AEs, pyrexia, chills, flu-like symptoms, general weakness and fatigue, and reactions at the injection site have been noted. Among serious AEs, cellulitis of the injection site with about 2% frequency has been noted. Immune-related AEs such as vasculitis, pneumonitis, and vitiligo have also been noted during talimogene laherparepvec treatment, all being nonserious and occurring in ≤7% of patients [21, 111]. Generalization of infection in the form of herpetic infection is extremely rare and is presented by single cases, and moreover, the study of the genome of the virus-caused generalized infection in those patients revealed it was a wild, but not a genetically modified strain [43].

Although talimogene laherparepvec is generally safe, it is recommended to take certain precautions to prevent the transmission of the virus to a healthy person in close contact. Among these measures, during the whole treatment and 30 days after the last dose, avoid any contact with injection sites and body fluids (use of a condom during sexual intercourse, avoid kissing in the presence of wounds on the oral mucosa in any partner, and use individual dishes and personal care items); for 8 days after each injection, wear water- and airproof dressings at the injection sites, which when utilized should be packed in plastic bags. At the same time, during the treatment, there are no restrictions for patients to visit public places, restaurants, baths, etc. [43].

Contraindications to the use of talimogene laherparepvec are the presence of clinical or laboratory signs of herpetic infection in the patient, current use of antiviral drugs (for example, acyclovir), and severe immunodeficiency (due to HIV, leukemia, lymphoma, immunosuppressive therapy). Patients taking low doses of corticosteroids (up to 10 mg in the equivalent of prednisolone) may be considered as candidates for therapy. The use of the virus in pregnant women and children is not recommended, since this group has not been investigated in clinical trials (although animal studies showed no adverse effect on the fetus) [43].

27.4.1.2 Oncolytic Adenoviruses

As oncolytic agents, serotype 5 adenoviruses are most commonly used. The best-known representatives of oncolytic adenoviruses are H101, which is approved for clinical use in China; ONYX-015, the effectiveness of which is limited; ICOVIR-5; CV706; CG0070; and OBP-301, which are now undergoing clinical trials [120].

The genetic modification of adenoviruses, aimed to increase tumor selectivity, consists in modifying the way of virus penetration into the cell and the process of its replication following the invasion. Adenovirus serotype 5 invasion into the cell occurs in two phases: binding of fiber protein of the virus to the coxsackievirus and adenovirus receptor (CAR) of the target cell [121, 122] and then penetration of the virus mediated by an interaction of arginine-glycine-aspartic acid (RGD) sequence of the penton base and αv integrins on the cell surface [123]. Genetic modification ordering to reduce adenovirus tropism to normal cells (detargeting) consists of deletion in RGD sequence (penton base) gene and induction of the mutation in the AB-loop of the fiber knob [124]. Increased tropism of the virus to tumor cells is achieved by modifying the viral capsid proteins—an insertion of tumor-specific ligands into C-terminus and HI-loop of fiber proteins, L1 loop of the hexon, RGD loop of the penton base, and minor capsid protein IX, which would bind to certain receptors that are present only or predominantly on the surface of the cancer cell [125–128]. The best modification is considered to be those consisting of the insertion of RGD-4C into the fiber knob of adenovirus [129, 130].

A possibility of not only systemic but also local administration of adenovirus is limited by its sequestration during passage through the liver, which is also associated with significant hepatotoxicity. Invasion of the liver cells occurs in a different, CAR-independent way, and therefore, the above-described method of detargeting is not sufficient to minimize the viral tropism to the liver cells [131]. Hepatocytes and Kupffer cells capture viruses by binding their HSPG and low-density lipoprotein receptors to the fiber knob domain but indirectly by the mediation of coagulation factor X and complement component C4-binding protein. Coagulation factor X binds to hypervariable regions (HVRs) of the adenovirus hexon [132, 133]. The genetic modification that prevents this is an induction of a mutation in the coagulation factor X-binding site of the HVR or replacement of the HVR gene with a homologous gene from another adenovirus serotype that does not undergo such sequestration in the liver [120].

Two main methods have been developed in order to limit the replication and cytolytic properties of adenovirus on tumor cells. The first method (or type 1 viruses) is to induce a mutation in the *E1* region. *E1B55K* gene normally functions as an inhibitor of p53 and, consequently, apoptosis of the infected cell. H101 and ONYX-015 viruses carry deletion in this gene, so they can effectively infect and replicate only in tumor cells that lost p53 during progression. *E1A* gene serves to block the Rb-binding domain in Rb/E2F complex of the host cell which results in the release of E2F. The latter in its free state is a transcription factor and activates expression of proteins of DNA synthesis machinery (e.g., DNA polymerase, thymidine kinase, dihydrofolate reductase), which allows the replication of the virus DNA. A mutation of *E1A* gene (*E1AΔ24*) limits replication of the virus only to those cells in which Rb is absent (e.g., malignant glioma or retinoblastoma cells). But this comes with a problem of toxicity: the virus contains an endogenous promoter of *E1A* gene, and therefore, enhanced expression of the defective *E1AΔ24* gene occurs ubiquitously, which becomes toxic (primarily hepato- and hematotoxicity) and creates an obstacle to systemic administration of the virus. To correct this effect, an insertion of the E2F-1 promoter near *E1AΔ24* gene site was performed. This promoter is activated by the free E2F dimer and is blocked by Rb/E2F complex (which is present in normal cells). Activation of the promoter in tumor cells enhances expression of *E1AΔ24*, and its block in normal cells inhibits this expression, which reduces the systemic toxic effects of the virus [120, 134]. The described

modification is present in the last generations of ICOVIR [12, 50].

The second method (type 2 viruses) is that a promoter is inserted into a genome of the virus, which is activated by a specific protein of the tumor cell, which limits the virus replication by a tumor or a specific tissue. This promoter regulates expression of E1A. For example, CV706 virus carries a promoter which is activated by the prostate-specific antigen and therefore multiplies primarily in prostate cancer cells. OBP-301 virus contains a promoter that responds to telomerase reverse transcriptase and, accordingly, multiplies in cells with a high amount of this enzyme [50, 120, 135].

27.4.1.3 H101

H101 virus (Oncorine) has been developed in China and approved by the Chinese State Food and Drug Administration for use as a chemotherapy-combined treatment for advanced stages of head and neck tumors. In the Phase III clinical trial that was conducted in 2000–2004, the virus in combination with chemotherapy showed a 79% positive response rate, compared with 40% for chemotherapy alone [19]. H101 carries a deletion of *E1B55K* (see above) and deletion of the *E3* genes. The latter is responsible for a synthesis of death protein and systemic toxicity of the virus. The mechanism of cell death caused by H101 infection probably lies in ICD, but immunological features and immune response to oncolytic adenoviruses are significantly less studied than that for talimogene laherparepvec. Monotherapy with H101 proves to be not enough effective, presumably because of the difficulties in overcoming barriers formed by the microenvironment of solid tumors by the virus [136, 137]. Therefore, currently, the possibilities of different types of combined therapy are being explored: e.g., a combination of transarterial chemoembolization with simultaneous intraarterial administration of H101 in patients with hepatocellular carcinoma showed 40% 3-year survival rate, while 22% in chemoembolization alone [138]. Histone deacetylase inhibitors in vivo have shown an ability to enhance CAR expression (see above) on the surface of tumor cells (e.g., esophageal squamous cell carcinoma) and, consequently, to increase the H101 infecting activity [137].

Besides H101, H102 and H103 viruses have been developed. H102 carries an alpha-fetoprotein-activated promoter and is therefore able to selectively replicate in hepatocellular carcinoma cells [134]. H103 carries a heat shock protein (HSP) 70 gene, which is a DAMP and enhances immunogenicity of tumor cytolysis. In 2009, the Phase I of H103 clinical trial ended. The results showed an objective response achieved in 11% of patients, and 48% had at least stabilization of the disease [139].

27.4.1.4 The Immune Response to Adenoviruses

The immune response in the context of oncovirotherapy usually consists of two aspects: elimination of the virus due to an activation of antiviral immunity and antitumor response, enhanced by the influence of the virus on the tumor and its microenvironment (i.e., ICD).

Studies with tumor-bearing animals infected with oncolytic adenovirus (VRX-007) have shown that in immunocompetent individuals (both those that were previously immunized with adenovirus and naive), neutralizing antibodies are formed by day 7 after virus administration and at the same time are detected in the tumor tissue; tumor growth stops for 2–3 weeks but then continues, and repeated injections of the virus no longer affect it [140].

On the other hand, the presence of anti-adenoviral immunity plays a role in preventing the dissemination of the virus to normal tissues and provides a certain safety for virotherapy.

Insertion of genes of pro-inflammatory proteins into the genome of adenoviruses in order to strengthen the immunogenicity of infection and cell death is investigated: the abovementioned H103 with an inserted HSP70; proteins GM-CSF, Fas ligand, and IL-27, enhancing maturation and the function of antigen-presenting cells [141]; IL-12, activating T cells [142]; and IFN-α, IFN-β, and IFN-γ, which have a direct antitumor effect

and stimulate the immune response [143–145]. A number of viruses expressing direct-acting antitumor molecules such as TNFα, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) have been developed [146–148]. Most of these options were investigated only in preclinical studies, because due to the success of talimogene laherparepvec, interest in adenoviruses somewhat subsided, but the rapid development of the industry will lead to the need to find the most effective and safe recombinants of viruses, and adenoviruses are the most suitable candidate due to their well-studied genome and great availability for modifications.

27.4.2 Naturally Occurring Oncolytic Viruses

Naturally occuring oncolytic viruses are strains of viruses that are normally not pathogenic to humans, and therefore have minor and easily predicted systemic toxic properties, but exhibit antitumor activity against many neoplasms. They basically do not require any modifications aimed to promote tumor selectivity of the virus, because they do not infect normal human cells, but are able to penetrate and multiply in tumor cells that have lost their mechanisms of antiviral protection. These viruses include Newcastle disease virus, reovirus, parvovirus, and coxsackievirus. A number of natural OVs have modifications that are not associated with an enhancement of their selectivity but with a change in immunogenic properties, for example, VSV with the insertion of IFN-β, tumor antigen libraries and others (Table 27.3).

27.4.2.1 Newcastle Disease Virus

Newcastle disease virus (NDV) is an RNA virus belonging to the *Paramyxoviridae* family. It is basically pathogenic to birds but occasionally can cause an infection in humans in form of conjunctivitis or a mild flu-like syndrome.

NDV is divided into lentogenic (avirulent), mesogenic (medium-virulent), and velogenic (highly virulent) strains depending on the degree of pathogenicity to birds. Such differences are associated with the peculiarities of activation of F (fusion) protein, which provides penetration into the host cell and basically is inactive in its F0 form [149]. F0 is activated by selective cleavage, which in lentogenic NDV is performed only by trypsin-like proteases of the respiratory and digestive tract and, in mesogenic and velogenic by various proteases, for example furin, that is present ubiquitously [123, 149, 150]. This division is important to be understood if talking about viral immunotherapy, since the pathogenicity of NDV is in line with its oncolytic properties. Mesogenic and velogenic NDV can multicyclicly replicate in the human tumor tissue, and they are defined as lytic strains. Lentogenic NDV is prone to be attenuated after the first cycle of replication, and it is a non-lytic strain [151]. Non-lytic strain is interesting mainly in the meaning of being an object for gene-engineering—the artificial modification of the F protein, for example an insertion of the polybasic cleavage site, increases fusogenic and oncolytic properties of the virus and increases the clinical effect in vivo [149, 152–154].

NDV, being an RNA virus, replicate basing on formation of a double-stranded RNA. This structure is a strong inducer of cellular defense mechanisms, consisting in the synthesis of type I (α and β subtypes) and type III IFN, which, by enhancing expression of IFN stimulating genes of innate immunity cells, exhibits antiviral activity in healthy tissues, limiting the spread of the virus. Increased secretion of IFN-α/β at the site of NDV infection has been shown in a number of studies in vitro and in vivo, and generally there is no doubt concerning it. In the tumor tissue, production of IFN and response to it are often disrupted: a weak response of the human fibrosarcoma cell line to IFN-β was shown, due to reduced phosphorylation of IFN-pathway proteins STAT1 and STAT2 and weak activation of IFN-regulated genes [155] and disrupted pathways of apoptosis and antiviral protection (defects of RIG-I, IRF-3, IRF-7), as well as the role of immunosuppressive microenvironment [156, 157]. Reduced production of IFN does not

allow an adequate antiviral response to develop within the tumor at the first stages, allowing the virus to replicate and further infect tumor cells. The defect of apoptosis of infected cells (for example, an excess of anti-apoptotic activity of Bcl-xL [158] and Livin protein [159]) does not allow the virus to be elicited or to limit its replication in the tumor.

Another mechanism that determines the relative insensitivity of normal human cells to NDV is the blockade of viral RNA replication on the basis of a newly produced anti-genome nucleocapsid, which occurs after penetration of the virus into the cell and transcription of its genes. In tumor cells, this stage almost always occurs without the resistance of the host cell.

Cell lines expressing H-Ras and N-ras oncogenes demonstrate greater sensitivity to NDV than their analogs without these oncogenes. Human fibroblasts after N-ras-transfection acquire tumorigenicity and become 1000 times more sensitive to NDV [160]. HaCaT cells are insensitive to NDV before their transformation with H-Ras [161]. All these natural differences form the basis of selectivity of the virus, and NDV replicates 10,000 times faster in human cancer cells than in normal human cells [162].

NDV seems to be an attractive oncolytic agent because its entry into the cell occurs due to binding to sialic acid residues on the membrane that are present on cells of almost all human cancers, which provides a wide range for the use of the virus [163]. In addition, the human population potentially lacks immunity to NDV, so it does not limit its effectiveness (as for adenoviruses). NDV is not inclined to spontaneous recombination and integration into the host's genome. Toxic properties of the virus even in the case of systematic administration are minimal, since it is not basically pathogenic to humans [149].

The mechanism of tumor cell death infected with NDV is similar to ICD induced by other OVs. Among the PAMPs that the NDV-infected cell releases are 5′-triphosphate viral RNA [164], HN protein [165, 166], and double-stranded RNA [161]. These substances react with the pattern recognition receptors (PRR) of innate immunity cells and an early phase of type I IFN response starts as previously described [167, 168].

Among the specificities of ICD caused by NDV is an exposure of hemagglutinin-neuraminidase (HN) and F viral protein to the cell surface. HN protein reacts with Nkp46 PRR of NK cells, which stimulates cytotoxic antitumor properties [165]. HN also activates monocytes and stimulates the release of TNF-related apoptosis-inducing ligand (TRAIL) [169]. HN on the surface of an infected cell enhances an adhesive ability for the better interaction with lymphocytes and is involved in stimulating CD4+ and CD8+ T lymphocytes [170, 171].

In vitro infection of normal and tumor cell lines demonstrated that on the third day after the infection the viability of normal cells ranged 69–95%, while the viability of different malignant cells lines did not exceed 44% [172].

Local intratumoral administration of NDV leads to the tumor infiltration by NK cells and CD8+ and CD4+ FoxP3 lymphocytes, but not by immunosuppressive Treg, and consequently to a significant increase in immunostimulating/immunosuppressive cells ratio. Particles of the virus can be found in a tumor undergone the direct administration of the virus for 96 h following an injection (and possibly further—depending on the method of detection). In a distant metastatic tumor, no virus particles can be detected, but the same lymphocytic infiltration is observed [173]. This indicates the formation of an antitumoral immune response, which confirms the theory of OV-induced ICD.

In preclinical studies, NDV showed its oncolytic effect on many solid tumors, including melanoma, colorectal carcinoma, hepatocellular carcinoma, pancreatic adenocarcinoma, pleural mesothelioma, and glioblastoma. In clinical trials, the virus was used both as a therapeutic agent and for the production of antitumoral vaccines in the form of tumor viral oncolysates (see below): for the treatment of glioblastoma multiforme [174, 175], colorectal carcinoma [176], pancreatic adenocarcinoma [177], breast adenocarcinoma [178], renal carcinoma [179], and others. A 10-year follow-up of patients with stage II malignant mela-

noma who received NDV as adjuvant postoperative therapy showed a 60% survival rate (while observations of such patients receiving standard treatment showed a survival rate of up to 33%) [180].

In 1993, Csatary tested MTH-68/HVVV strain in a placebo-controlled Phase II trial for the treatment of various advanced chemorefractory cancers, where a completely new route of administration of the virus was proposed: inhalations of viral particles at a dose of 4000 U/day, twice per week for 6 months, aimed on targeting pulmonary metastases. The effect was significant—a 2-year survival rate was 21% in the NDV arm and 0% in the control. The treatment was well tolerated, with no significant AEs [181].

In 2002, in Phase I clinical trial of the PV701 strain involving 79 patients with advanced chemoresistant tumors, a spectrum of the adverse effects of the virus was investigated. The most common AE was an influenza-like syndrome, occurring after the first dose but decreasing with subsequent administrations. Dose-limiting effects were dyspnea, diarrhea, and dehydration. Desensitization with minimal initial doses was proposed to address AEs, which increased the maximum tolerated dose tenfold [182, 183]. It is not completely clear how this desensitization affects the effectiveness of therapy, but its effect on toxicity was well-defined. The result of the trial demonstrated a complete response observed in one patient, a partial response in one patient, and minor responses in seven patients. Fourteen patients were progression-free for 4 months to over 30 months.

Non-lytic NDV strain was studied in 14 patients with glioblastoma. One patient had a complete response; all others had progressive disease [175].

To date, the evidence base is not sufficient for a final conclusion on the effectiveness of NDV as an immunotherapeutic drug. The available data clearly indicate that the virus has a potential and requires further research and more extensive clinical trials.

NDV is also studied as an antitumor vaccine in the form of oncolysates or whole-cell vaccines. These vaccines generally have proven to be safe and effective in uncontrolled clinical trials. A clear conclusion about the degree of clinical benefit is not yet available, and it is necessary to conduct controlled trials to make the final conclusion [149].

An interesting approach is proposed by Schirrmacher: a modification of autologous tumor cells taken during resection of the primary focus in a metastatic disease by NDV, to enhance the immunogenic properties and to use these tumor cells as a vaccine. In 2009, the results of the Phase II/III clinical trial of the autologous tumor vaccine modified with non-lytic Newcastle disease virus (ATV-NDV) for postoperative treatment of colorectal cancer with liver metastases were published. In patients with colon cancer, the 9- to 10-year survival rate differed significantly: 21.4% in the control group and 69.2% in the ATV-NDV group. It is interesting that no significant differences were noted in a rectal cancer subgroup [184, 185].

Later, Schirrmacher and others in the Immunological and Oncological Center in Cologne, Germany, modified the ATV-NDV vaccine by adding human DCs. The new vaccine was named viral oncolysate-pulsed DCs (VOL-DCs). This combination increases the efficiency of antigen presentation by cells, as the density of contact of the DCs with tumor antigens increases since the process begins in vitro even before administration to a patient. Exogenous antigen-presenting DCs stimulate maturation of tumor-specific T cells in the patient's body [168]. A proposed complex administration regimen is as follows: the patient receives injection of NDV and hyperthermia up to 38.5–40.5 °C as a pretreatment. After that, the VOL-DC vaccine is administrated [186]. Hyperthermia is a favorable background for enhancing immune responses. NDV triggers oncolysis and ICD of tumor cells that prepare the immune system by stimulation of the formation of a pool of VOL-specific lymphocytes, mostly CD4+ helpers. With the administration of the VOL-DC vaccine against a background of such an activated immunological status, the release of chemokines CCL3 is enhanced at the site of injection. This stimulates active migration of DCs to the regional lymph nodes, and CD4+ helpers increase efficiency of

lymphocyte stimulation by DCs during the antigen presentation, improving the effect of vaccination [187]. VOL-DCs in 2015 received an approval for individual use in cancer patients as an advanced therapeutic medicinal product [168].

Genetically modified strains of NDV are developed and show a good effect. Among the modifications, as mentioned above, are increased fusogenicity by changing the F protein; insertion of NS1 protein (from influenza A virus) that alters immune response by inhibiting the type I IFN response and apoptosis [188]; arming with pro-apoptotic rFMW/AP proteins from chicken infectious anemia virus [189]; cytokines IFNγ, GM-CSF, IL-2, and TNFα [152]; immunoglobulins against ED-B fibronectin [190]; and insertion of tumor-associated antigens genes [191].

27.4.2.2 Reovirus

Reovirus (respiratory orphan enteric virus, genus *Orthoreovirus*, family *Reoviridae*) is a non-enveloped RNA virus that is ubiquitous, affecting the upper respiratory tract and the gastrointestinal tract with minimal clinical manifestations [192]. There are no known serious human diseases associated with reovirus [193]. The asymptomatic course of infection and the ubiquitous prevalence of the virus cause a high frequency of seropositivity to reovirus among the human population [194].

There are three serotypes of mammalian reovirus. Their prototypes were isolated in children with different manifestations of infection or without them. Type 3 Dearing (T3D), isolated from a child with diarrhea, is most widely studied for its oncolytic properties today, although other serotypes also show these properties [195].

The selectivity of T3D reovirus on normal and transformed cells has been studied back in the 1980s, and it was noted that normal cell lines are resistant to infection of the virus, whereas the virus causes cell lysis in transformed cells and the HeLa cell line [196].

Selective oncospecificity of reovirus is associated with the surface receptor of epidermal growth factor (EGFR) and its signaling pathway Ras. The Ras pathway is a proto-oncogene; it is associated with the control of the cell cycle, proliferation, differentiation, and apoptosis of the cell. During transmission of the signal from the EGF membrane receptor, Ras changes from a guanosine diphosphate (GDP)-bound form into an active guanosine triphosphate (GTP)-bound form, triggering the subsequent pathway elements. Mutation of the Ras gene can lead to a stabilization of the active GTP-bound Ras, and the pathway remains active regardless of the presence of EGF stimuli [197], and the cell acquires an ability of uncontrolled proliferation. Such a transformation can occur in another protein of this signaling path—RAF, which leads to the same effect. Hyperactivity of the Ras pathway is often found in cancer cells: up to 30% of all tumors [198], up to 90% of pancreatic cancer, 50% of colorectal, and 40% of lung cancer [199]. Normally, the antiviral protective mechanism of the cell reacts to invasion of reovirus as follows: double-stranded virus RNA (dsRNA) activates protein kinase R (PKR) by binding to the N-terminal domain. Activated PKR inhibits translation of viral proteins, thereby realizing the viral replication blockade (as in T-VEC antiviral response; see Fig. 27.3). Hypothetically, the elements of the Ras pathway system (probably its Ras/RalGEF/p38 part) can inhibit PKR activity [198, 200, 201], and therefore tumor cells with a highly active Ras system are very susceptible to reovirus infection.

However, there is evidence that the mechanism of oncospecificity of the virus is associated with other features of cell biology. In vitro on the squamous cell carcinoma of the head and neck cell lines it was shown that sensitivity of the cells to reovirus did not correlate with a degree of activity of their Ras system, and stimulation or inhibition of EGFR and blockade of MAPK, PI3-K, and p38MAPK elements of the Ras pathway did not affect the cytotoxicity of the virus and the rate of growth of the infected tumor. Inhibition of phosphorylation of PKR (i.e., its artificial inactivation) also did not significantly increase sensitivity of primary resistant cells to reovirus. These data cannot be accepted as the only truth, but it should be remembered that based on this

information not only patients with biomarkers of increased activity of EGFR/Ras/MAPK pathway should be selected for reovirotherapy. Similarly, the criteria for selecting patients for clinical trials should not be a positive EGFR/Ras/MAPK status only [202].

One of the factors of cell's susceptibility to reovirus is the number of specific receptors on the cell surface—junctional adhesion molecule-1 (JAM-1) [203], but there are data that contradict this fact too [202]. The number of co-receptor sialic acid residues on cell membranes may also play role [193].

The mechanism of cell death under the influence of reovirus is thought to be caspase-dependent apoptosis that occurs with a participation of TRAIL and caspase-8 pathways, which was mainly observed for melanoma cells and for several other tumors [204, 205]. Additionally, necroptosis was shown in head and neck squamous cell carcinoma cell lines [206]. An immune response to tumor invasion by the virus and generally cell death occurs according to the common mechanism of ICD: recruitment of DCs, activation of NK and CD8+ T lymphocytes, and formation of antitumor immunity [207].

Due to the high degree of anti-reoviral immunity in the human population and rapid appearance of neutralizing antibodies even at the first contact of a nonimmune individual with the virus, the immune response is a significant limiting factor for systemic intravenous administration of reovirus [193]. The use of reovirus in animal models in combination with immunosuppressive cytotoxic agents such as cyclosporin A, cisplatin, and cyclophosphamide showed a better effect compared to monotherapy, partly because of reduced inactivation of the virus by neutralizing antibodies [208, 209]. Cyclophosphamide, in addition, selectively inhibited Treg activity and antibody formation in response to reovirus and at the same time somewhat modulated the antitumor adaptive response by increasing activity of the T cells. It was also shown that the combination of cyclophosphamide and reovirus with IL-2 can further increase efficiency, probably by enhancing the NK cell response to the tumor [210].

On the other hand, in the experiment with murine tumor models, injection of reovirus to naive mice had minimal effect, while mice immunized against reovirus 2 weeks prior to treatment and having specific antibodies showed a much better tumor response and survival [211]. It supports the significant role of immune response in reoviral oncolysis, and therefore, it is necessary to find a balance between the maximum possible immunosuppression and the minimum necessary immunocompetence for the effective use of OVs in general.

In Phase I clinical trials, a good tolerability and an absence of dose-limiting adverse reactions to reovirus were shown in both intratumoral (in patients with subcutaneous tumors, prostate cancer, and malignant glioma) and intravenous administration (various solid tumors, metastatic colorectal cancer, multiple myeloma), including in combination with chemotherapeutic agents [212–216]. The maximum administrated dose was set on the level of 3×10^{10} TCID(50) (tissue culture infectious dose 50) per injection for 5 days per week, repeated every 4 weeks. However, the maximum tolerated dose wasn't achieved. Among AEs noticed during Reolysin therapy are grade 1 and 2 flu-like symptoms—fever, fatigue, nausea and vomiting, and headache, which didn't depend on dose and cycle—and among grade 3 toxicities—flu-like symptoms and uncomplicated lympho- and neutropenia [217]. Combination of reovirus with chemotherapeutic agents like docetaxel also showed low toxicity: the frequency of grade 3 and 4 toxicities, like neutropenia, was relevant to those for docetaxel monotherapy [212].

A combination of reovirus with carboplatin and paclitaxel in 19 patients with refractory to preceded chemotherapy with platinum-containing agents in advanced head and neck malignancies (mostly squamous cell tumors) has shown an achievement of a complete or partial response in 42% and stabilization in 32%. The median overall survival was 8.9 months that is significantly longer than in other second-line regimens [218]. In a similar study with 13 patients, a partial response was achieved in 31% and at least stabilization during 12 weeks in 46% [219].

The same combination was studied in patients with metastatic non-small cell lung cancer with a mutation in the Ras system. The results are median progression-free survival of 4 months, overall survival of 13.1 month (95% CI: 9.2–21.6), and 1-year survival rate of 57% [220]. Phase II clinical trials were conducted for metastatic small-cell lung cancer; melanoma; ovary, peritoneum, and fallopian tube malignancies; and unresectable pancreatic cancer [221].

Phase III clinical trial of a combination of IV reovirus with carboplatin and paclitaxel in comparison with carboplatin and paclitaxel alone in patients with advanced or metastatic head and neck tumors involving 167 patients is being conducted. Of these, for 118 patients with locoregionally advanced tumors (with and without metastases), results were obtained: median progression-free survival was 94 days (13.4 weeks, $n = 62$) in the reovirus with chemotherapy arm vs. 50 days (7.1 weeks, $n = 56$) in the chemotherapy alone arm. In the 88 patients discontinued from the study so far the median overall survival was 150 days (21.4 weeks, $n = 50$) in the test arm vs. 115 days (16.4 weeks, $n = 38$) in the control arm. Results of a group of metastatic disease have not yet been published [221, 222].

27.5 Combined Immunotherapy

OVs show their effectiveness in preclinical and clinical studies. However, knowing the immunological basis of tumor biology and the mechanism of OVs action, it should be assumed that the combination of viruses with other immunotherapeutic agents will have a better effect. This is especially relevant for targeting of distant metastatic tumors that are not directly exposed to OV, and accordingly they are not subjected to direct oncolysis and additional stimulation of the immune response with PAMPs, but only immunomediated reactions. In vivo in bilateral flank experiment with implanted human B16 melanoma, Zamarin and co-authors achieved 50% of complete regressions of the primary tumor followed infection with NDV, while the distant tumor that wasn't directly exposed to the virus regressed completely in 20%. In total, long-term survival did not exceed 10%. In the combination of IV NDV with anti-CTLA-4 antibodies (Ipilimumab), the primary tumor was rejected in 90% and the distant tumor in 80% of observations. The long-term survival rate exceeded 70% (in the anti-CTLA-4 group only—no more than 35%) [173].

A combination of vaccinia virus with anti-CTLA-4 antibodies in an experiment with murine models of subcutaneous mouse renal adenocarcinoma and murine colon adenocarcinoma showed an interesting feature of constructing of combined therapy regimens: when the virus and antibodies were administered simultaneously (on day 0), survival and tumor growth rate did not differ from those with vaccinia virus monotherapy, and account for about 10% survival rate by day 30 and tenfold tumor increase on days 20–25. However, administration of antibodies on day 4 from the onset of virotherapy increases survival to about 75% by days 30–35 and reduces the rate of tumor growth—a four- to fivefold increase on day 25. This is attributed to the fact that stimulation of the immunity with anti-CTLA-4 antibodies during the primary replication phase of the virus enhances antiviral immunity (as an increasing amount of CTLs recognizing vaccinia epitopes has been detected in the first case) and does not allow the virus to fully carry out its effect [223].

Reovirus showed increased efficacy when was used in combination with GM-CSF and anti-VEGF. In an experiment with murine tumor models (B16 melanoma), preconditioning with GM-CSF prior to the reovirus injection increased the titer of viral particles in the tumor 100–1000 times through enhancing its delivery to the tumor. An explanation for this is an ability of GM-CSF to mobilize monocyte/macrophages and stimulate infiltration of the tumor with them, which can act as carriers of viral particles. Survival rate of mice preconditioned with GM-CSF was significantly higher than those which undergone administration of either reovirus or GM-CSF alone. It should also be noted that mice that had

antibodies to reovirus showed greater survival and the survival of naive individuals did not significantly differ from control groups [211]. Pretherapy of VEGF-secreting tumors carrying mice with anti-VEGF drugs followed by reovirus administration after 24 h twofold slows murine B16 melanoma tumor growth in the next 30 h compared to anti-VEGF only and to reovirus injected 48 h after anti-VEGF administration. Sunitinib and avastatin, in combination with reovirus, showed a high survival rate of mice, whereas in monotherapy each drug showed a low survival. However, in the same study on the VEGF-non-secreting tumor model, conditioning with the proangiogenic agent $VEGF_{165}$ increased the effect of reovirus and survival twofold. This fact is associated with increased delivery of the virus to a tumor due to the developed tumor vascular system under the influence of $VEGF_{165}$. The authors suggest two scenarios for possible applications of this data: for tumors producing VEGF, a combination of OV with an antiangiogenic agent, and for VEGF-non-secreting tumors—OV with proangiogenic $VEGF_{165}$ [224].

A combination of GM-CSF/reovirus and anti-PD-1 also significantly increases survival compared to GM-CSF/reovirus alone and anti-PD-1 alone in vivo. The same result was observed for a combination of VSV-ASMEL (altered self-melanoma epitope library, engineered VSV) and anti-PD-1. The best effect was shown for a combination of all components: GM-CSF/reovirus/VSV-ASMEL + anti-PD-1. This approach simultaneously covers several aspects of the immune response: GM-CSF/reovirus causes primary oncolysis and release of tumor antigens and stimulates Th1 cells, VSV-ASMEL again provides a spectrum of tumor antigen (ASMEL genes products) and stimulates Th17, and finally anti-PD-1 enhances already activated Th1 and Th17 pools [98].

A combination of T-VEC with ipilimumab in the Phase Ib clinical trial for the treatment of IIIb–IV stage melanoma (with T-VEC regimen as described above, and ipilimumab 3 mg/kg IV every 3 weeks up to totally four infusions starting at the sixth week of virotherapy) showed a satisfactory safety profile with grade 3/4 treatment-related AEs rate of 26.3%, which were mostly associated with ipilimumab. Eighteen-month progression-free survival was 50%, and 18-month overall survival was 67%, which is a better result than when using either T-VEC or ipilimumab as monotherapy [225]. In the Phase II trial of this combination compared with ipilimumab monotherapy, the grade 3/4 AEs rate was 45% and 35% for combination and ipilimumab alone, respectively. Objective response (complete response or partial response, according to the modified immune-related response criteria) was achieved in 39% of patients in the combined therapy arm and 18% in ipilimumab only arm [226].

27.6 Conclusion

Oncolytic virotherapy is a novel stage of the development of cancer immunotherapy. Despite more than a hundred years history of studying various pathogenic agents as a therapy for neoplasms, only with the development of genetic engineering and understanding of the underlying immunological processes of the immunotherapy, their profound study and practical application have become possible. However, there is still a great deal of questions remaining unsolved concerning theoretical and practical aspects of virotherapy, and it cannot be stated that we are close to answering yet.

The immune system plays a central role in realization of the oncolytic potential of viruses. When the cell is infected, stress of the endoplasmic reticulum occurs, which leads to a specific type of death—an immunogenic cell death. During the immunogenic death, the cell secretes pro-inflammatory stimuli that attract innate immune response cells, i.e., NK and dendritic cells. The latter present antigens of the destroyed tumor cell and trigger an adaptive immune response that attacks both the infected tumor and distant, initially uninfected metastatic foci.

The main challenge of adaptation of viruses for their therapeutic use is to increase their selectivity toward tumor cells and to decrease it toward

normal ones. This allows to enhance their effectiveness and to reduce systemic toxicity. Some viruses demonstrate this selectivity naturally and do not require genetic modifications. Mostly these are viruses that are basically nonpathogenic or mild pathogenic for humans: Newcastle disease virus, reovirus, parvovirus, and coxsackievirus. Other viruses require profound modifications, as they normally cause disease in a human or do not show sufficient affinity toward the tumor—HSV, adenoviruses, and vaccinia.

T-VEC (talimogene laherparepvec) is the first oncolytic virus approved by the FDA in 2015 in the United States as a treatment agent for advanced melanoma and in 2016 in Europe and Australia. The drug showed its effectiveness in Phase III trial OPTiM significantly increasing overall survival in comparison with GM-CSF.

Oncolytic adenovirus H101 has been approved in China for the treatment of advanced head, neck, and esophageal tumors. The genome of adenoviruses has been studied quite deeply, and a wide range of different modifications have been proposed for the virus adaptation, even some that allows virus to be activated only in certain types of tissues.

Newcastle disease virus shows its oncolytic properties even without genetic modifications and demonstrates low toxicity even in systemic administration. To date, clinical trial data do not allow us to make a final conclusion about its effectiveness because of the limited number of studies, but the available results clearly indicate the need for further investigation. Nowadays, NDV is being considered mostly in the context of cancer vaccines in the form of viral oncolysates and their various modifications.

Reovirus is currently undergoing Phase III clinical trial as a combined chemo-virotherapy for advanced head and neck tumors. The preliminary results have been published to argue in favor of the effectiveness of the drug.

The combination of oncolytic viruses with other immunotherapeutic agents is the key to enhancing the effect of both, as these drugs potentiate the action of each other. Such combinations remain relatively safe and do not show significant increase in the side effects rates.

Despite the apparent clinical effectiveness of oncolytic viruses and certain successes in understanding the theoretical aspects of their action, much remains not fully defined and contradictory. Further research is needed both for the development of new virotherapeutic agents and for an in-depth understanding of the current ones.

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Immune Targeting of Oncogenic HPV as Therapy for Cancer

28

Peter L. Stern

Contents

28.1 Introduction

It is estimated that around 5% of all cancers may be associated with oncogenic HPV infections [1, 2]. The implementation of prophylactic vaccination programs based on virus-like particle (VLP) vaccines is showing success but will take time to impact on cancer rates and critically depends on delivery to those at risk and prior to infection [3]. This is particularly challenging for those in the developing world, and the VLP vaccines have no therapeutic activity and thus do nothing for the existing burden of disease. It is increasingly apparent that the immune system is a significant factor in the natural control of cancers [4]. This chapter will review the natural history

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of HPV-associated neoplasia and ongoing strategies utilizing immune targeting of HPV for therapy of these cancers.

28.2 The Burden of HPV-Associated Cancers

It is now clearly established that particular human papillomavirus infection (with a high-risk (hr) type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, or 59) is a critical component in the development of cancers of the cervix, penis, vulva, vagina, anus and oropharynx [5]. The HPV attributable fractions (AF) of these malignancies worldwide [2] are 100%, 51%, 88% and 78% for cervix, penile, anal and vaginal carcinomas, respectively. There is an age dependency for vulvar cancer with AFs of 48%, 28% and 15% for women aged 15–54, 55–64 and >65 years. There are large disparities in AFs between regions/countries, for example, for oropharynx tumours 51% in North America, 42% in NW Europe, 50% in E Europe, 24% in S Europe, China (23%) and India (22%). Of the annual 608,000 HPV-associated cancers, only about 7% occur in males, while 87% are cancers of the cervix (Table 28.1).

Even before the involvement of HPV in cervical cancer was known, the availability of organized cervical smear screening programs enabled the detection of dysplastic cells from the cervix and could provide for secondary prevention [6]. To be fully effective, women need to attend multiple screening visits across their sexually active lifetime, delivered to populations by a well-organized health service capable of providing high coverage and quality-assured methodology plus the downstream treatment and follow-up services. Primary prevention through prophylactic vaccination against the most oncogenic HPV types using VLP vaccines is now being implemented with encouraging success in many countries worldwide [3]. The available bivalent and quadrivalent vaccines both target the HPV 16 and 18 types, which account for about 70% of cancers (quadrivalent vaccine also contains VLPs for HPV 6/11, which cause benign genital warts). In clinical trials with high-grade cervical intraepithelial neoplasia (CIN3) as the end point, protection is virtually 100% against the vaccine type lesions [7, 8]. However, cross protection against 16/18 related HPV types as best shown by the bivalent vaccine can raise the levels of protection against CIN 3 to about 93%. A nonavalent vaccine (quadrivalent plus VLPs for HPV types 31, 33, 45, 52, 58) offers a similar level of protection so no VLP vaccine is likely to be 100% effective since they do not necessarily provide protection against all oncogenic types [9]. A key rate-limiting feature is vaccination coverage which needs to be >80% to deliver maximal population protection [10]. The general policy is to immunize adolescent girls, and it will take >20 years for approaching the full impact on cervical cancer even with very efficient national programs. It is a fact that for the foreseeable future, many populations will simply not be vaccinated (or screened) and there will be many HPV-driven cancers that will need treatment for decades to come. The effectiveness of available treatments of lower genital tract neoplasia depends on early detection when surgical options can be curative. However, while chemoradiation therapy of cervical cancer can deliver 66–79% survival at 5 years, the outlook for patients with persistent or recurrent disease is very poor [11]. An increased understanding of the natural history of HPV infection and the mechanisms which lead to either immune control and viral clearance or immune deviation and viral persistence are illuminating opportunities to better harness the host immune response to treat HPV-associated disease.

Table 28.1 Annual worldwide incidence of HPV-associated cancers (×1000)

Tissue	Total cases	Attributed to HPV	Male (HPV)	Female (HPV)
Cervix	530	530	–	530
Vulva	27	12	–	12
Anus	27	24	11	13
Vagina	13	9	–	9
Oropharynx	85	22	17.6	4.4
Penis	22	11	11	–
Total	700	608	39.6	568.4

28.3 The HPV Infection Life Cycle

The 8Kb double-stranded DNA genome of HPV consists of early genes encoding the E1, E2, E4, E5, E6 and E7 proteins plus late genes L1 and L2 encoding the capsid proteins [12]. The virus requires the cellular machinery within the normal process of epithelial renewal to complete its infectious life cycle. In the target tissue, for example, the transformation zone of the cervix, micro-abrasion exposes the basement membrane where the 55 nM virus particles bind and undergo some conformational changes that ultimately provide for uptake by basal epithelial cells [13, 14]. An initial period of genome amplification follows, with the maintenance of 50–100 copies of the viral episome in the basal cells. The process of virus production only begins once the infected basal cell begins to migrate upwards where eventually they exit the cell cycle and terminally differentiate. The early proteins E6 and E7 stimulate proliferation of the parabasal cells and thereby the replication apparatus, providing for enhanced cellular survival and time and machinery to replicate the viral genome [15]. The E6 and E7 proteins cooperate to abolish cell cycle checkpoint controls through the deregulation of two major tumour suppressor pathways, p53 and Rb, respectively. The E7 binds to Rb and promotes its degradation, and this releases the transcription factor E2F (critical for progression from G1 to S phase) that forces cells into division. This would normally trigger apoptosis, but this is prevented by the action of E6 on directing degradation of pro-apoptotic proteins like p53. The p53 pathway senses damage to the host genome and enables the cell to have time for repair or be eliminated through apoptosis. In the productive life cycle of the HPV, the possible accumulation of genetic mutations in the epithelial cells through genomic instability is negated by the requirement for terminal differentiation to complete virus production. Thus, following the amplification of the viral genome to many thousands of copies, transcription of E6 and E7 is downregulated by the viral E2 protein, and that switches the HPV life cycle to the production of the capsid proteins. This is linked to the differentiation of the epithelial cells, and the new virions are assembled and are released from the terminally differentiated uppermost cells. Importantly, the viral life cycle is entirely within the epithelium, there is no viremia or virus-induced cell death, and this stealthy process can occur without activating any local inflammatory response [3, 12, 13].

In most cases of infection, some activation of the innate immune response occurs, and antigen-presenting cells sample the antigenic environment leading to activation of the adaptive immune response. The innate immune response detects danger signals through pathogen recognition receptors (PPR) leading to processing and presentation of the tumour antigens by antigen-presenting cells (APCs) [16]. Activated APCs (CD83+, CD80/CD86+) migrate to the secondary lymphoid tissues through a CCL19 /CCL21 chemokine gradient detected by CCR7 APCs with the expression of the matrix-metalloproteinase (MMP)-9 supporting their migration through the extracellular matrix [17, 18]. In the lymphoid organs, the APCs engage with the T-cells, activating those with appropriate specificity using the two-signal system comprising processed antigen in the context of major histocompatibility complex (MHC) molecules and CD80/CD86 with the specific T-cell receptor (TCR) and CD28 molecules, respectively [19]. Thereafter, a combination of cytokines and other specific cellular interactions control the balance of T-cell differentiation including for cytotoxic T-cells. Optimally activated and weaponized T-cells have the ability to migrate to and destroy the tumour. Subsequently, homeostatic processes use inhibitory signals (immune checkpoints) between T-cells and APCs (CTLA-4/CD28 and PD-1/PD-L1) to modulate the specific effectors when no longer required, while endogenous T regulatory cells (Tregs) act to maintain self-tolerance [20–22]. In the clinical setting of normal tissue, immune checkpoints have a vital homeostatic function. However, tumours can hijack these homeostatic pathways to evade the immune system and allow uncontrolled growth [23]. Checkpoint inhibitors that can block these regulatory pathways can promote immunosurveillance and tumour clearance. Recent work has

shown the efficacy of checkpoint inhibitors in some previously treatment-refractory cancers with the licensing of anti-CTLA-4 and anti-PD-1 antibodies for the treatment of metastatic melanoma and some other cancers [24].

There is strong evidence that T-cells specific for the viral oncogenes are required to clear the virus-infected cells [25–27]. This is supported by the reactivation of HPV infection and increased incidence of HPV-associated neoplasia in immunosuppressed individuals [28–31]. In addition, specific T-cells help provide for the optimal activation of specific B-cells that produce virus-neutralizing antibodies targeting the capsid proteins. The production of these antibodies is a relative late event, and the levels produced are often insufficient to prevent further infection, and they cannot influence an established infection [32]. De facto, in patients with HPV-associated cancers, natural HPV-specific T-cell responses are insufficient to effectively control tumour outgrowth. However, pre-existing specific T-cell responses against E6 and E7 in patients with HPV-related tumours have been associated with better outcome after treatment [25]. In such cases, these effector responses must overwhelm the negative influences of the cornucopia of immunosuppressive cells and factors which can populate the tumour microenvironment including both specific and non-specific induced Tregs, M2 macrophages, myeloid-derived suppressor cells, tumour cells and associated fibroblasts, all of which interfere with specific T-cells' function [33, 34].

28.4 HPV Carcinogenesis: Immune Deviation and Persistent HPV Infection

For oncogenic HPVs, if E6 and E7 expression are unregulated, then the epithelial cells will not differentiate and will stay in cell cycle with the possibility for the accumulation of mutations in the absence the actions of the guardians of the genome. Thus, while oncogenic HPV is necessary, it is not sufficient for malignant cancer development per se. Persistence of high-risk HPV infection is the definitive risk factor for cervix cancer leading to the development of high-grade CIN (Fig. 28.1). An important molecular change underlying progression of CIN is the integration of the viral genome into that of the host [12]. The most frequently disrupted open reading frame of the virus is that of the E2 gene which is the negative regulator of the E6 and E7 transcription. This event therefore keeps the HPV "infected" cells in cycle, with the increased likelihood of genetic compromise and the possibility of the selection of advantageous oncogenic mutations.

In parallel, viral oncogene expression also skews local immune activation with such immune deviation potentiating immune escape that further favours viral persistence and lesion neoplastic progression (Fig. 28.2) [34]. This begins with E6/E7 downregulation of the level of CCL20, the chemoattractant for epidermal antigen-presenting cells (APC) (Langerhans cells) leading to an early failure of optimal innate immune activation and loss of local APCs [39, 40]. In addition, STAT-3 is constitutively activated in HPV transformed cells [41], and this drives IL-6 production that acts on tumour-associated myeloid cells in a paracrine fashion [42]. Further activation of STAT-3 in the monocytes upregulates CCL2 production, which stimulates MMP-9 and other tumour-promoting factors with an autocrine CCL2/CCR2 loop reinforcing the inflammatory microenvironment [43]. IL-6 produced during cervical carcinogenesis also interferes with mature APC (dendritic cell (DC)) migration through downregulation of the CCR7 receptor as well as DC IL-12 production, therefore influencing the flavour of any T helper responses [44]. In advanced neoplasia, IL-6 paracrine effects on tumour-associated fibroblasts instruct the production of CCL20 by the stroma, which further magnifies the chronic pro-tumour milieu [45].

It is clear that high-grade precancers and cancers have a plethora of local immunosuppressive factors that can potentially limit anti-tumour immunity. Indeed, such immune factors are able to upregulate checkpoint inhibitor ligand, PD-L1, on both tumour and associated immune cells providing another mechanism to limit effective

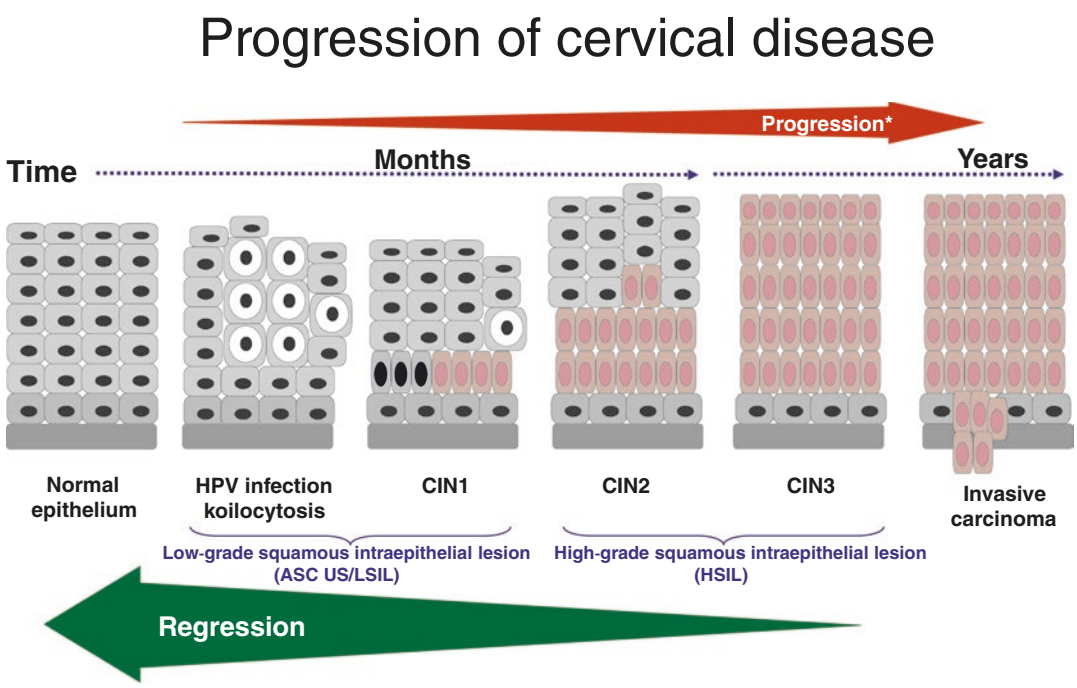

Fig. 28.1 Progression of cervical disease. The process of cervical carcinogenesis is illustrated schematically. After the cervix is infected with HPV, infection may cause mild pap abnormalities and/or mild CIN, which usually clear spontaneously. Koilocytosis is a distinctive histological feature of HPV infection and is the appearance of halo or koilocytotic cells in the differentiated layers of the squamous epithelium. The koilocytes are squamous epithelial cells that contain an acentric, hyperchromatic nucleus that is displaced by a large perinuclear vacuole [35]. Persistence of high-risk HPV is the key factor in the progression to precancerous lesions or high-grade dysplasia (CIN2/3) which has a greater likelihood of progression to invasion and cancer [36, 37]. Abnormal infected cells and CIN1 can also be termed low-grade squamous intraepithelial lesions (LSIL), while CIN2 and CIN3 can also be termed high-grade squamous intraepithelial lesions (HSIL) [36]. The progressive development of cellular changes from HPV infection to cervical cancer generally takes 10–20 years, although, in very few cases, it may only take 1–2 years [36]. Generally, CIN1 changes can arise within 3 months of infection, CIN2 within 6 months and CIN3 within 1–2 years

anti-tumour specific T activity [23]. Macrophages and myeloid-derived suppressor cells (MDSC) limit T-cell function both via PD-L1 expression and IL-10 production that modulates APC function with the induction of Tregs [46, 47]. Inhibition of cytotoxic T lymphocytes (CTL) further derives from myeloid cell production of TGFβ, reactive oxygen species (ROS), reactive nitrogen intermediates and arginase and nitric oxide synthase (NOS) that depletes the CTL function requiring metabolite, L-arginine. M2-type macrophages secrete TGFβ and IL-10 and together with IL-6 can attract immunosuppressive Th17 and Treg cells [38, 45]. Importantly, when Tregs migrate to the local LNs, they can provide protection for subsequently metastasizing tumour cells [48, 49]. Additional changes selected in progressing CIN 3 block anti-HPV cytotoxic T-cell function through HLA class I downregulation and failure of lesion entry of α4β7 CD8 T-cells through modulation in the expression of the ligand, MAdCAM-1 on the endothelium of lesion-associated neovasculature [26]. Any immunological therapeutic intervention strategy, even for CIN lesions, will need to combat significant challenges to deliver an efficacious outcome.

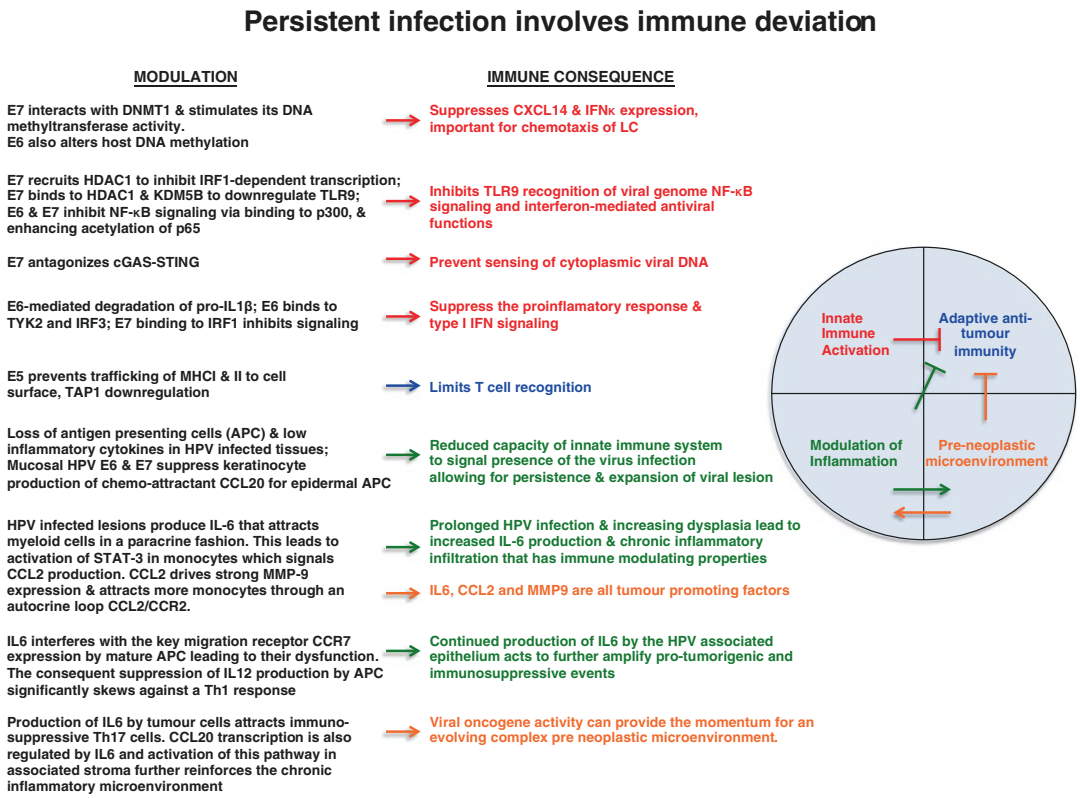

Fig. 28.2 Persistent infection involves immune deviation. The figure summarizes the consequences of viral expression that can lead to immune deviation, providing for viral persistence and risk of neoplasia [34, 38]. Early in HPV infection, oncogene activity can blunt the activation of innate immunity, the key to recruitment of the firepower of the adaptive immune response through specific antibody and cellular effector mechanisms. These events can lead to a modulation of inflammation, which is skewed, and self-reinforcing to yield a pro-neoplastic microenvironment

28.5 Therapeutic Vaccine Strategies

Attempting to utilize immune targeting of HPV gene expression for therapy of HPV-associated cancer dates back over 30 years. The principle strategies have focused on generating specific effector T-cells against the constitutive and functionally obligate expression of E6 and/or E7 oncogenes. Since then, HPV 16 (18) E6 and/or E7 oncogene vaccines employing various delivery technologies using viruses, bacteria, nucleic acids, peptides/proteins and cells, including dendritic cells, have been tested [50, 51]. While most of these vaccine approaches proved effective in preclinical animal models, data obtained in early-phase clinical trials were frequently underwhelming. Given our current knowledge of the complex interactions which may limit either endogenous or induced immunologically driven resolution of HPV-associated neoplasia, with hindsight, this is not very surprising. The lack of any consistent demonstration of significant medical impact results from not only the immunological escape mechanisms acquired during the cancer natural history but also the difficulty in designing appropriately powered clinical trials. For example, in cervical cancer, early-stage patients treated surgically have a high cure rate, while the chemo-radiation treatment of late-stage patients may interfere with vaccine immunogenicity complicating the interpretation [52].

Nevertheless, therapeutic vaccines targeting the HPV oncogenes have shown encouraging success in some recent early phase clinical trials tested in patients with high-grade anogenital lesions. There are many excellent reviews that document the extensive range of these therapeutic vaccine approaches [25, 50, 51]. This chapter will focus on some selected examples of sustained vaccine approaches with current clinical trial activities [53].

28.5.1 Protein/Peptide Vaccines

The design and delivery of cancer vaccines with the ability to induce strong CD8 T-cell responses is considered the benchmark for potential success. Vaccines incorporating the HPV 16 E6 and/or E7 proteins or synthetic long overlapping peptides (SLPs) can present the full spectrum of antigenicity to the recipient T-cell repertoire but may not be sufficiently immunogenic without the use of adjuvants and/or targeting to antigen-presenting cells. Protein antigens are mostly processed and presented through the major histocompatibility complex (MHC) II pathway, a T-helper-2-biased response favouring antibody production. Modifying the antigen and/or adding immunostimulatory molecules can shift processing through the MHC I pathway and stimulate a CTL response. Ideally, the vaccine platform needs to avoid antigen competition and provide for efficient processing by DCs to stimulate durable CD4 and CD8 T-cell responses with an adjuvant to deliver a T-helper-1-polarised response [54].

A fusion protein of HPV 16 E7 that targets to the endoplasmic reticulum (TVGV-1) with the adjuvant GPI-0100 stimulates a strong CTL response. A phase II double-blind, randomized, parallel-group, dose-ranging study assessing safety and efficacy of three vaccinations of the vaccine compared to its adjuvant in patients with HPV 16 cervical high-grade lesions (CIN2/3) is imminent (NCT02576561). Another phase II study is evaluating the efficacy and safety of PepCan (HPV 16 E6 peptides combined with *Candida* skin testing reagent called Candin®) in adult females with high-grade CIN over a 12-month time period (NCT02481414). The results from a phase I trial [55] demonstrated some efficacy against non-16 HPV types so Candin alone needs to be tested with participants receiving four vaccinations at three weekly intervals. Necessarily when using a CIN end point, any clinical, virological, or immunological responses need to be assessed within a relatively short time frame, typically, 6–12 months. Challenges for driving such approaches into phase Ill trials include the spontaneous CIN remission rates requiring the patient group size to be large to sufficiently power any efficacy studies and for long follow-up times. In addition, measures of vaccine-induced HPV-specific T-cell immunity sampled from the peripheral blood do not necessarily reflect the responses that will need to be active in the lesion itself [56]. The long-term objective of such vaccine regimens is to provide a safe, cost-effective non-surgical alternative for treating CIN2/3 that obviates any risks, albeit small, associated with surgery. However, the significant challenge is that treating CIN2/3 surgically is very efficacious, and so, the vaccine treatment must be as good if not better.

Encouraging results have been seen in clinical trials that tested HPV 16 vaccines in patients with HPV 16-associated vulvar intraepithelial neoplasia (VIN). In contrast to patients with CIN3 where surgical treatment can deliver approaching 100% resolution, in many cases, high-grade VIN lesion surgery is not an option, and/or the other limited treatments available are not curative [11, 57]. A combination of imiquimod followed by TA-CIN (a fusion protein of HPV16 L2E6E7) vaccination (without adjuvant) in patients with high-grade VIN lesions delivered 63% complete regression at 1 year [58]. Imiquimod is a topically applied innate immune response Toll-like receptor (TLR) 7/8 agonist that negates local immunosuppressive factors and could provide for an improved clinical impact of vaccination in VIN. Indeed, after treatment with imiquimod and vaccination, local infiltration of CD8 and CD4 T-cells was significantly increased in clinical responders whereas non-responders (with persistent VIN) showed an increased density of T regu-

latory cells. After vaccination, only the clinical responders showed significantly increased lymphoproliferation to the HPV vaccine antigens. A phase I study of TA-CIN to determine the safety of TA-CIN vaccine as adjuvant therapy is planned (NCT02405221). In the first part, 14 patients previously treated for HPV16-related cervical cancer in the past year, and with no evidence of disease, recurrence will receive three immunizations of TA-CIN vaccine at four weekly intervals either in arm or thigh. Pre- and post-vaccination levels of circulating antibody and proliferative responses of peripheral blood mononucleocytes to HPV16 E6, E7 and L2 as well as HPV16 E6- and E7-specific CD8+ T-cells and/or CD4+ T-cells will be determined. It is likely that further optimization of TA-CIN could be obtained by the use of an adjuvant and/or in combination with a checkpoint inhibitor strategy.

A vaccine composed of 13 synthetic long peptides of 25–35 amino acids derived from HPV 16 E6 and E7 oncogenic proteins and adjuvanted with Montanide (ISA101) showed very good T-cell immunogenicity and significant clinical impact on lesion responses in patients with high-grade VIN but did not impact on more advanced malignant disease [59, 60]. Recent preclinical studies have suggested some new opportunities for optimization of vaccination to impact more advanced cancers. Thus, treatment of tumour-bearing mice with standard carboplatin and paclitaxel chemotherapy plus vaccination significantly improved survival [61]. The mechanism was directly associated with the chemotherapy altering the myeloid cell population in the blood and tumour while having no effect on tumour-specific T-cell responses. Studies in advanced cervical cancer patients treated with carboplatin-paclitaxel confirmed a reduction in the high circulating myeloid cells and a concomitant improvement in the patient T-cell responses. It was observed that the nadir of circulating myeloid cells was at 2 weeks after the second cycle of chemotherapy. Using this point for vaccination was tested in patients, with robust and sustained HPV16-specific T-cell responses to a single dose of the vaccine demonstrable (see Ref. [4]). A clinical trial (NCT02128126) is now in progress that is assessing the safety, tolerability and the HPV-specific immune responses of different doses of the ISA101 long-peptide HPV16 vaccine with or without pegylated interferon alpha (IFN-α) as combination therapy with carboplatin and paclitaxel with or without bevacizumab (standard of care therapy). The rationale is that the chemotherapy could enhance the tumour-specific immunity and synergize with cancer immunotherapy with the addition of pegylated IFN-α aimed at further improving the immune response. Another proposed clinical study aims to evaluate whether anti-HPV responses are stimulated in metastatic anal cancer patients who made a complete clinical response following chemotherapy with docetaxel, cisplatin and 5-fluorouracil (NCT01845779).

Several other clinical trials are planned to further evaluate the optimal use of ISA101 SLP vaccine in combination with other treatments of HPV-related disease, for example, a phase II trial of nivolumab (anti-PD-L1) and HPV-16 vaccination in patients with HPV 16-positive incurable solid tumours. HPV-16 vaccination is given three times at 3–4 weeks intervals, and checkpoint inhibitor is administered intravenously (IV) every 2 weeks starting at 8 days of the first immunization. There are 3 weeks in cycle 1 and 2 weeks in cycles 2 and beyond. The goal is to see if nivolumab combined with the ISA101 SLP vaccine can help to control cancer that has spread. The safety of the study drugs will also be studied (NCT02426892). Another clinical research study is to learn whether utomilumab (humanized mAb recognizing 4-1BB (CD-137) protein receptor expressed by CD4 and CD8 T-cells plus NK cells, when given IV alone or combination with the ISA101 vaccine) is able to shrink or slow the growth of tumours in patients with incurable HPV 16-positive oropharyngeal squamous cell carcinoma (OPSCC). The rationale is that the anti-CD137 will stimulate and increase the number of immune cells and therefore enhance anti-tumour function (NCT03258008). A phase I/II study will also assess the safety and efficacy of the ISA101 SLP vaccine in HIV+ men with CD4 counts >350 × 10E6/l and HPV16-induced intra-anal

high-grade AIN (grade 2–3) that failed on or recurred after previous treatment (NCT01923116).

Another approach to optimize HPV16 peptide vaccination has used two of the HPV16 E6 SLP conjugated to Amplivant®, a synthetic Toll-like receptor (TLR) 2 ligand, with the goal of maximizing the induced Th1 response and obtaining more high-avidity cytotoxic CD8+ T-cells. The two peptide sequences are within the most immunodominant regions of the overlapping HPV16-SLP set and contain both T helper and CTL epitopes. In preclinical murine studies, Amplivant®-conjugated SLP showed 10–100 times higher bioactivity compared to unconjugated SLP, in terms of induced immune responses [62]. A phase I study to determine the biological activity of this vaccine (Hespecta) in patients treated for HPV16-positive tumours or premalignant lesions is in progress (NCT02821494).

28.5.2 Listeria-Based Vaccines

Attenuated bacterial vectors can be generated by transformation with plasmids allowing the expression of the selected genes of interest and their delivery to the host antigen-presenting cells. One example, which has made some progress to later-stage clinical testing, is *Listeria monocytogenes* (Lm), an anaerobic, Gram-positive facultative intracellular bacterium that is associated with foodborne disease in susceptible hosts. Immune responses are well documented and robust, with the activation of both the innate and adaptive arms [63]. Following phagocytosis by macrophages, Lm escapes the phagosome by secreting the pore-forming toxin listeriolysin O (LLO), a virulence factor that targets the phagosomal membrane for destruction [64]. This allows the bacterium to grow rapidly in the cytosol and for actin nucleator A (ActA)-dependent cell-to-cell spread. Allosteric changes in the master transcriptional regulator protein-related factor A (prfA) lead to the upregulation of the ActA protein and a 200-fold increase during intracellular bacterial growth. This facilitates the movement of the bacteria to the cell surface and their subsequent spread to other cells. Thus, the Lm life cycle is critically dependent on the coordinated expression of LLO and prfA. The innate immune response is activated during such infections via TLR-2 and TLR-5 recognition of Lm pathogen-associated molecular patterns (PAMPs) including peptidoglycan, lipoteichoic acid, lipoproteins and bacterial flagellins. In addition, nucleotide-binding oligomerization domain-like receptors (NLRs), NLRC4 and NLRP3, detect cytosolic Lm with the activation of the inflammasome, while AIM2 senses the bacterial DNA. These signals lead to the infiltration of neutrophils and macrophages that limit bacterial growth. Effective antigen presentation by macrophages that have phagocytosed any bacteria and dendritic cells stimulate strong CD4 and CD8 T-cell responses that clear the infection and provide for long-term memory.

These properties have supported the development of Lm as a bacterial vector for immunotherapy of HPV-associated cancers using an attenuated organism with deficiency in the master transcriptional regulator protein-related factor A, plus a truncated non-hemolytic listeriolysin (LLO) molecule which prevents escape from the phagolysosome but retains the adjuvant properties [65]. In the vaccine construct (ADXS11-001), the modified LLO is fused to HPV 16 E7. The engineered Lm is taken up by APCs and escapes the phagolysosome through the secretion of LLO. In the cytosol, many copies of the LLO-E7 are released, and the adjuvant properties of the bacteria effectively stimulate innate/adaptive immune responses to HPV 16 E7. There is also induction of pro-inflammatory cytokines from natural killer cells, recruitment of monocytes from the peripheral blood to the site of inflammation and maturation of local dendritic cells. LLO-fusion protein breakdown through phagocytosis leads to antigen processing by the MHC class II endosomal pathway stimulating CD4 T-cells, while LLO also potentiates ubiquitin-mediated proteasomal degradation and the cytosolic pathways leading MHC class I presentation activating CD8 T-cells.

Listeria-based E7 vaccines have been tested in syngeneic mouse models of HPV-driven cancer.

The vaccine construct has been shown to stimulate innate immunity with the production of IL-2, IL-12, TNFα and IFNγ and costimulatory molecules necessary for DC maturation and stimulation of CD4 and CD8 antigen E7-specific T-cell responses [65]. This T-cell immunity can overcome tumour-induced immune tolerance and generate immune memory able to maintain specific immunity and block tumour recurrence [66]. A more recent study showed that a combination of Lm-LLO-E7 with an anti-PD1 antibody that blocks the PD-1/PD-L1 interaction potentiates the efficacy of the immunotherapy in the TC-1 mouse model [67]. Most importantly, the combination treatment provides for a significant reduction in Tregs and MDSC cells in the tumour and tumour microenvironment plus enhanced antigen-specific CD8 T-cells in the periphery and the tumour leading to prolonged survival or complete regression. This type of study has led to the initiation of several clinical trials in patients with HPV-associated cancers.

The first study in 2009 assessed safety in metastatic or recurrent cervical cancer patients in phase I trials with dose escalation from 1×10^9 to 1×10^{10} of the vaccine given as an intravenous infusion followed by a second immunization 3 weeks later [68]. This trial reported an acceptable safety profile with flu-like symptoms shown by all the patients although at the highest dose, some recipients displayed severe fever and dose-limiting hypotension. While overall, 722 vaccine doses have been received by 290 patients with HPV-associated cancers, a few serious adverse events suggest a requirement for additional caution when using the live attenuated Lm vectors [69, 70]. A randomized phase III clinical trial (AIM2CERV) in high-risk locally advanced cervical cancer following chemoradiation is recruiting (NCT02853604). This aims to compare the disease-free survival (DFS) of ADXS11–001 to placebo administered in the adjuvant setting following concurrent chemotherapy and radiotherapy (CCRT) administered with curative intent to subjects with high-risk locally advanced squamous, adenosquamous, or adenocarcinoma of the cervix. In this study, subjects will receive a 7-day course of an oral antibiotic or placebo starting 72 h following the completion of study treatment administration. An interim analysis will be performed when there is at least one-half the number of DFS events required for full maturity of the study.

In order to shift the balance in favour of the functionality of cytotoxic T-cell responses in the tumour, ADXS11-001 vaccination in combination with the checkpoint inhibitor durvalumab is being tested. This monoclonal antibody binds to PD-L1 and blocks interaction with PD-1 on activated T-cells and has a modified Fc region to prevent either antibody-dependent cytotoxicity (ADCC) or complement-dependent cytotoxicity. An ongoing study in cervical or HPV+ oropharyngeal squamous cell carcinoma (OPSCC) patients will initially determine the safety and tolerability of the combination and identify any dose-limiting toxicity. In the phase II study, the primary objective is to evaluate tumour response, progression-free survival (PFS) and safety of either monotherapy or the combination (NCT02291055).

28.5.3 Vaccinia-Based Vaccines

Viral vectors have been seen as attractive candidates for therapeutic HPV vaccine delivery, and many have been explored in preclinical studies [50, 51]. Vaccinia virus has a very large stable double-stranded DNA genome and is highly infectious. It was used in the first HPV vaccine tested in a clinical trial (TA-HPV) and incorporated both HPV 16 and 18 E6 and E7 modified with slightly modified sequences to abolish any transforming function. However, there are two further issues of concern with such live vector vaccines: (1) the generation of antiviral neutralizing antibodies upon initial immunization that can limit subsequent HPV-related immune responses and (2) concerns about pathogenic risk especially with recipients with impaired immunity. An initial phase I/II study in which eight patients with late-stage cervical cancer were given a single dose of TA-HPV documented no significant clinical side effects or environmental contamination by live TA-HPV. An anti-vaccinia

antibody response was detected in all the patients, but only three developed HPV-specific antibodies and only one showed evidence of induction of HPV-specific cytotoxic T lymphocytes [71]. In a further trial of the vaccine in patients with early invasive cervical cancer. T-cell responses were detected in only 4/29 patients [72]. To contend with the riders to testing vaccines in either early- or late-stage cervical cancer. Patient's safety, immunogenicity and efficacy of TA-HPV were tested in women with high-grade VIN. In these patients, 5/12 showed evidence of 50% or more lesion size reduction, and increased T-cell responses were measured in 6/10, while all patients showed boosted vector-specific responses [73]. These types of result are a fair reflection of many attempts to test cancer vaccines at this time where it was often difficult to correlate measures of vaccine immunogenicity with clinical responses if any were seen [73, 74]. To avoid problems of boosted vector-specific responses, heterologous prime-boost vaccination schedules employing TA-HPV in combination with TA-CIN were tested. Ten women with HPV 16-positive high-grade VIN, previously primed with TA-HPV, received three booster immunizations with TA-CIN. All but one demonstrated HPV 16-specific T-cell and/or antibody responses following vaccination, but no link between clinical and immunological responses was observed [75, 76]. The reciprocal delivery of TA-CIN × 3 (at four weekly intervals) followed by a single dermal scarification of TA-HPV demonstrated immunogenicity but no simple relationship between the induction of systemic HPV-16-specific immunity and clinical outcome [77]. As discussed earlier, topical use of imiquimod followed by three doses of TA-CIN in women with high-grade VIN was shown to correlate lesion response with local immune infiltration and composition exemplifying the need to measure local factors in response to experimental immunotherapy [58]. This approach may yield the necessary insights to identify key factors for clinical response of patients, thereby ensuring sufficiency of momentum to provide the funding for optimally designed clinical trials that can establish useful efficacy. If used in a prime heterologous context, vaccinia vectors may still be useful, and constructs expressing E7 linked to calreticulin (CRT), LLO or lysosome-associated membrane protein have all been explored in preclinical studies in this type of approach [50, 51]. To deal with any safety concerns, an attenuated strain MVA can be utilized although this is operationally defective for growth in human cells, and the immunizing virus dose therefore needs to be high [78]. MVA expressing HPV 16 E6/E7 (TC4001) with human IL-2 is being tested in a phase I/II trial evaluating a combination of vaccine and avelumab (not only binds to PD-L1 and blocks PD-1 interaction but also mediates antibody-dependent cellular cytotoxic (ADCC) against PD-L1-expressing targets) in HPV-16-positive recurrent/metastatic malignancies and expansion cohort to OPSCC (NCT 03260023).

28.5.4 RNA Virus-Based Vaccines

RNA viruses such as Sindbis, Venezuelan equine encephalitis or Semliki Forest are attractive vaccine vectors because they are able to produce RNA replicons with self-replication capacity allowing for sustained target expression while being defective for viral particle production [79]. This maximizes vaccine target immunogenicity while minimizing vector-specific responses. VVax001 is a therapeutic Semliki Forest virus vector encoding HPV-16 E6 and E7 currently being tested in patients with CIN2/3 who will receive three consecutive doses, at intervals of 3 weeks with the assessment of E6 and E7-specific T-cell immune responses (NCT03141663).

28.5.5 Nucleic Acid-Based Vaccines

DNA vaccines avoid any issues of neutralizing antibodies that may be induced to the vector and are easy and cheap to manufacture. The use of electroporation has provided an immunization methodology able to deliver more consistent immunogenicity. Using IM injections of a DNA plasmid encoding HPV-16/18 E6/E7 (VGX-3100) followed by electroporation using the

CELLECTRA™-5PSP device, CIN2/3 lesions in vaccinated patients showed a significant regression including viral clearance. Importantly, such peripheral vaccination altered the composition, magnitude and quality of immune responses in the target lesions [80, 81]. This exemplifies the role of local factors in determining immunologically driven therapeutic outcomes, but in most clinical trial designs, they are at best very difficult or almost impossible to monitor. These studies have provided momentum for a prospective, randomized, double-blind, placebo-controlled phase III study to determine the efficacy, safety and tolerability of VGX-3100 adult women with HPV 16 and/or 18-positive CIN2/3 (NCT03185013). A clinical trial of treatment of patients with HPV-16 and/or HPV-18 high-grade VIN with a combination of VGX-3100 vaccination and imiquimod is in progress (NCT03180684). Studies in more advanced disease are also progressing including a prospective study of VGX-3100 vaccination in patients with HPV-associated head and neck squamous cell carcinoma (NCT02163057) and in patients with either inoperable invasive cervical carcinoma associated after standard chemoradiation therapy or with persistent/recurrent cervical cancer following salvage therapy (NCT02172911). A trial combining VGX-3100 vaccination with durvalumab in HPV-positive OPSCC is recruiting (NCT03162224).

Another HPV E6/E7 DNA therapeutic vaccine (GX-188) has oncogene E7 sequences fused to the extracellular domain of Fms-like tyrosine kinase-3 ligand and the signal sequence of tissue plasminogen activator. This design aims to promote antigen presentation and trafficking of the fused protein to the MHC I pathway. Electroporation-enhanced immunization stimulates E6/E7-specific T-helper-1-polarized responses and HPV16-specific CD8 T-cells in CIN3 patients. The majority of these patients (7/9) showed complete lesion regression and viral clearance within 9 months [82]. Combination strategies are likely to be required to induce sufficient high-quality T-cells that can traffic to the lesion and deliver a curative payload for all patients. Local delivery of imiquimod is one approach being tested that might provide the necessary boost of a T helper 1 response. Alternatively, interleukin-7 (IL-7), a T-cell growth factor used for treating lymphopenia patients, might enhance the expansion of the T effector populations. GX-I7 is a protein drug recombining human IL-7 and hybrid Fc (hyFc) with the recombined region not exposed and each region's characteristics able to reduce immunogenicity and improve the efficacy of the drug. A study to investigate the safety and efficacy of GX-188 administered IM by electroporation plus the application of GX-I7 either intravaginally or imiquimod topically in subjects with CIN3 is recruiting patients (NCT03206138).

A pilot study of the DNA vaccine pnGVL4a-CRT/E7 (detox) for the treatment of patients with HPV16+ CIN2/3 compared the immunogenicity of three different routes of administration: intradermal by gene gun, intramuscular and intralesional plus or minus imiquimod (NCT 00988559). pNGVL4a-CRT-E7(detox) was well-tolerated, elicited the most robust immune response when administered intralesionally and demonstrated preliminary evidence of potential clinical efficacy [83].

Another DNA vaccine construct, pNGVL4a-Sig/E7(detox)/HSP70, with targeting and adjuvant properties, is being used to prime HPV16+ CIN3 patients followed by a boost using the TA-HPV vaccine with or without imiquimod (NCT00788164). Animal studies have established increased immunogenicity of such prime-boost vaccination [84]. The recipients will receive pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine intramuscularly (IM) on days 1 and 29 and TA-HPV IM on day 57 with one group receiving topical imiquimod on days 1, 29 and 57. However, there may be some logistical challenges of imiquimod application in the cervix as well as the need for an efficacious outcome able to compete with existing treatment options for CIN3.

Another DNA vaccine, VB10.16, has been constructed to express molecules with a targeting module (e.g. human macrophage inflammatory protein-1 alpha) linked through a dimerization

module (composed of the hinge and constant regions of the CH3 domain of IgG3 which provides bivalency and flexibility) to an HPV 16 E6/E7 fusion protein. Upon intramuscular administration, VB10.16 expresses HPV16 E6/7 and a protein that targets receptors on APCs. Upon binding to APCs and subsequent internalization, the APCs mature, and the HPV16 E6/7 antigenic protein is optimally presented by the APCs with the prospect of excellent CTL induction. An exploratory, open, prospective multicentre study of VB10.16 immunotherapy in patients with CIN2/3 is recruiting across in Europe (NCT02529930). The previously stated limitations of such trials apply here, and it seems likely that the vaccine will have to have an extraordinary immunological and clinical impact to warrant further development with CIN as a targeted treatment.

28.5.6 Cell-Based Vaccines

The development of ex vivo methods for the production of dendritic cells from monocytes provided much optimism for maximal antigen presentation of target antigens by such cell-based cancer vaccines. Given that the HPV-driven oncogenesis requires additional genetic changes that might also be immunogenic, using tumour lysates, not just HPV early antigens, with dendritic cells could broaden the activation of the tumour-specific adaptive immune repertoire. Unfortunately, the demands of reproducible and sufficient production of APCs with appropriate longevity and to good clinical practice have thus far proved challenging in the clinical setting [84]. The optimal route of administration is also not clear. Similar problems have also limited the development of tumour-based vaccines including with modifications providing for the production of cytokines like IL-2, IL-12 and GMSCF. Additional approaches to maximize tumour antigen presentation are focused on delivering antigens either directly to professional APCs in vivo or through enhancing antigen-processing pathways [85].

28.6 Adoptive Cell Transfer (ACT)

Adoptive transfer of ex vivo-expanded tumour-infiltrating lymphocytes (TIL) can be efficacious with response rates of about 30% in patients with treatment-refractory metastatic melanoma [86–88]. Generally, attempts to enrich for antigen-specific populations were not shown to necessarily correlate with clinical responses. This is consistent with the view that the impact of the treatment is to provide a wide range of anti-tumour specific T-cells to re-exert tumour control. The latter had previously been inactive through multiple immunosuppressive factors in the tumour but are expanded and functional after ACT. One key to success is the preconditioning of the patients providing opportunity for preferential expansion of the adopted cells on transfer. The impact of immune checkpoint blockade targeting CTLA-4 or PD-1 with blocking antibodies and their use in combination may also add to the proportion of patients showing clear clinical benefit [89]

Metastatic cervical cancer patients, previously treated by chemo- or chemoradiotherapy, were treated with a single infusion of tumour-infiltrating T-cells (stimulated when possible for HPV E6 and E7 reactivity) with the cell infusion preceded by lymphocyte-depleting chemotherapy followed by IL-2. Three of nine patients experienced objective tumour responses with the two complete responses sustained on follow-up 15–22 months after treatment. Interestingly, a correlation between HPV reactivity of the infusion product and clinical response was observed (NCT01585428). The efficacy of TIL treatment ultimately depends on the balance of expanded effectors with anti-tumour activity overcoming the more negative influences both in the isolated TIL (by preferential expansion) and in the local tumour microenvironment. While spectacular clinical responses can occur, this is still unpredictable and requires individual patient TIL expansion with associated substantial cost and logistical issues.

More generic tumour antigen-specific cell therapies are being developed through the engi-

neering of T-cell receptors (TCR) or chimeric antigen receptors (CAR) effector lymphocytes [90–93]. Peripheral blood leucocytes can be genetically engineered to express a TCR with tumour antigen specificity albeit with a particular MHC restriction and expanded for ACT. For example, a TCR from an anal cancer patient's infiltrating T-cells recognizing an HLA-A*02:01-restricted epitope of HPV-16 E6 was cloned. Normal T-cells genetically engineered to express this TCR showed high avidity for the HLA-A*02:01-restricted epitope of HPV-16 and could kill HPV-16+ tumour cell lines [94]. The drawbacks to this approach include the continuing negative influences on T-cell effector functions in vivo through immunosuppressive factors and that targeting a single epitope in a particular MHC context provides ample opportunity for immune escape by HLA downregulation, a frequent event in cervical [95–97] and other cancers [98]. In addition, it is not always obvious that a cloned TCR is the potentially most effective target to deliver what has to be a knockout punch to all or most of the tumour cells. An alternative approach is to generate a synthetic structure composed of an extracellular recognition domain for antigen specificity (e.g. ScFv antibody) linked through a flexible hinge region to transmembrane and intracellular domains, which provides for signal delivery within the CAR T-cells. This approach necessitates a cell surface expression of the target antigen, for example, CD19 in CAR T-cell treatment B- ALL that has recently been licenced [93], and thus, there is no potential for targeting HPV antigens for this type of cancer treatment.

28.7 Optimizing Immune Intervention Strategies

28.7.1 Early Cancers

For HPV-associated anogenital cancers, early neoplastic stages (cervical, vulvar, vaginal, anal, penile intraepithelial neoplasia) have been identified, while as yet, no precursor lesion has been documented for OPSCC. At such an early stage, the size and relative homogeneity of the cancer plus its associated immunosuppressive influences are likely to be easier to overcome, perhaps by therapeutic vaccination alone. Some have favoured targeting early genes such as E2 [99], but this approach is compromised by the frequent loss of expression in the carcinogenic process [12]. Vaccination against HPV oncogenes could be effective, but to be acceptable, it would need to be safe, cheap, very straight forward and virtually 100% efficacious. To deliver this, further understanding is required of how to direct specific T-cell effectors to the site of the lesion where they can overcome any local immune suppression/escape and kill the neoplastic cells. The consequence of this must also be to reset the immune system so that it can fully utilize its adaptive immune repertoire to eliminate all elements of any residual HPV oncogenic threat which might include cells resistant to vaccine-induced activity. However, any useful therapeutic vaccine would need to incorporate activity against several high-risk HPVs oncogenes to be sufficiently effective against premalignant lesions like CIN and VIN. A critical question is whether this can be delivered by a simple immunization procedure alone or whether it will necessitate additional immune intervention steps to guarantee effectiveness.

28.7.2 Later-Stage Cancers

In any HPV-associated cancer that is not surgically operable, the prospects for more complex immune intervention strategies are more attractive and indeed desirable. The difficulties include the same issues for immune targeting as for early disease, but the problems are magnified by the increased genetic heterogeneity of the tumours, including those variants selected by immune pressure, the scale and diversity of the local and systemic immunosuppressive influences and the metastasis of the tumour cells [34, 38, 48, 100–102]. Figure 28.3 summarizes some of the many challenges for immune targeting of HPV cancers and some approaches to overcome these barriers. The diversity of the tumour microenvironment with its varying contributions of individual immunosuppressive factors provides formidable

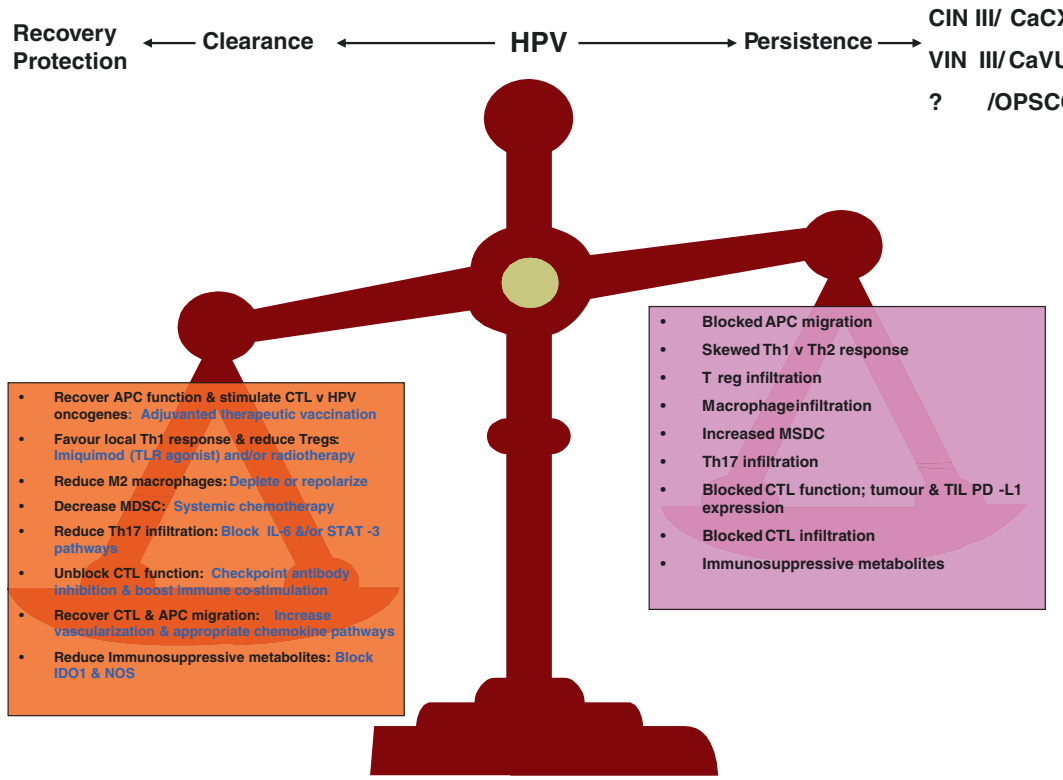

Fig. 28.3 Overcoming the barriers to effective immunity in HPV-associated cancer. To be effective, HPV targeted immunotherapies will need to overcome various components that influence the function and infiltration of immune effectors and antigen-presenting cells in HPV-driven neoplasia [103–105]. The principle approaches will need to focus on generating or recovering antigen presentation and anti-tumour T-cell effector migration and function, minimizing and reversing the immunosuppressive actions of other tumour-infiltrating populations including M2 macrophages, myeloid-derived suppressor cells, T regulatory cells and Th17 cells. The tumour microenvironment is also characterized by a suppressive inflammatory balance of chemokines, cytokines, metabolites and immune checkpoint ligand and co-stimulatory receptor expression

hurdles to defining particular treatment combinations of the therapeutic weapons available, their sequencing and timing. Hopefully, the ongoing clinical trials documented here will provide the means to identify those with the most potential. It is vital that future trial activity is focused on those strategies that have a credible likelihood of delivering a realistic clinical benefit.

28.8 Concluding Remarks

The remarkable impact of checkpoint inhibitors and other emerging immunotherapies in subsets of cancer patients where previously there was little, if any, clinical response to the available treatments helps to exemplify some of the problems that lay ahead for HPV immunotherapy [106]. While checkpoint inhibitor therapy clinical responses are very encouraging, it is by no means clear that the mechanisms are really understood—that treatment dosing or their use in combination, including in the context of standard of care (SOC), or the toxicities are anywhere near optimally elucidated. Most important is the need to know which patients are likely to respond. For example, tumour cell expression of the ligand for PD-1 has been claimed as a marker of response to checkpoint inhibition in some patients [107, 108]. Recent studies in OPSCC suggest that this is not necessarily true and prognostic factors can vary at a disease site as stratified by HPV involvement [109–112]. Predicting response to checkpoint inhibition or indeed any immune or other

therapy is not a simple issue [113]. Given our knowledge of the spectrum of immune factors involved in cancer per se which have been largely ignored until precipitated by the checkpoint inhibitor revolution, it is critical that future clinical trials seek to coordinate the collection of common data sets relevant to defining the immune characteristics of patient response [114]. This will undoubtedly necessitate the measurement of local tumour-related factors before and after treatments. The prospects are good for harnessing immunity to HPV-associated cancers to deliver more effective treatments than the current regimens. The recognition of the role of the chemo- and radiotherapeutic components of SOC in helping the recovery of effective anti-tumour immunity and thereby providing a key instrument of cure is an important insight. Understanding this can ultimately provide for a better-scheduled combination of treatment modalities for all cancers [103–105]

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29 New Advances in Radioimmunotherapy for the Treatment of Cancers

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Contents

29.1 Introduction

The Idea of Using Monoclonal Antibodies Directed to Tumour Markers Coupled with Nuclear Medicine to Deliver Ionizing Radiation against Tumours Appeared Just after Köhler and Milstein Developed Hybridoma Technology to Produce mAbs. Radiolabelled Antibodies Have Been Considered for the Treatment of Cancer since the Beginning of the 1980s [1], with the First Application Consisting of a Diagnostic Application with a mAb Directed against Carcinoembryonic Antigen Radiolabelled with Iodine-131 in Colorectal Tumours [2]. Therapeutic Applications Came Quickly after and Have Shown Real Efficacy in B-Cell Lymphoma Pathology [3]. These First Results Were Confirmed in Clinical Studies Demonstrating the Efficacy of Anti-CD20 mAbs like 131I-Tositumomab or 90Y-Ibritumomab Tiuxetan in the Radioimmunotherapy of Non-Hodgkin B-Cell Lymphoma (NHL) [4, 5]. The Recent Progress in Recombinant Humanized or Human Monoclonal Antibodies, the Disposability of More Stable Chelates, Improved Pretargeting Techniques, New and Innovative Radioisotopes and Administration Protocols Have Increased the Therapeutic Efficacy of Radioimmunotherapy (RIT) [6]. This Chapter Aims to Discuss the most Important Aspects and New Advances in RIT Practice for the Treatment of Cancers

29.2 Principles of Radioimmunotherapy

Radioimmunotherapy (RIT) consists of a targeted molecular therapy involving both radiobiological and immunological processes [7]. The key for RIT success consists of specific irradiation of tumour cells and irradiation of

healthy tissues as low as reasonably possible (e.g. RIT side effects). The vectorization of the radionuclides by the specificity of the mAbs conduces to a continuous, exponentially decreasing and low-dose-rate irradiation towards the targeted tumour. In comparison with conventional external-beam radiotherapy where the delivered dose is intermittent and high-dose-rate, RIT is dependent on the mAb pharmacokinetic distribution on the tumour site, and the dose-response relationship with patient outcomes, such as cell survival, has not been yet demonstrated. Whilst the exact mechanism of the radiobiological cytotoxicity is yet to be determined, it has been clearly demonstrated that we observe a synergy between the immunological cytotoxicity such as apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) due to the non-radioactive mAb injected prior to the radiolabelled antibody and RIT with bystander and abscopal effects [8].

Whilst RIT efficacy has been demonstrated in hemopathies such B-cell lymphoma and non-Hodgkin lymphoma, it is yet to be confirmed for solid tumours where the neovasculature is highly disorganized and presents anomalies like arteriovenous shunting or blood flow inversion [9, 10]. This deleterious phenomenon is compounded by the intratumoural high interstitial pressure and could limit the penetration of large-sized macromolecules such as mAbs [11, 12]. Fortunately, the low penetration of radiolabelled antibodies seems to be overestimated, and the autoradiography indicates that mAbs completely cover the tumour and bind to antigen-positive regions [13, 14]. Thus, the current RIT indications in clinical practice are small disseminated or minimal tumours, clusters of malignant cells or consolidation therapies. In minimal residual disease of solid tumours or hemopathies, the clinical setting, biodistribution and tumour dosimetry are more favourable because tumour cells are less hypoxic and more radiosensitive [15, 16].

The efficacy of RIT is mainly driven by the good correlation between the mAb and isotope choice [15]. Regarding therapeutic applications, nuclear medicine practitioners can use massive particle emitters such as beta minus particles, Auger electrons or alpha particles, which deliver their ionizing energy locally. The penetration path length, which depends on the initial energy of the radioactive emission, should match the size of the targeted tumours. This parameter, particularly with beta minus emission, produces effective irradiation over a few hundred cell diameters, resulting in a cross fire effect on nearby tumour cells as well as cytotoxic effects towards cells not necessarily targeted by the antibody. On the other hand, the choice of the mAb is crucial for RIT success. To circumvent the pharmacokinetic and biodistribution difficulties of using whole native mAbs, biochemists and immunochemists have developed numerous immunoconjugate derivatives such F(ab) and $F(ab')_2$ fragments and synthetic proteins (e.g. minibodies or single-chain variable fragment) [17–20].

The effectiveness of RIT in clinical practice has been demonstrated with non-ablative activities for therapy of relapsed patients, with refractory tumours or as consolidation after chemotherapy induction in follicular lymphoma or other hemopathies [21–23]. For solid tumours, RIT used as consolidation therapy targeting minimal residual disease (MRD) achieved promising clinical efficacy in colon-rectum carcinoma or prostate cancer [24, 25]. New RIT protocols such as pretargeting in medullary thyroid carcinoma [26] or dose fractionation approaches in metastatic castration-resistant prostate cancer [27] seem to be promising and are currently the mainstay of research in RIT with encouraging initial clinical results.

29.3 Radionuclides and Radiolabelling Techniques for Therapy

29.3.1 Radionuclides

Despite the large number of radionuclides available, only a few of them are used for RIT. Radioisotope choice is broadly driven by three criteria: physical characteristics, chemical characteristics and availability. The list of current radionuclides used (or considered) for RIT is summarized in Table 29.1.

Table 29.1 Radionuclides for antibody-targeted imaging and therapy

Radionuclide	Emission type	Half-life(h)	E_{max} (keV)	Maximum range in soft tissues (mm)	Production method	Secondary emission	Usual labelling method
Indium-111	Auger	67	2.72	Nanometre scale	Cyclotron	γ	Polyamino carboxylic acids: DTPA, DOTA
Iodine-131	β^-	193	606.3	2.9	Neutron reactor	γ	Direct labelling (tyrosine)
Yttrium-90	β^-	64	2280.1	12.0	Neutron reactor	/	Polyamino carboxylic acids: DOTA
Lutetium-177	β^-	162	498.3	2.0	Neutron reactor	γ	Polyamino carboxylic acids: DOTA
Rhenium-186	β^-	89.2	1069.5	5.0	Neutron reactor	γ	N_2S_2 or N_3S complexes (analogous with technetium chemistry)
Rhenium-188	β^-	17	2120.4	10.8	Neutron reactor	γ	N_2S_2 or N_3S complexes (analogous with technetium chemistry)
Copper-64	β^-	12.7	579.0	2.8	Cyclotron	β^+	Polyamino carboxylic acids: DOTA
Copper-67	β^-	62	561	1.8	Cyclotron	γ	Polyamino carboxylic acids: DOTA
Astatine-211	α	7.2	5.870–7.45	0.055–0.080	Cyclotron	X	Stannylated synthons: SAB, SAPS
Bismuth-213	α	0.76	8.4	0.1	Actinium-225 decay	γ, β^-	Polyamino carboxylic acids: DOTA, DTPA
Bismuth-212	α	1.0	6.3	0.080	Waste management	γ, β^-	Polyamino carboxylic acids: DOTA, TCMC
Actinium-225	α	240	8.4	0.1	Cyclotron or waste management	γ, β^-	Polyamino carboxylic acids: DOTA, HEHA
Thorium-227	α	448	6.0	0.080	Neutron reactor	γ	Polyamino carboxylic acids: DOTA Hydroxypyridin complex: HOPO

From a physical point of view, RIT uses non-penetrating radiation including beta minus particles, Auger electrons or alpha particles. These three modes of decay deliver their energy over small distances within an organism, an ideal situation for reducing irradiation of healthy non-targeted tissues. Both beta minus particles and Auger electrons are the same type of particle with a difference in terms of energy as a consequence of the different origin due to the radioactive mechanism.

The linear energy transfer (LET) in soft tissues for the electrons in the range of 0.1–1 keV

energy (e.g. Auger electrons) is in the range of 5–25 keV/μm. Consequently, the path length of penetration for an Auger electron is very short (subcellular irradiation of several nanometres from the point of emission) [28]. For the electrons in the range of 10 keV to 10 MeV energy (e.g. beta minus particles), the LET is in the range of 0.2–2 keV/μm [29]. Thus, the path length of penetration for beta minus particles is in the order of magnitude of a few millimetres to centimetres from the point of emission. Typical LET values for 5–10 MeV alpha particles are 100 keV/μm, and the path lengths of penetration for these alpha particles are close to 100 μm [30]. The choice of emission type is driven by the size of the tumour and the pharmacologic vector site of fixation. Auger electrons are more suitable for inner-cell irradiation close to DNA molecules, alpha particles permit irradiation on small cell clusters, and beta minus particles are used to irradiate microscopic tumours. Yttrium-90, for example, exhibits a long-range beta emission and can be used for larger masses, whilst lutetium-177 has a short-range, favouring treatment for smaller tumours [31, 32].

The radionuclide half-life must also be considered. Often, RIT is administered by systemic infusion, and the physical half-life must be matched with the time required for tumour uptake and clearance of unbound activity. A very short radionuclide half-life leads to non-negligible irradiation of healthy tissues during the pharmacokinetic biodistribution of the vector. The use of short half-life radionuclides requires small carriers that quickly reach the target cells, as proposed in peptide therapy, mAb fragments, pretargeting or small antibody-like vector approaches. Therefore, it is relevant in RIT practice to match the radionuclide physical half-life to the carrier biological half-life in order to obtain higher tumour-to-normal-tissue activity uptake ratios.

From a chemical point of view, radioisotopes for RIT must be chemically stable in vivo. Because radionuclides need to be bound to their immunological vector, the less-reactive species such as alkali, alkaline earth metals or noble gases cannot be used. Globally, they can be divided into two main categories, the radiohalogens and the radiometals, and each of these requires specific chemical protein radiolabelling approaches (for more specific details, see Sect. 29.3.2). The chemical nature of the radionuclides also influences the rate of its metabolism within a cell and therefore the pharmacological profile. For instance, when mAbs are internalized, residual metal radionuclides afford protracted radioactivity retention in tumour sites, whereas direct radiolabelling with radioiodine results in fast excretion of the radioactivity by the sodium-iodine symporter (NIS), thus reducing target cell exposure.

Finally, the method of radionuclide production is a very important aspect that determines the cost and availability of the radioisotope of interest. Parameters such as the final purity, total cost, specific activity, availability and the abundance of pre-irradiated material require special consideration in order to produce radionuclides perennially and of clinical quality. Currently, three production routes are used: neutron fission driven in a neutron nuclear reactor (direct and specific production or nuclear waste management), neutron bombardment (thermic neutron capture) also driven in neutron nuclear reactor, and charged particles (protons, deuterons, alpha particles) formed by bombardment in a particle accelerator (usually a cyclotron). In some cases, and for logistic reasons, a parent radionuclide is produced by one of the above ways and then used as a generator of the daughter radionuclide of interest. Some radiometals used in RIT are provided in a no-carrier added (n.c.a.) state in chloridric acid media. For these, it is very important to minimize contamination with trace metals (during production mode, glass contamination etc.) to improve the final radiolabelling yield and the specific activity of the final radiopharmaceutical mAb.

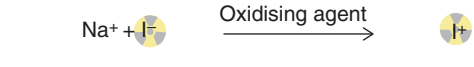
29.3.2 Labelling Techniques

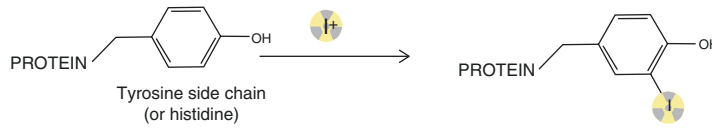
Historically, radiohalogens such as iodine radioisotopes were the first used for RIT applications. Iodine can react directly with proteins following oxidation from iodide (I^-) to I^+ form [33]. In this

case, the I^+ form reacts with the aromatic moiety of amino acids like tyrosine or histidine residues of the polypeptide chain (Fig. 29.1). This effective method nevertheless presents some disadvantages, and can't be used when the mAb is sensitive to oxidizing environments, when the radiolabelled tyrosine is close to the mAb affinity site (near to the complementarity determining region—CDR) or when the mAb is metabolized, resulting in the release of free iodine leading to nonspecific irradiation of normal organs such as the thyroid gland.

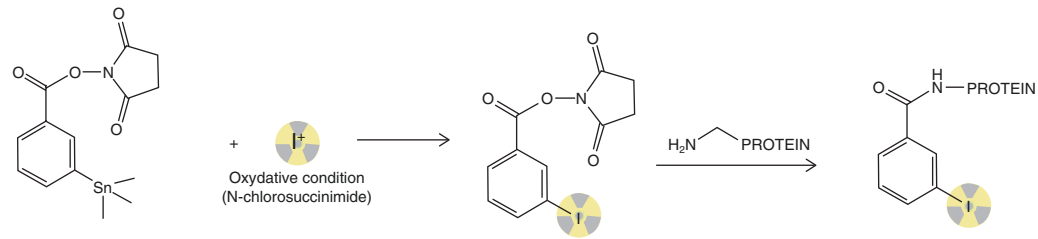
To circumvent the limitations of direct radiohalogenation of mAbs, immunochemists have developed several radiolabelling approaches using prosthetic groups like Bolton-Hunter reagent, organostannyl compounds or iodonium salts (Fig. 29.2). These groups are generally transformed into bioreactive compounds capable of forming covalent bonds with the protein [34, 35].

For radiometal isotopes, direct radiolabelling is also possible. For technetium or rhenium, it is possible to chemically couple via thiol groups after a mild reduction of the mAb, but the indirect radiolabelling is generally preferred. Radioactive metals can form very stable coordination complexes with a variety of ligands, including linear diethylenetriaminepentaacetic acid (DTPA) derivatives or macrocyclic 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA) polyaminocarboxylic derivatives (Fig. 29.3) [36]. These ligands are generally transformed into bifunctional compounds (bifunctional chelator agent—BCA) capable of reacting with proteins to form a covalent bond with lysine residues (activated esters or isothiocyanates), cysteine residues (maleimide) or synthetic bioorthogonal residues to perform click chemistry [37]. The chemistry of BCA compounds is an important development area, where the goal is to limit the transmetalation and transchelation phenomena that could occur in vivo when the radiopharmaceutical is in competition with metal complexed proteins such as transferrin or ceruloplasmin [38].





Oxidising agent = iodogen, chloramine-T, lactoperoxydase. N-halosuccinimide,...



Activated Tin Ester (ATE)

Iodinated Activated Tin Ester (IATE)
Succinimidyl Iodo Benzoate (SIB)

Iodinated protein

Fig. 29.1 Direct iodination of a monoclonal antibody

Fig. 29.2 Indirect iodination of a monoclonal antibody

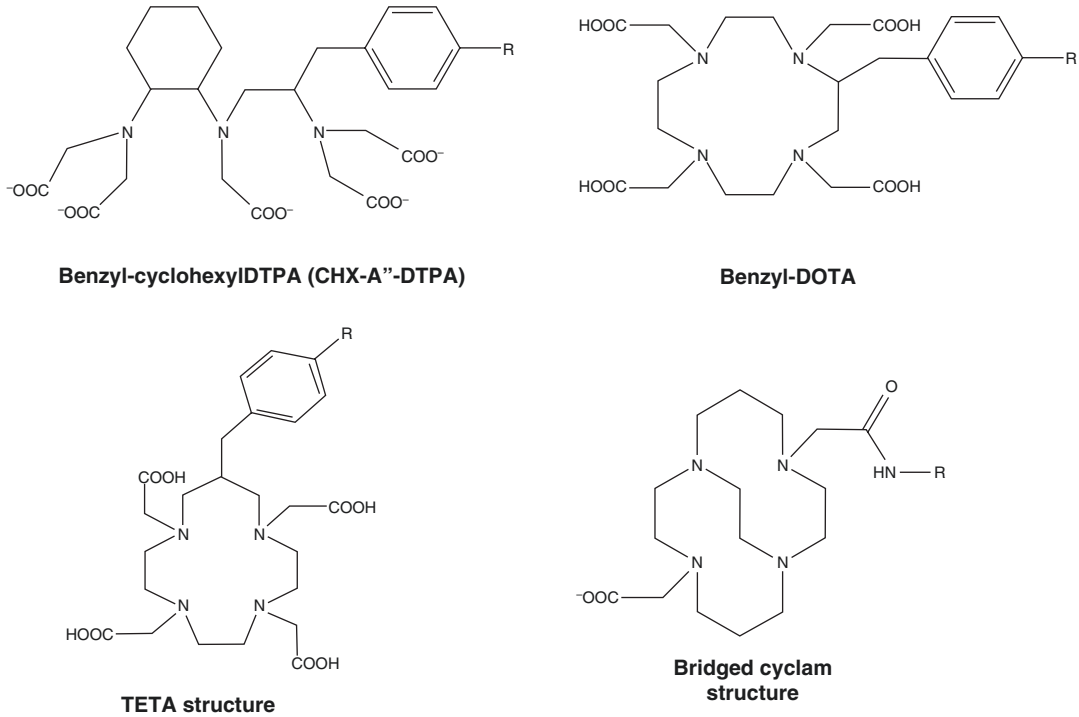


Fig. 29.3 Structures of chelators for complexation of radiometals

Chelating agents with high affinities and high kinetic stabilities are under development. The best approach is to limit this in vivo phenomena, requiring better chelation agent selection in order to improve both selectivity and stability. This choice integrates a stability constant and dissociation kinetic values which have to be for the latter as low as possible.

29.4 Treatment of B-Cell Lymphoma with Anti-CD20 Antibodies

Bexxar® and Zevalin® are administered 6–8 days after a pre-dose of cold mAbs, respectively, 2 × 450 mg of tositumomab and 2 × 250 mg of rituximab, to improve biodistribution and tumour targeting. Bexxar® and Zevalin® can be integrated in clinical practice using non-ablative doses for the treatment of patients with relapsed or refractory follicular lymphoma (FL) or as consolidation after induction chemotherapy in the front-line treatment in FL patients. Haematologic toxicity is the major side effect of RIT and depends on the extent of bone marrow involvement and prior treatment. Non-haematologic toxicity is generally low. Secondary myelodysplastic syndrome or acute myelogenous leukaemia (AML) was reported in 1–3% of cases [39–42]. The risk appears to be increased in patients previously treated by several lines of chemotherapy or radiotherapy. In a meta-analysis involving relapsed B-cell lymphoma patients treated with Zevalin® in four clinical trials, long-term responses (time-to-progression (TTP) > 12 months) were seen in 37% of patients [41]. At a median follow-up time of 53.5 months, the median TTP was 29.3 months. One-third of these patients had been treated with at least three previous therapies, and 37% of them had not responded to their last therapy. The estimated 5-year overall survival (OS) was 53% for all patients treated with Zevalin® and 81% for long-term responders. Using Bexxar®, a long-term meta-analysis performed on 250 heavily pretreated patients with indolent lymphoma

treated in five clinical trials, the objective response (OR) rates ranged from 47% to 68% and complete response (CR) rates from 20% to 38% [43]. Interestingly, poor prognostic patients showed durable responses (bone marrow involvement in 41%, bulky disease ≥5 cm in 49% and transformed histology in 23%).

Clinical results showed that Zevalin® or Bexxar® had a significant efficacy but moderate response duration as monotherapy in rituximab-refractory recurrence of FL. A higher therapeutic impact may be achieved using Bexxar® or Zevalin® in other indications. Recent studies showed that RIT can be administrated as a high-dose treatment. This approach consists of injecting a myeloablative activity of RIT or combining standard or escalated RIT activities with high-dose chemotherapy. In a recent prospective multicentre study, Shimoni et al. demonstrated that standard-dose Zevalin® (0.4 mCi/kg) combined with BEAM high-dose chemotherapy was safe and possibly more effective than BEAM alone as a conditioning regimen for stem cell transplantation (SCT) in 43 patients with relapsed/refractory aggressive non-Hodgkin lymphoma [44]. The 2-year progression-free survival (PFS) was 59% and 37% in the Z- BEAM and BEAM arms, and the 2-year OS was 91% and 62%, respectively.

RIT can also be administered as consolidation after induction therapy. The FIT randomized phase III trial *showed the benefits of* Zevalin® *as consolidation in* previously untreated FL patients [45]. A high conversion rate from partial response (PR) to CR of 77% was observed after RIT, leading to a high CR rate of 87%. Moreover, different studies suggest that RIT is a relevant option as consolidation therapy in different subtypes of B-cell lymphoma such as diffuse large B-cell or mantle cell lymphoma, in order to decrease the number of chemotherapy courses in elderly patients or as an alternative for stem cell transplantation in high-risk patients [46–49]. In 2014, Hohloch et al. published the results of 215 patients registered in the international RIT network. The median age of the patients was 62 years (range of 17–88), with 27% above the age of 70 years. Zevalin® was mainly used as consolidation after first-line or second-line chemotherapy (56.1%) The OR rate for the entire population was 63.3%. The complete response rate was 76.4% in patients treated as part of first-line therapy and 44.3% in patients with relapse.

RIT can also be considered alone in front-line treatment. Scholz et al. evaluated, in an international multicentre phase II clinical trial, the efficacy and feasibility of Zevalin® as first-line treatment in 59 FL patients [50]. Treatment indication resulted from B symptoms, grade 3A, organ compression or infiltration, rapid growth and/or bulky disease. The OR rate at 6 months after RIT was 87%, with 41% of the patients achieving CR, 15% unconfirmed CR, and 31% PR. Median PFS was 25.9 months. RIT was well tolerated, and the most common toxicity was haematologic and reversible.

Despite these promising results, RIT has failed to be widely adopted by haemato-oncologists [51]. In an interesting recent review on the treatment of lymphoma by RIT, Illidge regretted the low implementation of RIT in current clinical practice [52].

29.5 Promising Results for Hemopathies Using Other Antibodies

29.5.1 Targeting of Lymphoma with Anti-CD22 Antibodies

For lymphoma, targeting antigens other than CD20 using rituximab appears relevant, offering the possibility of targeting populations of cells not expressing CD20, or not responding to cold anti-CD20 mAbs. CD22 is a transmembrane glycoprotein expressed on mature B cells but not expressed on stem cells or plasma cells and functions in B-cell regulation/activation. CD22 is highly expressed across malignant B-cell histologies. The anti-CD22 mAb epratuzumab is well suited for RIT because it is humanized, internalized by target cells, stably labelled using DOTA and administered without a loading dose of cold antibody, in contrast to Zevalin® or Bexxar® [53].

^{90}Y-epratuzumab RIT has been improved by the use of repeated injections [54–56]. A multi-

centre phase I/II study was designed to assess fractionated ^{90}Y-epratuzumab in NHL relapsing patients [56]. Sixty-four patients with 1–5 prior therapies (median: 2) with different B-cell lymphoma histologies were enrolled. The total ^{90}Y activities ranged from 0.185 to 1.665 GBq/m^2, with comparable numbers treated at ≤0.37 (n = 17), >0.37–0.74 (n = 13), >0.74–1.11 (n = 16) and >1.11 GBq/m^2 (n = 18). Even at the highest total ^{90}Y activity of 1.665 MBq/m^2, grade 3–4 haematologic toxicities were manageable with support for patients with <25% bone marrow involvement. The overall OR rate was 62% (48% CR/unconfirmed CR). For FL patients without prior SCT, response rates increased with total ^{90}Y activity, with 92% CR/unconfirmed CR at the highest dose levels (>1.11 MBq/m^2). Patients with CR/unconfirmed CR achieved long-lived responses continuing up to 5 years, including 24.6-month median PFS for 12 FL patients receiving >1.11 MBq/m^2 total ^{90}Y activity.

Targeting of antigens other than CD20 appears particularly interesting in the context of consolidation therapy after rituximab-based therapy. A French phase II trial sponsored by the LYSA group is ongoing and is assessing front-line treatment using fractionated RIT with ^{90}Y-epratuzumab as consolidation therapy after chemoimmunotherapy in bulky or stage III/IV aggressive B-cell lymphoma. Another important perspective is the clinical evaluation of dual-targeted antibody/radioantibody therapy [53, 57, 58]. Combining an unconjugated anti-CD20 antibody therapy with a radioimmunoconjugate binding to a non-competing antigen might improve responses by allowing optimal uptake of each agent [58, 59]. Preclinical studies showed that efficacy increased when consolidation using anti-CD20 veltuzumab was delivered after anti-CD22 RIT [59]. The injection of cold mAb after the radioactivity dose provided higher efficacy than injection before RIT, and the amount of pre-dose cold mAb could be minimized [53, 58]. Thus, a re-examination of RIT in the treatment of B-cell lymphoma was proposed [57], emphasizing that in RIT clinical practice, nearly 900 mg of unlabelled anti-CD20 IgG antibody is pre-dosed to the patient before the anti-CD20 ^{90}Y or ^{131}I RIT.

29.5.2 Targeting of Multiple Myeloma Using Anti-CD138 Antibodies

Multiple myeloma (MM) is a malignant plasma cell disorder characterized by the proliferation of clonal cells in the bone marrow and in extramedullary sites at later stages of the disease [60]. The annual incidence is 4–6 cases per 100,000. The median survival of this previously incurable disease has markedly improved over the last decade due to the extensive use of high-dose therapy and autologous stem cell transplantation in younger patients and to the broad introduction of novel agents, i.e. thalidomide, bortezomib and lenalidomide used in combination with dexamethasone or alkylating agents [61]. Other drugs such as histone deacetylase inhibitors (vorinostat, panobinostat) or mAbs (elotuzumab) are under development and assessment in large prospective phase II or III studies [62].

Numerous immunotherapy approaches targeting MM cell surface antigens have been tested. Preclinical and clinical trials have been conducted with naked mAbs having an intrinsic cytotoxic action, interfering with ligand binding or involved in angiogenesis. Anti-CD20 rituximab [63], anti-CD38 [64], anti-CD54 [65], anti-CD74 [66], anti-CD317 [67, 68] and anti-CD319 [69] have been assessed as monotherapies or in combination with other therapeutic drugs or in preparation for autologous SCT. Because IL-6 is a major autocrine/paracrine growth factor for MM cells, immunotherapy with anti-IL-6 mAbs has been performed. A transient tumour cytostasis was obtained, which did not cure the tumour [70, 71]. Finally, Lee et al. have shown the expression of CD66a but not of other CD66 isoforms in MM. These findings open the possibility of using mAbs against members of the carcinoembryonic antigen (CEA) and immunoglobulin superfamily in RIT [72]. Erba et al. have performed an RIT clinical trial using a ^{131}I-L19SIP mAb specific to the EDB domain of fibronectin, reporting a stabilization of the disease in two patients at an advanced stage of MM [73]. Among targeted antigens, CD138 [74, 75] and CD38 [76] seem interesting as they are currently used as

standard markers in many laboratories for the identification and purification of myeloma cells. The feasibility of anti-CD138 (syndecan-1) RIT using ^{131}I-B-B4 was also reported, with encouraging dosimetry results [74]. Syndecan-1 belongs to the family of heparan sulphate bearing proteoglycans. Expressed on the epithelia, this molecule is also present on pre-B cells and plasma cells, and it plays an important role in regulating MM [77]. Syndecan-1 is expressed in all MM tumours within the bone marrow and is present at relatively high levels on MM cell surfaces [77–80].

In MM, tumour cells are mostly disseminated in the bone marrow either as isolated cells or as microscopic tumour cell clusters. Beta emitters with relatively long path lengths (1 mm to 1 cm) are not very suitable to target such isolated cells. In contrast, the high-linear energy transfer characteristics of alpha particles enable localized irradiation whilst preserving surrounding tissues, and cell toxicity is achieved with only a few disintegrations at the cell surface. In vitro and preclinical studies demonstrated the promising therapeutic efficacy of ^{213}Bi-labelled anti-mCD138 for the treatment of MM [75, 81]. CD138 targeting with a mAb coupled to a radionuclide emitting alpha particles thus represents a potential new therapeutic option for MM, and the use of alpha emitters with longer half-lives, such as ^{211}At (7.2 h), should be evaluated in the clinic.

29.6 RIT of Metastatic Prostate Cancer

PCa accounted for an estimated 70,347 deaths in Europe in 2013 [82]. Up to 40% of patients eventually develop metastases despite local therapy. Once metastases have developed, PCa is incurable, and all therapy is palliative. Medical castration is highly effective in shrinking tumour burden, decreasing prostate-specific antigen (PSA) levels, enhancing the quality of life, and improving survival [83]. However, most patients evolve towards progression despite castration, with a median duration of response of 12–24 months [83]. At the stage of castration-resistant PCa (CRPC), cytotoxic chemotherapy was the only therapy [84, 85] until 2012, when the European Medicines Agency (EMA) approved the use of abiraterone acetate before docetaxel. Within the past year, three new drugs were FDA approved for the treatment of patients with CRPC (cabazitaxel, sipuleucel-T and denosumab). However, the survival benefit of these drugs in CRPC is modest: respectively +2.4, +4.1 and +3.6 months, and more efficacious drugs are needed.

Radiotherapy is an established treatment for clinically localized PCa or for palliation of painful bone metastasis [86]. PCa is a solid malignancy for which RIT may be favourably used because it is a radiosensitive tumour with typical distribution to sites with high exposure to circulating radiolabelled mAbs (bone marrow and lymph nodes). In preclinical and clinical PCa therapy studies, radionuclides have been linked to antibodies or peptides with affinity to mucin, ganglioside (L6), Lewis Y (Ley), adenocarcinoma-associated antigens and prostate-specific membrane antigen (PSMA) [87–90], but PSMA appears to be the most specific.

PSMA is non-secreted type II integral membrane protein with abundant and nearly universal expression on prostate epithelial cells and is strongly upregulated in PCa [91–95]. Pathology studies indicate that PSMA is expressed by virtually all PCa [96]. The level of expression in non-prostate tissues is 100- to 1000-fold less than in prostate tissue [91], and the sites of PSMA expression in normal cells (brush border/luminal location) are not typically exposed to circulating mAbs. De-immunized J591 mAb, which targets the external domain of PSMA, giving an easy and rapid access to the antigen, seems to be the best clinical candidate for imaging and therapy of PCa [97, 98].

A phase I trial assessing ^{111}In/^{90}Y-J591 was performed in 29 patients [99]. Dose-limiting toxicity was seen at 740 MBq/m^2, and 647.5 MBq/m^2 was determined as the maximum tolerated dose (MTD). The overall targeting sensitivity of bone and soft tissue metastasis was 81%.

Decrease of PSA was observed for two patients as an objective measurable disease response with a decrease of lymph node size.

Thirty-five patients were enrolled in a ^{177}Lu-J591 phase I trial [100]. The 2590 MBq/m^2 level was determined as the MTD. Repeated dosing, with up to three doses of 1110 MBq/m^2, could be safely administered. Clearly identified sites of metastatic disease were successfully imaged by ^{177}Lu-J591 scintigraphy in 100% of patients. The median duration of PSA stabilization, after treatment, was 60 days with a range of 28–601 days. No immune response was detected. A phase II ^{177}Lu-J591 trial was initiated in CRPC patients (ASCO congress 2008). Fifteen patients (cohort 1) were treated with 2405 MBq/m^2. The second cohort (2590 MBq/m^2) enrolled 17 patients expanded to 15 patients (ASCO congress 2013). Sensitivity of known metastasis targeting was 93.6%. Reversible thrombocytopenia and neutropenia toxicity occurred, respectively, in 46.8% and 25.5% of patients. The second dose cohort (2590 MBq/m^2) showed not only higher PSA responses (46.9% vs. 13.3%, $p = 0.048$) associated with a longer survival (21.8 vs. 11.9 months, $p = 0.03$) but also more reversible haematologic toxicity.

These trials provide the support that radiolabelled de-immunized J591 is well-tolerated and non-immunogenic. Radiolabelled J591 effectively targets PCa metastases with high sensitivity and specificity, reduces PSA and declines with a dose-effect relationship.

29.7 RIT with Alpha-Emitting Radionuclides

Alpha particles emit a high LET of approximately 100 keV/μm and deliver a high proportion of their energy inside the targeted cells (the range in tissue is short and less than 100 μm), leading to highly cytotoxic effects on tumour cells [101, 102]. In vitro studies have demonstrated that between 1 and 20 cell nucleus traversals by alpha particles are sufficient to inactivate a cell as compared to thousands or tens of thousands for the same effect with beta minus particles. Alpha particles create multiple DNA double-strand breaks and have been shown to be independent of both dose rate and oxygenation of the irradiated tissue [103].

29.7.1 Therapeutic Indications

Related to these characteristics, the use of alpha-emitting RIT offers a promising alternative way to treat various cancer pathologies and making them particularly suited for the therapy of isolated tumour cells and minimal residual disease (MRD), micrometastatic diseases or haematologic tumours. Despite the discovery of alpha-emitting radionuclides in the early twentieth century, the first alpha-RIT clinical trial was performed in 1997 [104]. This first clinical trial application of α-RIT was performed with an anti-CD33 humanized monoclonal antibody labelled with Bi-213. The CD33 antigen is a 67 kDa glycoprotein overexpressed in most acute myeloid leukaemias (AML), and the ^{213}Bi-antiCD33 mAb was administered in 18 patients with AML. The results showed a reduction in circulating blasts in most patients (~80%), whereas no extramedullary toxicity was observed. More recently, several clinical trials were initiated to treat lymphomas [105], melanomas [106], glioblastomas [30] and ovarian carcinomas [18, 107]. These α-RIT clinical trials appear very promising, and larger phase II clinical trials have to be performed to fully demonstrate efficacy. Using α-emitters for therapy remains a challenge, even though $RaCl_2$ is routinely used for pain palliation and bone metastasis in castrated resistant prostate cancer (CRPC) patients, and large clinical trials will require high production and accessibility of α-emitting radionuclides [108–110].

29.7.2 Limited Availability

More than 100 α-emitting radionuclides are currently known, but once selected for appropriate characteristics, less than ten have been evaluated. The most promising are astatine-211, the lead-212/bismuth-212 generator, bismuth-213,

radium-223, actinium-225 and thorium-227. These isotopes are generated in association with nuclear weapon production, nuclear fuel waste reprocessing and cyclotrons. The major supply problem, which will need to be solved before their routine use in α-RIT, relates primarily to the low level of isotope production currently possible. For example, only a few centres in the world are able to produce Bi-213 [111], and combining all current production sources would permit annual treatment of only 200 patients. Astatine-211 and actinium-225 could be produced more easily by cyclotrons [112, 113]. This issue of availability was clearly identified, and recent analysis from the United States Department of Energy emphasized the need to develop infrastructures to produce α-emitting radionuclides. Currently, both actinium-225 and astatine-211 appear to be the most promising α-emitting radionuclides. Actinium-225 could be produced in a cyclotron from a radioactive target (radium-226) under a $^{226}Ra(p,2n)^{225}Ac$ reaction, whilst astatine-211 is produced from a natural and non-radioactive target (bismuth-209) by a $^{209}Bi(\alpha,2n)^{211}At$ cyclotron reaction.

29.7.3 Issues and Current Developments

The cytotoxic effects of α-particles provide very interesting opportunities for improving RIT efficacy in certain indications. The first clinical trials showed good efficacy and a good toxicity profile. The indications where α-RIT seems to be efficient are physically limited to a small cluster of tumour cells (micrometastasis, haematologic diseases, MRD), but this limitation could be overcome by therapeutic association with chemotherapy to obtain cytoreduction prior to α-RIT use. For the same reasons, multi-α emitters like actinium-225 which successively emit four α particles in their decay scheme may permit longer irradiation targeting larger tumours burdens.

Different optimization strategies like pretargeting or fractionated approaches may be used to enhance the therapeutic window (i.e. increasing the tumour-to-organ ratio in terms of activity delivery). In the same way, the use of short-lived α-emitting radionuclides like astatine-211 coupled to a mAb with a good pharmacokinetic profile allows to optimize the tumour-to-healthy tissue dosimetric contrast.

Finally, knowledge of α-particle radiobiologic subcellular effects is increasing, and different models for target organs (bone marrow, kidneys) are being developed to determine the dose distribution following an RIT treatment [114, 115].

29.8 High Efficacy of Pretargeting Approaches

29.8.1 Metastatic Thyroid Carcinoma

Medullary thyroid carcinoma (MTC) represents less than 10% of all thyroid carcinomas. Prognosis of metastatic disease varies from long- to short-term survival. Among the various prognostic parameters, advanced age, stage of the disease, EORTC prognostic scoring system mutations in the RET oncogene and association with multiple endocrine neoplasia (MEN) 2B are commonly accepted as prognostic factors [116–120]. Moreover, Barbet et al. demonstrated that calcitonin (Ct) serum level doubling times (DT) were an independent predictor of OS [121]. In this study, all the 41 patients with Ct DT >2 years were still alive at the end of the study, 2.9–29.5 years after the initial surgery. Eight patients (67%) with DT between 6 months and 2 years died of the disease 40–189 months after surgery, and all 12 patients with Ct DT < 6 months died of the disease 6 months to 13.3 years after the initial surgery. Giraudet et al. confirmed the prognostic value of biomarker DT in metastatic MTC [122].

Targeted therapy using multikinase inhibitors can be applied in progressive patients, and vandetanib has been approved [123–128]. MTC cells express high levels of CEA, and anti-CEA radiolabelled mAbs have shown promising results [129, 130]. Pretargeted RIT (pRIT) was developed to improve the tumour-to-normal tissue ratios and to deliver increased tumour absorbed doses to relatively radioresistant solid tumours.

This involves an initial injection of an unlabelled bispecific monoclonal antibody (BsmAb), followed by a second injection of a radiolabelled bivalent hapten-peptide [131–135]. Using this system, the radiolabelled bivalent peptide binds avidly to the BsmAb attached to the CEA antigen on the cell surface, whereas non-targeted hapten-peptide in the circulation clears rapidly through the kidneys.

A phase I/II clinical trial began in 1996 to evaluate pRIT using the murine anti-CEA × anti-indium-DTPA F6x734 BsmAb and a bivalent indium-DTPA hapten labelled with iodine-131, in 26 metastatic MTC patients [136]. Good tumour targeting was observed. Dose-limiting toxicity was haematologic, and the maximum tolerated activity was estimated at 1.8 GBq/m^2 in this population of patients with a high frequency of bone marrow involvement. Some tumour responses were observed, mainly in patients with a small tumour burden and after repeated courses of pRIT. Because of relatively high haematologic toxicity and frequent immune responses, the chimeric hMN-14 × m734 BsmAb was developed and assessed in a prospective phase I study performed in 34 patients with CEA-expressing tumours to determine the optimal BsmAb dose, hapten activity, and pretargeting interval [137]. A BsmAb dose of 40 mg/m^2 with a pretargeting interval of 5 days appeared to be a good compromise between toxicity and efficacy. HAMA elevation was observed in 8% of patients and HAHA (human anti-human antibody) in 33%.

In 2006, OS of a series of 29 MTC patients involved in the two phase I/II pRIT trials was retrospectively compared with that of 39 contemporaneous untreated patients (data collected by the French endocrine tumour group, GTE) [138]. A second objective was to examine whether post-pRIT Ct DT variation was a surrogate marker for survival. Patients with Ct DT < 2 years were considered as high-risk patients. This study showed that OS was significantly longer in high-risk treated patients than in high-risk untreated patients (median OS, 110 vs. 61 months; $p < 0.030$).

Following these encouraging results, a prospective phase II multicentre pRIT trial was designed in progressive MTC patients with Ct DT shorter than 5 years. Forty-two MTC patients received 40 mg/m^2 of hMN-14xm734 and 1.8 GBq/m^2 ^{131}I-di-indium-DTPA hapten 4–6 days later [139]. Disease control according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (objective response + stabilization) was observed in 32 patients (76.2%), including a durable CR of at least 40 months in one patient (2.4%) and durable stable disease (≥6 months) in 31 patients (73.8%). Tumour uptake assessed by post-pRIT immunoscintigraphy was a significant predictor of response. As previously reported, toxicity was mainly haematologic, requiring careful post-RIT blood monitoring. Pre-RIT biomarker DT and impact on DT after pRIT were predictors of OS, confirming the value of serum biomarkers in selecting patients and monitoring therapy.

New generation compounds are available today for pRIT. Humanized, recombinant, trivalent BsmAb (anti-CEA TF2) and bivalent histamine-succinyl-glutamine (HSG) peptides have been produced [140, 141]. The use of TF2, composed of a humanized anti-HSG Fab-fragment derived from the 679 anti-HSG mAb, and two humanized anti-CEA Fab-fragments derived from the hMN-14 mAb (labetuzumab, Immunomedics, Inc.) by the Dock-and-Lock procedure should reduce immunogenicity [140–142]. Moreover, the HSG peptide allows facile and stable labelling with different radiometals, such as ^{177}Lu or ^{90}Y, having favourable physical features that could improve pRIT efficacy [143].

29.8.2 Other Neoplasias

New generation recombinant humanized trivalent BsMAb and bivalent histamine-succinyl-glutamine (HSG) peptides have been produced. These can be labelled with a variety of radionuclides, including yttrium-90 and lutetium-177 for therapeutic purposes [141–143]. This new-generation pretargeting system using anti-CEA × anti-HSG bsMAb TF2 and ^{177}Lu-IMP288 has been performed and optimized in two clinical trials in patients with metastatic colorectal carcinoma and

lung carcinoma [144, 145]. Different schedules were studied to define the optimal molar doses of TF2 and IMP-288 and the optimal delay between the two infusions.

Three cohorts of three patients were included in the first part of a phase I/II clinical trial designed to optimize and assess anti-CEA × anti-HSG bsMAb TF2 in CEA-expressing lung cancer patients. Patients underwent a pre-therapeutic imaging session S1 (44 or 88 nmol/m^2 of TF2 followed by 4.4 nmol/m^2, 185 MBq, of ^{111}In-IMP288) and, 1–2 weeks later, a therapy session S2 (240 or 480 nmol/m^2 of TF2 followed by 24 nmol/m^2, 1.1 GBq/m^2, ^{177}Lu-IMP288). The pretargeting delay was 24 or 48 h. According to the pharmacokinetic and imaging analysis, the best dosing parameters corresponded to the shorter pretargeting delay (24 h) and to the highest TF2 molar doses. Whilst toxicity was quite limited in the eight patients evaluated, treatment efficacy was minimal in this optimization part of the study, with only two cases of disease stabilization for only short periods of time [145]. Thus, to improve treatment efficacy, the injected activity should be increased for the second part of the study, which is planned with an activity escalation. Overall, it was not expected that a single therapy cycle would be sufficient to deliver antitumour therapeutic doses, and the use of shorter half-life and higher intrinsic toxicity radionuclides, such as yttrium-90, could be preferable to that of lutetium-177. Taking into account these data, a prospective phase-I study is on-going, to assess fractionated injection of ^{90}Y-IMP288 in metastatic colorectal carcinoma patients.

29.9 Immuno-PET: The Future for Dosimetry Assessment and Patient Selection

For more than two decades, mAbs have been labelled with gamma-emitting radionuclides, such as ^{131}I or ^{111}In and subsequently used in planar or single-photon emission computed tomography (SPECT) imaging procedures. Whilst providing reliable and confident information, this modality suffers from several drawbacks including poor sensitivity, poor spatial resolution and complex scatter correction due to the collimator. Accurate quantitative information could be better achieved using PET for mAb imaging (immuno-PET). Indeed, immuno-PET has several advantages over conventional immunoscintigraphy with gamma-emitters. The improved spatial resolution makes the delineation of tumours and organs better compared with SPECT. Additionally, an exact attenuation correction, a precise scatter correction and, last but not least, a high sensitivity combined with the possibility to perform true whole-body imaging in a reasonable time constitute the key factors for the superiority of PET over SPECT or planar imaging. Immuno-PET images also take advantage of new advances in PET detectors [146, 147] and reconstruction algorithms [148]. Both spatial resolution and signal-to-noise ratio are greatly improved with these developments. The performance of immunotargeting depends on the choice of the mAb (specificity, affinity, dose) and the radionuclide. Combining mAb and PET emitters requires an appropriate match between the biologic half-life of the protein and the physical half-life of the isotope [149–151]. Table 29.1 shows different relevant PET emitters. The use of ^{18}F or ^{68}Ga with a short half-life is limited to small-size molecules such as antibody-based fragments or pretargeted peptides which distribute rapidly in the body [152–156], whereas ^{89}Zr [157, 158] and ^{124}I [159–161] are well suited to the labelling of large molecules such as intact mAbs. Copper-64 with an intermediate half-life of 12.7 h can be used for labelling of a large number of molecules with different sizes. Within the scope of a "theranostic" approach, pairs of beta+/beta-emitting radionuclides (^{124}I/^{131}I, ^{86}Y/^{90}Y, ^{64}Cu/^{67}Cu, ^{44}Sc/^{47}Sc) are very promising because the same distribution is expected both for imaging dosimetry and therapy with the same elements. Several added values for immuno-PET imaging have been highlighted [149–151].

29.9.1 Immuno-PET and Development of New Drugs

PET could provide information about tumour targeting, pharmacokinetics, accumulation in critical normal organs or optimal dosing. Immuno-PET constitutes a powerful tool to characterize new antibody-based drugs in early stages of development (phase 0/I/II) and then makes it easier to design phase III trials with the most promising mAbs [150, 151]. For example, it has been demonstrated recently that immuno-PET could be useful for visualizing CD138-expressing tumours with ^{124}I-B-B4 in the context of treatment of metastatic triple-negative breast cancer that cannot benefit from hormone therapy or anti-Her2/neu immunotherapy [162].

29.9.2 Patient Selection for Therapy

Until now, only invasive methods such as biopsies followed by immunohistochemical analysis could identify patients with lesions that had the highest chance of success with antibody-based therapy. Immuno-PET can offer a non-invasive solution to quantitatively assess target expression. For example, anti-Her2 therapeutic agents are only effective in patients who have Her2-positive breast cancer as determined by immunohistochemistry. It has been proven that mAbs labelled with ^{68}Ga or ^{89}Zr could non-invasively identify those lesions that are likely to respond to therapy [153, 163]. It is also a powerful innovation for improving knowledge about the efficacy and in vivo behaviour of mAbs. Based on immuno-PET, the treatment strategy could be tailored for individual patients before administering expensive medicines [164].

29.9.3 Determination of the Cumulated Activity Concentration for RIT

A study assessing a humanized A33 mAb labelled with ^{124}I in colorectal cancer clearly demonstrated in a clinical setting that the tissue concentration as measured by PET imaging and as derived from ex vivo measurements in a gamma-counter agreed well [165]. This offers a unique opportunity to determine the maximum injected activity considering the dose-limiting organs like bone marrow [150]. Similarly, the injected activity could be adapted for each patient given the desired dose to the tumour when mAb imaging is used as a prelude for RIT [166]. As an example, it has been shown that ^{90}Y-Zevalin distribution could be predicted by ^{89}Zr-Zevalin [167]. Thus, immuno-PET holds promise for allowing comparisons between different dosing regimens and mAb constructs [168].

29.9.4 Therapy Response

Immuno-PET represents a non-invasive technique for monitoring mAb-based therapy or other therapies by measuring early changes in biomarker expression before being detected using MRI or CT. For example, ^{89}Zr-ranibizumab-PET was found to be a potential VEGF-PET tracer allowing the visualization and quantification of VEGF signalling [169]. Moreover, immuno-PET could also be exploited as a new tool when multi-observation image analysis is considered. This emerging field aims at merging several PET acquisitions to assess tumour characterization (as metabolic volume, uptake variations or heterogeneity). The information brought by immuno-PET is complementary to other existing PET tracers and may certainly help to better stratify patients and eligibility to mAb therapy. A pilot study was recently proposed to assess this [170].

29.10 Conclusion

RIT appears as a most promising targeted therapy in the treatment of hemopathies and solid tumours, especially at the stage of MRD. For B-cell lymphoma, clinical results show that RIT has significant efficacy but moderate response duration as monotherapy in rituximab-refractory B-cell lymphoma. A higher therapeutic impact

may be achieved using RIT in myeloablative treatment, as consolidation after chemoimmunotherapy, or as a first-line treatment. Randomized phase III clinical trials should be performed in naïve or minimally treated patients to better identify the benefits and the role of RIT in B-cell lymphoma in the era of rituximab based-therapy.

For solid tumours, RIT should be developed in combination with several other drugs and in reiterated courses of treatment, just as chemotherapy is used. Today, in many cases, RIT is still assessed in the clinic as single agent, even if preclinical studies have shown synergy between RIT and chemotherapy or antiangiogenic agents. Immuno-PET and dosimetry studies could probably help to select patients for RIT and optimize the injected activity. Finally, RIT may have the potential of killing the last tumour cells, now identified as chemoresistant and radioresistant tumour stem cells. This may require the combination of all possible new developments, including new antibody specificities, pretargeting, fractionated administration and the use of alpha-emitting radionuclides.

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30 Radiation and Immunity: Hand in Hand from Tumorigenesis to Therapeutic Targets

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30.1 Introduction

Since the mid-twentieth century when linear no-threshold (LNT) theory developed [1], interest in understanding the biological mechanisms underpinning the link between radiation and cancer has been exponentially increased. As its name implies, the theory does not consider a threshold of radiation dose above which radiation becomes tumorigenic. However, the origins of the LNT appear to lie in the assumption that any doses of radiation are tumorigenic and the more the radiation dose, the higher the risk of cancer. Modern research not only removed the tumorigenic label from low-dose radiation but also related that to the activation of the repair system. Thereby, the body becomes prepared to mount early responses that control the initial DNA damage, block the spread of damage, and prevent genomic instability and tumor evolution. Among them are the immune system responses. The present chapter first enumerates different types and doses of radiation associated with cancer, then would track the role of immunity and radiation as codrivers of carcinogenesis, and finally moves to the effects of immunotherapy and radiotherapy as comanagers of cancer treatment.

30.2 Radiation and Cancer

30.2.1 Space Radiation

The galactic cosmic rays (GCR) are composed of high-energy heavy ions and secondary radiation, e.g., neutrons and recoil nuclei [2]. Due to their high energy density, the ability of shielding to decrease the rate of radiation absorption is still not satisfactory and estimated to be about 25%–35% [2]. Studies determine the absorbed dose Gy (effective dose Sv) for each of lunar mission (180 days), Mars orbit (600 days), and Mars exploration (1000 days) as follows: 0.06 (0.17), 0.37 (1.03), and 0.42 (1.07) [2]. The corresponding mortality rates for men and women are 0.68% and 0.82, 4% and 4.9%, and 4.2% and 5.1% [2]. Because of the scarcity of direct data, researchers rely on rather indirect calculations to predict cancer incidence and mortality rates following space radiation. Different approaches have been developed to reduce the uncertainties surrounding these indirect estimations [3]. For example, the excess relative risk (ERR) model predicts the incidence rates of radiation cancer proportional to background cancer rates, which are mainly affected by age, gender, and tissue. It can be functionally more fitted by including additional variables such as astronaut age at first flight and typically age at exposure. The details of this model and other aspects of space radiation cancer have been recently reported by NASA in [4]. These calculations estimate the risk of cancer following a Mars mission about 400%–600% [5], and consequently, a space mission for more than 90 days is not recommended [5].

30.2.2 Radiation Therapy

The diagnosis of cancer on a previously irradiated tissue is referred to as "radiation cancer." The earliest reports date from the 1930s. As reviewed in [6], radiation cancer of the neck usually develops a long time (mean of 25 years) after irradiation which might be due to thyrotoxicosis or tuberculous lymphadenitis. Occasionally, larynx and thyroid cancer might occur. Pharynx cancer is, however, the best-documented radiation cancer of the neck [6].

More important is, however, the increase in the occurrence of cancers subsequent to radiotherapy for a primary tumor as asserted through meta-analysis studies. When the primary tumor is, for example, located at the prostate, the second malignancies might occur in the bladder, colon, and rectum with HRs of 1.67, 1.79, and 1.79,

respectively [7]. Further, following radiotherapy for breast cancer, these malignancies mainly consisted of lung cancer, esophagus cancer, and sarcomas with corresponding HRs of 1.12, 1.53, and 2.53 [8]. An analysis of long-term outcomes demonstrated that patients would have a more than twofold increase in the risk of lung cancer when 10 years or more have passed radiotherapy for breast cancer [9]. Therefore, the incidence of second cancers seems to increase over time.

Intensity-modulated radiation therapy (IMRT) offers optimization of conventional radiotherapy by means of radiation concentration in tumoral tissues as well as the restriction in delivery of radiation to the adjacent healthy tissues. The development of second cancers is the main sequel to IMRT. Studies estimate that IMRT escalates the incidence of second cancers by 100% and even more in people who could survive primary cancer and live long to be able to be affected by second cancer [10].

30.2.3 Computed Tomography (CT) Radiation

It has been a hot topic of debate during the last two decades. Annually, CT-induced cancer is the leading cause of death for nearly 500 individuals under 15 years of age in the United States [11]. For each CT study, effective radiation dose (ERD) varies across different anatomic areas and types of diagnostic CT examinations [12]. A huge retrospective study of patients diagnosed with leukemia (n = 178 604) and brain tumors (n = 176 587) reveal the direct relationship of these malignancies with a radiation dose of CT scans performed in childhood with corresponding ERRs of 0.036 and 0.023 per mGy [13]. Studies assign the lowest median ERD of 2 mSv to a routine head CT study, whereas the highest median ERD of 31 mSvis is associated with a multiphase abdomen-pelvis CT study [12]. Additionally, radiation exposure associated with CT directly increases with the number of examinations, of course in different ways, with the lowest numbers of examinations associated with cancer for CT coronary angiography and the highest ones for routing head CT [12]. Generally, women seem more susceptible to CT-induced cancer [12]. More significant is the progressive decrease in CT-induced cancer rates with age [12]. There is a twofold increase in cancer rates among people undergoing CT scan at 20 years of age compared to those undergoing CT scan at 40 years of age, who, in turn, show a nearly twofold increase in cancer rates compared to those undergoing CT scan at 60 years of age [12]. In this manner, the highest median CT-induced cancer rate of 1 in 270 is reported for females who underwent CT coronary angiogram at 40 years of age, whereas the lowest median incidence rate of 1 in 14,680 is observed among males who underwent a routing head CT at 60 years of age [12].

30.2.4 High-Frequency (Radio Frequency and Microwave) Electromagnetic Radiation

People exposed to occupationally radio frequencies (RF) and microwaves (MW) generally exhibited higher incidence rates for all types of cancers than those not exposed during a 15-year study period (1971–1985) in Poland [14]. After controlling for the type of cancer, the difference between exposed and unexposed groups remained significant for the following types of cancer: hematopoietic system and lymphatic organ cancers, esophagus and stomach cancers, colorectal, nervous system cancer including brain, and skin cancer including melanoma. The observed/expected ratios (OER) – defined as the morbidity rate among people in the exposed than that among those in the nonexposed group – of 6.31, 3.24, 3.19, 1.91, and 1.67, respectively [14]. Of note, people exposed to RFMW were at greater risk for all types of hematopoietic system and lymphatic organ cancers with the OERs ranging from 2.96 for Hodgkin's lymphoma to 13.90 for chronic myelocytic leukemia (CML) [14].

30.2.5 Low-Dose Nuclear Radiation

To investigate the possible effect of exposure to low-dose nuclear radiation on death from cancer, Cardis and colleagues carried out a longitudinal analysis of data driven from three national cohort studies: the United States, the United Kingdom, and Canada [15]. The authors distributed partici-

pants who were nuclear industry workers into 11 according to the cumulative dose of exposure. Overall, no evidence of significantly higher mortality with increasing exposure existed. However, the mortality rate was shown to increase with increasing exposure dose particularly among patients with multiple myeloma and all leukemia except CLL.

30.2.6 Solar UV-B Radiation (280–320 nm)

The relation of this radiation to cancers is expounded in two main ways. Solar UV-B radiation is demonstrated to decrease the risk of cancers of colon, breast, ovary, prostate, and NHL. On the contrary, it has been revealed to increase the risk of cancers such as bladder, esophagus, kidney, lung, pancreas, stomach, rectum, and corpus uteri and related premature deaths. A study in the United States showed this negative aspect of solar UV-B radiation mainly affects white Americans rather than other ethnic groups such as black Americans and Asian Americans with greater than ten-fold increase in premature cancer mortality rates [16].

30.3 Radiation, Immunity, and Cancer: Cellular Pathways

30.3.1 When Radiation and Immunity Go Hand in Hand to Subvert

Deoxyribonucleic acid (DNA) harbors the effect of external ionizing radiation in mammalian cells from the initial radiation energy deposition and singly DNA base-damaged sites to possible double-strand breaks and eventually radiation-induced mutagenesis [17]. Reasonably, there is a linear relationship between DNA damage and radiation dose. It is rather astonishing that the average tendency of tumoral cells to repair radiation-induced DNA damage is the same as that of non-tumoral cells [18]. However, individual cells show heterogeneity in response to the DNA damage inflicted. Most cells begin to hurriedly revert the damage while there are cells that represent no attempt to repair the damage and even worse are cells attempting to aggravate the initial damage [19]. Transformed cells that possess the remaining DNA damaged sites signal to the innate immune system primarily via NKG2D (natural-killer group 2, member D) receptors. In the following, the immune cells, e.g., natural killer (NK) and T cells [20] expressing these receptors and signaling pathways such as nuclear factor-kappa B (NF-κB) [21] and signal transducer and activator of transcription (STAT) factors [22], begin to engage in the DNA damage response pathway.

Once the DNA undergoes damage that affects its replication or modify chromatin structure, tumoral cells from mice show the upregulation of NKG2D ligands [23]. Such serious DNA-damaging drivers are, for example, high doses of ionizing radiation and ultraviolet light that lead the expression of NKG2D ligands such as ULBP1, ULBP2, ULBP3, and MICA in human cells. Depending on the type of DNA-damaging driver, different serine/threonine-protein kinases act as upstream to the upregulation of NKG2D ligands. In the case of ionizing radiation, ataxia telangiectasia and Rad3-related protein (ATR) appears at least partly responsible for ligand upregulation, whereas ataxia-telangiectasia mutated (ATM) under ultraviolet C conditions. Commensurate with the activation of these kinases, tumoral cells constitutively express NKG2D ligands. After all, the cancer immunoediting process would determine the fate of tumor: elimination (cancer immunosurveillance), equilibrium (cancer persistence/dormancy), or escape (cancer progression) [24]. In the equilibrium phase, tumoral cells are, because of their genomic instability, destined to be in the shuffle between elimination and escape. The elimination of tumor entails innate and adaptive immune responses that mediate cancer cell death while its escape accompanies chronic inflammation. In this manner, inflammation exhibits cancer-promoting activities rather than cancer-preventing activities.

The origins of the link between inflammation and cancer largely lie in the extrinsic (radiation, carcinogen, stress, smoke, and infection) and

intrinsic (genetic and epigenetic changes) circumstances that motivate transcription factors such as NF-Kb [21] and STATs [22]. Ultraviolet (UV) radiation, as an extrinsic factor, leads to the activation of both receptor (JAK-associated cytokine receptors) and non-receptor tyrosine kinases (Src family kinases) that stimulate the phosphorylation ofSTAT3. Upon the activation of this transcription factor, the gene expression of pro-inflammatory mediators (cytokines, chemokines, and COX-2) is upregulated in parallel with the expression of genes that play a decisive role in tumorigenesis. Further, one of the key functions of the kinase ATM is to activate NF-κB. Although important to the expression of pro-survival and pro-senescence genes, the NF-κB is an active pathway in the production of pro-inflammatory mediators and, to a lesser extent, in the induction of pro-apoptotic genes. In this manner, radiation would ram the cellular microenvironment into a series of inflammation-promoting cancer and cancer-promoting inflammation cascades.

30.3.2 When Radiotherapy and Immunotherapy Work Hand in Hand to Treat

Before the cancer begins to disseminate, the possible remedy lies in the in situ collapse of cancerous cells. In this case, local radiation therapy by disintegration of the DNA of cancer cells is useful in the eradication of the primary tumor. It owns the ability to evoke the innate and adaptive immune responses that can seep through the body so that the effect of radiation might be seen at sites distant from primary tumor as well. This effect is referred to as the abscopal effect. Below is a view of the various ways radiation therapy and immune responses reciprocally influence each other.

The cancer-immunity cycle is composed of seven sequential steps: release of cancer cell antigens, cancer antigen presentation, priming and activation, trafficking of T cells to tumors, infiltration of T cells into tumors, recognition of cancer cells by T cells, and killing of cancer cells [25]. Radiotherapies serve as a stimulus to the first step of this cycle. More clearly, the tumoral cells begin to alter their immunogenicity once they sense the presence of radiation. In addition, different immune cells including antigen-presenting dendritic cells, macrophages and myeloid-derived suppressor cells, NK cells, and T cells would be influenced by radiation [26]. Therefore, it should come as no surprise that ionizing radiation is now considered as an immunological adjuvant that would help induction and modulation of immune responses [27, 28]. It generated immune-stimulatory effects including alteration in immunogenicity via the expression of MHC class I, Fas death receptors, NKG2D ligands, and heat shock proteins; activation of cell death-related signaling pathways by inflammatory mediators such as calreticulin, HMGB1, and ATP; and production of pro-inflammatory cytokines, chemokines, and adhesion molecules that assist immunogenic cell death which become successful [27]. Immunomodulatory effects of radiation which are mediated by anti-inflammatory cytokines such as TGF-β and IL-10, chemokines such as stromal cell-derived factor (SDF)-1α, and metabolic enzyme indoleamine 2,3-dioxygenase (IDO) would result in an increased number of regulatory T cells, activation of M2 immunosuppressive macrophages, and ultimately inhibition of immunogenic cell death [27]. Apparently, the complexity of radiation effects on immune responses can be reduced by considering a dose-dependent fashion so that anti-inflammatory, pro-inflammatory, and immunosuppressive effects are respectively observed within the low-, moderate-, and high-dose ranges [21]. The capacity to hit at both DNA and non-DNA targets pretends to be responsible for holding such broad-spectrum activity [29]. In this manner, radiation plays role to maintain the immunological microenvironment of tumors [30] as a determinant of response to therapy [31].

The effects of radiation on the immune system are not indiscriminate, but are carefully immune context-dependent [32]. In an endogenous immune system, the pervasive antitumor influence of radiation on the body includes the induction of tumor antigens and NKG2D ligands. The former would stimulate innate and adaptive immune responses particularly cytokine (IFN--α/β) production and recruitment of lymphocytes

and NK cells to the tumor microenvironment. The latter signal to their receptors expressed by cytotoxic T lymphocytes (CTL) that are, in turn, in aid of immunogenic cell death. In parallel, dendritic cells become mature and responsible for tumor antigen presentation, which is crucial to the induction of effector T-cell responses (antigen-specific CD8+ T-cell responses). In addition, the activation of TLR4-MyD88-HMGB1 pathway in DCs by radiation provides an alternative way to induce cross presentation of tumor antigens and CTL. It further fosters the antigen presentation pathway that radiation, in a dose-dependent fashion, would evoke the expression of major histocompatibility complex (MHC) class I and MHC class II molecules. However, radiotherapies might give the tumoral cells a nudge in the unwanted direction of radioresistance, with increasing the number of regulatory T cells.

On the side of radiation as an adjuvant for immunotherapy, there are evidences that radiotherapies promote the efficacy of adoptively transferred T and NK cells in cancers [32]. Overall, radiation therapy offers a favored strategy to allow access to tumoral nests [33]. Particularly, it reinforces the tendency of transferred T cells to infiltrate into tumor sites possibly through increasing the expression of pro-inflammatory cytokines (IFN-γ), anti-angiogenic chemoattractants (MIG and IP-10), NKG2D ligands, and Fas receptors. To recruit lymphocytes into tumors, IFN-γ arranges various activities such as the expression of MHC class I and ICAM-1 on tumoral cells and activation of STAT1. MHC class I molecule takes part in antigen presentation and cross-presentation, while the expression of adhesion molecule ICAM-1 determines the immunogenicity of tumoral cells. Taken together, MHC class I and ICAM-1 molecules maintain effector functions of T cells: antigen-specific T-cell responses. The first apoptosis signal (Fas) receptors result in the further amplification of signals thatNKG2D ligands send to CTL for cancer cell death. In the case of adoptive NK cell therapy, the role of radiation as an immune adjuvant on tumor control explicitly depends on the radiation dose. High-dose radiation caused NK cells to lose their cytotoxic capacity, whereas low-dose radiation enhanced the NK cell-mediated cytotoxicity. The former problem possibly lies in the sensitivity of NK cells to high-dose radiation. The latter opportunity occurs possibly because of the radiation-directed caspases that act as almost indispensable to the apoptotic machinery [34].

On the side of immunotherapy as an adjuvant for radiotherapy, studies elucidate that immunotherapies improve the efficacy of radiation. It is substantially fulfilled by setting low numbers of regulatory T cells [35], priming antigen-specific CD8+ T-cell responses [36], and stimulating the maturation of dendritic cells (DCs). Frankly, it is important for radiotherapy to optimize immune responses, which would not only contribute to the control of tumor growth but also might facilitate the killing of tumoral cells. Immunotherapy by accomplishing such optimization objectives improves the therapeutic efficacy of radiotherapy. Below present several modes of such accomplishment.

Different types of radiation-related cell death exist: mitotic catastrophe, apoptosis, necrosis, autophagy, and senescence [27]. Overall, necrosis is the most common profile of cancer cell death by radiation therapy. While apoptosis tends to occur from mid to high doses of radiotherapy, necrosis is particularly observed with high or ablative doses. The activation of the canonical pathway of NF-κB by tumor necrosis factor (TNF) and toll-like receptors unleashes a variety of inflammatory mediators within tumoral tissues and its neighboring tissues that underwent necrosis or apoptosis under radiation therapy conditions [21]. It is inclusive of not only overall antitumor immunity but also of some molecules such as damage-associated molecular patterns (DAMP) and apyrase-sensitive nucleotides that take part in the wound responses and pose a key challenge to sustainable antitumor immune responses [27]. Ultimately, the NF-κB pathway processes a reduction in the cellular sensitivity to apoptosis and therefore resistance to radiotherapies appears [37]. Altogether, as reviewed in [27, 38], the radiation-induced necrosis and inflammation might paradoxically contribute to

antitumor immune response and rapid tumor growth and so may become in or out of favor with the host evidences. NF-κB inhibitors have indicated synergic efficacy with radiotherapy in terms of an increase in apoptosis and of a reduction in inflammation [39].

Though both chemotherapy and fractionated radiation had the effect of nullifying the original advantage of ablative radiation therapy to tumor rejection, immunotherapy represented attempts surrounding the priming of T cells and maturation of DCs to amplify that [36]. Supporting this, research reveals no superiority of radiation therapy (comparable efficacy) over surgical resection of the primary breast tumor for improving the overall survival [34], while the combined therapy with anti-CTLA-4 antibodies and fractionated radiation therapy not only eradicated the primary tumor but also prevented lung metastasis and therefore was able enough to enhance the overall survival. The latter appeared to lie in the ability of CTLA-4 blockade to prime antigen-specific CD8+ T cells that promote the immunogenicity of tumor cell death.

Commensurate with its purpose of promoting tumor growth, the cytokine TGF-β serves to slacken NK cell-mediated cytotoxicity in tumoral cells by downregulation of NKG2D ligands [40] and circumvent DC activation induced by radiation. However, there have been reports of high rates of nonresponse and recurrence with radiation therapy alone or even in combination with anti-CTLA-4 antibodies or anti-TGF-β therapy that reflect resistance to the action of these therapies. The main mechanism of resistance seems to lie in the T-cell exhaustion that would prohibit necessary effector CD8+ T-cell responses. An increase in the expression of programmed cell death protein 1 (PD-1) might exacerbate the T-cell exhaustion. Supporting this, addition of anti–PD-1 antibodies has been shown to yield more promising results than when the combinations of radiation therapy with anti-TGF-β [41] or with anti-CTLA-4 antibodies [42] used.

As discussed above, the superior efficacy of the combined approaches consisted of both immunotherapy and radiotherapy might reflect nonredundant mechanisms for each treatment [42]. It marks a shift from isolated treatment with each of radiotherapy and immunotherapy to combined immunoradiotherapy [36] for the management of treatment resistance in cancer.

30.4 Radiation, Immunity, and Cancer: Clinical Implications

30.4.1 Curative Purposes

30.4.1.1 Radiotherapies

Patients with different types of cancer might profit from radiotherapies (alone or combined with other therapeutic options) in different stages of tumor development. For example, if the tumor is not resectable or tumor resection is deemed to be harmful, the stereotactic ablative radiotherapy (SABR) is suggested as a curative-intent therapy to patients with peripheral early-stage non-small cell lung cancer (NSCLC) [43]. Further, different options of radiotherapy, including internal radiotherapy, 3D-CRT, 3D-CRT and TACE, stereotactic body radiotherapy, and charged particle radiotherapy, have been used in patients with advanced hepatocellular carcinoma (HCC) (for review see [44]). Meanwhile, meta-analyses [45] show that radiotherapy concomitant with chemotherapy (chemoradiation) provides patients with cervical cancer a 16% boost in the progression-free survival and a 12% boost in the overall survival compared to when chemotherapy is given alone. It seems that patients with stage I and II are more likely to benefit from chemoradiation. Pooled analyses predict that preoperative administration of radiation with doses of above 60 Gy yields in more than 20% pathological complete response rate and nearly 90% resectability rate in patients with locally advanced rectal cancer (n = 487) [46]. Postoperative radiotherapy also appears effective in patients with early-stage breast cancer in terms of enhancing overall survival and reducing recurrence rates [47].

30.4.1.2 Radionuclide-Bearing Monoclonal Antibody Therapies

Radionuclides represent a potential surface to boost the cytotoxic effect in cancer cells by monoclonal antibodies. As reviewed in [48, 49], among numerous radionuclides available for therapeutic purposes, only iodine-131 and yttrium-90 have been approved by FDA to be used as conjugates to monoclonal antibodies Tositumomab (Bexxar®) and ibritumomab tiuxetan (Zevalin®). These anti-CD20 targets are used to treat non-Hodgkin's lymphoma (NHL).

30.4.2 Prognostic Purposes

A number of immunological markers such as lymphocyte infiltration can be used to predict response to radiotherapy [26].

30.4.3 Complications and Cautions

30.4.3.1 Adverse Events

Roughly speaking, radiotherapy as a standalone treatment approach or as a part of the combined approaches (chemoradiation) would result in an acceptable increase in severe and early adverse events especially hematological and gastrointestinal toxicities [45, 46]. IMPRT is, however, associated with fewer toxicities than conventional 3D-conformal radiation therapy (3D-CRT). Surprisingly, a systematic review recently revealed that the clinical end points in patients with pancreatic cancer would not be significantly improved by IMPRT as compared to 3D-CRT [50].

The aggravation of swallowing disorders by radiotherapy in patients with head and neck cancers is associated with acute as well as chronic serious sequels in feeding [51]. As described in [52], there have been developed different categories of precautions that oncology physicians and radiation oncologists must take to reduce the risk of radiotherapy-induced dysphagia.

In addition, meta-analysis reveals the fear of recurrence among patients with cancer would be instilled by radiotherapy [53].

30.4.3.2 Mortality

Postoperative radiotherapy predisposes patients with completely resected NSCLC (n = 2343) to an increase of 18% in the death risk [54]. In addition, the benefit postoperative radiotherapy brings for patients with early-stage breast cancer is variable, and so, radiation oncologists must beware of selecting potential candidates undergoing postoperative radiotherapy [47].

30.4.3.3 Immunodeficiency

It is immediately possible for radiation therapy to indulge in cytotoxicity not only in cells of the tumor but also in both mature and precursor cells of the immune system including NK cells, B cells, T cells, monocytes, and granulocytes [32]. The higher the radiation dose, the greater the risk of negative effects of radiation on the immune system. Even more worsening is that there are evidences that the acute radiation-induced defect in cellular immune responses might persist for many years after radiotherapy in patients with laryngopharyngeal cancer [55].

30.4.4 Emerging Modern Radiotherapy Protocols

Application of nanomolecules to enhance the efficiency of radiotherapies has been recently investigated. For example, Zhang and colleagues recently reported a 50% increase in the uptake of radiation by GSH-Au nanomolecules Au10–12(SG)10–12 [56].

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Hurdles in Cancer Immunotherapy

31

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31.1 General Hurdles

31.1.1 Limitations of Current Animal Models in Predicting Efficacy of Cancer Immunotherapy Modalities in Human Body

With regard to structural and physiological similarities between humans and animals, novel discoveries are initially evaluated with animal models and subsequently applied to humans. Among clinical trials on novel therapies, 85% fail in the early phase, and only half of those that pass phase III obtain approval for clinical use [1]. Moreover, the greatest failure rates belong to cancer drug trials [2].

Mice are the primary experimental model used in preclinical cancer studies. Nevertheless, there are important interspecies differences in mechanisms of cancer development between mice and humans [3], and thus, human disease may not be precisely simulated by animal models [4]. Although human tumors often develop in a concealed manner during months to years, transplanted tumors in animal models are grown within days that surely cannot show the complexity of human cancer. Besides, xenograft human models used for cancer introduction in animals would induce a stronger response to immunotherapy as the tumor is primarily a foreign antigen to the animal's immune system. Furthermore, the tumor cell lines used for inducing cancer in animal models are produced many years ago, and new animal models with probable evolutions in allelic frequency and alterations in histocompatibility antigens through generations may show stronger immunotherapy response [5]. Even though the production of transgenic mice is costly, they are better models of human cancer and thus are likely to produce more valid results. Also, animal studies with negative results are less likely to get published [6]. Therefore, survival and tumor burden data extracted from single mouse models may show high efficacy of treatment, which is most often not observed in a clinical trial [7]. Weak methodology is another issue with animal models. In animal studies, an unmasked researcher usually handles designing, execution, and data evaluation, which limits the translation of outcomes [8]. In fact, this can lead to the observer-expectancy effect. In addition, some studies report size differences between animal species that can cause some limitations such as limitation in maximal drug volume to be administered and the maximum volume of blood samples to be drawn [8]. Also, there are appreciable interspecies differences in drug metabolism that should be taken into account [9].

Since the evaluation of therapies in an animal model may not exactly mimic human response, researchers should identify important differences between the animal model and humans and also examine animals blindly in their studies (Fig. 31.1).

31.1.2 Complexity of Concepts and Mechanisms Pertaining to Cancer, Tumor Heterogeneity, and Immune Escape

When we are looking at a system in the human body, there are complex interactions between single elements to make it work. Cancer is one of

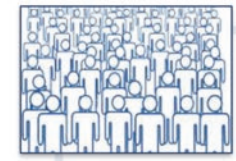
GAP IN BASIC SCIENCE

Insufficient exchange of information

Non-T cell-inflamed phenotype

BASIC SCIENCE TO ANIMAL

1. Instability of bispecific antibodies
2. Self-nature of most tumor antigens
3. Ideal CAR T cell population
4. Dependance on the in-vivo maintenance of T cell populations
5. Insufficient exchange of information



COMMERCIALIZATION

1. Limitation in combination immunotherapy
2. ACT is highly personalized and thus labor-intensive
3. Toxicities

ANIMAL TO HUMAN

1. Insufficient exchange of information
2. Complexity of cancer, tumor heterogeneity and immune escape
3. Lack of valid biomarker for assessment of clinical response
4. Conventional clinical response criteria do not work in immunotherapy
5. Prolonged time to obtain approval
6. Limited funds
7. Paucity of teams of scientists and clinicians
8. Differences related to CAR-T cells immunotherapy in Europe member states
9. Animal model may not predict safety and efficacy of immunotherapy

CONSIDERATION AS STANDARD THERAPY

1. Complexity of cancer and tumor heterogeneity
2. Lack of valid biomarker for assessment of clinical response
3. Conventional clinical response criteria do not work in immunotheraphy
4. Robust and cost-efficient manufacturing
5. CAR T cells dosing as a dividing drug
6. Immune regulatory cells in the tumor microenvironment
7. Low levels of co-stimulation
8. T cell allergic / T cell exhaustion
9. Tumor evasion / Tumor resistance
10. Immunoediting
11. Toxicities

Fig. 31.1 Glance over the potential hurdles that cancer immunotherapy is confronted in the different phase of clinical research

the most complex biological systems and involves abnormal genetic and epigenetic networks. Cancer develops almost always forming a single cell in multiple steps and microevolutionary processes, in which independent events lead to the accumulation of gene mutations over time. However, human tumors often exhibit prominent heterogeneity in many morphological and physiological characteristics [10] that determine tumor behavior, biologic intercellular interaction, and aggressiveness and might be very difficult to be distinguished in the molecular level. In fact, genetically different tumor cell clones present simultaneously within the same tumor mass, and there might be hundreds of different mutations in each cell. This complexity greatly influences therapeutic response in different patients. As a result, cytotoxic drugs may have a divergent effect on cancer clones. In addition, clonal interaction may potentiate or inhibit the response to therapeutic agents [10] that make pathophysiology of cancer more complex. Therefore, it is very important to consider clonal heterogeneity for the best treatment approach.

The complexity may explain the variable response of immunotherapies. Patients' own immune system characteristics are an important factor in response to immunotherapy, which is determined by many factors such as age, previously administered treatments, tumor-specific features, and tumor-associated immune cell (TAIC) density. There are reports of local immune activity in the tumor environment [11, 12] and mutation load [13] in cancer response to therapeutic intervention and outcome. Immune escape as a biological effect determines the response of cancer cells: either eliminated by the immune system or kept in an occult state of immune equilibrium as dormant cancer by immune resistance [14]. Recent studies demonstrated that along with the destruction of tumor cells, the immune system is able to sustain cancer cell growth and keeps silent cancer in an equilibrium state [15].

Another consideration is tissue sampling. Only a small region of tumor is sampled by tumor biopsy, and thus, it may not representative of the whole tumor [10]. As targeted therapy has become a very popular approach for cancer treatment, the absence of the targeted antigen in some clones can limit the therapeutic effect of therapeutic agents.

In the end, before the selection of therapeutic intervention, each patient should be selected according to the specific characteristic of his/her tumor and receives individualized treatment, so one approach may not be effective for all patients.

31.1.3 Lack of Specific Clinical Efficacy Biomarker(s) for Assessment of Cancer Immunotherapies

Although cancer immunotherapy is one of the most promising approaches in cancer treatment, the success rate is quite variable in different patients based on the characteristics of their tumors. Therefore, similar to conventional anticancer therapy, standard biomarkers to predict and evaluate responses in immunotherapy are critical before beginning the treatment [16, 17]. An extensive assessment of baseline immunity in the periphery and the tumor microenvironment is essential to predict the efficacy of cancer immunotherapy [18]. To solve the obstacle, the Society for Immunotherapy of Cancer (SITC) reestablished the Immune Biomarkers Task Force. Two important limitations for identification of biomarkers are as follows: (1) investigators are unable to determine the most important factor of immune responses in a clinical response to immunotherapy, which is partly due to cancer complexity, and (2) the optimal source to evaluate the immune response parameter is not clear yet [5]. Additionally, the discrepancy in different approaches and protocols to monitor T-cell responses in clinical trials may lead to inconsistent results and yield invalid results, which necessitate an internationally accepted definition and consistency in immune monitoring approaches [19]. Furthermore, a high clinical response to therapy is required to detect correlation, and low clinical response in immunotherapy is another issue that makes identification of well-established biomarkers difficult.

31.1.4 Conventional Clinical Criteria Do Not Delineate Different Response Patterns to Cytotoxic Agents and Immunotherapies

After the initiation of treatment, response is classified in three ways: (1) regression of tumor, (2) early tumor progression followed by tumor reduction, and (3) being stable with no noticeable change or progression. Response evaluation criteria in solid tumors (RECIST) are conventional criteria defined by the World Health Organization (WHO) that evaluate response of tumor to cytotoxic agents. Immunotherapeutic intervention in some patients can terminate tumor after the initial progression or make tumor to stop, which actually increase a patient's survival. However, these therapeutic effects are considered as no response to RECIST [20]. Hence, conventional criteria may be not applicable for the evaluation of response in immunotherapy.

There are a growing number of novel monitoring techniques arising from different labs and studies [21], but modified assay protocols produce divergent results, which complicates interpretation. Besides, variation in data analysis, quality of studies, and interpretation of results would lead to more chaos [22]. New comprehensive immune-related response criteria, harmonization of assays, and modified statistical model considering hazard ratios as a function of time are recommended to increase the efficacy of methods to assess clinical response immunotherapies [23].

31.1.5 Obtaining Approval to Initiate Clinical Trials Is Time-Consuming

Conducting clinical trials is a necessary step for the assessment of the efficacy of new discoveries in humans, and new agents should systematically be evaluated to translate from bench to the bedside. There are a growing number of clinical trials worldwide. However, obtaining approval for clinical trials is a time-consuming process and has been a real challenge for some researchers [24]. In some countries, the regulatory approval of a comparable application may take more than a year. In the United States and Canada, an investigator must receive first feedback from Food and Drug Administration (FDA) reviewers within 30 days of submission, but the trials may need rounds of revisions that prolong the time to obtain approval [5]. In multinational studies, obtaining assurances, local protocol approval, and informed consent documents from each enrolment site are additional hurdles scientists are confronting with [24]. In addition, clinical trials need to use products that are manufactured based on good manufacturing practice (GMP) regulation, which may not be available for many researchers [5].

A harmonized model to reduce ethics review process time and a single submission form are recommended to minimize approval time for the approval of clinical trials [25].

31.1.6 Challenges in Design of Clinical Trials

Maximum tolerated dose (MTD) or recommended phase II dose (RP2D) is typically determined with the presumption that increasing doses of drug yield superior efficacy. However, finding an MTD may not be feasible in cancer immunotherapies [26] and will likely vary from individual to individual based on genetic and biological differences. In addition, this approach may not work for immunotherapy as the overstimulation of the immune system can lead to autoimmune toxicity. Thus, for these types of studies, optimal biological dose (OBD) is recommended [27]. Moreover, a combination of immunotherapeutic agents with each other or other therapies make the determination of MTD more challenging. Therefore, to determine the therapeutic window in combination with immunotherapies, a prediction of the dose-response surface by model-based analyses is required that demands novel trial designs [26]. Nonetheless, model-based trial designs need reliable biomarkers, understanding the designs, real-time modeling, and sample assessments and expose the researcher to the complicated regulatory processes, which makes conducting such a design challenging [26].

Clinical response to therapeutic agents may be different between cytotoxic agents and immuno-

therapeutic approaches. Consequently, the traditional end point used for cytotoxic agents needs to be adjusted for immunotherapy [28]. According to the traditional end point, patients receiving immunotherapy may show no clinical response early after treatment, and it can lead to early termination of clinical trials. Hence, end points for immunotherapy clinical trials should be extended [29]. In addition, end points of immunotherapy studies should involve biomarkers related to the activity of the immune system against tumor cells [30].

Phase III clinical trial conduction requires a large group of patients to confirm the efficacy of a therapeutic approach. Tumor heterogeneity causes large variations in tumor-specific antigen among patients with the same cancer. Thus, only small subsets of patients with the same cancer type may be eligible for an immunotherapy agent targeting a specific antigen, and it may take years to recruit a large group of eligible cancer patients for the conduction of phase III clinical trial. As a result, novel clinical trial designs are in need to make it possible to conduct trials with a small group of patients with unique tumor characteristics [31].

31.1.7 Reagents for Combination Immunotherapy Studies Are Limited

It has been shown that combination immunotherapy approaches can have promising results in cancer and may provide synergic effects [32]. There are as many as 200 agents, including over 15 immunotherapy agents, approved by the FDA for the treatment of cancer, and evaluation of the efficacy of every possible combination is not feasible [26]. Hence, investigators need to select the most effective agent with the highest synergic activities to yield the best optimal outcome. Another issue in cancer immunotherapy is that combinations of agents expose patients to potential additive toxicity. Although combination immunotherapy may result in a better outcome, it can also increase the rate of adverse effects, which limits its application [33]. As genetic, biologic, and environmental elements are critical in the efficacy of various treatments in different patients, they also influence the potential toxicity of various treatments in different patients and need to be taken into account [33]. Therefore, it is important to balance the optimal effective dose of therapeutic agents with toxicity. In combinational trials involving two or more pharmaceutical companies or institutions, application for investigational new drug and regulatory process is performed only by one company or institution, and it releases information about the safety of new agents [5].

31.1.8 Limitation of Funding to Support Knowledge Translation

Although many new therapeutic agents with promising preclinical results have developed over time, the lack of funding makes it challenging to translate basic research into clinical research. Trials can impose a great financial burden at the expense of thousands to several hundred million dollars for small studies and large multicenter trials, respectively [34]. An assessment of cancer clinical trials in Korea revealed that nearly one-third of investigators had difficulties to provide funding [35]. In the United States and the United Kingdom, most funding belongs to breast cancer [36]. National Cancer Institute (NCI) is the largest funding source for cancer research in the United States, and $5.665 billion was considered for NCI budgets in 2018, $275.471 million increase over 2017 (https://www.cancer.gov/). Nevertheless, raising funds is still a challenging matter for investigators.

31.1.9 Limited Number of Groups with Both Scientists and Clinicians Aiming at Translation Research

A multidisciplinary team is an essential step and should be considered for translating innovation at a molecular level into clinical drugs [37]. However, a collaboration of multiple field experts is common in research, but real coordinated teamwork is rare [38]. Cancer immunotherapy as a high-technology intervention is highly interdisciplinary. Cancer immunotherapy necessitates a team of basic scientists to investigate the molecu-

lar aspect of immunotherapy, translational scientists to transform basic knowledge to clinical agents, company/industry to manufacture new drugs, and physician scientists to evaluate the efficacy of new therapies. Pharmacists, nurses, trial coordinators, and the IRB regulators shall also be added to this list among others. Previous literatures proposed different models for clinical and translational research training [37]. One of the important reasons is that PhD scientists working in cancer immunotherapy have limited capability or knowledge to translate their discoveries to the clinic. In addition, clinicians may have not been interested in immunotherapy due to previous negative experience of cancer immunotherapy [5]. Despite efforts to train PhD students as translational investigators [39], clinical researchers and translational PhD scientists acting separately would not be a solution. In addition, obtaining the initial approval for the trial, evaluation of study protocol, and data analysis may demand additional staffs, which confirm the importance of team-based working.

31.1.10 Insufficient Circulation and Exchange of Evidence Needed to Advance the Field

For a single group of researchers, it would not be feasible to study all aspects of cancer including the epidemiology of cancer, genetic components, chemical intracellular reaction, and developing therapies. Thus, the researchers need to share their knowledge and findings to decrease the workload for each other. Many efforts have been made to increase knowledge exchange between scientists in different fields [40, 41]. Peer-reviewed journal articles are introduced as the ideal way for the exchange of scientific evidence, whereas workshops and meetings may facilitate circulation system-level implementation information such as financial and policy information [42]. However, despite all emerging strategies, there are still significant barriers to information exchange.

31.2 Chimeric Antigen Receptor (CAR) T-Cell Immunotherapy

31.2.1 Hurdles Related to Mechanism and Process of Research

31.2.1.1 Limited Infrastructure for Efficient Knowledge Translation

CAR T-cell immunotherapy is a recent development, which requires a highly advanced gene-editing technology that is available only in a few countries. CAR T-cell immunotherapy requires multicenter efforts along with high capacities to produce vector stocks and CAR T cells. Literature showed that compared to the United States, translation of the CAR T-cell immunotherapy in Europe has faced difficulties, and authors blamed limited sources to manufacture CAR T cells of high quality as the primary reason [43]. Besides, CAR T-cell therapy as a new treatment approach needs educated and oriented nurses and personnel to know possible adverse effects of treatment and give patients the standard care [44]. Thus, effective infrastructure is one of the most important factors in CAR T-cell immunotherapy.

31.2.1.2 Need to Release Certificate Prior to Clinical Evaluation of CAR T Cells as Genetically Modified Organisms

In Europe, CAR T cells are a form of advanced therapy medicinal products (ATMPs) and classified as genetically modified cells. Thus, CAR T cells are considered as genetically modified organisms (GMOs). Clinical trials for CAR T-cell immunotherapies must be approved for the use of GMOs according to environmental risk assessment in some European member states, which consequently obligate risk assessment for each new type of CAR T cells [43]. Hence, to ease the risk assessment process, a standard conventional approach on the GMO requirement of CAR T cells, which reflect on all CAR T cells, should be provided.

31.2.1.3 Difference in Requirements Among Various Settings

Variation in the application process among European member states is another hurdle limiting the activation of clinical trials [45]. These variations lead to disparity in approval timeline and additional struggle, particularly in international clinical trials, which mandate obtaining approval from each participating site. [43]. Consequently, conduction of multinational trials enrolling patients from several European Union (EU) member states becomes very unappealing. Therefore, an integrated approach for safety risk assessment of the GMOs in Europe would result in a timely regulation process for clinical trials.

31.2.1.4 Lack of Standard and Specific Guidance

ATMPs as biotechnological products involve cell-, gene-, and tissue-engineered therapies, which are frequently patient-specific [46]. CAR T cells are considered a type of ATMPs. The growing demand for CAR T cells requires the manufacturing of highly individualized gene-edited T-cell products. Although CAR T-cell products need to be in concordance with academic research for essential knowledge, due to this personalized nature, pharmaceutical companies may be incapable to proceed according to clinical translation used for biotechnological products [43]. Diverse structure and wide-ranging functions of ATMPs imply that general guidance may be insufficient to translate into product-specific requirements [47]. Rapidly evolving nature of ATMPs and the lack of regulatory knowledge are major hurdles for scientists against using them [48].

To reduce delay, investigators are recommended to follow regulatory and scientific guidance with competent authorities for clinical trials in advance [43].

31.2.1.5 High Burden of Documentation Needed Even in Early Phase of Application for Clinical Trials

GMP regulations obligate pharmaceutical companies to present classified documentation and records on the manufacturing process in order to make all development, manufacturing, and activities accessible [49]. However, there is inadequate knowledge about the documentation process with regard to ATMP development academic institutions [48]. It's been demonstrated that the knowledge and documentation needed to approve clinical trials represent a substantial hurdle for principal investigators not by the ATMP GMP facility managers [48]. European Commission established new guidelines on GMP guidelines specific to ATMPs (https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-4/2017_11_22_guidelines_gmp_for_atmps.pdf).

31.2.1.6 Product Chain Identity

CAR T cells are individualized genetically modified T cells. Thus, accuracy in CAR T cells distribution from the pharmaceutical industry to the hospital to reach patients is very important. Even in some cases, the patient receiving the treatment may be in a different continent. The product should be tracked precisely to prevent a patient-product mismatch. The transport errors can occur in two levels: (1) transfer of leukapheresis materials from apheresis and cell-processing laboratories to the manufacturing company and (2) from the manufacturing company to the treating center. After the delivery of manufactured CAR T cells to the hospital, hospital staff should control the chain of identity to be in concordance to the manufacturing facility [50]. So far, product identifiers employed by hospitals may differ from the manufacturing company, which may result in uncertainty and loss of information [43].

31.2.1.7 Lack of Specific Regulatory Requirements for CAR T Cells to Facilitate Knowledge Translation

CAR T-cell therapy is one of the new promising therapeutic approaches, and like most of the novel procedures [51, 52], a specific regulatory process and requirement have not been defined. In fact, regulatory agencies frequently adjust the requirements as different aspects of therapy are made available. Thus, to prevent delay in approval and facilitate translation, the investigator should identify current regulatory requirements and get

adapted in advance. Therefore, establishment of specific guidelines as universal regulation for the manufacturing and application of CAR T cells seems pivotal. The approval process should be a balance between high-quality standards to minimize risks and lower limitation for the application of the CAR T-cell therapy [53]. There are limitations in existing guidelines such as the lack of a threshold for transduction efficiency, not considering individual variations among patients, and the lack of a specific and standardized method to assess the biological potency of CAR T cells. In addition, clinical considerations need to be adapted as CAR T-cell therapy is evolving [53].

31.2.2 Practical Hurdles

31.2.2.1 Labor-Intensive Nature of Adoptive Cell Transfer (ACT)

Adoptive transfer of genetically engineered cells is characterized by gene modification of patients' own immune cells to make the immune system to detect and fight cancer more efficiently and increase immune response. As a result, this approach for cancer treatment is highly individualized, and the products are specified for each patient. However, the product manufacturing process demands multilevel cooperation of many skilled and trained workforces and is considered labor intensive for many investigators [54]. In addition to the need for laboratory expertise, production of these products and gene-modified cells requires high-quality infrastructure to keep all environmental conditions under control and confirm sterility. Thus, these conditions and requirement increase the probability of failure in product manufacturing process [54].

Therefore, ACT, as a new therapeutic modality, demands a labor-intensive and patient-specific process, which precludes commercialization and limits extensive use in practice [54, 55], and can be considered as a service instead of distinct drug [55].

31.2.2.2 Limited Number of Cancers with Natural Tumor-Reactive Lymphocytes Eligible for Isolation and Expansion

An immunotherapeutic approach in patients with metastatic melanoma is to isolate tumor-infiltrating lymphocyte, produce a large amount of autologous T cells ex vivo, and reinfuse T cells to recognize and fight cancer cells. Previous studies reported that the use of tumor-reactive lymphocytes in ACT has had favorable outcomes even with curative potential for metastatic melanoma [56] and some other malignancies [57]. Although ACT of expanded tumor-infiltrating lymphocytes has been encouraging, isolation of tumor-reactive lymphocytes is limited in many cancers. This is mainly due to the presence of a negligible number of tumor-reactive lymphocytes in peripheral blood [58]. Notably, this approach may not apply to all types of cancer.

31.2.2.3 Dependence on the In Vivo Maintenance of T-Cell Populations

After infusion of engineered T cells into patients, they need to interact with environmental signals to proliferate and act against a targeted antigen. However, there are known and unknown factors that regulate immune cell induction and proliferation in the human body, which can influence the efficacy of ACT therapy.

Previous studies have reported that lymph depletion before ACT increases the antitumor activity of infused T cells. Host T cells can compete with transferred T-cells for available cytokine, and a limited amount of cytokine would reduce the proliferation of antigen-specific T cells. Besides, the existence of regulator T cells can suppress proliferation and reduce the activity of tumor-reactive T cells. Lymphodepletion before ACT is shown to increase the availability of proliferation cytokines and restrict the population of regulatory T cells. Although lymphodepletion by chemotherapy and irradiation will also decrease the number of antigen-presenting cells (APCs), tumor cell apoptosis leads to tumor antigens uptake and presentation by APCs and may increase the function of APCs [59]. This evidence confirmed that the status of a patient's immune system before immunotherapy is an important factor in the function of transferred T cells.

The natural selection of tumor cells in response to immunotherapy is another issue that may influence infused T-cell performance. The presence of high heterogeneity among tumor cells makes antitumor activity of engrafted T cells only against a pro-

portion of tumor cells. Subsequently, tumor cells with low immunogenicity would survive and proliferate and will show resistance to infused T cells [60]. Thus, tumor heterogeneity can minimize the persistence and efficacy of transferred T cells.

Activation of naive CD8 T cells to proliferate and generate effector cytotoxic T cells requires three signals, which include antigen presentation on major histocompatibility complex class I (MHC-I) molecule, a costimulatory signal, and inflammatory cytokines [61]. Many tumor cells acquire the ability to evade the presentation of MHC-I [62]. Downregulation of MHC-I in tumor cell decreases the ability of cytotoxic T cells to recognize and induce apoptosis of cancer cells [63]. On the other hand, downregulation of costimulatory molecules and expression of coinhibitory receptors by tumor cells can impede the effective activity of immune cells against tumor [64].

CD8+ T cells have been shown to have evolutionary distinct differentiation states including naive, early effector, intermediate effector, and late effector. In vitro developed late effector T cells have the most antitumor activity. This is while in vivo, these late-stage cells showed significantly lower antitumor activity than early-stage T cells. These findings are due to factors such as high proliferative potential, less apoptotic risk, and higher reaction to homeostatic cytokines in early-stage T cells. Therefore, late-stage differentiated T cells employed in ACT probably will exhibit low antitumor activity [59].

Looking at the complexity of the regulation of immune cell activation, proliferation, and persistence, it may difficult to predict the expansion and survival of engrafted T cell, and immunotherapy may show variable outcomes.

31.2.3 Some Other Pending Issues

31.2.3.1 Determination of Ideal CAR T-Cell Population Subset, Phenotype, and Construct

The majority of previous clinical trials have used autologous, unselected peripheral blood mononuclear cells (PBMC) for the production of CAR T-cell products and IL-2 for signaling stimulation leading to the generation of T-cell products containing both effector CD4+ and CD8+ T cells [43]. The proportion of CD8+ and CD4+ T-cell subsets in the peripheral blood is considerably variable in patients due to different factors including age, pathogen exposure, and the lymphocytotoxic effects of chemotherapy [65, 66]. Thus, it is not surprising that PBMC-manufactured CAR T-cell products have heterogeneous numbers of CD8+ and CD4+ T-cell subsets leading to variable responses to treatment and adverse events in clinical trials [67–70]. However, a robust bulk of studies have focused on the development of optimized CAR T-cell products, which possess T cells with boosted proliferation capacity and survival [71–76]. It is suggested that designing products from enhanced subsets of CD8+ and CD4+ T cells may potentially lead to increased treatment efficacy. There are different variants of CD8+ and CD4+ T cells including naive, effector, and memory T cells with distinct surface phenotype. Memory T cells can also be divided into central and effector memory T cells [77–79]. In this regard, a previous preclinical study showed that CAR T-cell products from purified CD8+ or CD4+ central memory T cells or naive T cells have higher therapeutic efficacy in comparison with effector CAR T-cell products [80]. In fact, administration of a predefined number of enhanced and purified CD4+ and CD8+ T cells could lead to synergistic potency. In conclusion, there is vast experimental data supporting the idea of defined CAR T-cell products. However, therapeutic efficacy and higher potency of these kinds of CAR T-cell products are not definitive, and any concurrent conclusion about their actual clinical therapeutic benefits would be premature. Technical improvements in the manufacturing of these products with a higher number of patients would reveal the potential benefit of defined CAR T-cell products to a greater extent.

31.2.3.2 Selecting Appropriate Animal Models to Investigate the Safety and Efficacy of CAR T-Cell Products

Over the past decades, mouse models have been used as an acceptable preclinical model making a bridge between *in vitro* experiments and clinical trials. Mice are small, easy-handling, and low-cost animals with a short propagation time. Nonetheless, they are not an ideal preclinical

model for cancer immunotherapy. A variety of mouse models have been employed for CAR T-cell studies: (1) Several CAR T-cell studies have been on human xenograft models [81], which are immunodeficient and tolerant to human cells. These models cannot distinguish between xenogeneic rejection, human CAR T-cell allogeneic response to the tumor, and the actual CAR T-cell therapeutic effects leading to tumor regression. Furthermore, as the host immune system is minimized in these models, they are incapable of investigation of tumor microenvironment or the host immune response to CAR T cells. (2) Syngeneic models have an intact immune system yet need murine cells [82, 83]. These models may cover some of the disadvantages of xenograft models, yet they have their own shortcomings. In fact, xenograft and syngeneic models could be used together to address the disadvantages of each other. (3) Transgenic mouse models are relatively new models for CAR T-cell studies [84], which can provide information far more than syngeneic and xenograft models. However, only three CAR T-cell tumor-associated antigens (TAAs) have been investigated with transgenic models. Although transgenic mouse models have not been able to reveal toxicities seen in the clinical settings, these endogenous cancer models could be of great value as their progression is similar to cancers in human individuals. Furthermore, humanized transgenic mouse models have been recently developed to recapitulate the human immune system in animal models [85]. In this regard, there are some CAR T-cell studies using mice engrafted with CD34+ hematopoietic stem and progenitor cell (HSPC)s; however, CAR T-cell studies on mice with concurrent CD34+ and tumor cells are lacking.

Primate models are the most recent animal models for studying the side effects of CAR T-cell treatment [86]. These studies have some limitations, including a small number of animals and the inability to assess antitumor effects of CAR T-cell treatment. It should be noted that primate models are potentially useful in the evaluation of TAAs because they are highly conserved. Macaques, which have an immune system comparable to that of humans, have been used for the investigation of neurotoxicity induced by CAR T-cell therapy [87]. Primate studies must undergo extensive ethical regulations and should be considered only after confirmation in mouse models. Finally, it is important to note that no animal model is perfect for CAR T-cell studies and a constellation of different animal models should be utilized in order to investigate various therapeutic and side effects of CAR T-cell treatment.

31.2.3.3 Feasible and Cost-Efficient Production Process

One of the greatest challenges in the development of CAR T-cell products on a massive scale is the design and development of cost-effective technologies for clinical manufacturing of CAR T-cell products in order to sufficiently supply the later clinical trial phases and perhaps commercialization [88]. Several technical and economical obstacles must be overcome in the way of CAR T-cell therapy. The manufacturing process of CAR T-cell products is highly complex and eventually needs to be simplified and automated. The manufacturing automation is necessary for standardization and control of product composition. Furthermore, automation and simplification of the process decreased operator-introduced errors, which may lead to a heterogeneous composition of products. Fortunately, leading biotech and pharmaceutical companies are highly interested in the CAR T-cell therapy platform, which guarantees the increased development of manufacturing tools and platforms required for clinical CAR T-cell production. In fact, simplification of manufacturing processes, enhancement of manufacturing robustness, and design of automated systems might contribute to a greater production scale and increased cost-effectiveness [54, 89].

31.2.3.4 Determining the Dose of CAR T Cells

There has been no consensus about the dosage of CAR T-cell therapies. CAR T-cell dose could potentially affect the immune-mediated adverse events following CAR T-cell infusion. CAR T cells can be administered in different routes, including intravenous, intratumoral, intracranial, intraperitoneal, hepatic artery, pleural, and transcatheter arterial infusion [90–94]. CAR T-cell dose is typically split to multiple injections (e.g., three injections each day apart) in order to reduce the probability of adverse effects and increase the treatment tolerabil-

ity [43]. Generally, the total dose of CAR T cells is between 7.5×10^7 and 3.4×10^8, yet it is common in clinical trials to apply a dose-escalation regime both inter- and intra-patiently. Regardless of the total dose, the number of infused CAR T cells is dependent on the percentage of CAR-positive T-cell. It has been revealed that this percentage is significantly variable in different trials and also within a specific trial. Overall, there have been various routes and dosages for CAR T cells in different clinical trials.

31.3 Immunological Hurdles Restricting the Efficiency of Antitumor Cytolytic T Cells

Indeed, T-cell-based immunotherapies demonstrate impressive results in targeting cancer cells. However, several hurdles make a barrier to achieve a successful immunotherapy. T-cell-based immunotherapy needs to address these hurdles to achieve the maximum efficiency that is expected [95]. To achieve a successful T-cell response against tumor, different strategies should be implemented, including the following: (1) optimizing the level of T-cell activation by using altered peptides or novel antigens; (2) blocking immunosuppressive cell and factors, (3) maintaining the activity of T cells with high number by homeostatic cytokines such as IL-7, IL-15, and IL-21; (4) accessibility of T-helper cells; and (5) avoiding T-cell overstimulation [96].

31.3.1 Self-Nature of Most Tumor Antigens

Cancer arises from normal host cells rather than exogenous pathogens. Therefore, the antigens that are recognized by the immune system in this disease are self-molecules or mutated self-molecules. The immune system is considered to ignore the self-molecules to suppress autoimmunity development. Therefore, most antigenic variations that occur in tumor cells are incapable of recruiting immune system reactions, representing an important hurdle in cancer immunotherapies.

Proto-oncogenes and tumor suppressor genes are normal cellular genes that play an important role in carcinogenesis. Loss of expression of these genes is poor immunogens: thus, they can hide from immune system detection. On the other hand, tumor cells express weak self-antigen to escape from T-cell-based immunity [60]. The possible mechanisms to evade recognition by host T cells are (a) a low level of host T cells against the self-antigen; (b) the tolerance of immune system toward T cells; or (c) low affinity between self-peptide and host MHC molecule, resulting in no response of naive T cells against antigen-positive tumor cells [97].

Enhancing the affinity between antigen and MHC-I could solve the issue regarding the low affinity of T-cell against weak self-antigens. A transgenic mouse, which expresses both human T-cell receptor (TCR) chains in T cells and human MHC-I domains, showed that a single amino acid substitution could cause a sixfold increase in the affinity of the peptide for MHC-I molecules, activating naive host T cells. However, the wild-type forms have a very low affinity with no activation of naive T cells. This study demonstrated that increasing the affinity of the interaction between a self-antigen and the MHC-I molecule may result in immune response and tumor regression [62].

31.3.2 Low Levels of Costimulation

A proper and functional T-cell-mediated immune response is not only governed with the interaction between MHC molecules and TCR, but also costimulatory and coinhibitory receptors are required for T-cell full function. An intact costimulation signal is necessary for an appropriate immune response against tumor. In fact, tumor cells could escape from the immune system responses through reduced expression of costimulatory molecules. A defective costimulatory signal in the tumor microenvironment can cause T-cell anergy, thereby limiting antitumor immune response and efficiency of immunotherapy [98].

The two major costimulatory molecules involved in T-cell activity belong to the B7/CD28 family and tumor necrosis factor (TNF)/tumor necrosis factor receptor (TNFR) family. B7/CD28 costimulatory factor triggers the T-cell immune response in the early phase. However, the TNF/

TNFR costimulatory molecule is induced within hours to a week after TCR engagement, involving in late-phase response [64].

CD28 receptors provide costimulatory signals, which are essential for T-cell function and activity upon interaction with B7-1 and B7-2 ligands that are expressed on APCs. After T-cell activation, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) receptors are constitutively expressed on T cells, inhibiting excessive activation of T cells. The lack of CD28:B7 signal interaction, which is particularly prominent in some tumors, results in T-cell anergic and immune evasion [99]. Preclinical studies reported that increasing the B7 expression on tumor cells could improve the efficiency of T-cell response. However, B7-1 and B7-2 also bind CTLA-4 with higher affinity than CD28. Thus, vaccine B7 might have the opposite result, limiting T-cell immunity [100].

CD40/CD40L is TNF:TNFR costimulatory molecule; CD40 is expressed in many immune cell types and interacts with CD40L on activated T and B cells [101, 102]. CD40/CD40L interaction induces the production of cytokines and costimulatory factors that are involved in the activation and differentiation of T cells [103]. Moreover, CD40 plays a crucial role in dendritic cell (DC) maturation, triggering effective cell-mediated immunity against tumor. However, a low level of CD40 expression on DC was observed in tumoral models, suggesting a new strategy for tumor cells to escape from immune response and inhibiting successful immunotherapy. Combination immunotherapy approaches could address these major concerns, providing meaningful clinical improvement [104–106].

31.3.3 Immune Regulatory Cells

The tumor microenvironment plays a major role in restricting immunotherapy efficiencies. Tumor-specific T cell, which is activated by active immunization or adoptive transfer, must be able to remain active in the immunosuppressive microenvironment of the tumor. Unfortunately, tumor cells harnessed the immune regulatory mechanisms, which are involved in self-antigen tolerance, to escape from immune destruction.

Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and immune checkpoint receptors are the main immune regulatory cells that are involved in preventing autoimmune disease. However, accumulation of these regulator cells has been observed in the tumor microenvironment, resulting in limiting the efficiency of immunotherapy, thus accelerating tumor progression (Fig. 31.2) [107].

31.3.3.1 Immunosuppression Activity of CD4+ Suppressor Cells

CD4+ Tregs are modulators of the immune system, rolling in the maintenance of peripheral tolerance in addition to suppressing the proliferation and excessive activation of effector T cells. It seems that CD4+ Tregs are recruited to the microenvironments of many tumors, associated with tumor progression and a poor prognosis [107].

The following are strategies to utilize CD4+ Tregs to suppress immune system activity against tumor [108]:

1. Inhibiting effector T-cell activation through cell-cell contact; expressing a high level of death receptors such as CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR)
2. Inhibiting effector T-cell activation through releasing immunosuppressive cytokines (TGF-β, IL10, and IL35), indoleamine 2, 3 dioxygenase (IDO), granzyme B, and adenosine
3. Suppressing the antigen-specific priming of naive T cells.
4. Developing immature effector T cell through interfering with the function of APCs

Targeting tumor-induced CD4+ Tregs fosters immune response against tumor cells as well as breaks the barrier to successful immunotherapy. Treating with anti-CCR4 and anti-CD56 is a preferred alternative approach in suppressing and eliminating tumor-induced CD4+ Tregs [107].

31.3.3.2 Immunosuppression Activity of CD8+ Suppressor Cells

In contrast to CD4+ Tregs, the role of CD8+ Treg cells in cancer has not been investigated

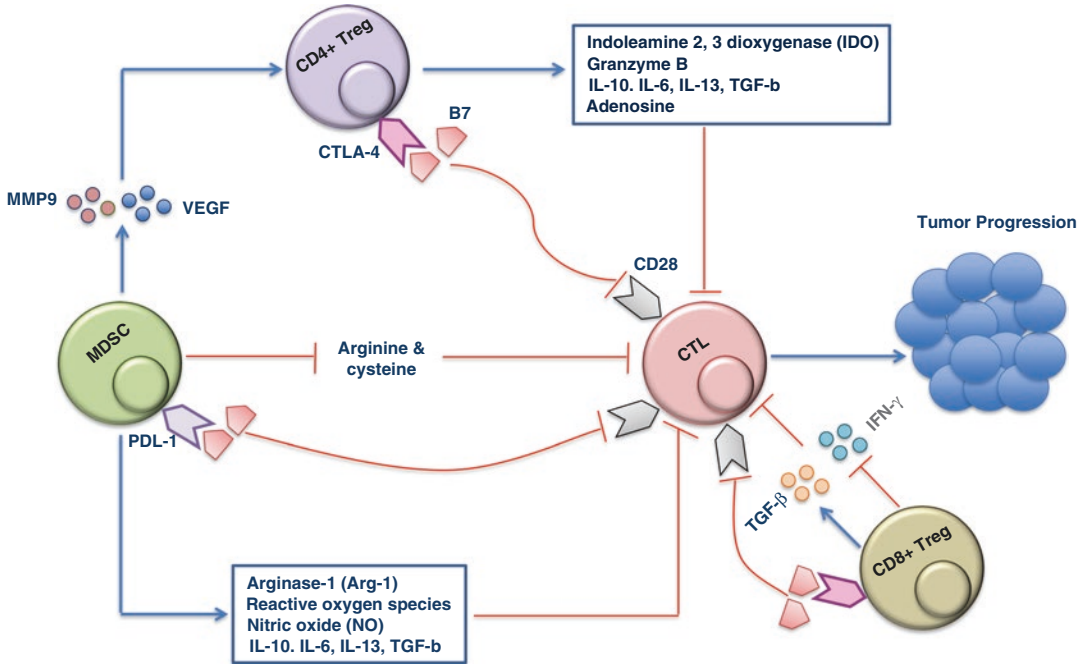


Fig. 31.2 Specific hurdles related to the presence of immune regulatory cells in the microenvironment of tumor, involved in the suppression of CTL-based immune response and limiting the efficiency of immunotherapy. MDSC, myeloid-derived suppressor cells; CTL, cytotoxic T cell; Treg, T regulatory; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PDL-1, programmed death-ligand 1; CD, cluster of differentiation), TGF-β, transforming growth factor beta; IFN-γ, interferon gamma; IL, interleukins); MMP 9, matrix metalloproteinases 9; VEGF, vascular endothelial growth factor

extensively. Both CD8+ and CD4+ Tregs express high levels of forkhead box P3 (FOXP3) and CTLA-4 as their major characteristic markers. However, in contrast to CD4+ Tregs, expression of CD28 is partially dispensable in CD8+ cells, which is at least partially due to low production of Il-2 [109]. The limited number of studies revealed high accumulation of CD8+ Treg cells (CD8+ CD25+Foxp3+, CD25+CD122+Foxp3+, and CD8+CD28) in the tumor microenvironment [110–112], which cause suppression of cytotoxic T lymphocytes (CTL) immune response in a CTLA-4- and TGF-β1-dependent manner [109].

In colorectal cancer, CD8+FOXP3+ Tregs can inhibit the proliferation of T cells and secretion of interferon-gamma (IFN-γ). Similarly, in coculture with ovarian tumor cell lines, CD8+ effector T cells converted into CD8+FOXP3+ Tregs suppressed T-cell proliferation. Moreover, a positive association between CD8+ Tregs infiltration and progression of disease in patients with ovarian cancer has been reported.

Although CD8+ Treg cells are a small population of CD8+ T cells, obstructing CD8+ Treg cells could potentially enhance immune response and the efficacy of immune-based therapies.

31.3.3.3 Immunosuppression Activity of Myeloid-Derived Suppressor Cells

MDSC are a heterogeneous population of immature myeloid cells that usually differentiate into DC or macrophages. However, during malignancy, they migrate toward tumor microenvironment, remain immature, and cause immune system suppression. MDSCs secrete different immunosuppressive components such as arginase-1 (Arg-1), reactive oxygen species (ROS), nitric oxide (NO), and cytokines (IL-1, IL-6, and TNF-α). Moreover, MDSCs induce Tregs and require suppressive tumor-associated mac-

rophages (TAM) to the tumor microenvironment. Target depletion of MDSCs in animal model studies could facilitate CTL-mediated tumor cell killing, highlighting the role of MDSCs in immune evasion and tumor progression [113].

IDO expression is responsible for recurring MDSCs toward tumor microenvironment. Moreover, IDO has a critical role in suppressing T-cell activation through the deprivation of tryptophan. Therefore, IDO can be a potential target for cancer therapy in inhibiting MDSC migration, promoting T-cell activity, and thereby maximizing the efficacy of immune-based therapies.

There are other major strategies for targeting MDSCs in cancer [114], including (1) blocking MDSC differentiation and recruitment, (2) inhibiting activation of MDSC, (3) MDSC depletion, (4) using cyclooxygenase-2 (COX2) and phosphodiesterase-5 (PDE-5) inhibitor to obstruct MDSC immunosuppressive functions [115, 116].

31.3.3.4 IL-13 Secreting Natural Killer T (NKT) Cells

NKT cells are a distinct T-cell population that comprises the characteristics of both T cells and natural killer cells. NKT cells develop under the restriction of the CD1-d molecule [117]. CD4+ NKT cells produce a high level of IL-13, which plays an important role in suppressing immunosurveillance through the IL-4R–STAT6 pathway. The lack of NKT cell in CD1-deficient mice results in reduced IL-13 secretion and thereby increase the CTL-based immune response against tumor. It is worth mentioning that the secreted IL-13 by NKT cells is not able to bind to the T cells itself. IL-13 interacts with IL-4Rα–IL-13R receptor via STAT6 pathway on other immune cells such as dendritic cells, to limit the CTL function and thereby downregulate immunosurveillance. In animal model studies, IL-13Ra2Fc causes tumor regression, introducing IL-13 inhibitors as a novel target therapy in cancer immunotherapy [118].

31.3.4 T-Cell Allergic Through Induction of Indoleamine 2,3-Dioxygenase

Indoleamine 2, 3-dioxygenase (IDO) is an intracellular enzyme that mediates the tryptophan degradation in immune cells. In T-cell-related immune response, IFN causes IDO expression on the surface of macrophage, resulting in catabolizing of tryptophan. Tryptophan is an important molecule for the proliferation and activation of T cells; therefore, depletion of tryptophan by IDO could cause T-cell tolerance and T-cell apoptosis and substantially limit T-cell activity against tumor cells [119].

The tolerogenic effect of IDO has been extensively reviewed elsewhere. In animal model studies, IDO expression could limit the ability of immunogenic mice to reject tumor cells. Moreover, IDO expression is associated with CTLA-4; a high level of CTLA-4 could upregulate IDO in dendritic cells [120].

IDO can interfere with the immune checkpoint inhibitor CTLA-4 treatment (ipilimumab). Mice bearing B16 melanoma did not respond positively to CTLA-4 therapy alone. However, they respond more in combination therapy of CTLA-4 and IDO inhibitor 1-methyltryptophan (1MT) [121]. A similar finding was observed in anti-programmed death-1 (PD-1) treatment. Negative IDO mice with B16 melanoma have better response and improved survival to an immune checkpoint inhibitor.

Some studies reveal that a combination treatment of radiotherapy and CpG oligodeoxynucleotide (a toll-like receptor 9 agonist) could increase IDO expression, resulting in the suppression of the immune system. However, adding D-1MT to the treatment regime could limit IDO activity and significantly decrease tumor progression [121, 122].

The combination therapy of IDO inhibitors with other treatments could increase the efficiency of immunotherapy. Epacadostat and indoximod are two major IDO inhibitors, which are under study in clinical trials. However, significant side effects were reported which need critical management [123, 124].

31.3.5 Exhaustion of T-Cells

In cancer, T cells can be overstimulated due to persistently high levels of antigens [125, 126]. In this condition, which is known as a state of exhaustion, T cells lose their ability to fight cancer and clear the tumor cells. In physiological conditions, T-cell exhaustion protects the host from immunopathology. However, exhausted T cells during cancer express several inhibitory immune receptors such as CTLA-4, PD-1, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3); they also suppress the effector cytokines necessary in immune response against tumor. Establishing new strategies by blocking these immunosuppressive markers could rescue T-cell exhaustion [127].

31.3.5.1 Inhibitory Checkpoints Associated with T-Cell Exhaustion

The coinhibitory molecules such as programmed death-ligand 1 (PDL-1) and CTLA-4 are expressed on tumor cells and immune regulatory cells, which interact with their receptors on activated T cells to cause T-cell exhaustion and prevent the formation of immune memory. The expression of inhibitory checkpoints is associated with immunosuppression, tumor progression, and thereby poor survival. In recent years, therapeutic targeting of checkpoint inhibitors showed impressive results in better survival and durable remission. However, failure of this immunotherapy has been observed in other trials [128].

After immune checkpoint blockade, T-cell activation and clonal proliferation are required in the tumor microenvironment [129, 130]. Moreover, a group of effector T cells should differentiate into memory T cells to perform long-term response against tumor antigens. Deficiency in any of these steps can result in cancer progression and resistance to inhibitor checkpoints. The defective pathways could be categorized into three main groups, including (1) insufficient generation of antitumor T cells, (2) inadequate function of tumor-specific T cells [131, 132], or (3) impaired formation of T-cell memory [129, 130]. Combination therapies are recommended to overcome resistance. For instance, in vivo studies in liver cancer reported that virotherapy using oncolytic viruses could mediate the systemic resistance to PD-1 immunotherapy; therefore, combining immune checkpoints with oncolytic viruses could be a more efficient target therapy in T-cell activation [133].

31.3.6 Mechanisms of Tumor Evasion in Late Stages of Tumor Development

In the early stages of cancer, tumor cells can be efficiently eradicated when exposed to T cells. However, in advanced stages, T cells ignore tumor cells, resulting in tumor escape and metastasis. Escaping from the effector mechanisms of the immune system leads to tumor progression, poor survival rate, and reduction in the efficacy of immunotherapy. Tumor cells have evolved several mechanisms, which influence both tumor cells itself or the host immune system to evade from immune response [134] (Table 31.1).

First, tumor cells try to remain concealed from immune detection through the impairment of antigen-presenting pathways. But if the immune system detects the tumor antigens, the tumor may proceed to adopt mechanisms in suppressing immune system response. A combination of factors such as the production of inhibitory cytokines and soluble factors, expression of inhibitory markers, and conversion of cellular infiltrates into tolerizing cells contribute to immune system evasion. Moreover, some tumor cells acquire apoptosis resistance through different strategies, and some cause the immune system to act against itself. All these immune escape mechanisms inhibit tumor regression and the effectiveness of immunotherapy [135].

Therefore, combinational immunotherapies are required to neutralize the different escape mechanisms of tumor cells and break the barriers to achieve successful immunotherapy.

Table 31.1 Possible mechanisms adopted by tumor cells to escape from immune system response

Immune escape mechanisms related to the host immune system	Immune escape mechanisms related to the tumor cells
Insufficient accessibility to T-helper cells	Tumor cannot activate quiescent precursors
Inadequate level of antitumor T cells	Low immunogenicity due to low expression of tumor antigen
Insufficient avidity of T cells for tumor	Lack or low expression of HLA
T-cell exhaustion and T-cell anergy	Producing immunosuppressive factors
Downregulation of TCR signal	Resistant to apoptosis pathways
Apoptosis of T cells in the presence of tumor	
Improper T-cell function	
Inability to infiltrate into the stroma	
Presence of immune regulatory cells in the tumor microenvironment	

TCR T-cell receptor, *HLA* human leukocyte antigen

31.4 Immunoediting

Cancer immunoediting refers to the adapted changes in the immunogenicity of tumor cells, to survive and escape from the immune system. In the late stages, tumor cells undergo Darwinian-like selection to gain different evasive mechanisms to block T-cell reactivity and promote tumor progress and metastasis [131].

Cancer immunoediting has three fundamental phases called elimination, equilibrium, and escape [136, 137]. In the elimination phase, known as immunosurveillance, the cooperation of innate and adaptive immunity can eliminate cancerous cells before they manifest clinically. In the elimination phase, high levels of immune-activator factors such as perforin, granzymes, first apoptosis signal (Fas) and TNF-related apoptosis-inducing ligand (TRAIL) receptor, IFN-α/β/γ, TNF-α, IL-1, and IL-12 in the tumor microenvironment could skew the immune system toward tumor eradication. If this step is successful, the tumor will be eradicated. But if cancer cells remain immunogenic, it may then enter the equilibrium phase. In this phase, new variants with various mutations are emerged and may last for many years. In this phase, immunological mechanisms try to prevent the outgrowth of tumor through adaptive immunity only. T cells, IL-12, and IFN-γ are the main players in this phase, whereas NK cells and other innate immunity components are not involved in this phase. Due to constant immune selection pressure, tumor cells continue to grow and enter the escape phase and eventually lead to malignancies. Various genetic and epigenetic changes in the immunoediting process could finally break the immune defenses and manifest clinically apparent disease. In the escape phase, adaptive immunity cannot recognize the tumor cells anymore; tumor cells become resistant to immune effector mechanisms and provide an immunosuppressive state. Different evasive mechanisms such as downregulation of costimulatory molecules, the lack or downregulation expression of MHC-I components, and suppressive microenvironment are determined to evade the immune system and immunotherapy [137, 138].

31.5 Tumor Resistance

Resistance of tumor to several antitumor mechanisms of the immune system could provide an escape route for tumor cells. Moreover, it can significantly affect the outcome of immunotherapy. The following mechanisms are defined to help tumor cells to escape from immune system and immunotherapy.

31.5.1 Defective Death Receptor Expression or Signaling

T cells and NK cells are two primary immune system cells that able to induce tumor-cell apoptosis upon death receptor pathways [139]. Lymphocytes express the death ligand FASL

(CD95) on the cell surface, which triggers cytolytic T-cell-mediated death upon interaction with death receptors FAS on the target cell [140]. In NK-cell-mediated death, the TRAIL ligand/receptor interactions play an important role [141]. The death receptors are members of the TNF receptor superfamily that contain an intracellular domain called as "death domain" (DD). The death domain is essential to induce tumor-cell lysis through the activation of caspase cascade pathways [142].

Tumor cells acquire apoptotic resistance and immunosurveillance evasion through different strategies. One strategy is the overexpression of antiapoptotic molecules such as $FLIP_{L,S}$, which can interfere with death receptor pathways and contribute to escape from T-cell-mediated immune response [143, 144]. Overexpression of $FLIP_{L,S.}$ has been observed in human melanomas and Burkitt's lymphoma cell lines [145]. Moreover, upregulation of B-cell lymphoma 2 (Bcl-2) expression is also associated with tumor resistance. However, its contribution to the immune system's escape is not clear, yet. In vivo and in vitro studies reported that Bcl-2 expression confers resistance to FasL and other apoptosis stimuli [146–148].

Another strategy that inhibits the death receptor-mediated apoptosis is the expression of soluble receptors that neutralize or impair death ligands. Soluble CD95 (sCD95) and decoy receptor 3 (DcR3) are the only two discovered soluble receptors, which inhibit the CD95 signaling pathway.

Loss of CD95 or TRAIL, as proapoptotic molecules, is another approach in death-resistant tumors. Oncogenic Ras and p53 aberration may contribute to this deficiency [149, 150].

31.5.2 Resistance to Perforin and the Granzyme B Pathway

The granule exocytosis pathway is another mechanism employed by the immune system to lyse tumor cells [139]. Granzyme B and perforin are two compounds secreted by NK and T cells to induce tumor cell apoptosis. Tumor cells employ different strategies to interfere with the perforin/granzyme pathway and thereby inhibit cell death, evade the immune system, and finally influence immunotherapies [151].

The major mechanism involves PI-9/SPI-6, a serine protease inhibitor that prevents granzyme B expression. Overexpression of PI-9/SPI-6 has been observed in different human and murine tumors. Another mechanism related to the perforin/granzyme pathway is an inappropriate interaction of perforin with the tumor cell membrane. Acute myeloid leukemia cells that are not able to bind perforin are completely resistant to NK-cell-mediated immune response [152, 153].

Overall, employing different mechanisms by tumor cells not only inhibits death receptor and granule exocytosis apoptosis but also limits the outcome of immunotherapies.

31.5.3 Genetic Instability as a Consequence of Malignant Transformation

Tumor cells are more genetically unstable compared to the normal cells. Genomic instability causes altered expression levels or mutation in cell-death-associated genes, rendering them elusive targets. Cancer cells usually employ different strategies related to genetic instability to evade immune response and immunotherapy [154].

31.5.4 Resistance to Apoptosis by Loss of Proapoptotic Regulator

31.5.4.1 P53 Expression

Mutation in tumor suppressor gene *TP53* is the most common form of loss of proapoptotic regulator in tumor cells. The wild-type of p53 (wtp53) activates several genes involved in cell proliferation, DNA repair, and cell death, thereby protecting cells from apoptosis in the context of genotoxic stress. Furthermore, there is evidence for the critical role of p53 in the immune system, specifically in the CTL-mediated immune response. P53 directly affects the antigen presentation via MHC-I by controlling critical genes

involved in the MHC-I generation, such as the transporter associated with antigen processing 1 (TAP1) and endoplasmic reticulum aminopeptidase 1 (ERAP1). Moreover, p53 is involved in the costimulatory signal formation, which is required for CTL activation. P53 reduces the expression of PDL-1 through the upregulation of microRNA, miR34, resulting in an appropriate immune response to cancer. Moreover, p53 increases the expression of Fas/APO-1 in tumor cells, which causes Fas/FasL-mediated apoptosis [155].

According to the function of p53 in migration and activation of CTL cells, a mutation in the p53 implicates tumor resistance to CTL immune response and immunotherapy. CTL-based immunotherapy could benefit more by restoring the wtp53 function in tumor cells [156].

31.5.4.2 Phosphatase and Tensin Homology Expression

Phosphatase and tensin homolog (PTEN) acts as a tumor suppressor gene, and its mutation results in tumorigenesis of many cancer types as well as resistance to immunotherapies [122]. PTEN has been shown to decrease cell proliferation and survival by regulating intracellular phosphoinositide 3-kinase (PI3K) signaling pathways. Therefore, the lack of PTEN expression accelerates tumor growth and increased tumor cell survival. In addition, tumors cells with defective PTEN are poorly immunogenic. Studies conducted on glioblastoma demonstrated that T-cell activity in lysing tumor cells decreases in PTEN-negative tumors, which was correlated with the upregulation of the B7-H1 cell receptor. Moreover, PTEN mutation could interfere with checkpoint immunotherapy in different cancers and affect the overall outcome of the treatment. The mechanism behind such resistance is not well defined yet. However, it was proposed that the production of anti-inflammatory cytokines, such as the chemokine (C-C motif) ligand 2 (CCL2) and vascular endothelial growth factor (VEGF) in PTEN-negative tumors contribute to reducing T-cell infiltration and substantially resistant to immunotherapies [157]. *In vivo* studies reported that transfecting PTEN mutant cells with the wild-type PTEN could facilitate T-cell function in killing tumor cells, making PTEN as a proper adjuvant target therapy in future immunotherapy.

31.5.4.3 Wnt-β-Catenin Pathway

The Wnt–β-catenin pathway has a major role in tumor resistance to immunotherapies. Wnt-receptor interaction promotes the transcription and accumulation of intracellular β-catenin, which inhibits dendritic cell recruitment toward tumor microenvironment, thereby suppressing T-cell infiltration. The mechanism behind is related to the low production of chemokine CCL4 due to Wnt–β-catenin activation [158]. CCL4 as a critical chemoattractant for DC, NK cells, and other cells of the immune system could improve response to immunotherapy, including ipilimumab, in melanoma [159]. In contrast, the lack of CCL4 causes resistance to immunotherapy by the inhibition of antigen presentation and T-cell stimulation by dendritic cells.

31.5.5 Dual Role of CTLs: Attacking Tumor Cells and Selection of Resistant Variants

CD8+ T cells are a major population of T cells and have a prominent role in inducing immune response against tumor. CTLs are MHC-I restricted that trigger the cytolytic killing of tumor cells. The positive association between the number of CTLs at the tumor site and a better prognosis has been reported in different studies [160]. However, tumor cells employ various strategies to stay alive and escape CTL-based immune response [161]. Despite the presence of tumor-associated antigens, which is required for CTL lysis function, tumor eradication by the immune system is often ineffective. In the concept of immunoediting, the immune system is developed to protect the body against tumor development, but on the other hand, it could sculpt the immunogenic phenotype of a developing tumor and resistant tumor cell variants [162]. Development of several malignancies in the pres-

ence of an intact immune system indicates the variant selective pressure utilized by the host immune system [162].

31.5.6 Actin Cytoskeleton

Actin cytoskeleton regulates the crucial process in cellular morphology, cellular movement, and cytokinesis. Studies reported that morphological changes related to the actin cytoskeleton might affect tumor cell susceptibility to cytotoxic treatments and evasion from the immune system. Moreover, the actin cytoskeleton plays a crucial role in NK-cell-mediated tumor lysis. NK cells are able to kill cancer cells through direct interaction with MHC-1 and release of various lytic granule contents. A well-defined structure called an immunological synapse (IS) between the immune system and tumor cells is essential for NK-cell-mediated immune response. The IS formation is due to the rearrangement of the actin cytoskeleton within NK cells. On the other hand, the actin cytoskeleton of tumor cells undergoes extensive remodeling, enabling tumor cells to escape from NK-cell-mediated cell lysis [163].

31.5.7 Events in Antigen Processing

The clinical efficacy of T-cell-based immunotherapy depends on the proper presentation of tumor-associated peptides by human leukocyte antigen class I (HLA-I) complex. Downregulation of HLA-I is associated with a poor prognosis in some cancer and resistance to some immunotherapies. The MHC-I molecule is a heterodimeric transmembrane glycoprotein that consists of two polypeptide chains, α- and β2-microglobulin (β2m). MHC-I triggers CTL-mediated immune response by presenting non-self-peptides to CTLs at the cell surface [164]. The formation of stable MHC-I is depended on the integrity of three essential pathways: (1) degrading the intracellular proteins into small peptides by the proteasome, (2) transporting the small peptides into the endoplasmic reticulum by intracellular peptide transport, and (3) loading the peptides to the nascent MHC and transporting to the cell surface [165]. Deficiencies in any components of the MHC-I antigen-processing pathway could affect their interaction with CTL, resulting in tumorigenesis, cancer progression, or resistance to cancer immunotherapies.

31.5.7.1 Impaired Proteasomal Mechanisms

In the MHC-I antigen-processing pathway, intracellular proteins are sent to the proteasome to be degraded into small peptides. The proteasome is a multimeric proteolytic complex that consists of 28 subunits, with subunits 61, 62, and 65 being responsible for the catalytic action. Recent studies indicated that a variety of stimuli such as IFNγ and TNF alter these subunits with LMP-2 (61i), LMP-10 (62i, MELC 1), and LMP-7 (65i), which form the so-called immunoproteasome [166]. The cleavage preference of immunoproteasome is different from proteasome, creating a different array of antigenic peptides. Recently, various studies reported the association between alteration in different subunits of proteasome and risk of different cancers. The lack of constitutive subunits δ, Z, and MB1 and the immunoproteasome subunits LMP2 and LMP10 were observed in premalignant and malignant multiple myeloma and breast cancer that was associated with a poor prognosis in some cancers. Moreover, it may contribute to limiting current immunotherapies by escaping through antigen loss and CTL lysis evasion [167].

31.5.7.2 Deranged Intracellular Peptide Transport

In the MHC-I antigen-processing pathway, transporter associated with antigen processing (TAP) delivers the small-peptide from proteasomes to the endoplasmic reticulum, where they bind to nascent MHC-I molecule. TAP is an ATP-dependent heterodimer that consists of two subunits TAP1 and TAP2. Many alterations in TAP subunits fail to transport peptides into the endoplasmic reticulum resulting in reducing the expression of MHC-I and subsequently disrupt the interaction between MHC-I and TCR [168].

The other side of the coin indicates that a low level of MHC-I expression due to TAP deficiency could increase the susceptibility of tumor cells to be killed by NK cells. NK cells recognize MHC-I molecules on target cells and are activated when the expression of MHC-I molecules declines. Therefore, in vivo studies demonstrated that the deficiency of TAP in lymphoma cell line makes them highly susceptible to NK cells and decreases their tumorigenicity [169].

31.5.7.3 Loss of β2-Microglobulin Protein Function

A proper immune response against tumor cells and a successful cancer immunotherapy depend on the recognition of the HLA-I on tumor cells with TCR on CTL cells. β2m is a major component of MHC-I molecule that mutation in β2m gene causes the lack or reduced expression of HLA molecules in different types of cancer. Immunotherapy usually increases the expression of HLA, unless the tumor cells have a structural genetic defect, such as β2m mutation. Deficiency in β2m destructs HLA-I formation, leading to cancer immune escape and decreasing the efficiency of immunotherapy [170].

31.5.8 Safety Concerns

31.5.9 Toxicities Related to CAR T-Cell Therapy

Serious toxicities and side effects are some of the major drawbacks of conventional cytotoxic agents [171]. A growing body of literature provides evidences of toxicity related to immunotherapy as well. In recent years, CAR T-cell therapy has shown an impressive clinical benefit; however, several deaths and major complications have been reported as well that have been attributed to a variety of toxicities that appear during treatment (Table 31.2).

Three possible causes contributing to the toxicity of CAR T cells have been reported [172]. The most common CAR T-cell toxicity is on-target, on-tumor toxicity related to the effects of binding CAR to the tumor antigen.

Cytokine release syndrome (CRS) is a potentially life-threatening on-target, on-tumor toxicity that appears after a large and rapid release of cytokines into the bloodstream. Symptoms include fever, nausea, rash, headache, chills, hypotension, and tachycardia. It is believed that Il-6, Il-10, and IFNγ cytokines are the major players in CRS-related symptoms [173].

In most cases, the symptoms could be rapidly alleviated by the systemic administration of corticosteroid [68, 174]. However, corticosteroid could limit the antitumoral effect of therapy through the ablation of the infused CAR T cells [68]. An appropriate alternative treatment is limiting the cytokine action by directly blocking the cytokine receptors. For example, treatment with IL-6 receptor-blocking antibody (tocilizumab) could overcome CRS complications without effecting CAR T-cell persistence [68, 175].

Tumor lysis syndrome (TLS) is another form of on-target, on-tumor toxicity that appears when cancer cells discharge their contents into the bloodstream. During rapid tumor cell death, several metabolic disorders such as hyperuricemia, hyperphosphatemia, hypocalcemia, and hyperkalemia may occur that required timely and proper management [176]. At least four different trials in various hematological malignancies reported TLS during their studies [175, 177–179]. The best approach to address risk stratification for TLS is reducing the size of tumor by other types of treatment before CAR T-cell therapy or controlling the amount of infused CAR T-cells.

Several neurological toxicities were reported in CD19-CAR trials. Neurotoxicity caused by cerebral edemas is a fatal toxicity responsible for several death cases. Moreover, reversible complications related to neurotoxicities such as delirium, encephalopathy confusion, expressive aphasia, and seizures were observed in patients receiving CD19-directed therapy. However, it is not yet clear whether neurological toxicities are specifically related to CD19 CAR T cells or CAR T-cell therapy in general [180].

The second major challenge in CAR T-cell therapy is on-target, off-tumor toxicity. CAR T

Table 31.2 The management of adverse events related to the adoptive therapy

Adoptive therapy	Type of toxicity	Management
CAR T-cell therapy	Cytokine release syndrome	1. Corticosteroid therapy 2. Blocking the cytokine receptors (e.g., tocilizumab)
CAR T-cell therapy	Tumor lysis syndrome	1. Reducing the size of tumor before CAR T-cell therapy 2. Controlling the amount of infused CAR T cells
CAR T-cell therapy	Neurotoxicity	Steroid therapy
CAR T-cell therapy	B-cell aplasia	1. Reducing the dose of the T cells 2. Using the second- instead of third-generation CARs
CAR T-cell therapy	Respiratory failure	Steroid therapy
CAR T-cell therapy	Risk of cancer in the transduction of retroviral and lentiviral	Suicide genes such as HSV-TK, iCasp9, and CD20
Immune checkpoint inhibitors	Thyroid gland disorders	Hypothyroidism: substitution with thyroid hormone Hyperthyroidism: treatment with beta-blocker
Immune checkpoint inhibitors	Hypophysitis	1. Treatment should be interrupted in any grade 2 or higher 2. Hormone replacement therapy 3. Steroid therapy
Immune checkpoint inhibitors	Gastrointestinal toxicity	1. Low grade: antidiarrheals and fluid and electrolyte supplementation 2. High grade: discontinue treatment and receive systemic corticosteroids
Immune checkpoint inhibitors	Pneumonitis	Immunosuppressive treatment
Immune checkpoint inhibitors	Cardiac toxicity	Corticosteroids therapy
Immune checkpoint inhibitors	Neurotoxicity	1. Steroid therapy 2. Myasthenia and Guillain-Barre syndrome: plasmapheresis or i.v. immunoglobulin (Ig)
TCR-modified T-cell therapy	TCR mispairing	1. Utilizing murinised TCRs 2. Inserting point mutations into the α- and β-chain C domains 3. Removing or reducing endogenous TCR chain expression
TCR-modified T-cell therapy	Risk of cancer in the transduction of retroviral and lentiviral	Suicide genes such as HSV-TK, iCasp9, and CD20

CAR chimeric antigen receptors, *TCR* T-cell receptor, *HSV-TK* herpes simplex thymidine kinase, *iCasp9* inducible caspase 9, *CD* cluster of differentiation

cells target the antigens that are expressed on normal cells in addition to malignant cells, which may cause healthy cells to be destroyed, and thereby limit the clinical approaches. The most severe case of on-target, off-target toxicity was reported in a trial targeting ErbB2 in lung carcinoma patients. Due to the expression of ErbB2 on normal lung cells, one patient died from respiratory failure and multi-organ dysfunction [181].

In CAR T-cell therapy for B-cell lymphoma, B-cell aplasia is a common adverse event that ranged from manageable lineage depletion to severe long-lasting toxicity [177, 182]. The CD19 and CD20 as common target antigens are present on normal B cells as well as cancerous cells leading to normal cell death and B-cell depletion. To avoid this type of toxicity, it is recommended to reduce the dose of the T cells and using the second instead of third-generation CARs [183].

The third potential side effect of CAR T-cell toxicity is related to the response of non-CAR T cells to the therapy [184]. The transduction of retroviral and lentiviral may pose the potential to

insert and enhance dormant oncogenes. To avoid this, suicide genes are a more preferred alternative approach that causes tumor cell to kill itself through apoptosis. The most commonly used suicide genes are herpes simplex thymidine kinase (HSV-TK), inducible caspase 9 (iCasp9), and CD20 [185, 186]. However, they can also result in the destruction of the modified T cells.

31.5.10 Toxicities Related to Immune Checkpoint Inhibitors

There needs to be a balance between the efficacy of a novel drug and a manageable safety profile. Despite astonishing clinical results of the immune checkpoint inhibitors in overcoming the tumor immunosuppressive signals, there are several toxicities (Table 31.2) [187].

Immune checkpoint inhibitors are largely cancer cell-specific. However, they could destroy other normal tissues and organs, where a high level of lymphocyte exists for controlling tolerance toward self-antigens. The drug-mediated hyperactivation of the immune system is no longer able to discriminate between neoplastic and normal cells, causing "auto-inflammatory" conditions known as immune-related adverse events (irAEs) [188].

The irAEs usually appear early in the treatment course, mostly within weeks to 3 months after the beginning of immune checkpoint therapy. Any organ or tissue can be involved, but the skin is the most commonly involved site in either CTLA4 (ipilimumab in 43–45% of the patients) or PD-1 (nivolumab and pembrolizumab in 34%) [189–192].

The other most frequently occurring irAEs are hypophysitis, hepatotoxicity, pneumonitis, neurological toxicity, rheumatological toxicity, renal toxicity, gastrointestinal toxicity, pneumonitis, and cardiac toxicity.

Moreover, animal and human models suggest that overactivation of T cells by immune checkpoint inhibitors could recruit autoreactive T cells and break the tolerance of self-antigens, resulting in autoimmunity. Several T-cell-associated autoimmune toxicities related to anti-CTLA-4 have been reported in preclinical models, including diabetes, colitis, and encephalomyelitis, highlighting the possible role of anti-CTLA-4 in the development of autoimmunity.

Anti-PD-L1 inhibitors appear to be safer compared to CTLA-4 inhibitors. The peripheral PD1/PD-L1 checkpoint interaction is specified at the tumor site. However, CTLA4/B7 interaction occurs in lymphoid organs and involves many organs resulting in more toxicity.

31.5.10.1 Ipilimumab

Ipilimumab is a monoclonal antibody that enhances T-cell activity by blocking CTLA-4. It has been reported that 60–85% of patients received ipilimumab at a dose of 3 mg/kg suffer from irAEs, and 2.1% ipilimumab-related deaths have been reported in the first phase III trial. These toxicities are dose-dependent as 30% grade 3–4 irAEs have been reported in a dose of 10 mg/kg ipilimumab. However, no toxicities were observed at a dose of 0.3 mg/kg [188].

31.5.10.2 Nivolumab

Nivolumab is a fully human IgG4 monoclonal antibody targeting the immune-checkpoint PD-1. For nivolumab, any treatment-related irAEs were documented in 74–85% of patients for metastatic melanoma patients [188].

31.5.10.3 Pembrolizumab

Pembrolizumab (previously known as MK-3475 or lambrolizumab) is an IgG4 humanized monoclonal antibody that targets PD-1. irAEs were more frequent (23%) with the highest pembrolizumab dose (10 mg/kg every 2 weeks) than that reported with lower doses (4% and 9% for 10 mg/kg every 3 weeks and 2 mg/kg every 3 weeks, respectively) [188].

Overall, the toxicity related to immune checkpoint inhibitors is mainly transient, and it could be controlled by temporary interruption of the treatment and administration of systemic steroid therapy (Table 31.2). Steroids are immunosuppressive agents that antagonize the pharmacological-mediated hyperactivated immune system. However, steroids could limit the antitumoral activity of immune checkpoint inhibitors.

Therefore, it is recommended that corticosteroid treatment should be avoided as long as possible but absolutely used when necessary.

31.5.11 Toxicities Related to TCR-Modified T-Cell Therapy

TCR-modified T-cell therapy showed many promising results in immunotherapy. However, major concerns related to this therapy exist. TCR mispairing between the transduced TCR and the patient endogenous TCR has proven to be an issue in TCR modifies T-cell therapy. This can increase the risk of generating autoreactive TCRs, which could react against peptides in normal cells in addition to malignant cells. To date, no toxicities associated with TCR mispairing have been reported in clinical trials. However, the preclinical studies demonstrated that TCR mispairing could reduce the interaction between cells and target peptide and substantially limit the functional properties of the genetically modified T cells. Moreover, it may increase the risk of autoimmunity due to the recognition of self-antigens [193].

Various strategies have been developed to minimize the TCR mispairing. Utilizing murine TCRs might be a preferable alternative option since related genes are more expressed in human T cells rather than human TCRs [194]. In this strategy, the constant domains in human TCR are substituted with murine sequences that result in preferential binding to each other rather than to the endogenous TCR. Another option is to insert point mutations into C domains of the α and β chains, which could improve specific pairing and limit TCR mispairing [195, 196]. Recently, an alternative strategy has attempted to minimize TCR mispairing by removing or reducing endogenous TCR chain expression [197, 198].

Another issue association with TCR-modified T-cell therapies is the transduction of retroviral and lentiviral which might pose a potential to insert and enhance dormant oncogenes. An alternative option is utilizing suicide genes that cause tumor cell to kill itself through apoptosis [193].

31.6 Hurdles of CAR T-Cell Cancer Immunotherapy in Solid Tumors

31.6.1 T-Cell Trafficking

Tumor-infiltrating lymphocytes (TLS), which can be found in the tumor stroma and within the tumor itself, can effectively eradicate the tumor cell. Previous studies have reported that the numbers of TLS are associated with a better prognosis and better antitumor responses in various solid tumors [199–201].

CTL infiltration plays a major role in killing cancer cells and providing a favorable outcome in T-cell-based immunotherapies. CTL trafficking is a major matter that could be affected by several factors, including impairment of chemokine-chemokine receptor, low expression of adhesion molecules, and abnormal vasculature [202]. Due to the hostile microenvironment of tumor, recruitment of CD8+ cell toward tumor cell is much more difficult compared to infectious disease. Therefore, new strategies are warranted to increase the level of CAR T cells into the tumor microenvironment.

In order to facilitate CAR T-cell trafficking, different strategies have been developed. One option is finding the best match chemokine-chemokine receptors. Successful CTL trafficking toward tumor cells is dependent on the chemokine produced by tumor cells and its appropriate chemokine receptor on the T effector cells. In melanoma, tumor C-X-C chemokine receptor type 2 (CXCR2) can efficiently direct T cells toward tumor cells [203]. In CD30+ Hodgkin lymphoma, CCR4 improved the homing of CAR-CD30-modified T cells [204]. In neuroblastoma, high CCR2b expression plays a major role in recruiting CAR-GD2 T cells [205].

Another strategy utilizes the local delivery of CAR T cells instead of systemic administration. In head and neck carcinoma, delivering ErbB-targeted CAR T cells into local stromal of tumor is currently under phase 1 clinical trial evaluation [206]. Moreover, ovarian cancer and malignant pleural mesothelioma are the next candidates for

local delivery because of their propensity for localized dissemination within peritoneal and pleural cavities.

31.6.2 T-Cell Infiltration

After appropriate accumulation of CAR T cells in the vicinity of the tumor, they infiltrate into the tumor mass and induce an effective antitumor response. For effective T-cell infiltration, different mechanisms should be considered such as adhesion of T cells to endothelial cells and chemokine-chemokine receptor interactions [207]. Some strategies have been suggested to enhance the T-cell infiltration and thereby the effectiveness of CAR T cells against tumor.

Engineered CAR T cells need to degrade heparan sulfate proteoglycans (HSPGs), a major component of ECM, in order to efficiently access the tumor. Caruana et al. have reported that CAR T cells, which express heparanase (which degrade HSPGs) are able to improve the T-cell infiltration and the immune response against tumor [208].

Moreover, the endothelin B receptor could inhibit proper infiltration of T cells in ovarian tumors; thus, blocking the endothelin B receptor could fascinate T-cell infiltration and thereby enhance the outcome of immunotherapy [209]. Another strategy to improve T-cell infiltration is blocking VEGF receptor-2, which is overexpressed by tumor-associated endothelial cells [202]. VEGF receptor-2 CAR T cells showed more antitumor effect, relating to high tumor infiltration rate.

31.6.3 Immunosuppressive Microenvironment

The microenvironment of solid tumors plays a critical role in suppressing the infiltration, activation, and effector activity of T cells and, thereby, restricting immunotherapy efficiencies. To have the maximum efficacy of immunotherapy, CAR T cells must withstand and remain active in the tumor microenvironment. Although CAR T cells can reduce tumor growth rate, they are not able to induce tumor regression or cure. The CAR TILs will lose their cytotoxicity activity and cytokine secretion capacity. Several immune suppressor cells and components in the tumor microenvironment, such as immunosuppressive cytokines and inhibitory immune checkpoints, can reduce the ability of CAR T cells in tumor eradication [172].

31.6.3.1 Inhibitory Cytokines

Immune suppressive cytokines in the microenvironment of tumor are one of the major barriers in immunotherapy of solid tumors. TGF-β and IL-10 are the main cytokines involved in mediating the immune system through different mechanisms. TGF-β suppresses the activity of CTLs and skew CD4+ T-helper cells toward Treg development. A TGF-b receptor inhibitor designed in CAR T cells as well as protection of activating cytokines such as IL-2, IL-12, and IL-15 by engineered T cells could improve the efficiency of CAR T-cell therapies. IL-12 secretion could kill antigen-negative cancer cells that may escape from T-cell therapy and shift tumor microenvironment toward T-cell-based immune response. Moreover, engineered T cells IL-2 and IL-15 improve the antitumor effects of CAR T cells

31.6.3.2 Inhibitory Immuno-Checkpoints

There are several inhibitory immune checkpoints such as PD1, CTLA-4, B7-H family members, or FasL, which could suppress TIL function and activity. CAR T cells could be suppressed in the microenvironment through the interaction between PD1 and its ligand, PDL1. Upregulation of intrinsic T-cell inhibitory enzymes and expression of surface inhibitory receptors could reactive CAR T cells. Targeting inhibitor checkpoint combing with CAR T-cell therapy could increase overall survival in patients with melanoma, renal cancer, etc. and improve antitumor effects.

31.6.3.3 Immune Suppressor Cells

Solid tumors are usually infiltrated with several immune suppressor cells such as MDSCs, M2 tumor-associated macrophages, and Tregs. These suppressor cells provide and evade mech-

anisms for tumor cells to be protected against the antitumor activity of the immune system. Animal studies demonstrated that integrating costimulatory molecules CD28 into CARs might help CAR-modified T cells to overcome the suppressive properties of Treg cells in the tumor microenvironment.

Moreover, MDSCs restrict the efficiency of anti-carcinoembryonic antigen (CEA) CAR T cells and increase in response to liver metastasis [210]. CAR T-cell efficacy was rescued when mice received CAR-T in combination with MDSC depletion. Tumor cells secrete high levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), which recruit MDSC toward the tumor microenvironment [211]. A combination of CAR T cells with neutralization of GM-CSF could be a more favorable alternative approach.

31.6.4 Toxicity

Several toxicities were reported during treatment with CAR T cells, making it a major challenge in CAR T-cell therapies. Three potently types of toxicity related to CAR T-cell therapies have been determined, which were described in detail before. The most common potential toxicity is on-target, on-tumor toxicity that is related to the effects of binding CAR to the tumor antigen resulting in CRS and TLS. The second major challenge is on-target, off-tumor toxicity, which involves CAR T cells binding normal cells in addition to malignant cells. It is related to target antigens that are expressed on both normal and malignant cells. The third potential side effect of CAR T-cell toxicity is related to the response of non-CAR T cells to the therapy. The transduction of retroviral and lentiviral may pose the potential to insert and enhance dormant oncogenes. To avoid this, suicide genes are preferred as they cause the tumor cell to kill itself through apoptosis [172].

Several strategies to overcome the major challenges related to the safety and efficiency of immunotherapy in solid tumors must be considered in forthcoming clinical trials.

31.7 Other Topics

31.7.1 Challenges in Antigen Selection

Immunotherapy is predicated on augmenting a patient's immune system against a tumor by stimulation of the patient's own immune system by transfusion of bioengineered tumor-specific T cells or antibodies. For the most effective activation of the immune system against tumor cells, one of the first steps is to identify an antigen with the highest specificity for tumor cells and with the least expression in normal cells. Although recent advance in immunotherapy has been greatly encouraging, selection of targeted antigen is still a major barrier to immunotherapy, particularly in solid tumors.

There are two categories of tumor antigens: (1) highly specific antigens including viral antigens in virus-associated cancers, mutated antigens, and cancer-germline genes and (2) antigens with low specificity including differentiation antigens and overexpressed antigens [212]. Most of the identified tumor-specific antigens are expressed on normal host cells to some extent [172] or have a shared epitope with self-molecules. Shared expression of targeted antigen on normal tissue can result in on-target off-tumor toxicity [213], in which immune cells attack normal host cells expressing the targeted antigen. Tumor heterogeneity is another issue that should be kept in mind for antigen selection [214]. A single tumor mass may contain genetically different tumor cell clones with the potential expression of different antigens [10]. Therefore, targeted antigens may not be equally presented on all tumor cells. Likewise, the presence of stromal cells in solid tumor may affect the behavior of tumor cells and make solid tumor more complex than hematologic malignancies [212]. Expressed antigens on tumor stroma were considered as potential targets in immunotherapy [215, 216].

Recently, various approaches such as costimulation CARs [172, 217], bispecific CARs [218], and inhibitory CARs [219] were adopted to limit on-target off-tumor toxicity. Although simultaneous targeting of multiple tumor-specific antigens

would significantly improve immunotherapy, scientists need to understand the complexity of tumor cells and identify most specific antigens with the expression on all tumor cells.

31.7.2 Hurdles Against Bispecific Antibodies

31.7.2.1 The Issues of Stability

For clinical use of bispecific antibodies, the products to be stable under storage and *in vivo* conditions in order to show therapeutic effect before degradation are essential. Bispecific antibodies may show variable stability based on their formats and under physiological conditions may aggregate and lose their activity [220, 221]. Many attempts have been made to increase bispecific antibodies stability [222, 223], but modest structural change in bispecific antibodies would significantly affect biologic activity of products [224]. Therefore, this issue obligates the production of a format with optimal activity and stability.

31.7.3 Need for New Interventions to Enhance Efficacy of Current Immunotherapies in Non-T-Cell-Inflamed Phenotype

Investigations have discovered that tumor-infiltrating T cells in the tumor microenvironment are primarily nonfunctional and possibly are attracted to tumor site because of local cytokines and chemokine [225]. This lack of activity of cytotoxic T cells is attributed to the upregulation of immunosuppressive factors such as PD-L1, IDO in the tumor microenvironment, and recruitment of regulatory T cells, which is actually induced by activated $CD8^+$ T cells [226]. Additional studies showed that immunotherapeutic approaches to block these checkpoints activate immune response and augment tumor regression [227]. However, this immunotherapeutic approach is only efficacious for patients with pre-existing antigen-specific CD8+ T cells in the tumor microenvironment, and evaluation of cancer patients demonstrates that only a proportion of tumors are infiltrated by tumor antigen-specific T cells. Therefore, for patients with the non-T-cell-inflamed tumor microenvironment, one must first identify factors that induce infiltration of tumor microenvironment by immune cells [228].

Studies evaluating recognition of cancer by innate immune revealed that production of type I IFN [229] and IFNγ by dendritic cells play an important role in the activation of CD8+ T cells and migration to the tumor microenvironment. The stimulator of interferon gene (STING) pathway, which is directly activated by cytosolic DNA [230], is identified as one of the main mechanisms of the activation of dendritic cells and production of IFNγ. In vivo, this cascade could lead to the infiltration of tumor by tumor-reactive T cells [231, 232]. Moreover, somatic mutation within tumor can cause variation in the stimulation of the immune system. Beta-catenin signaling is a recognized pathway that regulates immune response, known as T-cell exclusion, and its overactivation prevents tumor infiltration by lymphocytes [233, 234]. However, there are few studies reporting that β-catenin overexpression is associated with high levels of tumor-infiltrating lymphocytes [235]. Stimulation of the dendritic cell to induce T-cell priming against tumor antigens and strategy to overcome T-cell exclusion may be considered as new immunotherapeutic approaches in with the non-T-cell-inflamed tumor microenvironment [228].

31.8 Solid Tissue Cancer-Specific Hurdles

31.8.1 Melanoma

Melanoma is the most lethal skin cancer and is resistant to many cytotoxic therapies such as radiotherapy and chemotherapy, and the prognosis rate of this cancer is very poor especially in the late stages [236]. However, melanoma is the most immunogenic type of cancer, making it the most appropriate target for immunotherapy.

Several melanoma-specific antigens such as melanoma-associated antigens (MAGE) and NY-ESO-1 have been discovered, and high levels of specific antibody and functional lymphocytes can be found in patients with melanoma [237]. Moreover, metastatic melanoma is highly responsive to immune-stimulating agents, such as interferons and interleukin compared to other types of cancer [236].

Both antibody-mediated and T-cell-mediated pathways have shown promising results in immunotherapy of melanoma. However, in some cases, the disease progresses despite high accumulation of tumor-infiltrating melanoma-specific T cells, indicating the suppressive role of the tumor microenvironment in immunotherapy. Treg-mediated immunosuppression is one of the main hurdles in melanoma cancer. Treg is an important cell in maintaining immune homeostasis by inhibiting several physiological and pathological immune responses [238]. Murine model studies demonstrated that Treg depletion could enhance the immune response against melanoma, highlighting the role of Treg in melanoma progression. Similarly, a high level of Treg in patients with metastatic melanoma was reported compared to the age-matched healthy controls, and Treg cells were associated with lymph node and distant metastasis. Other immunosuppressive factors in the tumor microenvironment such as transforming growth factor β and interleukin 10 could recruit and activate Treg cells [239]. Moreover, expression of IDO on tumor cells triggers the conversion of conventional T cells to Treg [240]. GITR is a transmembrane protein, stimulation of which could directly block Treg function, making it a particular interest for cancer immunotherapy [241].

Inhibitory checkpoints are other hurdles in limiting CAR T-cell therapy in melanoma. CTLA-4 and PD-1 expression on CD4-positive, including Treg cells, could directly downregulate T-cell activation and, thereby, inhibit cancer regression. Multimodal targeting strategies using blocking inhibitor checkpoints could increase cytotoxic T lymphocyte infiltration [242].

31.8.2 Pancreas

Although CAR T-cell therapy has been very remarkable, CAR T-cell therapeutic approach in solid tumor is not encouraging, and there are challenging issues that should be solved [214, 243]. Heterogeneity in antigen expression within tumor cells, suboptimal trafficking to solid tumors, and suppression of CAR T-cell activity and survival in the tumor microenvironment are major barriers to the use of CAR T cells in solid tumors [214]. Pancreatic adenocarcinoma is associated with high mortality and the lack of effective treatment necessitating novel therapeutic strategies. As a result, there has been a push toward immunotherapy. There are several immunotherapeutic studies for pancreatic cancer targeting antigens, including mesothelin [244] (NCT03323944, NCT01583686, and NCT01897415), carcinoembryonic antigen (NCT00004178, NCT01212887), and prostate stem cell antigen [245] are conducting, but results are modest or pending.

Selection of tumor-specific antigen is a critical step in therapeutic approaches using CAR T cells. Antigens that are targeted in pancreatic cancer show minor expression on other tissues, and it may result in on-target/off-tumor toxicities [245]. In addition, factors such as a high level of regulatory T cells and immune evasion mechanisms provide a highly immunosuppressive microenvironment in pancreatic cancer [246, 247], which makes tumor resistance to immunotherapy. Therefore, investigators should explore pathways to centralize therapeutics on tumor antigen and neutralize tumor microenvironment.

31.8.3 Head and Neck Cancers

Advanced head and neck squamous cell carcinoma (HNC) shows a poor prognosis, and survival rate remained relatively unchanged during years. Hence, there is a need for novel therapeutic approaches. Seeing the high success rate of immunotherapy in other cancer, especially melanoma, researchers may consider immunotherapy for HNC [248].

Melanoma is a highly immunogenic tumor [249], and this is the main reason that melanoma shows a great response to immunotherapy. Evaluation of patients with HNC revealed that CD8+ T cell in circulation has upregulated expression of proapoptotic protein [250] and tumor-specific T cells in peripheral circulation underwent spontaneous apoptosis, which makes the immune system less effective against tumor and leads to tumor immune escape [251]. In addition, regulatory T cells are highly presented in the circulation of patients with HNC that with immunosuppressive function further impair tumor cell destruction by the immune system [252, 253]. MDSCs and TAMs are other associated factors in HNC, which regulate immune responses to tumor in HNC. MDSCs are immature CD34+ suppressor cells that normally differentiate into mature myeloid cells [254]. In patients with HNC, differentiation of MDSCs is disrupted and increases the risk of recurrence and metastasis in HNC [255]. Complementary studies confirmed that the inhibition of MDSCs trafficking to the tumor site may enhance the antitumor efficacy of immunotherapy [256]. TAMs are macrophage recruited to the tumor site and develop into either tumor limiting (M1) or tumor enhancing (M2) macrophage [257]. Previous studies showed macrophages that infiltrate tumor in HNC are primarily M2 and are associated with metastasis and low survival rate in patients with HNC [258]. Complex and high mutational load in HNC also may play a role in the feature tumor microenvironment and clinical response to immunotherapy [259, 260]. Besides all these and like other cancers, issues such as the lack of biomarkers for patient selection and adverse effects of combination therapy are barriers to immunotherapy in HNC [261]. Therefore, for the application of immunotherapy in HNC, investigators require to assess the tumor microenvironment accurately and address facing challenges.

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32 Ethical Considerations in Cancer Immunotherapy

Maurie Markman

Contents

32.1 Introduction

The concept of immunological therapy for cancer is not a new idea. Anecdotal reports of documented tumor regressions following local infectious episodes suggested an immune mechanism responsible for both clearing the invading pathogen and (as a *secondary effect*) favorably impacting the malignancy [1].

The quite rare but documented observation of spontaneous regression of malignant masses suggested a poorly understood immunological response to undefined tumor antigens [1]. In addition, shrinkage of metastatic lesions following the removal of the malignant primary (e.g., renal cell cancer) highlights the theoretical possibility that by surgically substantially lowering the tumor volume, there is a corresponding reduction in the concentration of an unknown factor (or factors) that has prevented a natural immune response from favorably impacting the course of the malignancy. An extensive body of laboratory-based research supports the potential role of immune cells and their products positively or negatively influencing the rate of cancer growth and spread [1].

More recently, prospective clinical trials have clearly documented the impressive clinical utility of several immunologically based treatment strategies to produce objectively measurable effects on existing malignant mass lesions and to improve disease-specific survival. It can be anticipated that the benefits of immunotherapy

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Table 32.1 Ethical issues with immunotherapy of cancer

1.	Unique toxicities
2.	Evaluation of efficacy in clinical trials and non-research settings
3.	Ethical justification for initiation of treatment in individual patients

demonstrated to date represents only the beginning of an exciting new era in cancer management that focuses on the unique immunological characteristics of a particular cancer and the immune system in individual patients.

A strong argument can be made that with this appropriate focus on the biological and clinical activity observed for immunotherapeutic strategies in clinical trials, there needs to be a corresponding robust discussion of a number of ethical issues surrounding this novel approach to cancer management. This chapter will briefly highlight a number of these issues and concerns.

32.2 Ethical Issues in Immunotherapy of Cancer

In the opinion of this commentator, a number of ethical concerns that are somewhat unique to the realm of cancer immunotherapy, in contrast to other approaches in the management of malignant disease (e.g., "standard" surgery, radiation therapy, and cytotoxic chemotherapy) require consideration. These issues fall into three general categories (Table 32.1).

32.3 Unique Toxicities

The side effects of cytotoxic and the more recent "targeted" antineoplastic therapeutic strategies are well described and include bone marrow suppression, emesis, and cardiac, hepatic, pulmonary, renal, cutaneous, and neurological dysfunction, as well as the development of secondary malignancies.

While hypersensitivity reactions are relatively common with certain drugs (e.g., the initial cycle of paclitaxel, multiple cycles of carboplatin), such events are relatively predicable within a population of patients (e.g., 10–15% incidence of allergic reactions in patients receiving >6 cumulative cycles of carboplatin) [2]. Further, these episodes are generally self-limited and are not associated with serious sequela, even if at the time they are quite anxiety-provoking.

In fact, therapeutic immunological manipulations may be associated with minimal side effects (e.g., tumor vaccines), assuming a substantial degree of specificity to the biological event or at least failure to activate or inhibit processes which may produce serious secondary effects. However, the potential for unexpected, severe, and life-threatening side effects associated with immunological strategies is very real, and in the absence of a clear understanding of both the incidence and overall seriousness of short-term and long-term effects, true informed consent may be problematic. One only needs to consider the now well-understood immune-mediated toxicity of acute and chronic graft-versus-host disease (GVHD) observed within the domain of bone marrow/stem cell transplantation to begin to appreciate the potential impact of immunological manipulation on both the quality and quantity of life.

Further, strong evidence suggests that the combination of immunotherapeutic agents (e.g., two checkpoint inhibitors) may be associated with an unprecedented incidence of severe toxic reactions while at the same time producing both impressive favorable short-term symptomatic effects and long-term survival benefits [3].

In addition, the uncontrolled release of potent cytokines and the accompanying impact of such events on a number of organ systems are a particular theoretical concern with novel immunological strategies previously untested in human trials [4].

Finally, in contrast to the large majority of side effects of cytotoxic chemotherapy, where symptoms are generally observed within "days or weeks" of the initiation of therapy it remains unknown if more delayed immune effects, perhaps occurring "months or even years" after treatment has been concluded will be observed [3].

As a result, until a relatively large number of human subjects have been treated with a particu-

lar immunological approach, the overall toxicity profile will remain uncertain and will mandate careful monitoring and regular updates to an ethical oversight committee responsible for ensuring subject safety. And when these strategies are employed in routine clinical practice, follow-up of patients employing public databases will be essential.

32.4 Evaluation of Efficacy in the Clinical Trial and Non-research Settings

Extensive preclinical evaluation has provided strong support for the conclusion that certain immunological mechanisms (e.g., vaccination) are most likely to be both biologically and clinically active in the presence of the smallest volume of active cancer.

Unfortunately, objectively evaluating efficacy may be problematic. If shrinkage of measurable tumor masses is not anticipated to be a likely outcome and the only acceptable measure of clinical benefit is a statistically significant improvement in overall survival in a phase III trial, this requirement will severely restrict both the types and quantity of immunotherapeutic strategies that can be moved forward for potential regulatory approval to become an acceptable "standard-of-care" therapeutic option. And when one considers the universe of possible immunological therapeutic approaches that may be clinically relevant, this concern is surely magnified by severalfold.

Further, even when such a study is conducted and completed, the result may not fit into the "standard" anticipated paradigm for a "positive trial" result, adding confusion to the research community, regulators, governmental and private payers of medical services, and patients themselves as regards the fundamental interpretation of a given trial's outcome.

Consider, for example, the randomized study of sipuleucel-T immunotherapy in the management of metastatic prostate cancer [5]. The study revealed the strategy to improve overall survival, but there was no statistically significant effect on progression-free survival, an unusual outcome in the realm of antineoplastic drug therapies. Whether this outcome is simply an aberration or this trial provides important insight into the nature of immunotherapeutic treatments of cancer remained unknown. In fact, other studies of immunotherapeutic agents have revealed similar outcomes making progression-free survival a challenging surrogate outcome. Unfortunately, the absence of a definitive answer to this question may result in decision-making for regulatory approval or use in an individual patient difficult.

Finally, in an era where molecularly targeted therapy has been generally accepted as the future of cancer medicine, it remains uncertain how exactly this concept will impact the development of immunologically based therapeutics. In fact, studies exploring exciting novel biomarker approaches for the selection of appropriate patients to receive particular immunotherapeutic drugs suggest the major potential clinical relevance of this idea [6–8].

However, such data raise two related and quite relevant ethical questions:

1. Is it ethical to enter patients into a trial whose cancers do not possess the biomarker that laboratory evaluation suggests is required for a favorable therapeutic effect?
2. Will it be appropriate to continue to conduct immunotherapy trials solely based on the "site of origin" when there is strong evidence that this is an insufficient criterion to define an appropriate target population, despite the continued regulatory agency mantra to examine efficacy based on histology/"site of origin" rather than on individual cancer's identified molecular signature?

32.5 Ethical Justification for Initiation of Treatment in Individual Patients

The concept of "off-label" administration of antineoplastic agents is not a unique problem. In fact, the rigidity associated with deciding whether payment will be provided for a particular drug

in a given situation varies remarkably between governmental agencies in different countries and among private insurers in societies where such payment strategies exist. However, the question of the appropriateness of employing a given immunological strategy in the management of a specific cancer patient only further magnifies the complexity of the questions.

For example, in addition to the issue of "off-label" use (for a tumor type not specifically approved by the drug regulatory agency), one needs to inquire if it is reasonable to apply an immunotherapeutic strategy in a setting where a patient is not predicted to be "immunocompetent" (e.g., presence of cancer cachexia). Moreover, what if this is the only approach that has any "hope" of providing a favorable result?

And what if a patient has the correct histology where an immunotherapeutic approach has been shown to be of benefit but the cell surface antigen whose expression is suggested to be necessary for a favorable effect is not completely absent but only minimally expressed (e.g., +1 staining)? If the patient wishes to proceed with the treatment despite this laboratory observation, should this be permitted considering the limited opportunity for benefit but with no other options likely to be more efficacious?

Finally, how would antineoplastic strategies based on the manipulation of an individual patient's immune cells be rationally initially investigated and subsequently evaluated by governmental regulatory/payment agents? Single patient experiences will surely fail the test of an adequate sample size to demonstrate "efficacy" for a regulatory agency or likely even a peer-reviewed journal.

However, one can make a strong argument that tumor vaccines created by stimulating immunoregulatory cells present within a specific microenvironment of an individual patient may be a highly relevant strategy for the future. It is most unlikely that any type of "randomized trial" will be relevant in such a setting.

In addition, one must ask the question that is being addressed in many other areas of oncology where it is increasingly recognized that unique molecular features discovered within small patient populations will mandate novel approaches to evaluate effectiveness: In the future, will all patients who receive a personal vaccine created based on molecular characterization of the individual cancer require ethical committee (IRB) review? Will all such individual patient efforts be considered "research" or possibly innovative clinical care? Moreover, if the rational argument is made that not all such approaches are "research," will the results of such individual patient efforts be permitted to be published (including side effects, responses, and the survival observed) to inform others (patients and physicians) who may wish to consider this strategy?

Conversely, will a rather rigid ethical review philosophy in many jurisdictions argue against permitting such professional peer-reviewed communication? And if that is the response, is it not the case that future patients will potentially be denied knowledge of the benefits, risks, or actual harms associated with these management strategies, and is this an ethically acceptable outcome?

Developing a reasonable evaluation strategy in the highly innovative but complex arena of cancer immunotherapy which honors the dual ethical mandates of generating knowledge helping future patients (clinical research) while, at the same time, insuring the particular patient undergoing treatment that she/he has been provided with the greatest opportunity (clinical care) will present the oncology community with a unique challenge.

32.6 Concluding Remarks

With the advances in the management of cancer based on immunological strategies, unique ethical issues will need to be carefully considered.

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Correction to Aging and Cancer Prognosis

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Correction to:
Nima Rezaei, Correction to Aging and Cancer Prognosis of Cancers
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The spelling of the author name was inadvertently published as **Arvin Hajmirzaeian** in the Table of Contents, List of contributors and Chapter 24.

This has now been amended throughout the book as Arvin Haj-Mirzaian.

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