Nima Rezaei
Editor

Cancer Immunology

Bench to Bedside Immunotherapy of Cancers

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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 the 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.

Foreword

Several empirical observations suggested a long time ago that established human tumors could melt away in response to perturbations of the immune system such as during acute infection. Such regressions of tumors occurred most often but not exclusively when infection occurred at the tumor site and sparked the interest of investigators in identifying the mechanism leading to such occurrences based on the assumption that infection acted as an adjuvant to boost existing but insufficient immune surveillance against neoplasms. These anecdotal observations are reflected not only in the scientific literature such as the classic reports of William Cooley in the late 1800s but even discussed by classic authors such as the doctor-writer Anton Chekhov.

It took time, however, to elevate these concepts derived from empirical observations to a science of molecular precision. Skepticism dominated the scene for a long time including during the late 1980s, when the introduction of systemic IL-2 therapy for the treatment of advanced melanoma and renal cell carcinoma provided consistent and reproducible evidence that some advanced

cancers could regress and remain in long-term remission with a treatment that had for sure no direct effect on cancer cells. Retrospectively, as too often occurs in science, this skepticism was unwarranted, and the detractors of cancer immunotherapy made a disservice by slowing the progression of this budding discipline. Common criticisms were not directed against the observation that cancers could regress but rather focused on denial about the overall effectiveness of treatment, the sporadic nature of the regressions, and the relatively high toxicity. In other words, the skeptics confused the clinical effectiveness of a treatment with the value of a promising scientific observation.

I am emphasizing this because it is important to remember those difficult moments now that books as sophisticated and comprehensive are presented on a topic that was not even considered true science by most just a few decades ago. Fortunately, several investigators did not give up, but focusing on the value of an uncommon but reproducible observation carried the field forward.

Thus this book! An achievement difficult to predict only two decades ago!

A book series that encompassed 77 chapters spanning biological aspects of innate and adaptive immune responses to system biology approaches to biomarker discovery, to portrays of clinical successes and discussion of regulatory processes that are about to revolutionize the development and licensing of new investigational agents.

A significant change occurred after the identification and molecular characterization of antigens recognized by antibodies and/or T cells. Moreover, the characterization of molecular mechanisms controlling the cross talks between cancer and non-neoplastic somatic cells expanded the field and understanding of the mechanistic bases of immune-mediated tumor rejection. These unarguable observations gave molecular precision to what was previously perceived as pointless practice. However, the true revolution came with the clinical demonstration that some of the novel biological agents could significantly improve the survival of patients, receiving, therefore, acceptance and recognition as standard therapies through regulatory licensing.

Yet, challenges remain, and it is not the time to relax. Still, the benefits, though reproducible, are marginal both in terms of number of patients benefiting from the treatment and in the length of survival for those who benefit. Most importantly, the outcomes are capricious and unpredictable. Predictive and surrogate biomarkers are missing in spite of novel technologies and strategies that could help in the identification and stratification of patients. Still, most clinical trials are designed to look at outcomes rather than comprehensively learn in case of failures. Still, a gap exists between the potentials for what we could do to better understand the biology of immune responsiveness and what we actually do.

This book is written for those who want to move the field forward both at the clinical and the scientific level. Such a compendium can provide a contemporary overlook at what has happened lately, which is remarkably logarithmic on a time perspective. Yet, we wonder how elemental this edition may seem just within a few years if the field continues to evolve at the current pace. We hope that a second edition will follow soon. Perhaps the editors should have asked for a clairvoyant's chapter. Hopefully, one of the young readers of this edition may step forward and help define the new frontiers of cancer immunotherapy.

Francesco M. Marincola, MD

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.

Cancer Immunology Series, a three-volume book series, is intended as an up-to-date, clinically relevant review of cancer immunology and immunotherapy. The book *Cancer Immunology*: *A Translational Medicine Context*, is focused on the immunopathology of cancers. *Cancer Immunology*: *Bench to Bedside Immunotherapy of Cancers*, is a translation text explaining novel approaches in the immunotherapy of cancers. Finally, the book entitled *Cancer Immunology*: *Cancer*

Immunotherapy for Organ-Specific Tumors, thoroughly addresses the immunopathology and immunotherapy of organ-specific cancers.

In the book: *Cancer Immunology*: *Bench to Bedside Immunotherapy of Cancers*, 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 a clinical perspective, with the aim to educate clinicians on the most recent approaches used in tumor immunotherapy.

Twenty-seven chapters are allocated in this regard. At the very beginning, an overview on frontiers in cancer immunotherapy is given in Chap. 1; then, novel strategies in cancer immunotherapy are discussed in Chap. 2. Thereafter, immunologic biomarkers possessing prognostic importance as well as tumor antigens valuable in the treatment and clinical evaluation of tumors are outlined in Chaps. 3 and 4, respectively.

Due to the importance of overcoming tumor immunosuppression and cancer tolerance when treating tumors, Chaps. 5 and 6 aim to tackle these crucial and challenging issues. From this point, more precise focus is given to introducing novel immunotherapeutic approaches by allocating Chaps. 7, 8, 9, and 10 to gene therapy, virus-based vaccines, cancer stem cells, hematopoietic stem cell transplantation, and lymphodepletion. Chapter 11 provides the readers with the most important details on the combination of chemotherapy and cytokine therapy in tumor management. Various aspects of the role of T lymphocytes in cancer immunotherapy are explained in Chaps. 12, 13, and 14, with special attention to their synthetic biology, clinical application, and roles in immunosurveillance and immunotherapy as well as in optimizing chemokine receptor–mediated homing of T cells in cancer immunotherapy.

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

The implication of photodynamic therapy and polarization of the tumor milieu are brought up in the following two chapters, Chaps. 21 and 22, followed by Chap. 23 which discusses targeting 5T4 oncofetal glycoprotein as an immunotherapeutic approach. Novel biomarkers discovered during anti-CTLA4 antibody therapy are described in Chap. 24. Chapters 25 and 26 discuss radioimmunotherapy and psychoneuroendocrinoimmunotherapy, respectively. Finally, the book concludes by pointing to the ethical considerations crucial during cancer immunotherapy.

The Cancer Immunology series is the result of valuable contributions of more than 250 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 for providing me the opportunity to publish the book.

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

Nima Rezaei, MD, PhD

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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 and lectin-like receptors
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
KIRs	Killer inhibitory 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	NOD-like receptors
NLRs	Nucleotide-binding domain and leucine-rich-repeat-containing proteins
NMC	NUT midline carcinoma
NOD	Nucleotide-binding oligomerization domain
NP	Normal prostate
NPC	Nasopharyngeal carcinoma
NPY	Neuropeptide Y
NSCLC	Non-small cell lung cancer
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	Prostate adenocarcinoma
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
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
RHOH	Rho GTPase
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 Ca2+ 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 Ag presentation
TAP	Transporter associated with Ag 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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Contents

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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

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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 proinflammatory 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 (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 co-stimulatory 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 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, -β, -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

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	

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:

- 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 chemo-radiotherapy 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 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

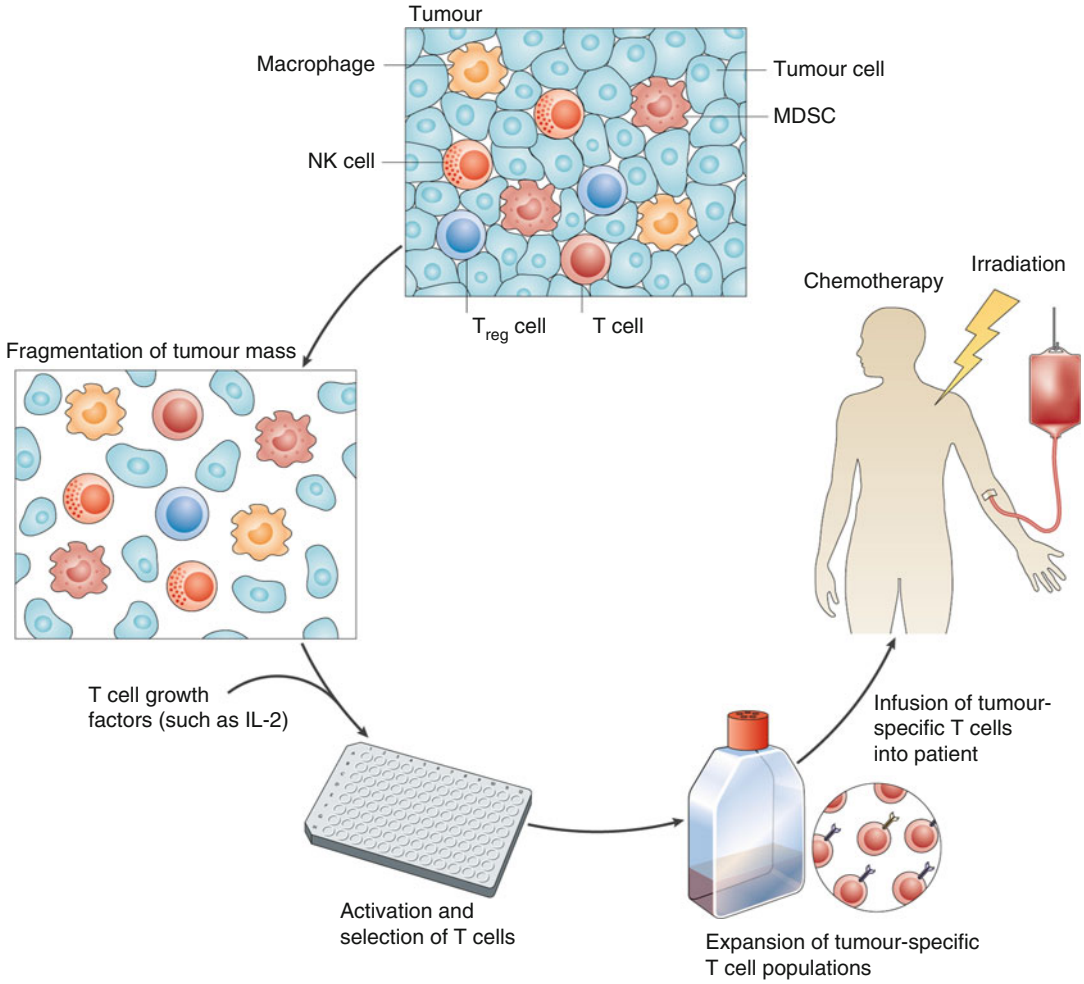

Fig. 1.1 Tumors are often complex masses containing diverse cell types. These masses can be surgically resected and fragmented, and the cells can be placed in wells into which a T-cell growth factor, such as interleukin-2 (IL-2), is added. T-cell populations that have the desired T-cell receptor (TCR) 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])

used method; it utilizes sera of cancer patients to detect over expressed antigens from tumor cDNA libraries [28]. Furthermore, several RNA-based methods have also gained importance: transcriptome analysis that include 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 nonprotein 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 maturation process that involves the upregulation of T-cell co-stimulatory molecules (e.g., CD80, CD86), 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 induce 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. DCs 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 [41]; 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 [42]. Both allogeneic and autologous tumor material have been used to load DC with clinical trials carried out using preparations using both types [43].

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 [44]. A phase I/II trial using genetically modified DC showed that

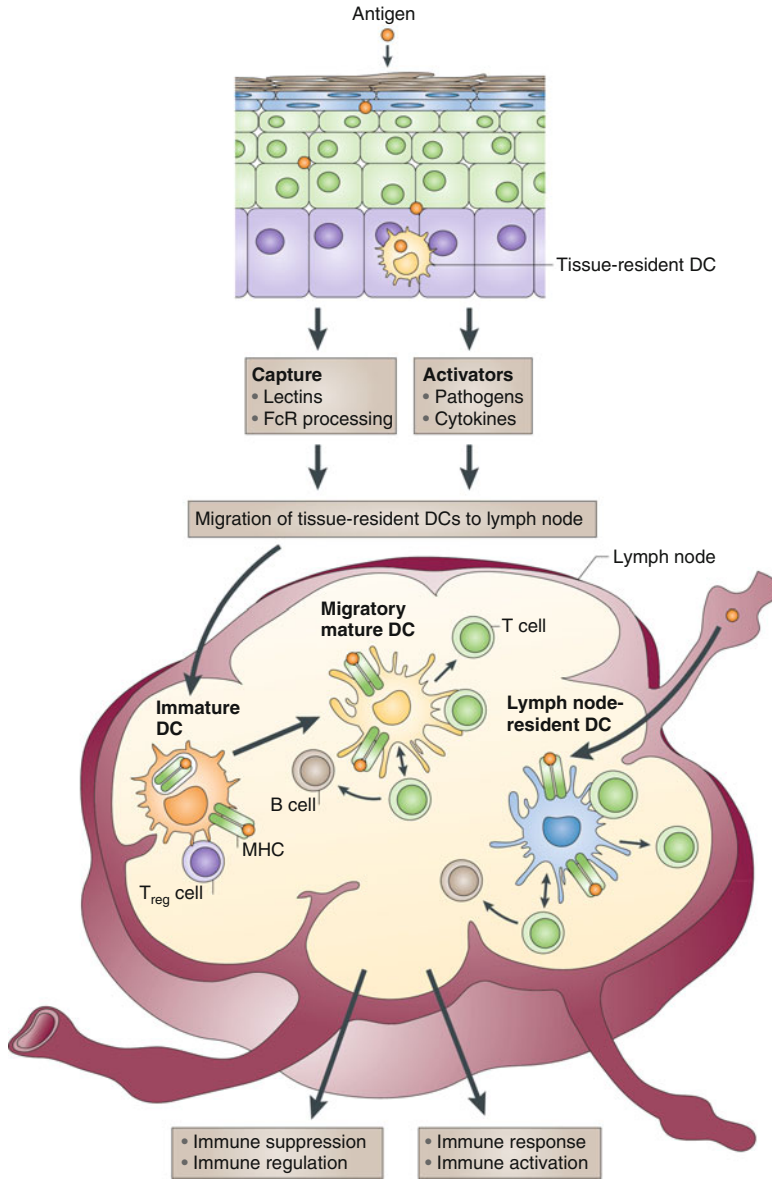

Fig. 1.2 Antigens can reach lymph nodes through two pathways: via lymphatics, where the antigen is captured by lymph node-resident dendritic cells (DCs), or via tissue-resident DCs. These immature DCs capture antigens, and DC activation triggers their migration toward secondary lymphoid organs and their 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 [136])

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 [45].

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* [46]. Other approaches include genetically modifying DC using adenoviral or retroviral vectors to directly express TH1 cytokine IL-12 [47], an adenovirus encoding *CD40L* [48] and modifying DC to express costimulatory molecules CD40L, CD70, and TLR4 called "TriMix" [49] and heat shock protein [50]. Furthermore, vaccines coupled to TLR ligands lead to efficient CTl activation by endogenous DC [51], and the use of oncolytic viruses also looks particularly promising [52].

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 [53, 54]). 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 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 [55], 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 [56, 57].

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 [58]. 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 [59]. 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 [60]. 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 [61, 62]. Conventional cancer treatments, such as de-bulking 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 [63]. 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 [64].

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 [65, 66]. 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 [67], while adoptively transferred tumor-specific $CD8^+$ T-cells destroy the vasculature of established tumors via an antigen-independent, IFN-γ-dependent mechanism [68].

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 [69]. 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 [70]. 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 [71, 72]. 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 [73, 74].

Other cancer-related mechanisms underlying tumor immune escape include loss of TAA expression [3], lack of co-stimulatory molecules expression [75], inactivating mutations of antigen presentation-related molecules [76], 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 [77]. Treg suppress T-cell responses and provide another mechanism compromising the development of effective tumor immunity [78]. 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 [79]. 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 [80]. 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 [81]. 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 [82]. 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 [83]. 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 [84]. 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 [85]. 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* [86].

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 [87]. Although suppressive myeloid cells were described more than 20 years ago in patients with cancer [88], 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 [89]. 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 [90].

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) [91], reactive oxygen species (ROS) [92], peroxynitrite [93]. Moreover, it has been reported that MDSCs promote de novo development of the FOXP3^{+} Treg cells *in vivo* [94]. 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 [95], inhibition of MDSC expansion [96], inhibition of MDSC function [97], and elimination of MDSC [98]. 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 [99]. 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 [100], and conversely, Tregs have been reported to induce alternative activation of human mononuclear phagocytes [101]. Cancer has thus served as a paradigm of *in vivo* M2 polarization [102].

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 [103]. 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 [104]. 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 [105]. 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 [106, 107]. 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 [108]. However, many promising immune-based therapies have been ineffective in human clinical trials [109]. 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 [110]. 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 [111].

IL-2 is approved in the USA for the adjuvant therapy of stage III renal cancer [112]. 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 [62].

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 [113]. In addition to surgery, nanotechnology [114] and molecular imaging [115] 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 [116]. 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 [117]. Clinical trials of chemotherapy and mAb have resulted in some efficacy against cancer in patients [118]. 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 [119]. 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 [120]. The combination of ipilimumab with chemotherapeutics (dacarbazine) [121] or docetaxel [122], with IL-2 [123] or with melanoma-associated peptide vaccines [124] 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) [125], colon cancer [126], pancreatic cancer [127], prostate cancer [128], and small-cell lung cancer [129]. For example, Wheeler et al. [125] 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 [130]. In a study by Dudley et al. [131], 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-cells 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 [132]. 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 [133], as does cetuximab, which completely inhibits the binding of epidermal growth factor (EGF) [134]. 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 [135].

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, antiangiogenic, 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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2.1 Introduction

The first 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. Though not a frequent but real fact of regression of primary skin melanoma or lung metastases of renal cell carcinoma occurs in one third of cases as partial spontaneous regression and in 1–2 % as complete regression for melanoma and 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, lymphocytes, etc.
- AIDS-associated tumors.

Mechanism of tumor escape from the immune attack is primarily due to the lack of specific antigens on tumor cell surface and loss or downregulation of the expression of molecules of major

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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 against tumor development. In addition, NKs play the crucial role as they can recognize and lyse transformed cells in 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 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 make 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 neutrophiles, 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 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 by 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

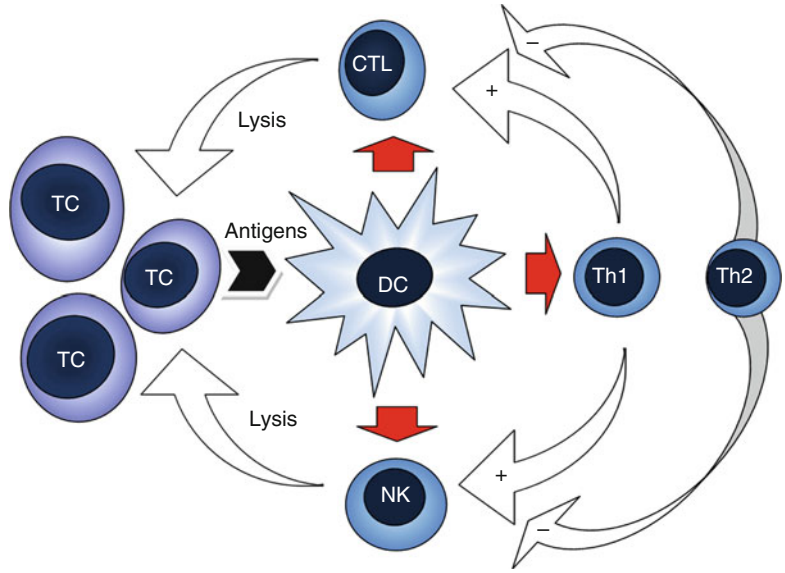

Fig. 2.1 Schematic interaction of the effectors of innate and adaptive immunity. *Abbreviations*: *TC* tumor cell, *DC* dendritic cell, *Th1* and *Th2* T helper cells of types 1 and 2, *NK* natural killer cell, *CTL* cytotoxic T-lymphocyte

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 cultured *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 true 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 even observed 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 those 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 capabilities, 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 *vs*. 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) 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 in disease-free survival [21–23].

To improve IL-2/LAK immunotherapy effectiveness, local and locoregional infusions were performed, allowing for 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 immunostimulating doses cause no marked side effect, including immune- and/or 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 little to 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 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 biodegradable in nuclear 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 nonmutated p53); (2) non-mutated differentiation antigens (e.g., MART-1, overexpressed in melanoma and found in normal melanocytes); (3) cancer-testis antigens (CTA), consisting of nonmutated genes expressed during fetal development, 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]. Tissues reactivated in cancer cells across multiple malignancies (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 with malignant effusions—primary tumor types included lung cancer, breast cancer, mesothelioma of pleura and other cancer localizations. Autologic 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 500–2,800 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 % of patients of whom 60 patients had complete remission and 10 experienced partial reduction of effusion (Fig. 2.2a, b). Recurrence of effusion occurred in 10 (11.8 %) patients after 1.2–2.5 months of the treatment completion. However, repeated one to two courses of IL-2/LAK therapy resulted in regression of malignant effusion. It is important to emphasize that delay or cessation of effusion was achieved only in cases which pleural liquid contained essential number of activated lymphoid cells including immunoblasts and mitoses.

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

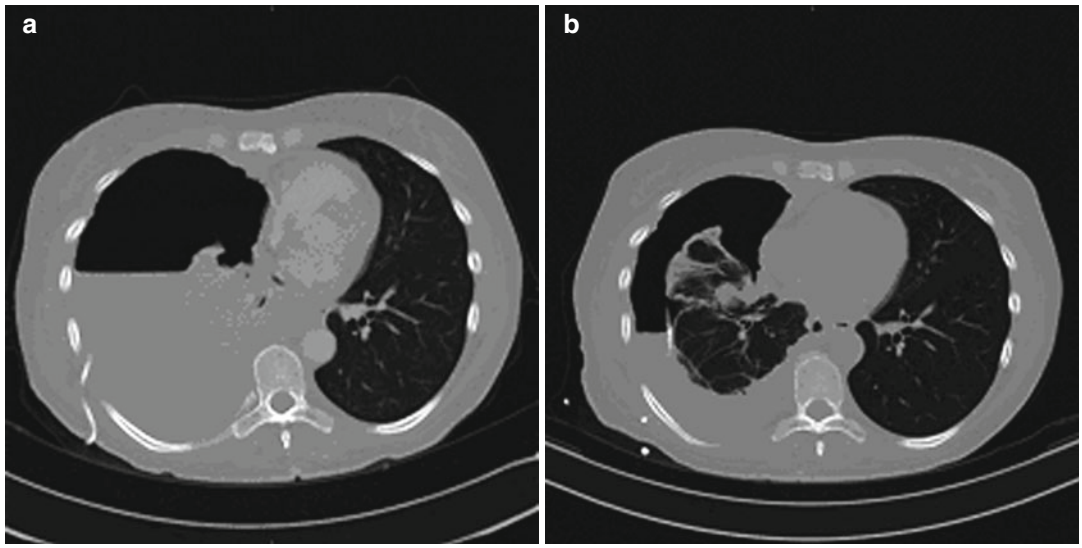

Fig. 2.2 CT of the chest during the course of IL-2/LAK immunotherapy of malignant pleural effusion. Patient Sh. Lung cancer (the right lung), right-sided pleuritis. (**a**) Prior to IL-2/LAK intrapleural immunotherapy; (**b**) Two months after the immunotherapy. Partial effect

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 6,000 r/min 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: One patient had a successful radiation therapy and fifteen 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. Only if 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 Autologic 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 thus playing 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 E_2 [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 SA 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 will develop metastases after nephrectomy or nephron-sparing surgery [52]; therefore such patients need effective adjuvant therapy. A recent **r**andomized controlled trial of adjuvant combined immunotherapy by TL-DC-CIK cells showed that all patients tolerated the TL-pulsed DC-CIK cells 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 cells 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 Spiral Up

Despite the theoretical rationale and experimental basis of antitumor cytotoxicity of induced lymphocytes, adoptive immunotherapy with lymphokine-activated lymphocytes designed by S. 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 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 or/and 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 heterogenicity of tumor cell population, where tumor cells have different expression of target tumor-associated antigens, should always be kept in mind. 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.

2.8 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 the 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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Novel Prognostic Biomarkers for Personalized Cancer Treatment

3

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3.1 Introduction

Discovery of the complexity of the biology of our physiological system, in the normal healthy state and in specific diseases, aids in discriminating between the physiological mechanisms, characterizing homeostasis, and the pathological ones leading to the loss of this equilibrium, index of illness state, and of its progression.

This information is underlined by the substances that result in modification of some specific characteristic during pathological states in comparison with the physiological one: for this reason, these substances could be used as clinical indicators for the risk of contracting a disease, as indices of the pathology progression. Moreover, they could also be used for the quantification of the risk/benefit in the therapeutic selection.

Therefore, these substances are called "prognostic biomarkers" for their decisional effect on the diagnosis and treatment determination in clinical practice. In addition, they determine a significant foot forward in the administration of medicine.

Improvement of the clinical/therapeutic strategies concretizes in the passage from a generic medicine structure, where the therapy is the same for all the patients, to a stratified system, where the patients are subdivided in clinical/therapeutic subgroups: specific biomarkers correspond, in fact, to specific pathological diagnosis and specific treatment possibilities. Otherwise, in a more specific way, the aim of the actual clinical practice is the passage to a personalized conception of treatments, where therapy is specific for the single individual, because it is adjusted on their physiopathological characteristics. These new lines of actions, however, underline the urgency for the identification of useful biomarkers for these procedures.

This urgency is also a priority of the actual administration of the health system, where this new clinical and therapeutic conception will lead to positive benefits, improving the quality of the health through early diagnosis and personalized treatments. The actual health system, in fact, is even more difficult to administrate for the changes in the age range that is most representative of the population: the mean age and the life span are constantly growing, and this increase of elderly people is related to a parallel increase in patients with a chronic inflammation and then of chronic-degenerative diseases as neoplasias, cardiovascular, autoimmune, and neurodegenerative diseases. The reasons have to be attributed to the complex diagnosis of chronic-degenerative diseases and to the difficulty in relevant treatments. In addition, they consume a growing portion of the health system budget, a phenomenon that will lead to biomedical, social, political, and economical challenges for the next generations [1].

The recent developments in the modern medicine, in particular in genetic, proteomic, and informatics areas, indicate a direction for the resolution of these issues: they are leading to the discovery of suitable biomarkers which indicate the risk for these pathologies in healthy subjects, for more effective programs of prevention and treatment. Hence, identification of suitable biomarkers, determinable in a simple and early

way in relation to the pathological onset, seems necessary: they will enable us to define significant indices for the quantification of the disease risk and the therapy risk and/or benefit (risk/benefit) in the selection of treatments.

3.2 Presentation

The intent of this chapter is to underline new and suitable biomarkers that could be evaluated in translational protocols of the clinical practice (1) as risk indicators for chronic-degenerative diseases such as neoplasias in the healthy subjects and, in this direction, for more efficient sanitary programs of prevention and treatment, and (2) as risk/benefit indicators in the selection of individual clinical treatments, for the stratification in clinical/therapeutic subgroups and the development of personalized therapies, that could lead to an increase in the success rate in the clinical practice.

For this aim, it is necessary to pick out biomarkers detectable in the peripheral blood, because the emetic withdrawal actually constitute the most practical and less invasive prognostic tool in the diagnosis and therapeutic response of the disease [2]. In addition, these biomarkers have to be highly prognostic for the specific pathophysiological conditions related to the pathology. Moreover it has to be emphasized that being the chronicity of the inflammation, the base for genesis and progression to chronic-degenerative pathologies, and to be suitable in the prevention and treatment of these diseases, these markers have to be informative for (a) the normal healthy state, (b) the transient inflammatory state, and (c) the chronic inflammatory state. The reason of these considerations is that the significance of these biomarkers depends on their prognostic capacity for the identification of passage (Fig. 3.1): (1) from the healthy condition, a state of homeostasis whose biomarker is defined as the *a type* and it is an index of *no risk* of pathology, (2) to the transient inflammation, a state in which the chance of recovery of the homeostatic equilibrium is still very probable, whose biomarker is defined as the *b type* and it is an index of *low risk* of pathology, (3) to the chronic inflammation, in which the recovery of the homeostatic equilibrium is physiologically improbable and the risk of degeneration or progression in the different chronic-degenerative disease is very high (the typical biomarker of this situation is defined as the *c type*, and it is an index of a *high-risk* of pathology and of pathological progression).

These biomarkers could lead to advantages in the organization of the health system for improvement in the health quality, related to early diagnosis and personalized therapeutic treatments. They are, in fact, suitable for the prevention of healthy subjects as they allow the selection of individuals with a low or a high risk of pathology. An accurate evaluation of the clinical preventive procedure over these people is justified. Therefore, thanks to these indices, these procedures can be avoided in case they are unjustified. They are, also, prognostic for the stratification of the patients in clinical/therapeutic subgroups and for the development of the personalized medicine, allowing the quantification of the risk/benefit for the specific treatment that will surely lead to positive transformations in different clinical strategies.

3.3 New Biomarkers for the Treatment of Tumors

In oncology, the urgency for new suitable biomarkers is relevant: in spite of significant improvements in the clinical results of last decades, specific biomarkers that could be prognostic for the diagnostic and therapeutic monitoring and for the definition of personalized therapies are lacking. In fact, biomarkers are required in order to select the patients that, in the initial phase of the disease, could benefit from adjuvant therapies and to better define the clinical/therapeutic subgroups in advanced pathological phases.

The principal prognostic factor that is index of survival or recurrence after a surgery of a localized disease is, actually, the stage of the tumor [3, 4]. Adjuvant therapy is an antiblastic precautionary treatment, performed after a radical surgery (macro/microscopically), in order to eradicate any micrometastasis or cell in transition. At any rate, while stage I is usually cured with the

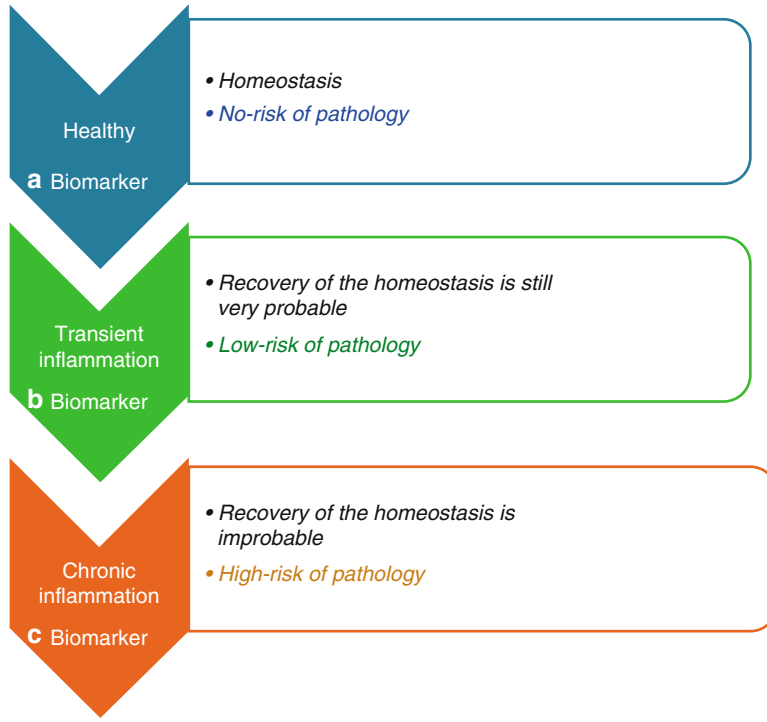

Fig. 3.1 Biomarkers have to be very prognostic for the specific physiopathological conditions inherent with the pathology. Being the chronicity of inflammation, the base for the genesis and the progression of chronic-degenerative pathologies, as tumors, the prognostic biomarkers have to be informative of a few points in order to be suitable in the prevention and treatment of these points. These pathologies include (**a**) the normal healthy state, (**b**) the transient inflammatory state, and (**c**) the chronic inflammatory state. The reason of these considerations is that the significance of these biomarkers depend on their prognostic capacity for the identification of the passages (1) from the healthy condition, a state of homeostasis, whose biomarker is defined as the *a type* and it is index of *no risk* of pathology, (2) to the transient inflammation, a state in which the chance of recovery of the homeostatic equilibrium is still very probable, whose biomarker is defined as *b type* and it is an index of *low risk* of pathology, (3) to the chronic inflammation, in which the recovery of the homeostatic equilibrium is physiologically improbable and the risk of degeneration or progression in different chronic-degenerative diseases is very high (the typical biomarker of this situation is defined as *c type*, and it is a index of a *high-risk* of pathology and of pathological progression)

surgery alone, the adjuvant chemotherapy is actually recommended for tumors at stage III and at stage II with a relatively high risk. However, about 75 % of patients in stages I–III could be treated with surgery alone. In stage III, 40–50 % of the individuals are cured in this way, while about 35 % of patients recur, in spite of receiving adjuvant chemotherapy [5]. Therefore, in clinical procedures performed on stage III, the majority of patients selected for adjuvant chemotherapy are cured, despite the fact that the majority of them do not require an adjuvant therapy or could not benefit from this treatment. The role of adjuvant chemotherapy is even more difficult to be defined in stage II disease: 60–70 % of these patients are treated with the surgery alone, and relapse is verified in 15–20 %, despite receiving adjuvant chemotherapy [6]. The QUASAR study randomized 3,239 colorectal cancer patients with a low risk for disease recurrence, to either observation or 5-fluorouracil/folinic acid (5-FU/FA); 92 % of these patients had stage II colorectal cancer. The 5-year beneficial effect of 5-FU/AF was only 3.6 %, indicating that 96 % of patients had received useless chemotherapy [7]. A surly valid contribution would be improvement in the quantification of the risk/benefit for a better treatment selection.

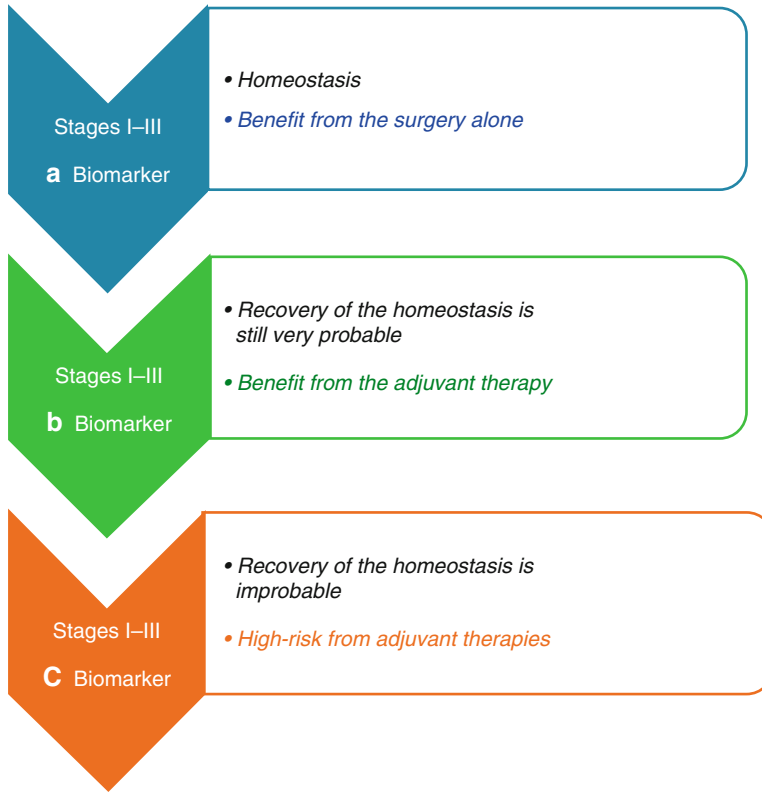

Fig. 3.2 Quantification of the risk/benefit for adjuvant chemotherapy. Adjuvant therapy is an antiblastic precautionary treatment, performed after a radical surgery (macro/microscopically), in order to eradicate any micrometastasis or cell in transition. At any rate, while the stage I is usually cured with the surgery alone, the adjuvant chemotherapy is actually recommended for tumors at stage III and at stage II with a related high risk. The role of adjuvant chemotherapy is even more difficult to be defined and a valid contribution would be the improvement in the quantification of the risk/benefit for a better treatment selection. The upper defined biomarkers could contribute to this improvement. More specifically the *a type* biomarkers, which are homeostasis indices, are suitable for the identification of the subject that could benefit from the surgery alone; the *b type* biomarkers, indices of a transient inflammatory state and then of the possibility to restore their homeostasis, are suitable for the selection of the subjects that could benefit from the adjuvant therapies in combination with surgery; on the other hand, *c type* biomarkers, indices of a chronic inflammatory state in which the restore of the homeostatic equilibrium is improbable, are suitable for the selection of the subjects for which the adjuvant therapies represent an increase of the risk of worsening rather than a benefit

The aforementioned biomarkers, in the presentation of the intention of this chapter, could contribute to this improvement. More specifically (Fig. 3.2), *a type* biomarkers, which are homeostasis indices, are suitable for the identification of the subject that could benefit from the surgery alone; the *b type* biomarkers, indices of a transient inflammatory state and then of the possibility to restore their homeostasis, are suitable for the selection of the subjects that could benefit from adjuvant therapies in combination with surgery; and on the other hand, *c type* biomarkers, indices of a chronic inflammatory state in which the restoration of the homeostatic equilibrium is improbable, are suitable for the selection of subjects for which the adjuvant therapies represent an increase in the risk of worsening rather than a benefit. For these same characteristics, *a, b, c type* biomarkers are also suitable for the risk/benefit monitoring in the selection of a better treatment for the subjects in an advanced stage, for the stratification in clinical/therapeutic subgroup (Fig. 3.3).

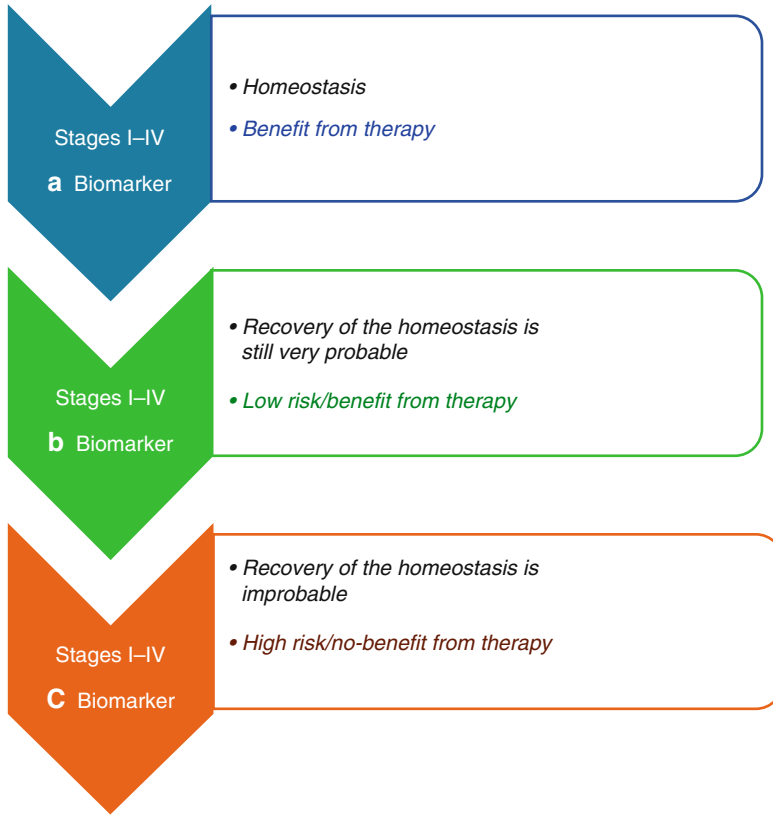

Fig. 3.3 Quantification of the risk/benefit for a better treatment selection. (**a**–**c**) Type biomarkers are also suitable for the risk/benefit monitoring in the selection of a better treatment for subjects in an advanced stage, for the stratification in clinical/therapeutic subgroup

3.4 Guidelines for the Identifications of "Suitable" Biomarkers: A Healthy Longevity Is Linked to an Healthy Function of the Immune System. The Pathology Is Generated by Alterations of This System

As mentioned above, since aging of the population is a basilar cause of the increase in degenerative diseases such as tumor, in order to select "suitable" biomarkers, it is necessary to discover which substances bring to: (1) a healthy longevity, for the definition of prognostic biomarkers of *a* and *b types,* respectively related with a *no-risk* and a *low-risk* of pathology; (2) the degeneration in the pathology, to discover the prognostic biomarkers of the *c type,* indices of a *high-risk* of pathology.

For the identification of a correct research guideline in the comprehension of the relationship between the aging and a healthy longevity or with its related dysfunctions, it is important to consider that a healthy longevity is related with a healthy function of the immune system [8, 9] and that the pathology is generated by alterations of this system. In this contest, it is inevitable to consider that men and women not only follow different pathways for the regulation of the immune response homeostasis, but these pathways, specific for each gender, also suffer alterations during aging and also differently predispose the two genders to disease and to treatments [8].

Therefore, for the identification of prognostic suitable biomarkers for the development of concrete preventions and treatment strategies for men and women, it is necessary to separately perform the study in the two genders, examining significant parameters for (1) a healthy longevity and (2) a pathology degenerations.

3.5 The Importance of the Evaluation of Both Genders as Independent Groups

This necessity is based on clinical and experimental data showing that, when the immune system is involved, men and women could not be assessed in only one group, because the results wouldn't be real, since in the immune response, there is a natural gender dimorphism [10–13].

During the reproductive ages, women have, for example, a more vigorous cellular and humoral immune response compared with men, and they also have more possibilities to reject transplants and tumors [14–19]. In addition, it has been proved that the immunosenescence affects both men and women, but it afflicts them differently. Men of every age and postmenopausal women present a less efficient T-cell response compared to premenopausal women [20]. Moreover, decrease of androgens in men during aging could contribute to their immunosenescence, but the loss of T-cell function, compared with women, is significantly less dramatic [21–23].

Multiple estrogen forms have been reported: estrone, estradiol, and estriol are the most common circulating forms. Estradiol binds both estrogen receptor-(ER)α and ERβ with high and equal affinities, while estrone preferentially binds ERα at a five times stronger affinity than ERβ [24]. Both pathways are involved in mediating estrogen effects, but ERα and ERβ present different functions inside the immune cells [25]. In premenopausal women, the estradiol derived from ovaries is the most common circulating estrogen, while estrone is the most common circulating form in postmenopausal women and in men. In men, testosterone is the primary substrate for estrogen production by the peripheral aromatization of androgens precursors, but it presents a modest difference related with age. However, the majority of studies have not revealed a significant correlation between age and the total level of estradiol in men [23].

Furthermore, it has been demonstrated that female and male hormones affect the immune system in opposite ways [26, 27]. For example, the Th1 and Th2 types of immune responses are respectively influenced, in prevalence, by androgens and/or estrogens: androgens favor the development of a Th1-type response and the activation of $CD8^+$ cells [28], while estrogens seem to direct the immune system to a Th2 dominance, where B-lymphocytes are activated and antibodies are produced [27]. Pregnancy, a state in which estrogen level is high, is characterized, in fact, by a prevalence of Th2: when this condition is not preserved, there is an increased risk of abortion [29, 30]. Research has shown that gender, male or female, is associated with relevant incidence and prevalence of different types of age-related diseases and is an important variable in the genetics of longevity [31–33], indicating an important observation: men and women follow different strategies to reach longevity.

3.6 Men and Women Follow Different Strategies to Regulate the Homeostasis of the Immune System

3.6.1 Variations of Pro- and Anti-inflammatory Cytokine Levels Regulate the Immune Response and Could Influence the Healthy State

Research on cytokines is a valid tool for the study of the immune system as they are crucial for regulations of its correct function, being substances that regulate the transfer of information between cells thanks to the activation of membrane receptors.

Moreover, these results [33] confirm that during aging, there is a remodeling of the network level profile of cytokines produced by T helper cells (Th) in our physiological system, which is essential in the correct regulation of the immune system. Moreover, variations of the relationship between the levels of pro- and anti-inflammatory cytokines regulate the immune response which could influence the longevity and the healthy state during aging.

For these reasons, the analysis of the network level profile of the cytokines is a useful tool to define the prognostic biomarkers for aging [8, 9, 33–35]. In this area, results of recent studies [35–37] are relevant which indicate, for the first time, that the immune response is regulated by cytokines that differ between men and women, attributable to these dissimilarities the different trend, between male and female genders, of (1) the immune response, (2) the susceptibility to diseases, and (3) the therapeutic response, opening a new area for the translational research at this level. This study has shown that the gender dimorphism in the Th cytokine pathways is normally present in the regulation of the immune response homeostasis of the healthy state: the antigen-presenting cell (APC) regulates the differentiation and the homeostasis of different Th cell types in rest state and during activation of the immune system of both sexes, but this effect is exerted through Th cytokine pathways specific for each gender (gender-specific) but also through pathways that are common for both (gender-common).

These results [35–37] show, in fact, that the IFN-γ cytokine regulates the male immune system, while IL-6 cytokine regulates the female immune system. The correct functions of the IFN-γ in men and IL-6 in women are biomarkers for the ascertainment of a healthy state of the immune system and then of a healthy longevity. It has been shown, in fact, that an altered functioning of these pathways is a prognostic biomarker for the passage from a healthy condition to the genesis of an adenoma and the progression to a colorectal tumor [35]. On the other side, correct functioning of the IL-10 cytokine regulates the return of the immune system to the rest state, after the response, both in men and women, and, for this reason, it is a prognostic for a healthy longevity in the both sexes but only if IFN-γ in men and IL-6 in women are correctly functioning. If this condition is not respected, IL-10 is a biomarker for the progression of the tumor [30] both in men and women. These results have also revealed that [35] (i) the immune cell production of IL-6 in women and IFN-γ in men decrease during aging; (ii) the age could be a significant and independent factor for the pathways of IFN-γ and IL-10 in men (iii) and for the pathways of IFN-γ and the soluble molecule of the IL-6 receptor (sIL-6R, that modulate the action of IL-6) in women [38].

3.6.2 "Double Prognostic Biomarkers": Appropriate Variations Between Pro- and Anti-Inflammatory Cytokines Assure the Success of the Immune Response but Following Different Gender Pathways

It has been shown that variations between pro- and anti-inflammatory cytokines could influence the success of the immune response [33–35]. However, the most relevant discovery for the definition of these suitable prognostic biomarkers is the identification of "double prognostic biomarkers": they are constituted by couple of pro- and anti- inflammatory cytokines that differ between men and women and assure the success of the immune response varying in appropriate correlation with each other [35, 36] and following different pathways in each gender.

These results [35, 36] have been shown that the early evolution of the immune response is influenced by the positive correlation between the production of IFN-γ——IL-10 and IL-6——IL-4 in men and the negative one between IL-6——IL-10 in women. The evolution of the late response is also influenced by the positive correlation between the production of IFN-γ——IL-4 in men and IL-6——IFN-γ in women. More specifically, the "double prognostic biomarkers"

for men are related by direct proportionality (they both increase or decrease together with the same trend, both positive or negative) between the IFN-γ——IL-10, IL-6——IL-4, and IFN-γ——IL-4 cytokine levels, while they are interconnected in women by an inverse proportionality (an increase of the first correspond to a decrease of the second and vice versa) between the IL-6——IL-10 cytokine levels and with a direct proportionality between IL-6——IFN-γ cytokines (Fig. 3.4). These variations between the couple of inflammatory and anti-inflammatory cytokines, specific for each gender, are to be considered "double biomarkers" gender-specific, in the valuation of relationships (1) between aging and a healthy longevity, to define the *a* and *b types* of indices, respectively of no and of low risk of pathology, for the healthy population prevention; of no and of low therapeutic risk for a better treatment selection of tumor patients; (2) between aging and pathology, to define the indices of *c type* with a high- risk of pathology for the healthy population prevention and a high therapeutic risk, for tumor patient treatments.

The relevance of the gender-specific differences in the regulation of the immune response is underlined by the evidence that homeostasis is preserved and there are no differences between men and women in the outcome of this response when the pathways of the gender-specific cytokines (IFN-γ e IL-6) still normally work [35, 36]. When alterations occur in the pathways of the gender-specific cytokines, the consequences for men and women are different, in terms of disease development. This event is related to the impairment of the immune system homeostasis, because the alterations of gender-specific cytokine pathways cause a pathologic polarization of specific T-cell types, different for each gender. The reason of this fact is related to the different effects on the generation of Th cell subtypes during the immune response generated by IFN-γ and IL-6 cytokines, present in the cellular environment. IFN-γ sustains the development of the Th1 type [39], while IL-6 supports the Th2-type differentiation and the activation of B-lymphocytes with the related production of antibodies. In addition, research in this area showed that it is not a single cytokine to determine a particular response but the interactions between different individual cytokines, organized in a network. These results [35, 36] indicate that the different predisposition to gender-specific diseases and the

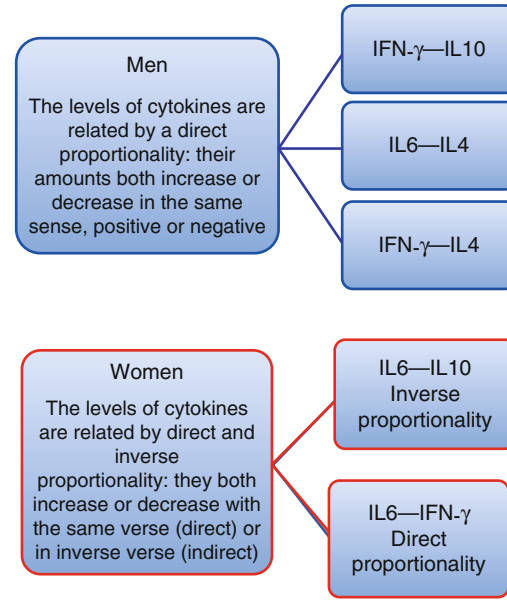

Fig. 3.4 The variations between pro- and anti-inflammatory cytokines influence the success of the immune response. The most relevant discovery is the identification of "double prognostic biomarkers," which are constituted by couple of pro- and anti- inflammatory cytokines that differ between men and women and assure the success of the immune response varying in appropriate relationship with each other and following different pathways in each gender. These results have shown that the "double prognostic biomarkers" for men are related by direct proportionality (they both increase or decrease together with the same trend, both positive or negative) between the IFN-γ——IL-10, IL-6——IL-4, and IFN-γ——IL-4 cytokine levels, while they are interconnected in women by an inverse proportionality (an increase of the first correspond to a decrease of the second and vice versa) between the IL-6——IL-10 cytokine levels and with a direct proportionality between the IL-6——IFN-γ cytokines. These variations between the couple of inflammatory and anti-inflammatory cytokines, specific for each gender, are to be considered "double biomarkers" gender-specific, in the evaluation of relationships (1) between aging and a healthy longevity, to define the *a* and *b types* of indices, respectively of no and of low risk of pathology, for the healthy population prevention; of no and of low therapeutic risk for a better treatment selection of tumor patients; (2) between aging and pathology, to define the indices of c type with an high risk of pathology for the healthy population prevention; and a high therapeutic risk, for tumor patients treatments

related disease progression are related to different cellular gender-specific polarization of the Treg, Th17, and Th9 cells, determined by interactions between TGFβ, IL-6, IFN-γ, IL-10, and IL-4 cytokines that vary between men and women [36]. These results are confirmed by the evidences of other researches that indicate the existence of mutual relationships in the Treg, Th17, and Th9 cells, because (i) TGFβ triggers the expression of Foxp3 transcription factor in naïve T cells, generating Treg cells, but (ii) IL-6 inhibits the TGFβ-driven expression of Foxp3, and TGFβ together with IL-6 induces ROR-gt transcription factor, triggering the developmental program of Th17 cells [36, 40], while (iii) IL-4 also inhibits TGFβ induction of Foxp3 expression, but TGFβ together with IL-4 induces Th9 cells' differentiation that produce IL-9 cytokine. The co-expression of IL-9 and IL-17 has been identified as a new function of Th17 in the mediation of tissue destruction and of the neurodegeneration [41, 42]. The female greater susceptibility to autoimmune diseases, as multiple sclerosis, could be attributed to the dominant influence of IL-6, in the inhibition of the regulatory cells that play a fundamental role in these diseases [40, 43–45]; the greater male susceptibility to, for example, the primary form of progressive multiple sclerosis (MS) [46] could be the result of the prevalent influence of IFN-γ in the control of Th9 cells that predispose to neurodegeneration. The IL-9 receptor complex is constitutively expressed on the astrocytes, and IL-9 induces the expression on these cells of CCL-20 but not the other chemokines [47], determining the migration of Th17 cells in the CNS where they promote neurodegeneration. Treg, Th9, and Th17 cells have shown to be important in the autoimmune diseases, as rheumatoid arthritis [48] and MS [49].

3.6.3 The Efficiency of the Treatment Is Related to a Reestablishment of IL-6 Pathways in Women, and IFN-γ Pathways in Men

Results in the research on MS [36] confirm these data, showing that alterations of the IL-6 pathways are involved in the loss of homeostasis of the immune system which is evident in a disequilibrium of Treg cells and in an increase of the neurological deficit in both gender, underlining the autoimmune etiology of MS. Further support of the existence of gender-specific pathways is provided by this study, observing that the benefit of the treatment with IFN-β (in the reestablishment of the immune system homeostasis and in the inhibition of the neurological deficit) is related to the reestablishment of IL-6 pathways in women and IFN-γ pathways in men. Moreover, the serum levels of the soluble molecule sCD30 and of the TGFβ cytokine could be used in both genders as "double biomarkers," for the assessment of the therapeutic success in terms of reestablishment of the immune homeostasis of the Th cells and the absence of the progression of neurological deficit.

Overall, these results shed light on the necessity of (a) suitable gender-specific biomarkers and (b) gender-specific drugs, whose activity could consider the different regulation system of the immune response between the two genders, assuring the same therapeutic results: return to the physiologic homeostasis thanks to the passage from an activation pathological state to a rest one.

On these bases, it is possible to affirm that the definition of suitable gender-specific biomarkers could lead to new strategies of preventions and to personalized treatments [37].

3.7 The Valuation of the Thioredoxin and CD30 Systems for the Prognostic, Diagnostic, and Therapeutic Stratification of Patients

It has to be considered that specific mechanisms responsible for different disease susceptibility between men and women have still to be clarified. However, research suggests that response could be found in different capacities of male and female cell to defend themselves from the oxidative stress [50, 51]. The cells of men and women differ in terms of production of reactive species of oxygen (ROS) and susceptibility to the oxidative stress [51]. This concept constitutes a new enthusiastic research area.

Oxygen metabolism could lead to the production of ROS in every type of cell, including cells in the immune system, that present antioxidant compounds and enzymes (as glutathione and thioredoxin reductase) [52, 53] and able to neutralize ROS and preserve the cellular oxidative equilibrium. However, the activity of ROS seems to be regulated in different ways between men and women and could be directly influenced by sexual hormones [51].

In vivo studies have also revealed the incapacity in male, but not in female, in the maintenance of reduced intracellular redox condition, essential for normal cellular functions [50], and this circumstance explains, at least in part, the differences between the two genders of the Th gender-specific cytokines pathways in the regulation of the immune system homeostasis. IFN-γ pathways, specific for male, is, in fact, a direct stimulator of the thioredoxin (Trx) and thioredoxin reductase (RTrx) gene expression in human T cells [53, 54], and there is a positive feedback between IFN-γ and the genetic expression of Trx/RTrx in the intracellular oxidative reduced regulation that is essential for the immune response. For these reasons, it is correct to suppose that the immune response through the IFN-γ pathway in men is indispensable for the activation of the Trx1/RTrx1 system and for the decrease of the intracellular oxidation levels, in order to preserve the oxidative cellular equilibrium. Indeed male cells, as previously pointed, are not able to maintain the intracellular oxidative reduced condition.

Therefore, these results indicate that for the identification of male and female gender-specific targets and biomarkers in an easy and early way in association with the onset of the disease, it is also required to valuate, in the peripheral blood, the factors that regulate the redox system and that intervene in multiple cellular process, as proliferation, cellular cycle, and death or survival signal pathways [55–58]. Indeed for the neoplasia, these processes are really relevant because they both interfere in the regulation of the host response to the tumor and the tumor to the host [59–63], and for these characteristics, they are potential targets/biomarkers with an ample predictive capacity in the clinical, diagnostic, and therapeutic stratification of oncologic patients. The use of these targets to identify new drugs to reestablish the physiologic homeostasis of the apoptotic process between the immune cell and the tumoral tissue is also fundamental for the definition of therapies specific for the regression of the neoplasm.

The immune-modulatory role of the oxidative stress is a support to the importance of the redox system in this contest. The oxidative stress is defined as cellular toxicity caused by oxygen derived free radicals and it determinates the loss of homeostasis in the redox cellular system and the functional body, as it makes the extra/intracellular environment balance impossible, which is vital for the normal functional body response [61]. Indeed, in homeostasis, the cell is able (1) to balance the activity of the oxidant/antioxidant factors, (2) to maintain reduced intracellular redox environment [56, 57], and then (3) to assure the normal functionality. In case of alterations in the redox system, the cell is no more able (1) to balance the relationship between oxidant/antioxidant factors, (2) to adapt the extra/intracellular environment and, for this reason, (3) the intracellular redox system is not reduced and the organism could not respond to the environment necessity, or it perform these activities in an inappropriate way [64]. In addition, it has to be underlined that for the biggest part of tumor agents, including the radiant therapies, the cytotoxicity for the tumor regression is concretized trough the induction of oxidative stress that originates from cellular injury caused by intermediary oxidant factors of the redox system [65]. A support of this hypothesis is furnished by the evidence that in tumors, including colorectal cancer, alterations in the physiological pathways of redox system regulations have been identified, and the results show that the functional inactivation of the immune cells produced by free radicals represents a significant immunosuppressive mechanism of the cellular response for the defense against the tumor [66–70].

Essential redox factors for the immune response are the ones appertaining to the thioredoxin system (Trx) which is a physiologic and fundamental regulator of the redox-mediated cellular reactions. Trx1 is a protein-containing selenocysteine and it catalyzes the NADPH-dependent reduction of the thioredoxin reductase (RTrx1)

with other numerous oxidized cellular proteins [71–73]. After an oxidative stress, Trx1 produces different cellular signals that activate the specific transcription factors regulating the nuclear decode of genes involved in the production of substances able to defend the cell against the oxidative stress induced by free radicals [74, 75].

On these bases, it is clinically relevant that CD30 (a membrane receptor of the immune cells as T and B cells, monocytes, dendritic cells, NK, eosinophils, and granulocytes) is the specific Trx1 immune receptors [76]. The potential of Trx1 and CD30 as single targets and biomarkers has yet been explained in the literature and in tumors, but the innovative hypothesis is the combined use of Trx1 and sCD30 (the soluble molecule of CD30 receptor) as a double target/biomarker (*Trx1-CD30*) [77]. The rational of this new direction is that the double target/biomarkers together with immunological and genetic-related parameter concretize (1) the availability of a composition of "suitable prognostic biomarkers" that are gender-specific and gender-common, for the prevention programs on healthy population and for the stratification of patients in clinical/therapeutic subgroups or to obtain personalized treatment; (2) it could also be the guideline for pharmacological interventions on the redox, immunological, and neurological systems, in chronic-degenerative states as tumor [77] and aging [37].

3.7.1 Trx1/RTx1 System

The redox control of the cellular physiology is one of the most relevant regulatory mechanisms in every living organism. Mammal cells contain two Trx-systems. The first is the Trx1/RTx1 that is normally localized in the cytoplasm but, in stress condition, could migrate into the nucleus (inducing the genetic codification) or it could be secreted in the extracellular environment [78] and, in this way, it attends the immune system network. The second system (Trx2/RTrx2) is localized in mitochondria and in the cytoplasmic reticulum, and it regulates cellular apoptosis [79]. There are two other system: the first is called TRXs testis/sperm-specific and is localized on the spermatids (Sptrx-1, Sptrx-2, and Sptrx3), and the second is the Txl-2 localized in lungs and in other ciliate tissues [80].

Trx1 is a thermostable protein of 108 amino acids largely distributed in all organisms from the bacteria to the mammals, containing a disulfide bridge, without metals and with a catalytic site that uses hydrogen as a donor for the oxide-reduction reactions [71, 81]. Its reduced form is able to reduce ribose and phosphate ribonucleosides. The oxidized form is reconverted in the reduced one thanks to the flavoprotein thioredoxin reductase (TrxR), with the participation of NADPH, forming the "thioredoxin redox system." Trx1 regulates the activity of enzymes as the "apoptosis signal-regulating kinase 1" [82], the caspase-3 protease that promotes apoptosis [83], and the "protein kinases C" [84]; it also promotes the binding and activation toward the DNA [85] of transcriptional factors as AP1 [86, 87], the "nuclear factor KB" (NF-κB) [88], the "glucocorticoid receptor" [89], and p53 [81]. Human T cells transformed by virus produce a factor called ADF, and it is identical to the human Trx1 [90]. Trx1 is also secreted by activated B-lymphocytes, the B-lymphocytes of the B-type chronic leukemia, fibroblast, and T-lymphocytes [91, 92]. Trx-1 is a powerful growth and survival factor for the cell [84, 87], and its expression is increased in different tumor types, especially in the most aggressive forms [90, 91], and it is related with the tumoral aggressiveness and with the inhibition of the immune system [81, 93]. Increased levels of Trx-1 have been associated with a decrease of survival in pulmonary tumor patients. In tumors, the increased expression of Trx-1 has been identified as an independent prognostic factor for the progression and the expression of VEGF, and Ref-1 is also associated with this datum [94]: these are important assumptions for the new therapies with monoclonal antibody that are specific for these cellular receptors.

3.7.2 The CD30/CD30L/sCD30 System

The receptor (R) CD30 is a member of the TNFR/NGFR superfamily, and it was originally identified on primary culture cell of Hodgkin and

Sternberg [95]. CD30R is also expressed in other different T- and B-cell lines after viral transformation; normally mononuclear cells of the peripheral blood (PBMCs) do not express CD30R, but a subgroup of T $CD45^+$ RO^+ express CD30R after mitogenic stimulation [96]. It could make us able to say that CD30R could be used as a marker of the autoimmune cells. The physiological function of CD30R has not yet been clarified, but there are evidences that it could behave as a signal-transducing molecule. The interaction between CD30R and its ligand (CD30L) on the T- and B-activated cell, monocytes, neutrophils, and eosinophils induces the rapid activation of genetic transcriptional factors such as JunN-kinase and NF-κB [97, 98]. Indeed, it has been shown that CD30 signals induce and regulate the integrated lymphocytary genetic expression of molecules that have a cytotoxic effect. In addition, they control lymph nodal traffic, proliferation, and apoptosis [96].

CD30R is generally defined as a molecule that mediates the regulation signals. The results [98–102] clarified and underlined the importance of its physiopathologic function: the pathways that are regulated by the interaction between CD30R and sCD30 (its soluble component is released in the environment when RCD30 interact with its ligand CD30L) control the physiologic homeostasis in the immune and in the neurologic system, and they regulate the functions of monocytes and dendritic cells, mature and immature, to direct the T-helper cell (Th) differentiation in the respective subtypes (Th1, Th2, Th3, Th9, and Th17). These results clarify, then, that the functional link between Trx1 and CD30 is a very important step in the physiologic homeostasis and it underlines the big potentiality of these elements as clinical diagnostic and therapeutic targets (Fig. 3.5).

Indeed, results of the research explained that in addition to Trx1, sCD30 is also able to influence the CD30R capacity to mediate the activation of intracellular signals, due to the inhibition of the binding between CD30L and RCD30: Trx1 makes this function catalytically, modifying the stoichiometric structure of RCD30 [76]; sCD30 makes the same function binding and blocking the binding site of CD30L, with which it has a strong affinity [102]. During inflammatory situations, RCD30 is strongly expressed on the immune cells, and as a consequence, there is an increase of the sCD30 levels that is released in the extracellular environment, and then it has a blocking function toward CD30L, inhibiting the mediation of the RCD30 signals [102].

The results have, also, underlined that the sCD30 level variations in the cellular environment (serum, tissue or tumoral microenvironment) could be used as biomarkers of the correct functioning of the immune system and the therapeutic response [77, 98–103]: the sCD30 level within the normal physiological ranges is a positive index of the immune system homeostasis and of the therapeutic benefit. A significant increase of the sCD30 level is, on the other hand, a negative index because it denotes an immunological deficit and the lack of a therapeutic response. For these reasons, they have both to be considered for the use of RCD30 as immunological and therapeutic biomarkers, because Trx1 and sCD30 could both influence the capacity of CD30R to mediate the activation of intracellular signals. Accordingly, changes of the Trx1 and sCD30 levels are functional extracellular biomarkers of the new Trx1/CD30 target (Fig. 3.5), while the Treg/Th1/Th9/Th17 cytokines levels are functional biomarkers of the intracellular pathways for the prognostic and diagnostic/therapeutic stratification of patients [77, 104–106]. The explanation of the Trx1 and sCD30 molecule regulation could lead to a therapeutic noninvasive tool for the reestablishment of the immunological homeostasis toward a therapeutic response benefit.

3.8 New "Double Biomarkers" That Are Prognostic for Patient Stratification and for the Personalized Therapies

Using the upper described "double biomarkers" and determining the variation in the peripheral blood of the molecule levels within the couples (Fig. 3.6):

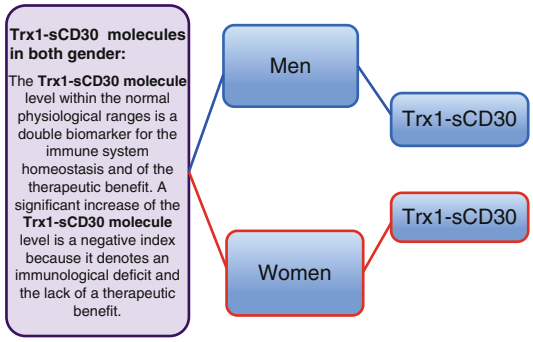

Fig. 3.5 The functional link between Trx1 and CD30 is a very important step in the physiologic homeostasis, and it underlines the big potentiality of these elements as clinical diagnostic and therapeutic targets. Trx1 and sCD30 are able to influence the CD30R capacity to mediate the activation of intracellular signals, due to inhibition of the binding between CD30L and RCD30: Trx1 makes this function catalytically, modifying the stoichiometric structure of RCD30 [76]; sCD30 makes the same function binding and blocking the binding site of CD30L, for which it has a strong affinity. Trx1 and sCD30 (Trx1-sCD30) level variations in the cellular environment (serum, tissue, or tumoral microenvironment) could be used as double biomarker of the correct functioning of the immune system and therapeutic response [98–103]: the Trx1-sCD30 levels within the normal physiological ranges are a positive index of the immune system homeostasis and of the therapeutic benefit. On the other hand, a significant increase of the Trx1-sCD30 levels is a negative index as it denotes an immunological deficit and the lack of a therapeutic response

Trx1—sCD30 molecules in both gender
IFN-γ—IL-10, IL-6—IL-4 and *IFN-γ—IL-4* cytokines in men
IL-6—IL-10 and *IL-6—IFN-γ* cytokines in women

it is possible to define (1) the disease risk in healthy subjects for prevention programs and (2) the disease progression and therapeutic risk/benefit in tumor patients that are suitable for new strategies in stratified and personalized medicine (Fig. 3.6).

More exactly the double biomarkers are:

1. Of *a* type that are prognostic of homeostasis and indices of
   (a) No risk of pathology in healthy subjects
   (b) The necessity of surgery only, in the early phases of the disease (I and II stages)
   (c) Therapeutic benefits in the advanced phases (III and IV stages)
   if the levels of *Trx1—sCD30* molecules in both genders are within the normal physiological ranges and:
   - The levels of *IFN-γ—IL-10*, *IL-6—IL-4* and *IFN-γ—IL-4* for men are in the physiological range and are related by a direct proportionality: their amounts may be equal, above, or under the mean, and they both increase and decrease in the same sense, positive or negative;
   - The levels of *IL-6—IFN-γ* and *IL-6—IL-10* for women are in the physiological ranges, and they are respectively related by direct and inverse proportionality: their quantities are equal, above, or under the mean; they both increase or decrease (1) with the same verse, positive or negative, in case of direct proportionality; and (2) in inverse verse, positive the first and negative the second or vice versa, in case of inverse proportionality;
2. Of *b* type that are prognostic for a transient inflammation and indices of:
   (a) Low risk/benefit of pathology in healthy subjects
   (b) Benefit of the adjuvant therapies in addition to surgery in early phases of the disease (I and II stages)
   (c) Low risk/benefit of therapeutic treatment in the advanced phases (III and IV stages) if the levels of *Trx1—sCD30* molecules in both genders are or are not in the normal physiological ranges and:
   - The levels of *IFN-γ—IL-10*, *IL-6—IL-4,* and *IFN-γ—IL-4* in men are not in the physiologic range but are still related by the same direct proportionality, corresponding to the homeostatic condition.
   - The levels of *IL-6—IL-10* and *IL-6—IFN-γ* in women are not in the physiological range, but are still related by the same proportionalities, inverse and direct, corresponding to the homeostatic condition.
3. Of *c type* that are prognostic for chronic inflammation and indices of:
   (a) High risk of pathology for healthy
   (b) High risk/no benefit for the adjuvant therapies associated to surgery in the early phases of the disease (I and II stages)
   (c) High risk/no therapeutic benefit in the advanced phases (III and IV stages) if the levels of *Trx1—sCD30* molecules in both

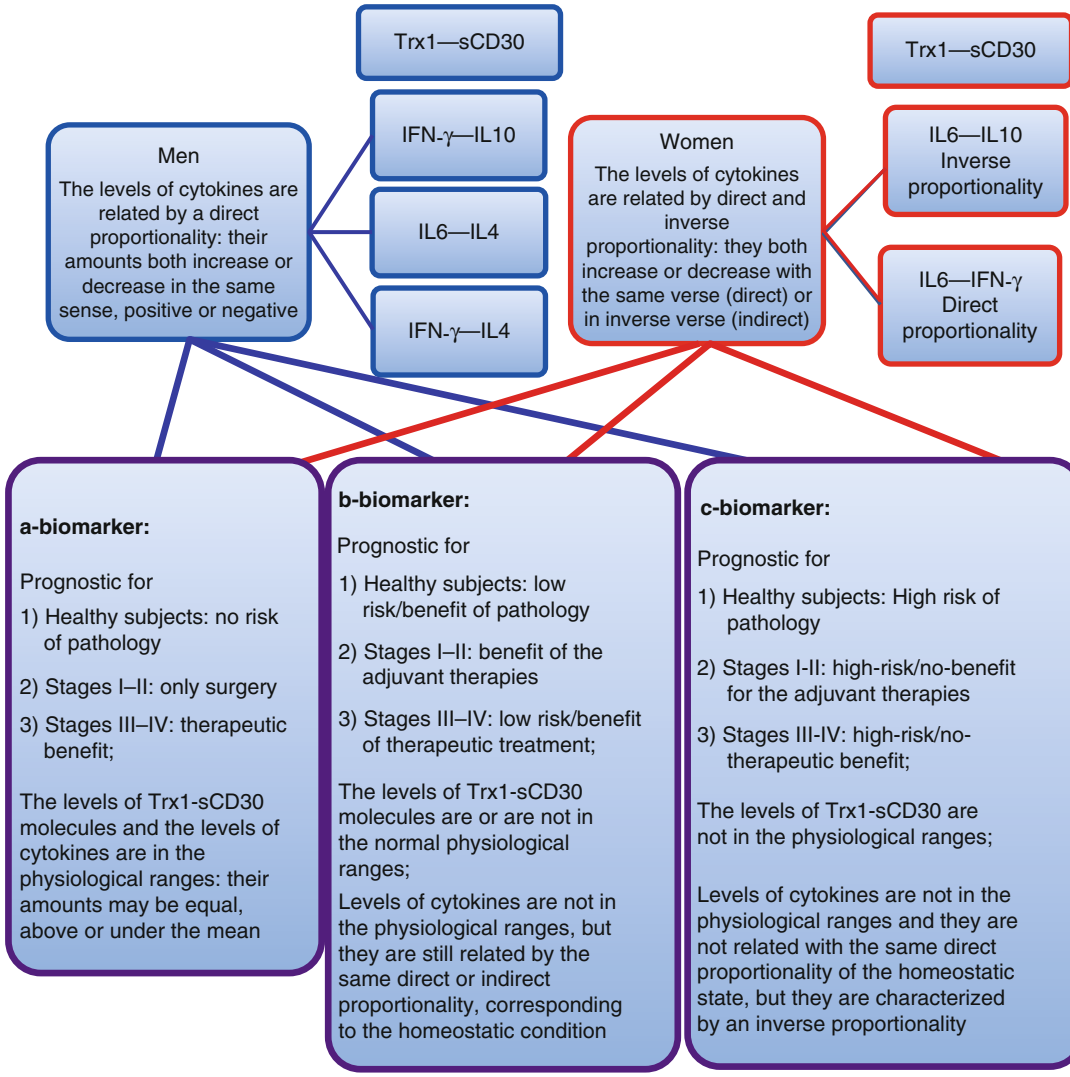

Fig. 3.6 New prognostic biomarkers for personalized therapies. Using the "double biomarkers" and determining the variation in the peripheral blood of the molecule levels within the couples make it possible to define (1) the disease risk in healthy subjects for prevention programs and (2) the disease progression and therapeutic risk/benefit in tumor patients who are suitable candidates for new strategies in stratified and personalized medicine. More exactly the double biomarkers are of (1) *a* type if levels of IFN-γ—IL-10, IL-6—IL-4, and IFN-γ—IL-4 for men are in the physiological range and are related by a direct proportionality (their amounts may be equal, above, or under the mean, and they both increase or decrease in the same sense, positive or negative); the levels of IL-6—IFN-γ and IL-6—IL-10 for women are in the physiological range, and they are respectively related by direct and inverse proportionality (their quantities are equal, above, or under the mean, and they both increase or decrease with the same verse, positive or negative, in case of direct proportionality and, in inverse correlation, positive the first and negative the second or vice versa, in case of inverse proportionality); (2) *b* type if the levels of IFN-γ—IL-10, IL-6—IL-4 and IFN-γ—IL-4 in men are not in the physiologic ranges, but they are still related by the same direct proportionality, corresponding to the homeostatic condition; levels of IL-6—IL-10—IL-6—IFN-γ in women are not in the physiological range, but are still related by the same proportionalities, inverse and direct, corresponding to the homeostatic condition; (3) *c* type if levels of IFN-γ—IL-10, IL-6—IL-4 and IFN-γ—IL-4 in men are not in the physiological range, and they are not related with the same direct proportionality of the homeostatic state, but they are characterized by an inverse proportionality; the levels of IL-6—IFN-γ and IL-6—IL-10 in women are not in the physiological range and are not related by the same inverse and direct proportionality of the homeostatic state, but the first couple by an inverse proportionality and the second by a direct one

gender are not in the normal physiological range and:

- The levels of *IFN-γ—IL-10*, *IL-6—IL-4,* and *IFN-γ—IL-4* in men are not in the physiological range and are not related with the same direct proportionality of the homeostatic state, but they are characterized by an inverse proportionality;
- The levels of *IL-6—IL-10* and *IL-6—IFN-γ* in women are not in the physiological range and they are not related by the same inverse and direct proportionality of the homeostatic state, but the first couple by an inverse proportionality and the second by a direct one.

3.9 Concluding Remarks

These results underline the necessity of (a) suitable biomarkers, specific for each gender and (b) gender-specific drugs, whose activity consider the different regulation system of the immune response between the two genders, assuring the same therapeutic result: the return to the physiologic homeostasis due to the passage from a pathologic activation phase to a rest one.

The network level profile analysis of cytokines is a valid tool to reveal the biological complexity of our physiologic system in the normal healthy state and in the pathological altered one. The variation of the pro- and anti-inflammatory cytokine levels regulates the inflammatory response, and they could influence the healthy state. "Double prognostic biomarkers" are formed by couples of pro- and anti-inflammatory cytokines that differ between men and women which assure the success of the immune response varying in appropriate relation with each other.

For men, these "double prognostic biomarkers" are defined by a direct proportionality relationship (they both increase and decrease with the same trend, positive or negative) between the levels of *IFN-γ——IL-10*, *IL-6——IL-4,* and *IFN-γ——IL-4* cytokines; while in women they are defined by an inverse proportionality relationship (when the first increases, the second decreases and vice versa) between the levels of *IL-6——IL-10,* and by direct proportionality between *IL-6——IFN-γ* cytokines.

These variations between gender-specific inflammatory and anti-inflammatory cytokines have to be considered as "double gender-specific biomarkers" in the evaluation of (1) the aging processes and the healthy longevity, to define the *a* and *b* indices that indicate no or low risk of pathology for the healthy population, respectively, and no or low therapeutic risk/benefit for tumor patients; (2) aging processes and pathologies, to define the *c* type index of high-risk of pathology for the healthy population and of high therapeutic risk/no benefit for tumor patients.

Trx1/CD30 is surely a promising target for new therapies in immunological pathologies as tumor. The interactions between sCD30 and Trx1 molecules regulate the normal immune homeostasis because both factors regulate the normal function of the CD30 receptor, although with different pathways (the first blocking the ligand CD30L and the second stoichiometrically, deforming the receptor). These substances have, then, an homeostatic function on redox immunological and neurological systems, in which *Trx1——sCD30* molecules are a suitable "prognostic double biomarker".

The new target Trx1/CD30 is a concrete prospective for new drugs. The *Trx1——sCD30* double biomarker and the related changes of the "double gender-specific biomarkers" defined by couples of inflammatory and anti-inflammatory cytokines that differ between the genders, open to new and important clinical diagnostic and therapeutic prospects for the predictive medicine of immune diseases including tumor (Fig. 3.6).

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Tumor Antigen and Epitope Identification for Preclinical and Clinical Evaluation

4

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4.1 Introduction

With the maturing of our insights in tumor biology and basic immunological mechanisms, we are now equipped to rationally develop immunotherapy approaches against cancer. The identification of human tumor antigens (TA) has provided the basis for tumor-targeted immunotherapy [1], especially that mediated by T lymphocytes recognizing MHC-associated target peptides. Several families of cancer antigens are now recognized (summarized in Table 4.1) and include cancer–testes antigens (CTA), viral antigens, oncogene and tumor suppressor gene products, differentiation antigens, fusion proteins, and overexpressed normal gene products such as cell cycle genes. Although more than 1,000 different antigens have been reported in the literature, not all represent appropriate target antigens, either because of their restricted expression profile or high levels of expression in vital normal tissues.

Major histocompatibility molecules (MHCs) present on the cell surfaces allow the immune

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Table 4.1 Potential TAAs and targeted cancer

Groups of tumor antigens	Antigens	Cancer type
Cancer testis (CT)	MAGE1-3 and -6	Melanoma, breast, head/neck
	HAGE, GAGE, NY – ESO – 1, BAGE, XAGE	Bladder, gastric and lung, head/neck, many cancers
Differentiation antigens	Tyrosinase, gp- 100, TRP-1, and -2, MART-1	Melanoma
	NY-BR1	Breast cancer
Viral antigens	EBV	Burkitt's lymphoma
	HepB	Hepatitis B
	HPV	Cervical and penile cancer
	HTLV	T-cell leukemia
Oncofetal antigens	CEA	Colon, breast, pancreatic
	α-fetoprotein	Liver cancer
	5T4	Many carcinomas
	Oncotrophoblast glycoprotein	Many carcinomas
Tumor-specific antigens	CDK4	Melanoma
	Caspase-8	Head/neck
	b-catenin	Melanoma
	BCR/ABL	CML
	p53 (mutated)	Breast, colon, other cancers
	Ras (mutated)	CML, AML, ALL
Overexpressed/mutated antigens	HER-2/neu	Breast, ovary, lung
	MUC-1	Breast, adenocarcinoma colorectal
	p53 (nonmutated)	Lung, bladder, head/neck
	WT-1	Pancreatic, colon, lung
	Proteinase-3	CML
	PAP, PSA, PSMA, survivin	Prostate
Idiotype antigen	Ig idiotype	B-cell NHL, MM

system to distinguish between self, modified, or nonself antigens. MHCs present cleaved protein fragments in the form of peptides following processing via the proteasome (class I) or the endosomal compartment (class II). Class I peptides induce cytotoxic effector T cells (CTLs) with specificity against tumor antigens, whereas class II peptides induce T helper (Th)-mediated immune response, which are also important in assisting the development of CTL memory. Hence it is recognized that both class I and class II epitopes should be included in peptide vaccine strategies.

Following the first report in 1991 that vaccinations with a single MHC class I binding CTL peptide epitope in IFA-protected mice against a subsequent challenge, many studies focused on the efficiency of this mode of vaccination [2]. This method proved to be beneficial in some preclinical models, for example, protection against the outgrowth of HPV16 in mice, but failed to show a good clinical correlation. Further studies on the peptide vaccination strategy showed that increasing the length of the peptide to include multiple CTL and Th epitopes significantly enhanced the efficiency of peptide vaccinations. The use of peptides where the anchor residues are substituted or "mimotopes" to enhance MHC–antigen interaction is a current strategy [3].

It has been observed that bulky tumors (developed at later stages of cancer) elicit tolerizing conditions within their tumor microenvironment, providing an escape mechanism for the tumor. Tumors are also responsible for suppression of immunosurveillance which inhibits the local antitumor immune response. Suppression of the immune system takes place through different mechanisms such as impairment of antigen pre-

sentation, activation of negative co-stimulatory signals, active biosynthesis of immunosuppressive molecules, recruitment of regulatory T cells (Tregs), and transformation of T cells locally into Tregs. Thus, suppressor cells produced within the tumor can migrate to lymph nodes and can give rise to immunosuppression which may represent an important mechanism for failure of immunotherapies in clinical trials. Using adjuvants/agonists has provided a new avenue, leading to the improvement in recurrence-free survival. This allows us to conclude that vaccination at early stages of disease progression is advantageous for inducing a stronger antitumor immune response.

Improved methodologies have significantly contributed to tumor antigen identification and assessment of functionality. The most commonly used techniques can be broadly divided into two, namely, the reverse immunology approach and the direct approach. In this review we consider these approaches in some detail, outlining the basis for their use. Finally, we will discuss the way in which immunogenic peptides derived from tumor antigens are being used in cancer immunotherapy trials.

4.2 Reverse Immunology Approach to Peptide Identification

The steps involved in this approach are well established (Fig. 4.1) and are less time consuming compared to the direct approach (Fig. 4.2). Firstly, candidate genes are identified/selected based on tumor-restricted expression. This can be achieved using PCR assays to determine RNA expression levels or antibody staining of cancer *vs*. normal tissues to identify differentially expressed antigens. Secondly, immunogenic peptide epitopes of cancer antigens are predicted by in silico analysis using several different computer-based algorithms. On successful validation on the immunogenicity of the identified peptide epitopes *in vitro* and *in vivo*, they would be selected for clinical trials. To date, reverse immunology has resulted in the identification of several MHC class I and class II peptides, which are recognized by antigen-specific T lymphocytes. These include peptides derived from MAGE-1, MAGE-2, MAGE-3, TRP2, gp100, HER-2/neu, SSX-2, PRAME, and EphA.

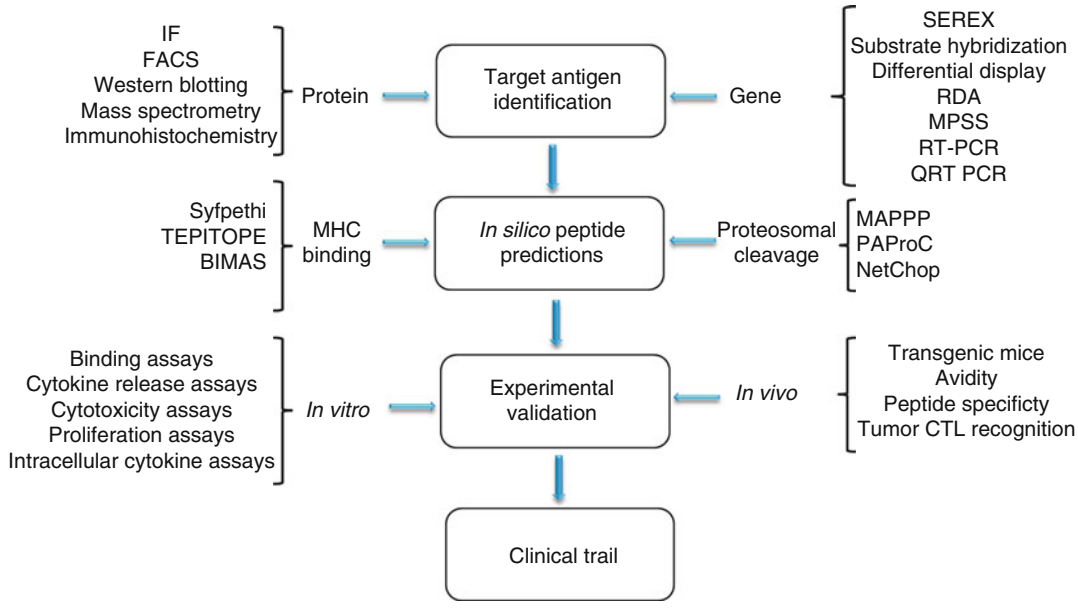

Fig. 4.1 Steps involved in reverse immunology approach

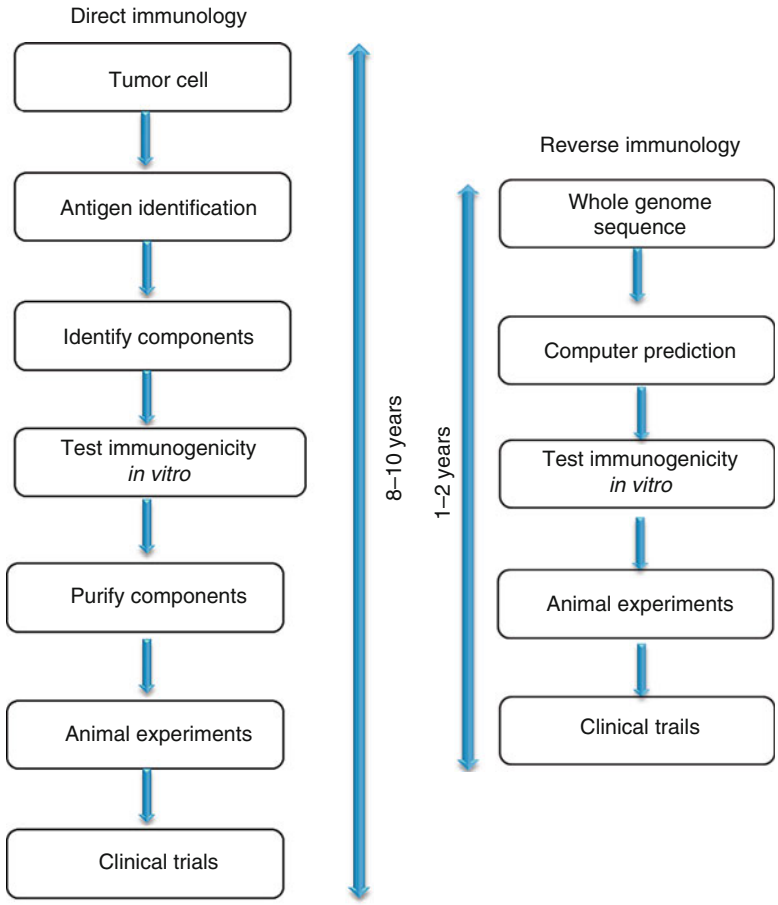

Fig. 4.2 Comparison of direct and reverse immunology approaches. Reverse immunology is well established and less time-consuming compared to direct approach

4.2.1 Target Antigen Identification

The identification of cancer antigens for immunotherapy represents a crucial step towards clinical immunotherapy. Tumor antigens are divided into tumor-unique antigens, whose expression is restricted to tumor tissue- or tumor-associated antigens which are usually overexpressed in tumors, but may show low levels of expression in normal tissues. In functional terms, TAs can be broadly divided into antigens that are required for tumor development and progression (indispensable antigens) and those antigens that are nonessential. Immunotherapy approaches targeting antigens that are not crucial for tumor development may eventually result in antigen-loss variants arising within the tumor, eventually leading to tumor escape. Therefore an ideal TA candidate would be quintessential for tumor development and would also be expressed in a wide variety of tumors making it a "Universal TA" [4]. The National Cancer Institute recently conducted a program to prioritize cancer antigens to establish a list of "well-vetted," priority ranked TA targets based on predefined unprejudiced criteria [5]. Adopting a pairwise approach, the criteria weighting for antigens, in descending order, was as follows: (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and the percentage of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression [5].

The initial step in reverse immunology is the search for protein/gene expression patterns selectively observed in tumor cells. Protein overexpression could be detected by using techniques such as immunofluorescence, western blotting, flow

cytometry, etc. The shortcoming of these techniques is that they are time consuming and depend on the availability of antibodies with high sensitivity and specificity. Also, with this method it is difficult to estimate protein turnover [6]. New large-scale gene expression assays such as cDNA microarrays, oligonucleotide chips, cDNA library sequencing, serial analysis of gene expression (SAGE), massively parallel signature sequencing (MPSS), subtractive hybridization, differential display PCR, and representational difference analysis (RDA) are a few of the techniques widely used to decipher complex expression patterns and identify new candidate antigens. SEREX (serological analysis of recombinantly expressed clones) has been a widely used technique that relies on the use of cancer patients' sera to screen selected tumor cDNA libraries [7, 8]. Tumor antigens identified via this method usually contain $CD4^+$ T-helper lymphocyte epitopes. The first tumor antigen, MAGE-1, was discovered through autologous typing and application of a newly developed DNA-cloning technique for defining the targets of T-cell recognition [9]. A melanoma patient with unusually favorable clinical course was identified to have CTLs that recognized autologous tumor cells. With antigen-specific T cells as a reagent and through the use of cosmid gene libraries, it was possible to identify and clone the *MAGE-1* gene. Studies on *MAGE-1* showed for the first time that the human immune system can respond to TA, and the findings transformed the study of tumor antigens and stirred a dynamic effort to discover tumor antigens, which has resulted in a long and still-growing list of antigens from a variety of tumors which potentially serve as targets for immunotherapy.

The use of antigenic epitopes/peptides to promote antitumor immunity represents one of the simplest and most applicable ways of targeting cancer cells expressing the respective protein. Recent studies have shown that immunotherapy approaches targeting multiple epitopes at the same time [10] or use of long synthetic peptides that would comprise multiple epitopes [11] can result in delivering clinical benefits to a greater number of patients. This strategy would broaden the clinical response in several ways:

1. In principle this would stimulate both CTL and T helper cell epitopes simultaneously.
2. Targeting different HLA types will increase the number of patients eligible for the vaccine.
3. This approach would decrease the risk of immune escape by tumor cells.
4. In addition, it would allow the synthesis of an "off-the-shelf vaccine" that could be used for different types of tumors.

4.2.2 In Silico Peptide Predictions

A number of computer-aided tools have been developed for the prediction of T-cell epitopes. These algorithms are based on the natural processing and presentation of proteins; the number of computer-aided algorithms available for the prediction of better T-cell epitopes reflects the existing level of understanding antigen processing. Thus, the tools designed for T helper epitope prediction are comparatively less advanced than for CTL epitope prediction.

For a target protein to be successfully presented on an MHC class I or class II molecule, it must undergo a number of processing steps resulting in the transport and cleavage of the peptide. Epitopes presented on MHC class I molecules are 8–11 amino acid length chains and are predominantly derived from intracellular proteins. A cytosolic multi-subunit proteolytic complex, known as the proteasome, degrades proteins to peptides which are later transported into the endoplasmic reticulum (ER) by the adenosine triphosphate (ATP)-dependent transporters associated with antigen processing (TAPs) [12]. The alpha chain of MHC I binds to the beta-2 microglobulin unit with the help of calnexin. Once stable, calnexin is replaced with calreticulin and tapasin [13]. Within the ER, peptides undergo further N-terminal trimming before their subsequent loading into the empty MHC-binding cleft. The epitope binds tightly to the epitope-binding cleft stabilizing the trimeric complex transported to the cell surface via the ER and Golgi network [13]. Exogenous proteins are processed mainly by the MHC class II pathway. The alpha and beta subunits of the MHC class II molecule are preoccupied by a non-polymorphic

invariant chain (Ii) which acts as a chaperone for class II folding and prohibits binding of intracellular proteins to MHC II [14]. The extracellular proteins are engulfed by endosomes which transport them to the Golgi apparatus. The inactive MHC II molecule is also transported to the Golgi where proteolytic degradation of Ii occurs, leaving the class II-associated invariant peptide (CLIP) in the peptide-binding cleft. The MHC II–CLIP complex can then interact with human leucocyte antigen (HLA)-DM (H-2M in mouse) which activates the dissociation of CLIP, allowing the loading of peptides into the empty MHC class II cleft [14]. The development of software prediction algorithms is based on peptide–MHC interactions or on proteasomal degradation.

4.2.2.1 Peptide–MHC Interactions

This is one of the early prediction tools developed and is based on the fact that MHC molecules would bind to peptides with similar "motifs." This fundamental principle led to the development of computer-based algorithms which screen potential peptide sequences of defined lengths and with similar binding motifs.

Broadly, the MHC-binding peptide prediction methods can be divided into three main groups: (a) motif-based methods, (b) statistical/mathematical expression-based methods, and (c) structure-based methods. Motif-based methods consider every amino acid within a peptide and assign it a positive or negative value, depending on the characteristics of the MHC groove with which it will interact [15]. SYFPEITHI (www.syfpeithi.de) is one of the widely used evidence-based motif matrix, as the data used within the algorithm are derived from the knowledge of actual natural ligands and can predict both class I and class II epitopes [16]. Another matrix-based prediction tool widely used is TEPITOPE (www.vaccinome.com), in which matrices are constructed based on the interaction of every amino acid with the MHC-binding cleft [17]. Nonetheless, instead of determining this empirically for each HLA allele, it combines these data with HLA sequence variation data to form virtual matrices. Even though the program is restricted to MHC class II, it allows prediction of highly promiscuous peptides within one search [17]. Structure-based methods calculate the binding energy of the peptide–MHC complex, and peptides energetically favored are predicted as binders. BIMAS is such a prediction system (http://bimas.cit.nih.gov/) that generates results expressed as estimated peptide dissociation values [18]. Structure-based methods utilize the power of artificial neural networks. The predictive accuracy of this method is very high, but these are more complex, nonlinear self-learning systems and require large amounts of data for learning [18]. PREDICT (http://sdmc.lit.org.sg:8080/predict/) and nHLAPred (http://www.imtech.res.in/raghava/nhlapred/) are examples of epitope selection methods.

4.2.2.2 Proteasomal Degradation

As described earlier, the proteasome is charged with recycling proteins and, hence, plays a major role in deciding whether a peptide is likely to be available to bind to MHC molecules. The proteasome has at least three different catalytic activities: trypsin-like (cleavage after basic amino acids), chymotrypsin-like (cleavage after hydrophobic amino acids), and peptidyl-glutamyl peptide-hydrolyzing activity (cleavage after acidic amino acids) [19]. The overall enzymatic activity (cleavage, inhibiting or enhancing) is a result of interaction between all subunits making the process complex. At present, three proteasome cleavage prediction methods are publicly available: PAProC (www.paproc.de) developed by Tubingen University, MAPPP (www.mpiib-berlin.mpg.de/MAPPP/) developed at the Max Planck Institute in Berlin, and NetChop (www.cbs.dtu.dk/service/NetChop/) developed at the Center for Biological Sequence Analysis at the Technical University of Denmark. Prediction Algorithm for Proteasomal Cleavages (PAProC) is a method for predicting cleavages by human and yeast (wild-type and mutant) proteasomes [20]. The influence of different amino acids at different positions is assessed using a stochastic "hill-climbing" algorithm. The PAProC server also allows the identification of peptides cleaved by the immune proteasome [21]. This is highly advantageous since current data suggest that

some tumor antigens, such as MAGE-3 (melanoma-associated antigen 3), would only be produced by the immune proteasome [22]. Also components of the immune proteasome have been found to be associated with tumor-infiltrating lymphocytes (TILs) in spontaneously regressing tumors. Though the complete role of the immunoproteasome has yet to be deciphered, selection of epitopes from both types of proteasomes will unveil the epitopes more suitable for immunotherapy [23].

MAPPP (MHC-I Antigenic Peptide Processing Prediction) is another approach that combines the proteasomal cleavage with MHC-binding prediction [24]. FragPredict is a component of the MAPPP package that deals with the proteasome cleavage prediction and consists of two algorithms; the first algorithm uses statistical analysis to predict potential cleavage sites, while the second uses results of the first algorithm as an input and predicts the fragments most likely to be generated. The second algorithm is based on the time-dependent degradation of a kinetic model of the 20S proteasome [24].

NetChop is a neural network-based method anchored on MHC class I ligands generated by the human proteasomes [25]. The rationale behind this approach is that every MHC ligand has to be generated by the proteasome; therefore, these ligands bear the closest resemblance to naturally processed *in vivo* cleavage products. The MHC class I ligands used to develop NetChop were compiled from public databases [20], two versions of which are available, 1.0 and 2.0, and the later version is trained with a data set that is three times larger.

A comparative study of PAProC, MAPPP, and NetChop showed a sensitivity ranging from 40 to 80 % [20]. The three programs varied in both sensitivity and specificity, but in general, programs with higher sensitivity had a lower specificity and vice versa [20].

4.2.3 Epitope Validation

In the validation phase of reverse immunology, the natural presentation and immunogenicity of the selected epitopes should be corroborated. Using cell lines or tumor tissues expressing the appropriate antigen and HLA allele, biochemical methods can be used to elute peptides from the cell surface or from isolated HLA antigen. The purified products are then analyzed by mass spectrometry [26] to derive sequence information and identify the target peptide (as discussed in detail below). Though this technique confirms the expression of HLA-bound ligands, it does not allow the assessment of peptides' immunogenicity. Assessing the immunogenicity of predicted peptides relies on demonstrating their ability to stimulate MHC class I- or class II-restricted T-lymphocyte responses. These may be either a primary response, where naïve T cells respond to antigens in culture or secondary, where, for example, patient $CD8^+$ or $CD4^+$ T cells, already exposed to antigen *in vivo*, demonstrate a secondary response. However, patient response to self-(tumor) antigens is generally quite weak, and T lymphocytes may become tolerant towards this antigen. Tolerance may be overcome by exposure of patient lymphocytes to a combination of interleukin (IL)-2 and IL-12 *in vitro*, which enhance the tumor-specific $CD8^+$ T-cell response and additionally prevent overgrowth of nonspecific, less-effective lymphokine-activated killer cells [27]. IL-12 is a potent inducer of tumor-specific CTLs and promotes the production of Th1 cytokines [28].

The immunospot assay, which is based on the detection of cytokine secretion in response to antigen, is used to detect antigen-reactive T-cell responses. Most current assays for measuring T-cell cytotoxicity are based on alterations in plasma membrane permeability and the subsequent release (leakage) of components into the supernatant (51Cr, lactate dehydrogenase assays) or the uptake of dyes (CFSE), which are normally excluded by viable cells. Another alternative is the use of flow cytometry to detect the expression of CD107 in the membrane, which is transiently expressed during the process of cell killing [29]. Use of cytokine-secretion assays, intracellular cytokine assays, HLA class I multimer staining (e.g., peptide-specific tetramers), etc. are among the techniques that have been thoroughly validated and established recently [30]. The use of tetramers has proved to be especially successful for the identification of peptide-specific $CD8^+$ T lymphocytes and to a lesser extent for $CD4^+$ T helper cells.

4.3 Direct Immunology Approach

The inherent weakness of reverse immunology is the incredibly low probability of identifying a peptide that is naturally processed, presented, and sufficient to induce CTL activity and tumor lysis. As a consequence, low-throughput attempts to screen limited numbers of peptides are typically unsuccessful. In addition, MHC peptides with low MHC receptor-binding affinities or those carrying post-translational modifications cannot be predicted with this approach. Hence, the laborious approach of direct biochemical isolation of T-cell epitopes still remains invaluable. Tumor cells (isolated from solid tumors or blood) or tumor cell lines could be used as a source for MHC–peptide isolation. Studies on tumor cell lines are advantageous due to their unlimited expansion capacity *in vitro*. However, the variations induced by *in vitro* passaging should be taken into consideration. Hence direct analysis of uncultured tumor cells should be performed where possible. The steps involved in direct immunology approach are (1) isolation and purification of peptide–MHC complexes, (2) analysis of purified epitopes, and (3) assessment of the immunogenicity of epitopes.

4.3.1 Isolation of Peptide–MHC Complexes

The techniques commonly used for isolation of MHC-associated peptides usually involve immunoaffinity chromatography and acid elution. Immuno-affinity chromatography (IAC) combines the use of LC with the specific binding of HLA antigens to antibodies or related agents [31]. The source material for this approach is usually frozen tissue, blood cells, or cultured cell lines. Solid tissue is first mechanically dissociated in the presence of protease inhibitors (to avoid any cleavage of MHC complexes) at a stable pH value (usually between pH 7.0 and 8.0). After washing (by centrifugation and filtration) the lysate is passed over MHC-specific monoclonal antibodies bound to sepharose beads. The beads are then washed to remove excess detergents, and MHC complexes are released from the antibodies by acid treatment. The peptides can be separated from the proteins by ultrafiltration, and the flowthrough is usually lyophilized before fractionation and sequence analysis. Though the technique provides highly pure isolates, it suffers disadvantages such as high cost due to the requirement of large amounts of antibodies (10–30 mg per isolation), complexity of the protocol, and inability to distinguish intracellular and extracellular MHC complexes [32]. Recent studies that tried to include desalting and inclusion of specific ions in desalting buffer have been shown to enhance peptide yield [33].

The acid elution technique is based on the release of MHC–peptide complexes from the cell surface by short acid treatment at pH 3.3 [34]. The source materials for this technique are cells from dissociated tissue or adherent or suspension cell cultures. Having intact cells is a prerequisite for the technique since cell damage will lead to the release of proteases generating peptide fragments from highly abundant cell proteins. The major advantage of the technique is that it is cost effective, simple, and it could differentiate intracellular and extracellular MHC complexes. This method has been successfully employed to identify T-cell epitopes from melanoma cells [35], as well as from the bcr–abl fusion protein expressed at the cell surface [36].

4.3.2 Analysis and Sequencing of MHC-Associated Peptides

HPLC fractionation can be performed prior to tandem mass spectrometry, which in combination allows high-resolution separation and sequencing of single peptides from complex samples [37]. Mass spectrometry (MS) is based on precise determination of molecular masses of analyte molecules. Following determination of the molecular mass of the analyte by various means (depending on MS instrumentation), the peptide sequence can be derived by fragmentation of the analyte ion. Hence MSMS analysis allows the detection of a single peptide from a complex mixture of peptide pools. Figure 4.3 illustrates an example of MSMS spectrum of peptides.

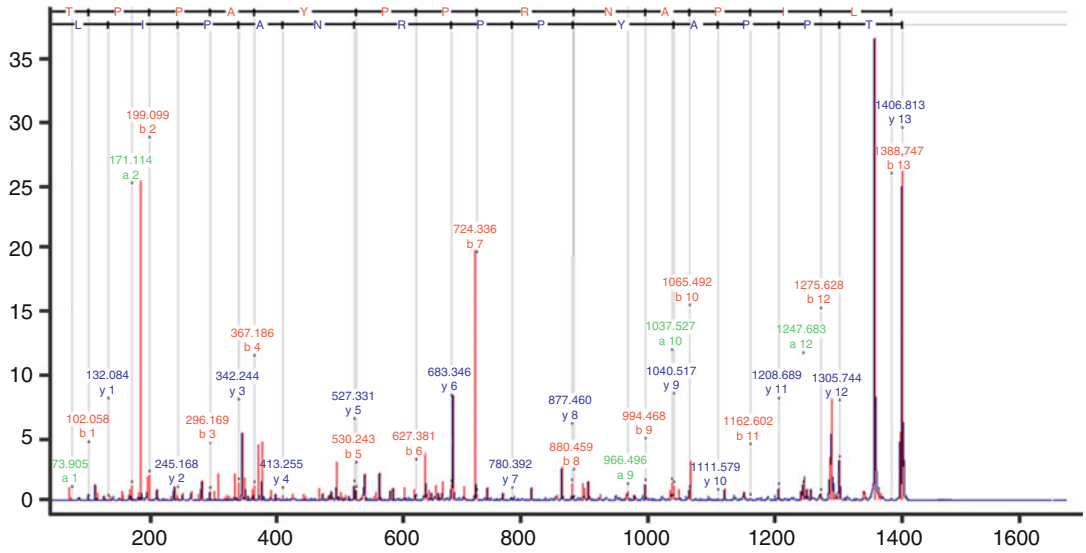

Fig. 4.3 MSMS spectrum showing HepB peptide of mass 1406.813. Fragment ions derived from the precursor (peptide ion) allow the assignment of the amino acid sequence of the peptide

4.4 Human Immunotherapy Against Tumor-Associated Peptides

Identification of the tumor-specific antigens and peptide epitopes expressed on MHC class I antigens on the cancer cell surface has facilitated the development of new approaches to immunotherapy. These cancer-specific antigens possess the potential to be used in the vaccine-based therapies, targeting the respective antigen; however, extending life expectancy and survival are necessary criteria for FDA approval and acceptance of these treatments. Figure 4.4 illustrates the steps involved in the discovery and identification of TA and translation of TA into clinical trials. It is often the case that phase I and II clinical trials demonstrate a degree of efficacy, but in randomized phase III clinical trials, patients failed to demonstrate statistically significant survival benefit [38]. Sipuleucel-T (Provenge), developed by Dendreon corp., is the first vaccine therapy to gain FDA approval and relies on programming dendritic cells (DC) *in vitro* against recombinant PAP protein. This approach demonstrated an overall extended life expectancy, but no significant effect on time to progression [39]. Results of this trial did not identify the peptide potentially targeted by this treatment. Although results are encouraging, there is still a need to improve the design of vaccination based on a better understanding of the mechanisms which promote and sub verse T-lymphocyte responses. DC-based vaccines are also relatively expensive and at present have questionable cost–benefit advantages.

Conceptually, having clearly defined the target peptides recognized by $CD8^+$ T lymphocytes and providing the appropriate stimulation of T helper lymphocyte responses, vaccines based on the use of synthetic peptides can then be used. This offers a simpler way of production and application of therapeutic vaccination on a wide scale, which would also prove cost effective. From recent studies we now recognize the need to abrogate Tregs and myeloid-derived suppressor cell (MDSCs) activities and provide appropriate co-stimulation, via adjuvants, to aid T-cell-mediated antitumor activation.

Following the discovery of *MAGE*, a number of other CTAs and differentiation antigens were reported [40]. The recent classification of TAAs further divides them into (1) cancer testis (CT) antigens, e.g., MAGE; (2) differentiation antigens, such as MART-1; and (3) widely occurring, overexpressed, or aberrantly expressed gene products, e.g., HER2/neu, hTERT, and survivin.

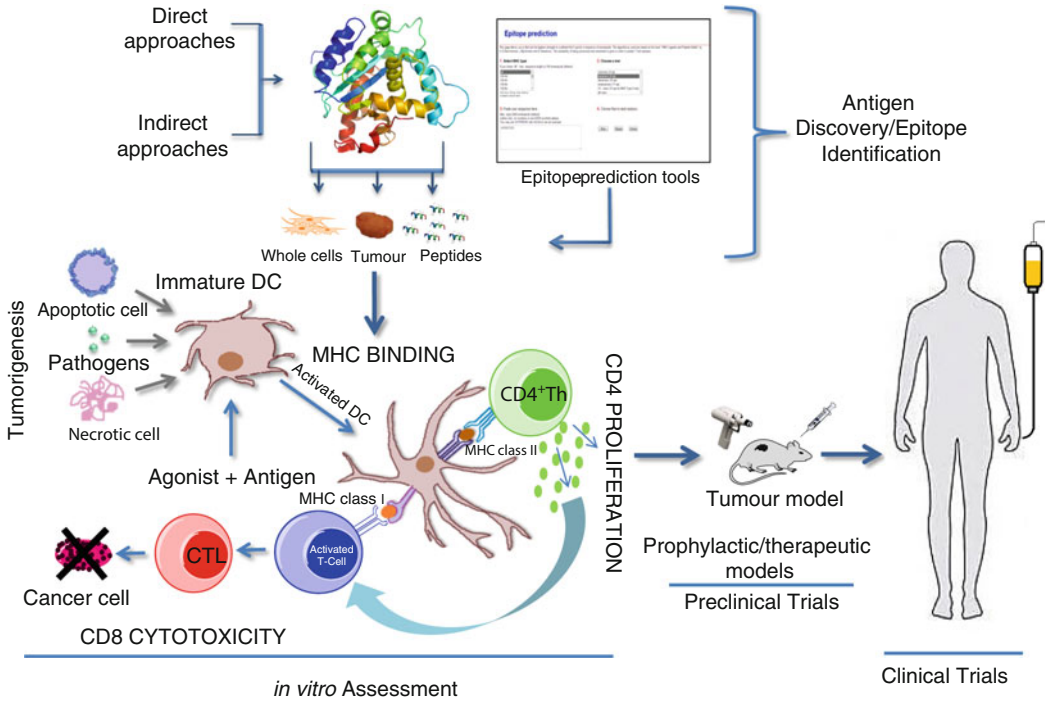

Fig. 4.4 Cancer immunotherapy: translation from bench to bedside. The figure illustrates the steps involved in the discovery of antigen and identification of immunogenic epitopes using direct and indirect approaches, *in vitro* screening, preclinical trials to assess the efficacy of the peptides, and translation of the immunogenic epitopes in the clinical trials

Additionally, patient-specific tumor antigens, such as k-Ras and B-Raf, arise through somatic gene mutation. Table 4.1 lists various TAAs, currently considered as target antigens for active immunization against cancer.

CT antigens are normally expressed in the testis of male germ cells and in some cases in the ovary and in trophoblast. When gene regulation of CT antigen is disrupted (transcriptional activation), it leads to CT antigen expression in tumor development. With the help of SEREX and differential mRNA expression analysis, a wide range of TAs have been identified. CT antigens commonly share characteristics such as highly tissue-restricted expression, the existence of multi-gene families, induction of expression by hypomethylation and/or histone acetylation, frequent mapping to chromosome X, and often being associated with tumor progression and immunogenicity in cancer patients. Spontaneous cell-mediated and humoral responses to several CT antigens including NY-ESO-1, MAGE-A, and SSX antigens have been observed [40]. If CT antigens are immunogenic and found to be highly restricted to tumor cells, these will be useful in the development of antigen-specific cancer vaccines; the list includes NY-ESO-1, MAGE-A, BAGE, CAGE, and HAGE antigens. HAGE, for example, is a potential candidate for cancer immunotherapy, for which immunogenic epitopes are being identified as potential candidates in cancer immunotherapy (unpublished results). In studies conducted by the authors, it has also been shown that HAGE plays a vital role in the ATP-binding cassette (ABC) subfamily B member 5 (ABCB5), associating with malignant melanoma-initiating cells (MIMCs)-dependent tumor growth through promoting RAS protein expression [41].

Differentiation antigens are expressed by tumor cells, as well as the normal cell precursor.

For example, in melanoma, tyrosinase, a rate-limiting enzyme in melanin biosynthesis, is stabilized by tyrosine-related protein-1 (TRP-1/hgp75) and can be considered as a tumor antigen target. In addition, serum IgG antibodies that immunoprecipitate TRP-1 protein have been detected in melanoma patients, inferring that other members in the melanin synthesis pathway are also recognized by the immune system [42].

Overexpressed or abnormally expressed antigens are also good targets for cancer immunotherapy. Tumoral transformation is linked to the overexpression of proteins involved in the control of cell cycle or in the natural senescent evolution of cells. Telomerase enzyme, known to protect the ends of chromosomes, prevents the progressive shortening of chromosome during successive cellular divisions [43]. Cells senesce once telomerase activity reduces [44]. Moreover, most tumors strongly express telomerase, making them unable to senesce [45]. Thus, overexpressed antigens such as p53, survivin, Ras, hTERT, and WT-1 are suitable candidates as they are often found upregulated in the tumors, which also correlates with the over presentation of the corresponding peptides at the surface of the tumor cells as peptide–HLA complexes. The consequence of peptide processing and presentation needs to be understood, since in some instances, presentation of MHC class I peptides by tumors cannot only lead to antitumor immunity but also states of tolerance as a result of clonal T-cell deletion or autoimmunity through increased T-cell activation.

TAAs found so far are classified into groups of tumor antigens listed in Table 4.1.

Many of these antigens demonstrate widespread expression in many different human cancers, and there is considerable knowledge of the peptides that are naturally processed and presented by MHC antigens.

The inclusion of class I and class II peptides in vaccines is proving to be a promising strategy. Synthetic long peptides from 15 to 35 amino acids long cover a wider region of the antigen and can include multiple CTL and/or Th peptides making such sequences ideal candidates for inclusion in vaccines. Having multiple CTL/Th epitopes helps in eliciting a more potent immune response, preventing tumor cells from undergoing immune escape. PAP peptides have been identified in our laboratory that consist of both CTL and Th epitopes; preclinical studies have proven these to be immunogenic and elicit prophylactic and therapeutic benefit in mouse models [46].

Th cells are found to be essential in generating antitumor memory CTLs. A peptide analogue of HER-2/neu has been shown to elicit strong immunogenicity [47]. The analogue contains multiple CTLs, which bind to the HLA-A2.1/A68, HLA-A11, and HLA-A3 alleles with intermediate to high affinity. The $CD4^+$ T cells generated in response to the HER-2/neu analogue exhibited an extensive capacity to synergize with syngeneic CTLs, rejecting HER-2/neu-positive tumors [47]. This analogue contains multiple CTL peptides and can be used as a multi-peptide vaccine containing overlapping Th and CTL epitopes for breast cancer immunotherapy.

A novel method of peptide delivery to antigen-presenting cells has been developed using HER-2/neu as a target antigen. This method involved linking a 4-amino acid moiety (LRMK Ii-key) to the N-terminal of the peptide directly or by using a simple polyethylene spacer (-ava-). Ii-key is derived from the MHC class II-associated invariant chain and helps in binding to MHC class II molecules. This was shown to improve the potency of antigen presentation. Ii-key is also believed to trigger the release of cytokines and chemokines from DCs after MHC class II binding. The candidate hybrid HER-2/neu-Ii-key (AE37) is undergoing clinical trials targeting breast cancer [47].

4.5 Strategies to Enhance the Immunogenicity of Peptide Epitopes

Ever since their discovery, vaccines are widely used to induce immunity against infectious agents, saving lives of newborns and adults alike from the deadly diseases. Vaccines recruit the immune system to target the antigen and produce memory to

avoid recurrence in the future. Peptide-based vaccines have been shown to be most effective as they present the antigens to the immune system in the form of peptides eliciting cytotoxic (CTL) response via class I peptides and/or Th response through class II peptides. However, peptides alone are not always potent enough to produce a strong, long-lasting immune memory. There is growing interest and ongoing research to develop a new generation of vaccines containing recombinant proteins or synthetic peptides. These new candidates promise to be less toxic than their more virulent phenotype, but they are poorly immunogenic when administered without adjuvant. This may be due to the fact that the antigen itself may readily be cleared by the organism without fully engaging with the immune system to generate "the secondary signals" required for immune activation. The half-life of the antigen can be extended to several hundred days when emulsified in the adjuvant in comparison with injection alone. Potential adjuvants currently used in preclinical and clinical trials are discussed below.

4.5.1 Potential Adjuvants for Boosting Immune Responses

Adjuvants are of interest in the context of cancer immunotherapy in order to boost the vaccine-specific immune response and are known to enhance T- and B-cell responses on administration and to engage components of the innate immune system [48, 49]. They serve to enhance the magnitude, breadth, quality, and longevity of specific immune responses to the antigen with minimal toxicity and are capable of boosting the immune response of weak antigens [50]. Adjuvants are being used clinically to increase the response to a vaccine in the general population, increasing the mean antibody titers and/or the fraction of subjects that develop protective immunity, concomitant with an increase in seroconversion [51]; also an increase in the seroconversion rate in populations with reduced responsiveness due to age, disease, or therapeutic interventions has been observed [52]. The use of adjuvants also allows reduction in the quantity of antigen being used, as well as reduction in the frequency of vaccination [53, 54]. Freund's incomplete adjuvant (IFA) is the most commonly used adjuvant in animal models, which in this setting induces a weak, Th2 immune response.

Various adjuvants have been trialed for their efficacy along with cancer vaccine administration and are listed in Table 4.2.

In general, adjuvants may achieve a qualitative alteration of the immune response [51], provide conversion of a Th2 response to Th1, enhance $CD8^+$ as well as $CD4^+$ T-cell responses, and increase the generation of the effector T-cell memory response [55, 56]. It has been shown that many adjuvants promote the speed of response after initial vaccination [55–57] and influence the breadth, affinity, or specificity of the response [57, 58].

4.5.2 TLR Agonists in Cancer Vaccine Trials

TLR agonists are potent adjuvants, activating DCs, augmenting T-cell responses and downregulating the suppressive effects of regulatory T cells. They promote both adaptive and innate antitumor immunity and affect the tumor microenvironment. TLR 3, 4, 7, 8, and 9 are the most promising TLR agonists for use alongside vaccination strategies [59]. The agonists approved by the FDA for human use are listed in Table 4.3 [60].

The growth of transplanted and viral tumors can be prevented by coadministration of BCG [61, 62]. It has also been observed that inoculation of BCG into established tumors leads to regression and prevention of metastasis [63]. Monophosphoryl lipid A (MPL) is a chemical derivative of *S. minnesota* endotoxin, which acts as a potent TLR4 agonist maintaining its immune-stimulatory properties. MPL has been incorporated in Cervarix (human papillomavirus [HPV]-associated cervical cancer vaccine) in the form of AS07 (MPL + aluminum salts) [64]. BCG is being used in phase I and II clinical trials, targeting melanoma, colorectal and breast cancers, and neuroblastoma. It is used in phase III clinical trials targeting melanoma and colon and lung cancer [60].

Table 4.2 List of potential adjuvants

Adjuvants	Innate receptor or pathway activated	Immune responses
Alum (licensed)	NLRP3 inflammasome	Th1 and Th2
AS04 (licensed)	TLR4 and inflammasome	Th1
MF59 and AS03 (licensed)	Tissue inflammation	Th1 and Th2
Incomplete Freund's adjuvant (IFA)	p24 stimulation	Induction of T-cell responses against HLA-A2 restricted epitopes in melanoma patients
Montanide ISA51		Strong T-cell lymphoproliferative response
Microbial derivatives (natural and synthetic)		
Monophosphoryl lipid A (MPL)	TLR4	Enhanced Th1 responses
Detox (MPL + CWS) OM-174 (lipid A derivative, E. coli), OM-triacyl	*In vitro* maturation of human dendritic cells	Induction of cellular and humoral responses in melanoma patients
Modified LT (genetically modified bacterial toxins [heat-labile enterotoxin, cholera toxin] to provide nontoxic adjuvant effect)		Balanced Th1 and Th2 responses
CpG ODN	TLR9	Th1 immunity with $CD8^+$ T-cell induction
Immunoadjuvant		
Cytokines: (IL-2, IL-12, GM-CSF, Flt3)		Enhanced antibody responses
Accessory molecules (B7.1)		Enhanced cellular responses by providing co-stimulatory signals to T lymphocytes
Poly-IC	TLR3, MDA5	Th1, $CD8^+$ T cells
Flagellin, flagellin antigens, flagellin proteins	TLR5	Th1+Th2
Imiquimods	TLR7, TLR8, or both	Th1, $CD8^+$ T cells (when conjugated)
CAF01	Mincle	Th1, Th17
ISCOMs and ISCOMATRIX	?	Th1+Th2, $CD8^+$ T cells

Table 4.3 TLR agonists approved by FDA for use in humans

FDA-approved agent	Targeted TLR(s)	Malignancy
Bacillus Calmette-Guerin (BCG)	TLR2/4	Bladder carcinoma
Monophosphoryl lipid A	TLR2/4	HPV-associated cervical cancer
Imiquimod	TLR7	Basal cell carcinoma

Imiquimod is a small non-nucleoside imidazoquinoline commonly known as S-26306 or R-837. Imiquimod was found to exert immune-stimulatory and anticancer effects by binding to TLR7, predominantly expressed at the endosomal membranes of monocytes, macrophages, plasmacytoid DCs (pDCs), and mast cells. It has been approved by the FDA for the treatment of actinic keratosis and basal cell carcinoma in humans. Imiquimod is particularly shown to stimulate pro-inflammatory cytokines and also promote chemokine ligand 2 (CCL2)-dependent recruitment of the pDCs into the tumor and their conversion into cytotoxic effector cells inducing regression in TLR7/MyD88, which is dependent on the expression of interferon α/β receptor 1 (IFNAR1). TLR7 is essential for pDCs to produce IFN-α/β leading to TNF-related apoptosis-inducing ligand and granzyme B secretion via IFNAR1 signaling [65]. Imiquimod is in phase I and II clinical trials as a single agent or in combination with other therapies for the treatment of various types of cancers including brain, breast, cervical, and colorectal cancer, as well as melanoma, neuroblastoma, sarcoma, and non-small cell lung carcinoma (NSCLC). It is also being used in phase II and III trials as a single agent targeting cervical cancer and head and neck squamous cell carcinoma (HNSCC) [60].

Cytotoxic chemotherapy is widely used to treat cancer, and combining immunotherapy with chemotherapy could create a number of valuable synergistic effects. Thus, while drugs kill most cancer cells, drug-resistant cells could be targeted by vaccination-induced T cells. On the other hand, chemotherapy-induced cancer cell death may allow the uptake of dying cells (and their antigenic proteins) by APCs. In addition, following processing and presentation, it activates the immune system against tumor antigens, a phenomenon known as "immunogenic cell death (ICD)." This effect can be achieved by an appropriate combination of chemotherapy with immunotherapy, for example, anthracycline-treated tumor cells can elicit an anticancer immune response via translocation of calreticulin to the cell surface, thus enhancing tumor cell recognition and uptake by DCs [66].

Immunotherapy combining the use of TLR agonists together with chemotherapy or radiotherapy seems to be a promising approach, especially with chemotherapy which promotes the potential of immunotherapy, for example, the use of cyclophosphamide to control Treg cells, antiandrogens that enhance T-cell infiltration into the tumor, and anthracyclines that appear to increase the potential for antigen presentation within the tumor environment. It is also recognized that monoclonal antibody (mAb) therapy, recognizing cell surface antigens on tumor cells, promotes T-cell responses via tumor destruction and increased tumor antigen processing and presentation by DCs. This indirect mechanism, combined with targeted vaccine therapy, may represent a promising approach to immunotherapy.

4.6 Future Prospects

Human cancer develops as a result of genetic, epigenetic, and/or environmental factors and apart from virally induced tumors that express tumor antigens of viral origin, and human cancer antigens are the result of aberrant gene expression. These proteins can be recognized by the immune system making them appropriate targets for vaccine-based immunotherapy.

Our increasing understanding of peptide epitopes and tumor antigens expressed across multiple tumor types now empowers researchers and clinicians to develop and implement vaccination strategies that enhance adaptive immunity, targeting multiple cancer-associated proteins. Furthermore, since many antigens are associated with the malignant process, it is likely that the use of vaccines against these proteins will circumvent the problem of genetic and phenotypic heterogeneity and diversity that exists within tumors. Two considerations which influence the effectiveness of T-cell-based immunotherapy must be measured. Firstly, the precise role of cancer stem cells which potentially self-renew and give rise to subpopulations of cancer cells within the tumor mass is unclear. Although the existence of these cells in solid tumors and leukemias has been demonstrated, little is known about their immune biology, especially with regard to tumor antigen expression and susceptibility/resistance to CTL attack. Since they are associated with a highly aggressive and resilient cell phenotype, they may well prove difficult to effectively target. Secondly, we are confronted with a variety of tumor escape mechanisms which allow cancer cells to avoid destruction by effector T cells, for example, loss of MHC class I and tumor antigen expression as well as tumor resistance through the production of immunosuppressive proteins, e.g., TGF-β, PD1, and IL-10. Research in these areas is beginning to show a clinical impact, and the recent clinical findings with anti-PD1 mAb therapy infer that abrogating the main elements of immune suppression allows antigen-specific immunity to take effect. Thus combining such strategies with active immunization is an obvious route to follow.

This review highlights some of the important features associated with adaptive T-cell immunity to cancer and how an enhanced therapeutic effect can be achieved when combined with other treatment modalities. Therefore, future clinical trials should result in treatments combining targeted vaccine therapy with antibody and/or chemotherapy in an effective way, resulting in enhancement of both MHC class I- and II-mediated T-cell responses. In addition, there is a need to consider the most appropriate way of

inducing effector T cells which produce memory T cells in the absence of regulatory T-cell mechanisms. Here the avidity of $CD8^+$ T cells may be crucial, and it is unclear at present whether high- or low-avidity T cells provide the most effective long-term benefits.

A sustained effort to introduce multi-epitope vaccination therapy, incorporating MHC class I and class II peptides of same and/or different TA, together with strategies that regulate mechanisms compromising host immunity offers the most realistic chances of achieving wide-scale benefit to patients. This will require further understanding of tumor–host immunity and appreciation of how we might improve patient well-being, so that vaccination has the best possible chance of success. Apart from working to develop new therapeutic strategies, it is important to prepare the patient for treatment to ensure that vaccination provides optimum patient benefit. Thus, the host's physical, nutritional, and psychological well-being is equally important in allowing activation of the immune system and increases the likelihood of effective destruction of secondary cancer. Proper diet, exercise, reduced stress levels, and emotional health all play vital roles in the patient's tolerance to therapy and the effectiveness of therapies utilizing a "healthy" immune system.

4.7 Concluding Remarks

Immunotherapy as a means for promoting antitumor immunity and especially combining well-defined peptide vaccines with conventional cancer treatments emerges as an excellent strategy for treating cancer patients in the future. In formulating peptide vaccines, promoting a T-cell memory response is essential and may only be achieved by boosting both MHC-restricted $CD8^+$ and $CD4^+$ T-cell responses, where the use of appropriate immune adjuvants plays a critical role. The host–tumor relationship and the interplay between cells and events occurring within the tumor microenvironment have enhanced the understanding of how immunity to cancer is governed. Therefore, in order to achieve success in the clinic, it will be important to include treatments that decrease the regulatory mechanisms that restrict immunotherapy, such as removing regulatory T cells and myeloid suppressor cells and checkpoint blockade therapy using PD-1 and PD-L1 mAbs.

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Strategies to Target Tumor Immunosuppression

5

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5.1 Introduction

At the beginning of the twentieth century, the concept of a vigilant immune system that can be manipulated to counteract with tumor development has emerged. In the first half of the century, the "immunosurveillance theory," describing the existence of a complex immunological mechanism capable of eliminating potentially malignant cells, was postulated [1]. In later years, several studies describing interactions between the immune system and the developing tumor have further refined this theory [2].

Indeed, both specific T lymphocytes able to recognize tumor-associated antigens (TAAs) as well as antibodies directed against these antigens are commonly observed in patients with cancer [3,4]. Nonspecific antitumor immune effector cells, such as macrophages or natural killer (NK) cells, are also present in the circulation and tumors of cancer patients. However, the presence of these various components of the host immune

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system capable of engaging an effective antitumor immune response does not generally translate to tumor growth arrest or tumor eradication.

To explain this phenomenon, the theory of tumor "immune escape" has been put forward. According to this theory, tumors are complex systems capable of sidetracking or completely blocking the host antitumor immunity, by interfering with various components of the immune system, thus affecting all stages of the antitumor immune response. Some of these immune escape mechanisms have been identified only recently [5]. Overall, they hamper antitumor immune responses: on one hand by reducing the homing of immune effector cells to the tumor site and on the other by suppressing the antitumor immune functions. Immunotherapies directed against cancer can attempt to stimulate the antitumor immune cells and/or to deplete protumoral immune cell populations or mechanisms.

Currently, tumor immunologists are searching for biomarkers that can be used to describe the "immune signature" of the tumor [6]. Defining the intratumor immunologic profile unique for every tumor type may enable personalized immunotherapeutic strategies for the effective control of tumor progression.

The aim of this chapter is to give an overview of novel strategies to target immunosuppression in the tumor microenvironment, illustrating their targets and the underlying mechanisms responsible for their therapeutic antitumor activity. Prior to this, 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 intervention are eliminated, but in some cases, allowing immune-resistant variants to survive and further develop [7]. The mechanisms of immune escape can be functionally divided into two main categories: tolerance and immunosuppression.

5.2.1 Tolerance Mechanisms

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 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 [8]. In contrast, IL-4-polarized [9], type 2 $CD4^+$ T cells (Th2) secrete cytokines which induce neutralization of antibody production by B cells, thus directing immunity towards a tumor-promoting type 2 response, prevalent in the context of tumor immunology.

One major mechanism of tumor-induced cytotoxic lymphocytes (CTLs) apoptosis is cross-linking between the overexpressed death receptor FasR (CD95) located on the surface of activated effector T cells and its correspondent ligand FasL located on the surface of human tumor cells [2]. Direct tolerization of antitumor T cells by tumor cell-induced TGF-β signaling is another highly effective mechanism, leading to significantly decreased function and frequency of CTLs [10].

5.2.1.2 Defects in the Antigen Presentation Process

The main factors involved in the antigen presentation process are DCs, tumor cell antigens, and HLA class I antigens. Tumor-induced alterations can affect the functionality of any of these factors.

Decreased numbers and function of DCs and a semi-mature phenotype of these cells are at the basis of the deficit in antigen presentation. A study on DCs isolated from renal cell carcinoma

patients indicates that less than 10 % of the total DC population represents activated cells capable of antigen presentation and T-cell stimulation [11]. The situation proves to be similar in patients with both advanced breast cancer [12,13] and non-Hodgkin lymphoma [14]. Moreover, DCs exposed to indoleamine 2, 3-dioxygenase [15], transforming growth factor-beta (TGF-β), or prostaglandins have been shown to induce tolerance and anergy leading to failure to recognize tumor cells.

A result of genetic instability of human tumors over time is the change of their antigenic profile and selective development of "epitope loss" tumors [16], which fail to be recognized and further on eliminated by the antitumor immune system. One other effect of this genetic instability is diminished or abolished expression of HLA class I antigens, with a frequency of antigenic loss or downregulation ranging from around 15 % in melanoma lesions up to more than 50 % in primary prostate carcinoma [17].

Lastly, it has been previously shown that both DCs in patients with cancer as well as human tumors express very low levels of co-stimulatory molecules [18]. Downregulation of these molecules on the surface of DCs may interfere with the process of cross-presentation, thus result in death or anergy of antigen-specific CTLs.

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. The most widely encountered suppressive cell populations within the tumor environment are myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and macrophages. 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 briefly addressed below.

5.2.2.1 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs ($CD11b^+CD14^-CD33^+$) [19], represent a heterogenic, bone marrow-derived, immature cell population [20] with an increased frequency in the peripheral circulation and tumors of patients with different types of cancers [21–23]. As a consequence of their heterogeneity, they have the capacity to inactivate both $CD4^+$ and $CD8^+$ antitumor T cells through a variety of mechanisms, involving amongst others the production of arginase [24,25] and reactive oxygen species [26] or iNOS activity [27], leading to inhibition of MHC class II expression [28] or blockade of STAT-5 signaling cascade [29].

5.2.2.2 Regulatory T Cells (Tregs)

Similar to MDSCs, Tregs have also been shown to accumulate in the periphery and tumors of patients with cancer [30]. Tregs can suppress the antitumor immune responses through their high surface expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), the main T-cell inhibitory signal [31] that mediates attenuation of intercellular association. Moreover, $FoxP3^+$ naturally occurring Tregs (nTregs) are well-known negative regulators of antitumor immunity through different mediators, such as FoxP3 [32]. Intratumor accumulation of FoxP3 leads to poor prognosis of gastric [33] and ovarian [30] carcinomas. Tregs can also reduce the immune activity of effector T cells by secreting immunosuppressive cytokines, such as IL10 and TGF-β [34].

5.2.2.3 Tumor-Associated Macrophages (TAMs)

TAMs are cells belonging to the innate immune system "alternatively" activated by Th2 cytokines such as IL-4 or IL-13 [35] towards an M2 noncytotoxic phenotype. These M2 macrophages are frequently found in solid tumors, where they promote remodeling of the extracellular matrix and secrete growth factors, therefore inducing tumor-specific neoangiogenesis [36]. Several studies have underlined their capacity to cause tumor growth both directly, by production of cytokines that stimulate proliferation of tumor cells [37], and indirectly, by stimulating

proliferation of endothelial cells [38]. 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 [39], while their secreted IL-10 subsequently induced a regulatory T-cell phenotype [40].

5.2.2.4 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 misbalance T-lymphocyte surveillance of tumor development [41] by inhibiting proliferation of antitumor effector T cells. Granulocyte-monocyte colony-stimulating factor (GM-CSF) is another cytokine with immunosuppressive properties due to its capacity to promote generation and expansion of TAMs [42] and recruitment of MDSCs at the tumor site [43,44].

Enzymes. Together with arginase and iNOS, which are central for two of the mechanisms of immunosuppression exerted by MDSCs, indoleamine 2,3-dioxygenase (IDO) and cyclooxygenase 2 (COX2) also present immunosuppressive properties. IDO inhibits T-cell activation by depleting tryptophan [45], one of the essential amino acids necessary for T-cell development, whereas COX2 stimulates prostaglandin E2 (PGE2) production, a prostaglandin involved in conversion of human DCs into immunosuppressive MDSCs [46].

Negative regulatory factors. Programmed death-1 (PD-1), a member of the CD28 superfamily of T-cell regulators [47], is one of the main negative regulators of antitumor immunity. High levels of PD-1 have been found on chronically activated $CD8^+$ T cells and during chronic infections [48]. PD-1 has two corresponding ligands, PD-L1 and PD-L2, members of the B7 family [49]. Within the context of tumor immunology, the ligand PD-L1, which presents an almost ubiquitous expression profile, is most relevant. Co-inhibitory signaling via PD-L1 (but not PD-L2) is necessary for conversion of naïve $CD4^+$ T cells to adaptive $CD4^+FoxP3^+$ Tregs. The PD-1/PD-L1 signaling pathway is viewed as yet another immune escape mechanism of solid tumors [50], due to its capacity to inhibit T-cell activation [51] through various downstream signaling effects. Although not as disputed as the PD-1/PD-L1 system, the lymphocyte-activation gene (LAG-3), member of the immunoglobulin superfamily and expressed on the surface of activated regulatory $CD4^+$ and $CD8^+$ T cells, B cells, and NKT cells have also been shown to contribute to tumor immunosuppression, as Tregs from LAG-3 (–/–) mice present reduced regulatory activity [52]. Lastly, CTLA-4 is a protein receptor expressed on the surface of helper T cells and transmits an inhibitory signal counteracting the stimulatory effects of CD28 [53].

Endothelin receptors. Both endothelin receptor type A (ETAR) and type B (ETBR) are G-protein-coupled receptors that belong to the endothelin system. Synthesis and secretion of endothelin-1 (ET-1), the corresponding ligand of ETAR, can be induced by a large array of stimuli within minutes. Upon binding of its ligand ET1, ETAR promotes vasoconstriction and tumor cell proliferation through a phospholipase C-dependent mechanism [54,55]. On the other hand, ETBR was shown to regulate T-cell adhesion and tumor homing via NO and ICAM-1 [56]. High expression of ETAR has been reported in prostate cancer patients with bone metastasis [57] and HPV-induced neoplasia [58], whereas ETBR expression was associated with the absence of tumor-infiltrating lymphocytes and decreased survival of ovarian cancer patients. Also, upregulation of ETBR in patients with vulvar squamous cell carcinoma has been correlated with tumor progression and early metastasis [59].

The above-described spectrum of strategies developed by human 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, at the same time, to build up mechanisms which enable them to hide

from an immune-based attack. Different types of tumors develop diverse immune escape mechanisms, translating into different 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 to the 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 tumor load. More recently, the importance of cancer-induced immunosuppression is being taken into consideration. The main challenge of these strategies is the unique immune signature of tumors, which 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 unimodal 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 immunosuppressive mechanisms imposed during tumor development (Table 5.1).

5.3.1 Strategies Targeting Homing of Effector T Cells

Some of the tumor immune escape mechanisms described above interfere with 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

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

Type of therapy	Targeted pathway	Achieved effect
Local tumor irradiation	Antigen presentation and processing Release of TAAs Production of inflammatory cytokines	Enhanced intratumor homing of effector CTLs
Endothelin receptor blockade	Upregulation of ICAM expression	
Chemotherapy Taxanes	Inhibition of angiogenesis Induction of programmed cell death	
Ab-mediated targeting of CTLs	Tumor and T-cell concomitant antigen binding	
Depletion/inactivation therapy MDSCs Tregs TAMs	 Inhibition of DNA replication Inhibition of tyrosine kinase signaling Enzyme inhibition	Enhanced activity of intratumor effector CTLs
Cytokine therapy IL-15 IL-7 IL-12	 T-cell growth factors DCs activation Vaccine adjuvants	
Blockade of negative factors Anti-CTLA-4 (ipilimumab) Anti-PD1/Anti-LAG3 Anti-TGF-β Anti CD40/CD40L	 Blockade of T-cell checkpoints Inhibition of receptor signaling Induction of T-cell activation	

intratumor homing of effector cells have been proven effective such as 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 the standard cure for various types of cancer, with the potential to eradicate tumor cells and induce modifications within tumor stroma with an end curative or palliative result, depending on the type and site of disease. Irradiation is frequently used as adjuvant therapy, in association with other therapies such as surgery, hormonal therapy [60], or bone marrow transplantation. Recently, irradiation has come to the attention of tumor immunologists due to its immunogenic properties. One major immunological effect of local tumor irradiation is the release of large amounts of tumor-associated antigens (TAAs) and danger signals that attract immune cells to the tumor site [61]. Furthermore, it has recently been demonstrated that cancer cells which remain after irradiation present high levels of co-stimulatory molecules and MHC components that render them more immune stimulatory. Other beneficial effects of local tumor irradiation are induction of pro-inflammatory cytokines such as TNF-α and IL-1β [62] and adhesion molecules and death receptors that can enhance CTL responses [63]. These changes within the tumor microenvironment seem to facilitate recruitment of effector T cells to tumors via two distinct mechanisms: first, by promoting vasculature normalization [64] and second, by stimulating overexpression of endothelial adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) [65].

The last decade brought forward substantial clinical evidence that local tumor irradiation has the capacity to activate the immune system. A recent clinical study performed on patients with nonmetastatic prostate cancer reported development of treatment-associated autoantibodies in approximately 14 % of patients undergoing external beam radiation therapy, compared with 0 of 14 patients who received radical prostatectomy [66]. To further substantiate this, another study reported that tumor-specific CTLs increase in prostate cancer patients after irradiation [67]. Local tumor irradiation has also been reported to enhance the efficiency of the antitumor treatment of choice. A recent case report of a patient suffering from metastatic melanoma with disease progression on ipilimumab (a monoclonal antibody targeting CTLA-4 activity) indicates a tumor shrinkage and antibody responses to tumor-specific antigens only after treatment with radiotherapy [68].

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 by facilitating antigen presentation and processing, causing the release of TAAs, increasing production of inflammatory cytokines, chemokines, and receptors involved in the recruitment of effector CTLs, thus enhancing migration of these active effector CTLs to the tumor site.

5.3.1.2 Blockade of Endothelin Receptors

Various studies demonstrate that endothelial cells from a variety of human cancers overexpress endothelin receptors. Blocking these receptors seems a promising strategy to delay tumor development or stop tumor cell proliferation. In fact, selective ET_AR blockade by the experimental drug atrasentan has been shown to delay progression of hormone-refractory prostate adenocarcinoma [69] and enhance the effect of paclitaxel/docetaxel used for treatment of prostate cancer [70] in patients. In a mouse model of HPV-induced cervical carcinoma, blockade of ET_AR caused inhibition of tumor growth [71]. Although it can be hypothesized that the effect of ET_AR blockade on tumor growth is mediated by an increase in T-cell homing to the tumor site, further studies need to be performed to elucidate the underlying mechanisms. On the other hand, in the context of ovarian and also other cancers, overexpression of ET_BR was associated with the absence of tumor-infiltrating lymphocytes and short-term patient survival [72]. Moreover, it was shown that interaction between ET_BR and its corresponding ligand ET-1 induces downregulation of intercellular adhesion

molecule 1 (ICAM-1) expression, a molecule involved in transmigration of lymphocytes into tumors. This effect is rescued by administration of an ET_BR small molecule inhibitor BQ-788. Neutralization of ET_BR by administration of the inhibitory peptide BQ-788 suppressed intercellular communication and cell growth in melanoma nude mice [71] and significantly increased T-cell homing to tumors [72].

5.3.1.3 Taxane-Based Chemotherapy

Originally, taxanes have been categorized as a class of chemotherapeutic drugs which block tumor development by inducing mitotic inhibition through disruption of microtubule functionality. Other studies suggested towards 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 [73]. More recently, the idea of taxanes as enhancers of effector CTL homing into the tumor site came into place. A recent study aimed at investigating whether inhibition of angiogenesis could contribute to overcoming tumor escape from immunity. The results clearly indicated that the angiogenesis inhibitor paclitaxel was capable of increasing leukocyte rolling on the tumor wall vessel and thus infiltration of circulating effector T cells to the tumor [74].

5.3.1.4 Antibody-Mediated Targeting of Effector CTLs

Monoclonal antibody (mAb) therapy is a method most commonly used to functionally inactivate or deplete suppressive immune populations such as myeloid-derived suppressor cells (MDSCs) or regulatory T cells (Tregs). Various studies using bispecific mAb suggest that they can also be successfully used as a tool with antitumor therapeutic application. These antibodies are artificial proteins composed of fragments of two distinct mAbs that can bind to two different types of antigen. For use in cancer immunotherapy, they are engineered to simultaneously bind to a cytotoxic cell (e.g., using a receptor like CD3) and a tumor cell, which needs to be eradicated. Different studies have shown that they display potent *in vitro* [75] and *in vivo* [76] effects against tumor cells.

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 do effectively migrate to tumor metastases are often found to be anergic or otherwise dysfunctional. Multiple mechanisms within the tumor microenvironment, as also indicated in the paragraph above, such as MDSCs or Tregs, negative regulatory factors such as CTLA-4, inhibition by ligands such as PDL-1 or factors such as TGF-β, and metabolic deregulation by enzymes such as IDO have all been implicated in generating this immunosuppressive tumor environment. To develop effective strategies to increase activity of intratumor effector T cells, both inhibition of tolerance mechanisms and restriction of tumor-induced immunosuppression should be addressed. To effectively target the above-described negative regulatory mechanisms, several strategies have been studied. Some of the strategies that are most widely studied preclinically as well as in patients will be addressed.

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

One commonly used mechanism to target innate as well as adaptive immunity that leads to tumor regression [77] is manipulation of the immunosuppressive 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.

Depletion of MDSCs was achieved either by treatment with tyrosine kinase inhibitors, such as sunitinib [78,79], which also induced reversal of Treg elevation or by treatment with inhibitors of DNA replication, such as 5-fluorouracil [80] or gemcitabine [81]. Sunitinib is a broad-spectrum tyrosine-kinase inhibitor capable of inducing selective MDSCs apoptosis in patients with metastatic renal cell carcinoma [82,83].

The reported response rate of sunitinib as a frontline drug is 48 % [83], thus representing one of the most promising drugs for reducing tumor-induced immunosuppression. Examples of chemotherapeutic agents which effectively target the immunosuppressive MDSC populations in preclinical studies include gemcitabine and 5-fluorouracil.

Another immunosuppressive population that has been intensively targeted for improving antitumor response is represented by Tregs. To date, several methods to deplete Tregs have been developed over time. Depletion of $CD4^+CD25^+$ Tregs by mAb therapy has been shown in both tumor-bearing animal models and clinical trials [84,85]. In animal models, Treg depletion was obtained by the administration of anti-CD25 mAbs before inoculation of tumor cells [81]. In line with this approach, it was recently reported that selective depletion of $FoxP3^+$ Tregs in transgenic DEREG (depletion of regulatory T cells) mice, in combination with therapeutic immunization against melanoma, greatly enhances the antitumor effect [86]. However, the potency of a combination of immunization and Tregs depletion not only depends on the involvement of Tregs in the tumor model studied but also on the involvement of Tregs 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 human papilloma virus (HPV) viral proteins E6 and E7 (SFVeE6,7) in a mouse model of cervical carcinoma [87]. In the clinical setting, one method to deplete Tregs by targeting their high CD25 expression is by employing the immunotoxin denileukin diftitox (Ontak™ Ligand Pharmaceuticals), approved for clinical use as therapy against cutaneous T-cell lymphoma [88]. In combination with immunization, it has also been used for the treatment of other types of tumors [89]. Daclizumab (Hoffmann-La Roche) is another anti-CD25 agent, previously used in patients with T-cell leukemia [90,91] and more recently in combination with a peptide vaccine for treatment of metastatic breast cancer [92]. 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 (GITR) antibodies or low doses of Treg-depleting cyclophosphamide [93].

Regarding TAMs, selective depletion is promoted by IL-15/TGF-α in human primary colorectal adenocarcinomas [94]. In other studies, IL-15 has been shown to reverse T-cell anergy [95] and rescue the tolerant phenotype of $CD8^+$ T cells [96]. Although TAMs depletion can be achieved by different approaches, such as blockade of TAMs chemoattractant chemokines (e.g., blockade of CCL-2 with the inhibitor molecule bindarit [47] or immunization with a legumain-based minigene vaccine [97]), 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 both murine teratocarcinoma and human rhabdomyosarcoma mouse tumor models [98] and in a mouse model of human cervical carcinogenesis, respectively [99]. 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.

5.3.2.2 Immunostimulatory Cytokines: Cytokine Therapy

In addition to the above-discussed IL-15, various other cytokines are viewed as promising immunorestorative drugs. IL-7, a survival cytokine crucial for T-cell development [100], increases the numbers of peripheral $CD4^+$ and $CD8^+$ T cells in patients [101]. 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 [102].

5.3.2.3 Blockade of Negative Regulatory Factors: Antibody Therapy

Antibody therapy against developing tumors has been employed in clinics for many years and belongs to the category of "molecular targeted therapy" of cancer. Despite the emergence of a large palette of monoclonal humanized anticancer antibodies, only a small number are approved for patient use, such as trastuzumab (Herceptin) for HER2-positive breast cancer and rituximab (Rituxan) for B-cell lymphoma. Due to their low toxicity profile and capacity to activate several distinct host effector mechanisms [103], these mAbs are seen as very promising anticancer drugs. The mechanisms most commonly 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 [104].

Another antibody that has been very recently approved for treatment of late-stage melanoma is ipilimumab (Yervoy), a human mAb directed against the CTLA-4 inhibitory molecule expressed on the surface of activated T cells [53]. CTLA-4 is a negative regulatory factor that competes for the same APC-expressed ligands as the T-cell co-stimulatory molecule CD28, however presenting significantly higher affinity [105]. Thus, it downregulates the antigen-specific immune responses initiated by interaction of APCs with T cells. 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 [106].

Programmed death-1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) are yet two other negative regulatory factors of T-cell functions [107]. Recently, PD-1 blockade has been shown to increase the induction of effector T cells in the spleen, prolong T-cell proliferation, and enhance the recruitment of effector T cells to tumor sites [108]. In multimodality therapy regimens, PD-1 blockade increased therapeutic efficacy of total body irradiation and DCs transfer therapy [109]. 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 [110]. 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 [111].

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. In a xenograft mouse model of prostate cancer, transfer of tumor-reactive, TGF-β-insensitive $CD8^+$ T cells leads to a 50 % decrease in average tumor weight, when compared with tumors of mice which underwent transfer of naïve $CD8^+$ T cells [112]. Also, mAbs against TGF-β which are nowadays in clinical trials seem to be very promising antitumor candidates as they present little systemic toxicity [113]. 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* [114].

Another class of antibodies capable of stimulating antigen presentation functions of APCs are agonistic antibodies against CD40 and/or CD40L [115]. As signaling via CD40 has been repeatedly shown to increase HLA expression and production of IL-2 by APCs, thus leading to T-cell activation [116], agonistic anti-CD40 antibodies presently tested in phase I clinical trials have a promising therapeutic potential.

5.4 Concluding Remarks

In the last few decades, major progress has been achieved within the field of cancer immunotherapy. However, despite this progress, the outcomes of clinical trials performed so far are significantly lower than expected. Contrary to the excellent therapeutic antitumor responses observed in animal tumors, clinical results in patients are modest,

likely due to the variety and complexity of immune inhibitory mechanisms present within the tumor microenvironment. Some explanations that can account for this outcome are insufficient homing and activation of antigen-specific immune effector cells within the tumor or development of immunosuppressive mechanisms, capable to inhibit their cytolytic activity. This paradigm calls for strategies that could suppress the suppressors themselves, thus introducing the necessity of multimodality treatment regimens to achieve long-term tumor regression. Emerging clinical trials indicate towards the development of potent immunization strategies, leading to generation of high levels of effector T cells with a proper phenotype and specificity, as a possible answer to the problem. A desirable, highly effective immunization strategy should 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, combination of immunization regimens with ways to enhance homing of these 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 a promising strategy. On the other hand, targeting only 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 anergyzed or tolerized by diverse immunosuppressive mechanisms developed by the tumor itself or by secondary immunosuppressive 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 immunosuppressive populations and blockade of negative regulatory factors, are necessary.

In conclusion, 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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Overcoming Cancer Tolerance with Immune Checkpoint Blockade

6

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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

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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, recent 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 histocompatibility (MHC) complexes that the antigens are presented on [8, 9]. Tumor tissue can promote lymphocyte anergy, or unresponsiveness, by downregulating necessary costimulatory 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. Rather, the failure appears derive from the inability of the immune response to overcome the 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 one of these checkpoints, CTLA-4, has led to a commercially available therapeutic drug in patients with advanced melanoma. 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 or together with standard therapies or cancer vaccines remains great. This chapter will review some of the most prominent therapeutic targets to overcome tumor-mediated immune suppression through targeted checkpoint modulation.

6.2 Cytotoxic T-Lymphocyte-Associated Antigen-4 (CTLA-4): A Paradigm for Immune Checkpoint Blockade

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, CD152) was the first recognized and is the best characterized inhibitory immune checkpoint molecule [12, 13]. CTLA-4 is the only checkpoint currently targeted with an FDA-approved therapeutic drug, ipilimumab. The success of this drug has dramatically increased interest in cancer immunotherapy generally and immune checkpoint blockade more specifically. Many lessons have been learned in the journey from CTLA-4 discovery until efficacy was proven for ipilimumab, not the least of which is that immune checkpoint modulation can treat cancer in a clinically meaningful way. 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.

6.2.1 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 [12]. 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 [14]. 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 a 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, costimulation with CD28 (on the T-cell) binding to B7-1 (CD80) or B7-2 (CD86) on the antigen presenting cell (APC) is perhaps

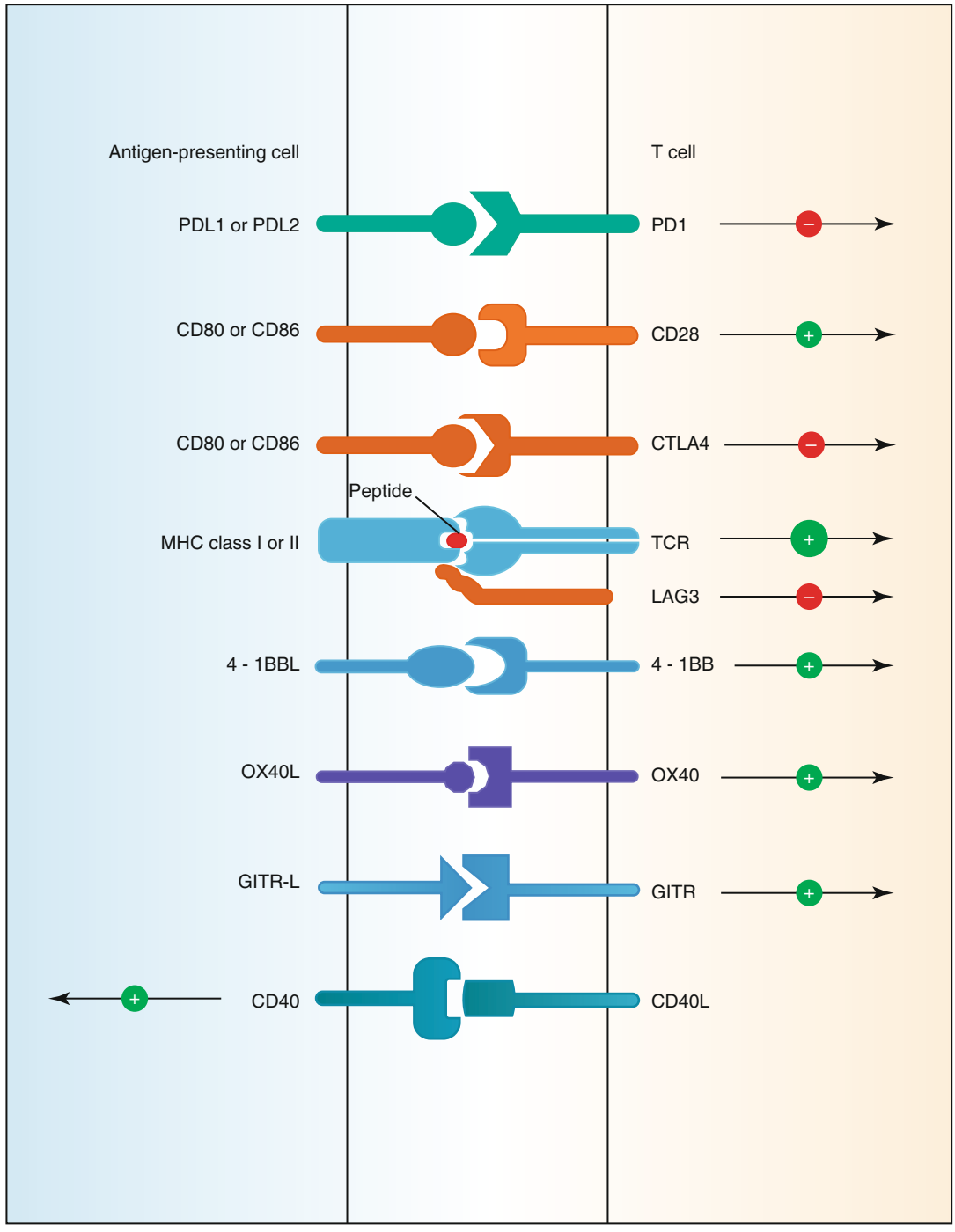

Fig. 6.1 Multiple immunomodulatory coinhibitory and costimulatory receptor-ligand pairs have been identified (although not all are depicted here). These pathways set the immunologic context when an antigen is presented on a T-cell receptor (*TCR*) to a major histocompatibility (*MHC*) complex

the most important and best known; B7-1 and B7-2 are expressed on APCs and are typically upregulated after activation [15, 16].

As the negative counterpart to CD28, CTLA-4 is an inhibitory checkpoint molecule expressed on activated T-cells and constitutively expressed on regulatory T-cells (Treg) [13]. After TCR-antigen mediated activation of T-lymphocytes, expression of CTLA-4 on the cell membrane increases dramatically. CTLA-4 appears to suppress the 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 [17]. Additionally, the importance of CTLA-4 in long-term immune memory has yet to be fully elucidated [18]. These remain areas of active research.

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 20–100 times greater avidity and can accommodate two ligands, whereas CD28 can only bind one [19–21]. CTLA-4 appears to blunt T-cell responses by not only competitively binding the CD28 ligands, B7-1 and B7-2, but also by receptor-mediated induction of cell cycle arrest, decreasing production of IL-2, limiting T-cell dwell time, and enhancing Treg function, among other mechanisms [18]. 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 CTLA-4-deficient mouse models with CTLA-4-immunoglobulin fusion protein (CTLA-4Ig) can abrogate the lymphoproliferative autoimmunity which would otherwise be fatal [22]. 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 [23]. 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 [20, 24] (Fig. 6.2).

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 [17]. The exact role that Treg-mediated immune suppression plays in the overall context of CTLA-mediated immune control is not entirely clear although it is an area of active research [16]. There is evidence from lymphocytes treated with anti-CTLA-4 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) [25, 26].

The important role of CTLA-4 in Treg homeostasis and immune control has become clear in multiple experiments. Treg-mediated CTLA-4 inhibits B7-1 and B7-2 expression on dendritic cells [27]. Murine models with CTLA-4-deficient $CD4^+$ $FOXP3^+$ (Treg) lymphocytes developed lymphoproliferative disease [27]. 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 [28–30]. 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 [16, 28]. However, expansion of Tregs with CTLA-4 blockade does not appear to lead to increased Treg function [31]. 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 [31].

As work progresses in deciphering the mechanisms of the CTLA-4 receptor's complex interplay within broader immune homeostasis, the

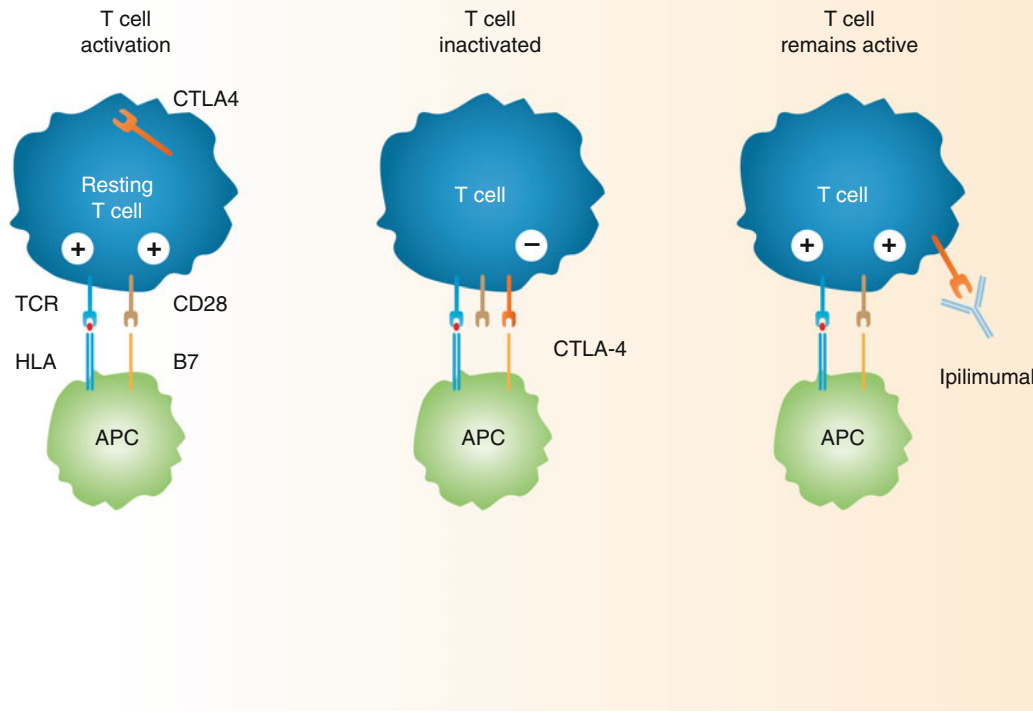

Fig. 6.2 Mechanism of action of CTLA-4 in suppressing activated T-cells and proposed mechanism of action for ipilimumab

CTLA-4 receptor is an attractive therapeutic target with potential for revolutionizing immunotherapy for cancer as well as other disease conditions. 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 [32–35].

6.2.2 Preclinical Development of CTLA-4 Blocking Therapy

Initial work to determine the expression patterns and function of CTLA-4 used fusion proteins of immunoglobulin with the extracellular domain of CTLA-4 (CTLA-4Ig) [36–40]. The CTLA-4 Ig protein was noted to bind to B7 with high avidity and, by competitively binding with B7 molecules, prevent CD28 mediated T-cell activation. Treatment with CTLA-4 Ig led to increased survival of transplanted xenografts, inhibition of experimental autoimmune encephalitis in murine models, and blocked antitumor immunity in murine models [36, 37, 41, 42]. Taken together, these studies supported the idea that CTLA-4 played a role in suppressing the body's T-cell response by competitive binding. Additionally, the CTLA4 Ig fusion protein was developed into FDA-approved therapeutic drugs by Bristol-Myers Squibb (New York, NY) for treatment of rheumatoid arthritis (abatacept) and immunosuppression after renal transplant (betalcept) [43–45]. Anti-CTLA-4 mAbs were first created by Walunas et al. Their uses further supported the notion of

an inhibitory role for CTLA-4 [46, 47]. The functional role of CTLA-4 was later confirmed with the aforementioned CTLA-4 knockout mice [14].

Soon thereafter, work with anti-CTLA-4 blocking mAbs for therapeutic purposes proceeded. Initial animal studies confirmed that the mAbs could indeed augment immune response to peptide antigens [47]. Not long after this, CTLA-4 mAbs were used to experimentally overcome antitumor immune tolerance. In 1996, Leach et al. reported results of experiments in mice injected with 51BLim10 colon cancer cell lines. Treatment with CTLA-4 mAbs resulted in regression of the tumors, whereas the control mice succumbed to the cancer by 35 days. Additionally, prior treatment with CTLA-4 mAbs provided protective immunity to a secondary challenge of tumor cells [48]. Additional work by Yang et al. confirmed the antitumor effect of CTLA-4 mAb in CSA1M fibrosarcoma and OV-HM ovarian carcinoma mice models and also demonstrated that the effectiveness was dependent on the stage of tumor growth, with later stages being less susceptible to mAb-enhanced eradication [49]. Since then, multiple murine models of various cancer types, specifically glioblastoma, sarcoma, breast, prostate, and colon cancer models, have proven the efficacy of anti-CTLA-4 mAb [18, 50–54]. However, in multiple other murine tumor models, including different cell lines from the same types of cancer that responded in previous experiments, anti-CTLA-4 mAb monotherapy was shown to have low or no antitumor effect [55–64]. Some have suggested, and it stands to reason, that less immunogenic tumors are less likely to respond to CTLA-4 mAb blockade alone [18]. Less immunogenic cancers may require additional antigenic stimulation, through active immunotherapy or through radiation or chemotherapy-induced cell death to realize its full potential as will be discussed further.

6.2.3 CTLA-4 Blockade Monotherapy in Melanoma

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.

6.2.3.1 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 [65]. 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 [66]. 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 polyclonal T-cell activation or autoimmunity [66]. Based on this work, ipilimumab proceeded with human trials.

Phase I Trials

Two initial phase I pilot studies of ipilimumab monotherapy were conducted in 2002 in castrate-resistant prostate cancer (CRPC) and unresectable malignant melanoma. In both studies, a single dose of ipilimumab was given at 3 mg/kg to each patient. This dose was selected based on pharmacokinetics from prior animal studies [43]. In the first study, 2 out of 14 patients with CPRC had a transient prostate-specific antigen (PSA) response of >50 % lasting up to 5 months [67]. In the second trial, 2 of the 17 patients with metastatic melanoma had a partial response to the treatment without any serious adverse events [67]. In 2003, a phase I trial of ipilimumab monotherapy was conducted in previously immunized metastatic melanoma and ovarian cancer patients also at a dose of 3 mg/kg. Five of the nine patients (three melanoma patients and two ovarian cancer patients) were previously immunized with autologous, irradiated, GM-CSF secreting tumor cells (GVAX), and all demonstrated some objective

response to ipilimumab with some developing "extensive tumor necrosis." Four melanoma patients previously immunized with a multiple peptide vaccine did not appear to respond. There were no serious toxicities reported [68]. From there, a series of phase I trials were conducted both in metastatic melanoma as well as prostate cancer [43]. An additional phase I trial, reported in 2003, tested ipilimumab in 11 patients with colon cancer, prostate cancer, or lymphoma at 3 mg/kg initial dose followed by three monthly doses at 1.5 mg/kg. A partial response was noted in lymphoma patients and appeared to coincide with observed autoimmune toxicities [69].

Phase I/II Trials of Ipilimumab Monotherapy in Melanoma

Based on these experiences, the drug proceeded with phase I/II trials in melanoma. Melanoma was selected as a cancer of interest for CTLA-4 blockade therapy based on observed responses in phase I trials and also its historical consideration as an immunogenic tumor based on cases of spontaneous regression and its response to IL-2 and other immunotherapies. Notable trials in this category helped determine the biologic dose used later in phase III trials. One phase I/II trial enrolled 88 patients with unresectable stage III or IV melanoma and gave them a single dose at 20 mg/kg, multiple doses at 5 mg/kg, or multiple doses of 10 mg/kg. All doses were tolerated, although more immune-related adverse events (irAE, discussed further below) were noted in the group receiving 10 mg/kg serially. A disease control rate (DCR), defined as patients with an objective response or stable disease, was 39 % (9 of 23 patients) in the 10 mg/kg serial dosing group and less than 15 % in other groups [70]. Another NIH-sponsored study enrolled 139 patients with metastatic melanoma and treated them with ipilimumab and a peptide vaccine ($n=54$) or ipilimumab monotherapy with intrapatient dose escalation ($n=84$). The doses were escalated from 3 mg/kg every 3 weeks to up to 9 mg/kg or until disease progression or unacceptable toxicity. An objective response rate of 17 % was observed for all patients [71]. Both trials noted significant rates of irAEs with 86 and 62 % of patients, respectively, experiencing some toxicity. Rates of ≥ grade 3 toxicity were 36 and 19 %, respectively. Of note, both trials found a positive correlation between the presence of irAEs and clinical response.

Phase II Trials of Ipilimumab Monotherapy in Melanoma

Multiple phase II trials have been conducted on ipilimumab monotherapy and ipilimumab with other agents in melanoma. The first series of phase II trials were initially sponsored by Bristol-Myers Squibb in 2006 [43]. The first trial was a multicenter, single-arm trial of previously treated, unresectable stage III or IV melanoma patients treated with ipilimumab monotherapy at 10 mg/kg every 3 weeks for four cycles, followed by repeat dosing every 3 months. The primary endpoint of best overall response rate (BORR), defined as the proportion of patients with compete response (CR) or partial response (PR). The study defined response rate by the traditional modified World Health Organization (WHO) criteria. The trial reported a 5.8 % BORR with a higher rate of response (27 %) when stable disease was included (defined as disease control rate, DCR) [72].

The second phase II ipilimumab monotherapy trial evaluated different dosing rates in randomized, double-blinded fashion. Again, pretreated unresectable stage III or IV melanoma patients were enrolled and dosed at 0.3, 3, or 10 mg/kg every 3 weeks for four cycles followed by maintenance therapy every 3 months at the same dose. As in the previous study, the primary endpoint was BORR by WHO criteria. This trial noted a dose-dependent relationship in both efficacy and irAEs. The trial found a BORR of 0 % for 0.3 mg/kg dosing, 4.2 % for 3 mg/kg dosing, and 11.1 % for 10 mg/kg dosing. The rates of serious (≥grade 3) irAEs were 0, 7, and 25 % for the dosing groups, respectively. This trial helped establish that 3 mg/kg was the minimum biologically active dose [73].

The third phase II trial examined the effect of inclusion of budesonide specifically to prevent treatment-induced diarrhea, along with ipilimumab. The trial included both treatment-naïve and pretreated patients with unresectable stage

III and stage IV melanoma. Ipilimumab was given open-label at 10 mg/kg every 3 weeks for four doses followed by maintenance therapy every 3 months. Patients were randomized and blinded to receive daily budesonide or placebo. Budesonide treatment did not affect the efficacy of ipilimumab which demonstrated a similar BORR in both arms and also comparable to the previous phase II trials. The BORR were 12.1 % (ipilimumab with budesonide) and 15.8 % (ipilimumab with placebo), with similar 1- and 2-year overall survival (OS). Prophylactic budesonide did not affect the rates of ≥ grade 2 diarrhea or other irAEs [74]. A retrospective evaluation of the data from this trial determined that treatment-naïve patients had significantly longer survival (median OS 30.5 months) compared to pretreated patients (median OS 13.6 months) [75].

An additional phase II trial, reported after ipilimumab was FDA-approved, looked specifically at melanoma patients with brain metastases, a group that had been excluded from most of the previously performed studies. The patients were divided into groups depending on whether their brain lesions were symptomatic or not. Patients were given ipilimumab at 10 mg/kg every 3 weeks with maintenance therapy. The trial found both groups responded at a similar low rate to previous trials with comparable toxicity. Patients with small, asymptomatic brain metastases generally responded better than larger or symptomatic lesions [76].

Phase III Trial of Ipilimumab Monotherapy in Melanoma

The first phase III study of ipilimumab, sponsored by Bristol-Myers 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 [71, 77–79]. 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 [80]. 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 [81]. 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 [82]. Significant questions remain and are currently under evaluation regarding the treatment of melanoma with ipilimumab. As discussed previously, a randomized, double-blinded 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 [73]. This data was not available at the initiation of the phase III trial. A phase III trial comparing the two dosing levels in both pretreated and untreated metastatic melanoma patients is currently underway (NCT01515189).

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 [72–74, 83]. 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.

Since ipilimumab has been approved, another drug, vemurafenib, a BRAF inhibitor, has proven effective and been FDA approved in patients with BRAF-V600E mutated (present in about 50 % of patients) metastatic melanoma [84]. The presence of a BRAF-V600E mutation does not appear to affect responsiveness to ipilimumab [85]. The optimal timing and sequence of these drugs in patients eligible for both has yet to be determined. Finally, the effectiveness of ipilimumab in patients with resectable high-risk stage III and stage IV melanoma is currently under investigation in two clinical trials (NCT01274338, NCT01274338). Other areas of active investigation in ipilimumab monotherapy include identification of the subset of patients who benefit most from CTLA-4 blockade and biomarkers predicting response to therapy.

6.2.3.2 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 [18, 86]. Tremelimumab is an IgG2 antibody that, similar to ipilimumab, blocks the binding site of CTLA-4. It has a longer half-life of approximately 22 days compared to 12–14 days for ipilimumab [86]. *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 [87].

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 [88]. 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 four doses and allowed up to four 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 [89].

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 [90]. 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 [91]. 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 [26]. Despite its lack of proven effect in this trial, tremelimumab remains under active investigation in other patient populations (discussed further below).

6.2.4 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, 7 of the deaths were from irAEs [80]. In a pooled analysis of 325 patients treated with ipilimumab at 10 mg/kg every 3 weeks for four doses, 72.3 % experienced irAEs and 25.2 % were ≥ grade 3 [92]. 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 [92].

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 [74].

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 [80, 91, 93]. 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 [74].

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 [92].

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 [94].

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 is 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 [95].

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 [71, 74, 78, 79, 96–99].

A similar correlation was not addressed in the phase III trials of CTLA-4 blockade antibodies, and further evaluation may help clarify this as well as the underlying mechanisms.

6.2.5 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 standardize definitions of tumors responsive to cytotoxic therapy and not as a surrogate for survival [100]. 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 constitute 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 [101]. Immune-related response criteria have been used alongside WHO criteria in multiple ipilimumab trials since it was first introduced [72, 102]. Further prospective validation will be needed to determine to what degree it correlates with overall survival.

6.2.6 CTLA-4 Blockade in Cancers Other than Cutaneous Melanoma

As previously discussed, melanoma was a logical first target for CTLA-4 blockade, given the evidence that it is an immunogenic tumor [103]. Given the success found in treatment of cutaneous melanoma, the use of CTLA-4 blockade alone or in combination with other therapies to treat other tumor types is an active area of investigation.

Table 6.1 Comparison of World Health Organization (WHO) and immune-related response criteria (irRC) for tumor response [101]

	World 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

6.2.6.1 Uveal Melanoma

Uveal melanoma is a rare cancer that, like cutaneous melanoma, shares melanocytes as the cell or 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 [104]. Uveal melanoma was excluded from most previous studies of ipilimumab in melanoma. A retrospective look at 14 patients with metastatic uveal melanoma treated with ipilimumab at 10 mg/kg from seven European centers' compassionate use program revealed a 29 % rate of partial response or stable disease with response behavior similar to those seen in cutaneous melanoma [105]. Phase I/II trials looking at ipilimumab in the adjuvant setting for high-risk uveal melanoma after completion of standard treatment and for the treatment of metastatic uveal melanoma are underway (NCT01585194).

6.2.6.2 Prostate Cancer

Castrate-resistant prostate cancer (CRPC) has limited treatment options available; however, it has proven susceptible to immunotherapy. As previously discussed, one of the original phase I trials of ipilimumab was conducted in CRPC patients and demonstrated a transient decline in the PSA in a number of patients [106]. An additional phase I dose escalation trial was conducted using tremelimumab in combination with androgen deprivation (with bicalutamide) in patients with PSA-recurrent prostate cancer after primary surgery or radiation. Out of 11 patients, 3 were noted to have late prolongation in their PSA doubling time, and toxicities were generally mild [107].

There have been five phase II trials of ipilimumab in prostate cancer with androgen deprivation therapy, radiation, chemotherapy, or immunotherapy and are described further in subsequent sections [108]. Two phase II trials have included an ipilimumab monotherapy arm that produced PSA declines of >50 % in a minority (13–25 %) of patients [109, 110].

There are currently nine ongoing phase II and two phase III trials involving ipilimumab in prostate cancer, most of which involve combination therapy [108, 111]. In addition, there is a randomized phase III trial of ipilimumab monotherapy in metastatic asymptomatic or minimally symptomatic CRPR currently underway and scheduled to have results in November 2015 (NCT01057810). The results of the phase III trials will be interesting, as they will examine overall survival. This is particularly relevant in light of the fact that sipuleucel-T, a cancer vaccine approved for treatment of metastatic CRPC, proved to be effective at improving overall survival without significantly decreasing PSA levels, which has been the primary endpoint for the phase II trials of CTLA-4 blocking therapy [5].

In addition to monotherapy, CTLA-4 blockade has been tried in combination with androgen deprivation therapy. Androgen deprivation is the first-line therapy for recurrent or metastatic prostate cancer and is associated with prostatic tissue apoptosis and lymphocytic infiltration. There is also evidence that androgen deprivation may stimulate thymopoiesis and specifically the production of naïve T-cells which may enhance antitumor response [112]. CTLA-4 blockade may augment the natural immune response elicited by this treatment. A phase I trial of tremelimumab in combination with androgen deprivation (with bicalutamide) was conducted in patients with PSA-recurrent prostate cancer as discussed earlier [107]. A phase II trial was conducted in which 108 patients with advanced prostate cancer were randomized to receive ipilimumab, given as a single dose of 3 mg/kg, in combination with androgen deprivation or androgen deprivation alone. Fifty-five percent of patients with combination therapy had a PSA reduction >50 % compared to 38 % in the arm undergoing androgen deprivation only [113]. Additional trials of this combination are currently underway (NCT01377389, NCT01498978).

6.2.6.3 Breast Cancer

Similar to the aforementioned trial in prostate cancer, tremelimumab has been used in combination with exemestane, an aromatase inhibitor, in patients with advanced breast cancer. Twenty-six patients with advanced estrogen and/or progesterone receptor-positive breast cancer, all of whom had progressed on previous hormonal therapy,

were treated with various doses of tremelimumab in combination with exemestane as part of a phase I dose escalation trial. The maximum tolerated dose was determined to be 6 mg/kg dosed every 90 days. This is a lower dose than used in previous trials, likely because dose-limiting toxicity was defined to include some grade 2 toxicities if they were immune related. The best response to therapy was stable disease for at least 12 weeks, which was noted in 42 % of patients. There was a correlation between patients with disease stability and peripheral lymphocytes expressing ICOS (inducible costimulator), a member of the CD28-superfamily of costimulatory molecules expressed on activated T-cells [114].

6.2.6.4 Renal Cell Carcinoma

A phase II trial of ipilimumab monotherapy was conducted in patients with metastatic renal cell carcinoma, a tumor thought to be immunogenic given rare observed cases of spontaneous regression and its observed response to cytokine therapy. Ipilimumab was dosed at 3 mg/kg initially followed by either 1 mg/kg or 3 mg/kg dosing every 3 weeks. Five out of 40 patients had partial response by RECIST criteria, including patients who had not previously responded to IL-2 therapy. As in other trials, they reported a correlation between response to ipilimumab therapy and autoimmune toxicities [97].

6.2.6.5 Gastrointestinal Cancers

There has been one phase II trial of tremelimumab as second-line therapy for metastatic gastric or esophageal carcinoma. Patients were given tremelimumab at 15 mg/kg every 3 months and monitored for response by RECIST criteria. One of 18 patients had a partial response and remained well on treatment for 33 months, while 4 others had stable disease. There was one treatment-related death from autoimmune colitis [115].

Metastatic and unresectable pancreatic adenocarcinoma is another disease process with few effective treatment options. A phase II trial of ipilimumab monotherapy was conducted in 27 pancreatic cancer patients, with treatment given at 3 mg/kg every 3 weeks for four doses. No patients responded by RECIST criteria, although there was one late response observed [116].

A phase II trial of tremelimumab monotherapy was conducted in patients with heavily pretreated metastatic colon cancer. Tremelimumab was given at 15 mg/kg every 3 months until disease progression. Of the 45 evaluable patients, only 44 received a single dose secondary to disease progression ($n=43$) or discontinuation ($n=1$). One patient had a partial response and received therapy for 15 months [117].

6.2.6.6 Hepatocellular Carcinoma

CTLA-4 blockade showed a particularly promising effect on patients with advanced hepatocellular carcinoma and chronic hepatitis C infection, another cancer with limited treatment options and a tumor that does have a history of both spontaneous regression and response to immunotherapy [118]. Tremelimumab was given at 15 mg/kg every 3 months to 20 patients with advanced hepatocellular carcinoma, many of whom were Child-Pugh class B cirrhotics (class C patients were excluded) and were not amenable to surgery and chronic hepatitis C infection; treatment was given until disease progression or severe toxicity. RECIST criteria PR were seen in 17.6 % of patients with a reported DCR in 76 %. Interestingly, along with the impressive tumor responses, a significant drop in hepatitis C viral load coupled with enhanced specific antiviral immunity was observed, raising the question as to whether CTLA-4 blockade may be beneficial in virus-associated malignancies [119].

6.2.6.7 Other Cancers

In addition to the trials listed above, phase I trials of CTLA-4 blockade have also been conducted in lymphoma, ovarian cancer, and as neoadjuvant therapy in urothelial carcinoma [120–122]. Additional trials are currently underway [108].

6.2.7 CTLA-4 Blockade 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.

6.2.7.1 CTLA-4 Blockade 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 [123, 124]. 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 [125]. Additionally, chemotherapy, when given appropriately, may enhance the effect of specific immunotherapy [126]. Evidence from clinical trials reveals that combining chemotherapy with cancer vaccination can be more effective than either therapy alone [127–129]. 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) [123]. 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 [130]. 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 [131].

Based on these results, the concept was tested in a randomized phase III trial evaluating ipilimumab with dacarbazine *vs*. dacarbazine alone [73]. 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 [101, 132]. 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 [93]. 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 [133].

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 [124, 134]. 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) [101]. 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 is being conducted using phased ipilimumab and chemotherapy in patients with squamous NSCLC, the group that derived the greatest benefit in subset analyses [102] (NCT01285609).

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 [135]. 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 phased combination of ipilimumab and paclitaxel/carboplatin is currently being tested in a phase III trial with an anticipated enrollment of 912 patients (NCT01450761).

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 3 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 was small with three patients having a decrease of >50 % in each arm [109]. 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 [136].

6.2.7.2 CTLA-4 Blockade 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 [137]. 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 [137–140]. 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 [141]. 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, CTLA-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 [141]. 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 [56]. 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 [55].

The abscopal effect refers to the regression of tumors in remote areas following localized radiation of tumors. These phenomena have been

documented in melanoma, renal cell carcinoma, and lymphoma [142–144]. More recently, 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 [145]. Similar cases of abscopal regression of metastatic melanoma in patients on ipilimumab have since been reported [146].

A phase I/II 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 [110]. 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 [110]. A phase III trial examining radiation with ipilimumab compared to radiation alone in advanced CRPC is currently underway (NCT00861614). Additional phase I/II and II trials evaluating the effect of ipilimumab with and without radiation in multiple cancer types are currently underway (NCT01689974, NCT01769222, NCT01449279).

6.3 Programmed Death 1 (PD-1) Pathway

6.3.1 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 [147]. 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 [148]. 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 have become more clear [149]. Like CTLA-4, PD-1 is a transmembrane protein expressed on effector immune cells [150]. 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 [151–153]. 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) [154]. PD-L1 is constitutively expressed in certain tissues such as lung and placental macrophages [155]. Its high level of expression in the placenta has been implicated in mediating materno-fetal tolerance [156, 157]. PD-L1 expression can also be induced on a broad range of hemopoietic, endothelial, and epithelial tissues in response to pro-inflammatory cytokines, such as interferon, GM-CSF, IL-4, and IL-19 [151, 158–161]. PD-L2 expression is more limited as it is inducibly expressed on dendritic cells, macrophages, and mast cells [155].

The PD-1 receptor pathway is an important negative regulator of the immune system. PD-1 appears to play 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 [155]. As mentioned before, PD-1 knockout mice helped initially reveal the function of PD-1. The initial B6-PD-$1^{-/-}$ oncogenic 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 [14, 148, 162]. 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 [163, 164]. 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 [165].

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 [166, 167]. PD-1 signaling decreases the production of several proinflammatory cytokines such as IFN-γ, TNF-α, and IL-2 [155]. 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 [168–170]. 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 [171]. Interestingly, PD-L1 was discovered to not only function as a ligand for PD-1 but also as a receptor bound by B7-1 (CD80) capable of delivering an inhibitory signal [172]. This finding not only demonstrates the complexity of lymphocyte regulation but suggests that blockade of these molecules could result in functionally different outcomes [162].

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 [149]. 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 [155, 173].

6.3.2 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 [174–185]. 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 [186, 187]. $CD8^+$ 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 [181, 186, 188, 189]. Circulating NK cells in cancer patients have been noted to express PD-1, while healthy control NK cells do not [190]. 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 [191–194].

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 [187, 195]. In these cases, the higher levels of PD-L1 expression may be in response to INF-γ signaling, as observed in normal human tissue [196, 197]. Thus, upregulation of PD-L1 expression may represent an adaptive tumor response to tumor-specific immunity, termed "adaptive resistance" [195, 198]. 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 [176]. 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.

6.3.3 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 concomitant with cancer vaccination [199–204]. Similarly, *ex vivo* blockade of PD-1 or PD-L1 improved the ability of human lymphocytic function against tumor tissue in multiple studies [191, 205–207]. 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.

6.3.3.1 Nivolumab

Several PD-1 blocking mAbs are currently under development in human trials. 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 [208]. 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 [208]. This data raises the possibility that PD-L1 could serve as a biomarker for response to therapy, an idea that is being actively investigated.

Nivolumab monotherapy is currently being investigated in multiple clinical trials, including phase I trials in hematologic malignancies (NCT01592370) and hepatocellular carcinoma (NCT01658878), phase II trials in renal cell carcinoma (NCT01354431), and phase III trials in NSCLC (NCT01642004, NCT01673867) and melanoma (NCT01721772). A phase I trial of nivolumab combined with ipilimumab (CTLA-4 blockade) has been published and is discussed below [209]. A phase III trial of nivolumab alone or in combination with ipilimumab in melanoma is planned (NCT01844505). As previously stated, PD-1 blockade demonstrated ability to augment cancer vaccines in preclinical studies. In addition, nivolumab is also being tested in a phase I trial combined with cancer vaccines in melanoma (NCT01176461). Nivolumab is also being tested together with chemotherapy in NSCLC (NCT01454102) and renal cell carcinoma (NCT01472081).

6.3.3.2 Other PD-1 Antibodies

A second mAb under development, MK-3475 (Merck, Whitehouse Station, NJ), is a humanized IgG4 with high-affinity binding to PD-1. MK-3475 was tested in a phase I dose escalation study in nine patients with advanced malignancy. The drug was given at 1, 3, or 10 mg/kg and redosed every 2 weeks in patients with NSCLC ($n=3$), rectal cancer ($n=2$), melanoma ($n=2$), sarcoma, and carcinoid ($n=1$, each). Initial results reveal that the drug is well tolerated with no $\geq$ grade 3 toxicities. A partial response was seen in one melanoma patient and stable disease was noted in several others [210]. MK-3475 is currently undergoing a large phase I trial in melanoma and NSCLC with an anticipated enrollment of 439 patients to be completed in 2015 (NCT01295827).

CT-011 (CureTech, Yavne, Israel/Teva, Petah Tikva, Israel) is a humanized IgG1 anti-PD-1 antibody that has demonstrated encouraging preclinical results. A phase I dose escalation study in 17 patients with advanced hematologic malignancies was conducted with a single dose of 0.2–6 mg/kg. The drug was well tolerated with no dose-limiting toxicities. There was evidence of clinical response with one complete response observed in a patient with follicular B-cell lymphoma and several other patients having stable disease. A phase II clinical trial of CT-011 in diffuse large B-cell lymphoma is currently underway (NCT00532259). CT-011 has shown a synergistic effect in preclinical studies when combined with cancer immunotherapy [203, 211]. CT-011 is undergoing multiple clinical trials in combination with vaccine therapy in multiple myeloma, acute myelogenous leukemia, and combined with sipuleucel-T in prostate cancer (NCT01096602, NCT01067287, NCT01420965). Phase II trials combining CT-011 with chemotherapy in pancreatic cancer, colorectal cancer, and relapsed follicular lymphoma are also ongoing [161] (NCT0131416, NCT00890305, NCT00904722).

An additional anti-PD-1 therapy under investigation is AMP-224 (Amplimmune Inc., Gaithersburg, MD/GlaxoSmithKline, London, UK), a PD-L2-IgG1 fusion protein, which is currently in phase I testing [16, 108] (NCT01352884).

6.3.4 PD-L1 Blockade

As previously discussed, because PD-L1 is capable of acting as both a PD-1 ligand and as an inhibitory receptor (bound by B7-1), blockade of this protein may have therapeutic effects different from PD-1 blockade. Based on these findings, development of a PD-L1 blocking antibody, MDX-1105 (BMS-936559; Bristol Myers Squibb, New York, NY), proceeded. MDX-1105 has been tested in a large phase I dose escalation clinical trial on 207 patients with advanced malignancies. Treated patients had NSCLC ($n=75$), melanoma ($n=55$), colorectal cancer ($n=18$), renal cell carcinoma ($n=17$), gastric cancer ($n=7$), and breast cancer ($n=4$). Patients received 0.3, 1, 3, or 10 mg/kg of the study drug every 2 weeks for up to 96 weeks or until unacceptable toxicity or disease progression.

Overall, MDX-1105 was well tolerated. A maximum tolerated dose was not achieved. Serious adverse events (≥grade 3) that were treatment related were seen in 9 % of patients. Drug-related adverse events were observed in 39 %; only 5 % were serious that were likely irAEs; common adverse events included infusion-related reactions, rash, diarrhea, and hypothyroidism, all of which were generally well tolerated.

Objective responses were seen in patients with NSCLC, melanoma, renal cell carcinoma, and ovarian cancer at doses of at least 1 mg/kg. Patients with melanoma had objective response rates of 6–29 % at various doses with three complete responses seen. Patients with NSCLC had objective responses at 3 mg/kg (8 %) and 10 mg/kg (16 %). Additionally, two (12 %) patients with renal cell carcinoma and one (6 %) with ovarian cancer demonstrated objective responses. Additional patients, including patients with colorectal cancer and pancreatic cancer, but not gastric or breast cancer, demonstrated disease stability [212]. No ongoing clinical trial has been currently registered for this drug.

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 CTLA-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 [191, 203, 205, 213]. 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 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.

6.4.1 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 [59]. 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 [29, 52, 54, 59, 60, 63, 214–221]. 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 [222]. Gp100 peptides have been shown to be immunogenic and elicit an antigen-specific T-cell response in the majority of melanoma patients [77]. 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 [223]. 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 [71, 78, 79]. 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 [80]. 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 [223]. 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 [26]. 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) have been combined with ipilimumab in a dose escalation trial [99]. 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 [96, 224]. In all 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).

6.4.2 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 [225]. 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 [98]. A phase II trial examining intratumoral injection of IL-2 combined with ipilimumab is currently underway (NCT01480323). 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 NCT01708941, NCT00610857). 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 [226]. Additional trials of ipilimumab and GM-CSF in CRPC and melanoma are currently underway (NCT01134614, NCT01530984).

6.4.3 Combination Checkpoint Blockade

There is ample preclinical data supporting dual checkpoint blockade in murine cancer models [58, 220, 227–230]. 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 (CTLA-4 blocking mAb) in patients with advanced melanoma demonstrate the potential of this combination [209]. The trial treated 86 patients with concurrent ($n=53$) dose escalation of the two agents or sequenced treatment ($n=33$) with nivolumab in patients previously treated with ipilimumab. In the concurrent arm, treatment was dosed at 0.3 mg/kg of nivolumab and 3 mg/kg of ipilimumab (cohort 1), 1 mg/kg of nivolumab and 3 mg/kg of ipilimumab (cohort 2), 3 mg/kg of nivolumab and 1 mg/kg of ipilimumab (cohort 2a), 3 mg/kg of nivolumab and 3 mg/kg of ipilimumab (cohort 3). Dose-limited toxicity was observed in cohort 3; therefore, cohort 2 was treated as the maximum tolerated dose. The concurrent treatment, perhaps not surprisingly, demonstrated considerably higher rates of adverse events than previous trials of either drug in monotherapy. Treatment-related adverse events were noted in 93 % of patients, serious treatment-related adverse events (≥grade 3) were seen in 53 % of patients, and 21 % of patients discontinued therapy secondary to these toxicities. The types of irAEs observed were similar to those seen in both nivolumab and ipilimumab monotherapy trials. That being said, the adverse events were reportedly well managed with immunosuppressant medication and hormonal replacement therapy (for endocrinopathies) and there were no treatment-related deaths observed. In the concurrent arm, 21 of the 53 patients (40 %) were noted to have a response by WHO criteria (the primary endpoint) with the suggestion of a higher response rate when irRC and unconfirmed responses are included. Remarkably, 16 (76 %) of those with an objective response had a tumor reduction of 80 % or more with five complete responses noted.

In the sequenced therapy arm, patients previously treated with ipilimumab were given nivolumab at 1 or 3 mg/kg. The majority of patients (73 %) had progressed on prior ipilimumab therapy. Treatment with nivolumab in sequence was better tolerated than the concurrent therapy with 18 % of patients exhibiting serious treatment-related adverse events (≥grade 3). Objective responses were seen in six of 30 (20 %) evaluable patients with four of the six responses comprising greater than 80 % reduction in tumor volume. Interestingly, no definitive correlation between tumor PD-L1 expression and treatment response could be made in either arm of the trial [209]. A phase III trial comparing the combination of each therapy individually has been designed (NCT01844505).

6.5 Other Checkpoint Pathways Under Development

6.5.1 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 1990 on activated T-lymphocytes and NK cells [231]. LAG-3 is structurally similar to CD4 and, like CD4, binds to MHC II complexes on antigen presenting cells (APCs), but with greater affinity [232]. 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 [198, 233–236].

LAG-3 is expressed on a subset of Treg cells that secretes immunosuppressive cytokines and is more potent that other LAG-3$^-$ negative cells of the Treg phenotype (CD4$^+$, CD25^{high}FoxP3$^+$). They are preferentially expanded in patients with cancer [237]. 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 [238, 239]. 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 [236].

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 [198, 240, 241]. Increased expression of LAG-3 on TILs, corresponding with increased CD8$^+$ T-cell anergy, has been noted in Hodgkin lymphoma, melanoma, and ovarian cancer [242, 243]. 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 [244].

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 improved CD4$^+$ lymphocyte cytokine production [245]. In melanoma, anti-LAG-3 mAb blockade improved the antitumor function of tolerized CD8$^+$ lymphocytes when coupled with a viral cancer vaccine [246]. 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 [227]. 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 [227, 229, 247]. 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].

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, 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 [248]. Based on these findings, a fusion protein of the extracellular portion of LAG-3 and the Fc portion of IgG1 was recognized as IMP321 [249]. IMP321 has been tested as a vaccine immunoadjuvant where it was well tolerated and produced encouraging immunologic results [250]. 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 [251]. 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 [252]. A phase I/II trial of IMP321 along with peptide vaccines in melanoma patients is underway (NCT01308294).

6.5.2 4-1BB

4-1BB (CD137), unlike the inhibitory molecules CTLA-4, PD-1, and LAG-3, is a costimulatory 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 [253, 254]. The 4-1BB receptor is bound by the 4-1BB ligand (4-1BBL) expressed on antigen presenting cells [254, 255]. 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 [256–259]. 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 [260, 261]. 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 [254, 262]. 4-1BB also appears to be functionally important in inhibiting Treg function and promoting antigen priming by dendritic cells [253]. 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 [263–265].

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 [256, 258, 266, 267]. 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 [268]. Treatments were well tolerated in animal models, although polyclonal T-lymphocyte accumulation in the liver was noted [269]. Combination of agonist 4-1BB mAb treatment with immunotherapy appears to function synergistically with immunotherapy and chemotherapy [230, 270–273]. 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 [253].

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 [274] 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 [274]. 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. Two phase I trials are currently enrolling patients in which urelumab is given as monotherapy in advanced solid malignancies or non-Hodgkin lymphoma (NCT01775631) and in combination with rituximab in non-Hodgkin lymphoma or chronic lymphocytic leukemia (NCT01775631). A second drug, PF-05082566 (Pfizer, New York, NY), is currently in phase I trials as monotherapy in solid tumors or in combination with rituximab in non-Hodgkin lymphoma (NCT01307267).

6.5.3 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 [275]. OX-40 is predominantly expressed on activated $CD4^+$ lymphocytes; however, lesser degrees of expression are observed on other cells such as activated $CD8^+$ lymphocytes, Tregs, NK cells, and neutrophils [276, 277]. The only known

ligand to OX-40 is the OX-40 ligand (OX-40L), which is primarily expressed on activated APCs [277]. OX-40 stimulates $CD4^+$ lymphocyte clonal expansion, survival, and cytokine production, particularly in late phases of activation [278–280]. OX-40 is also important in the generation of functional memory T-cell pools [281]. Signaling through the OX-40 pathway does expand Treg populations but the expanded cells are functionally impaired with an exhausted phenotype [282, 283]. 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 [284]. This is in contrast to OX-40 $L^{-/-}$ knockout mice or mice treated with OX-40 L blocking mAbs, which demonstrate impaired lymphocyte priming but normal lymphocyte localization and humoral immune responses [280, 285]. While OX-40 appears to function primarily through $CD4^+$ lymphocytes, there is evidence that this ultimately leads to augmented $CD8^+$ lymphocyte function as well [286–290].

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 [291, 292]. The antitumor effect was dependent on both $CD4^+$ and $CD8^+$ lymphocytes [293]. 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 [294]. Finally, OX-40 mAbs have been shown to function synergistically with other cancer immunotherapies, surgery, and radiation in murine models [295, 296]. 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 [275, 297, 298].

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 and combined with low-dose cyclophosphamide and radiation in metastatic CRPC (NCT01642290, NCT01303705).

6.5.4 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 [299]. Also like OX40 and 4-1BB, GITR is instrumental in modulation of T-cell responses to infection and cancer; however, it operates through nonredundant pathways [300, 301]. 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 [302, 303]. GITR ligation enhances T-lymphocyte activation, proliferation, resistance to activation-induced cell death, and resistance to Treg-mediated suppression [300, 304–307]. 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 [308–311].

In preclinical studies, agonistic GITR mAbs were shown to stimulate T-lymphocytes and overcome Treg-mediated tolerance [191]. This finding led to a series of experiments in mice that demonstrated agonist GITR mAbs enhance antitumor immunity [295, 312–314]. Agonistic GITR mAbs have also shown to improve the

effectiveness of cancer vaccines in animal models [315–317]. 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 [299] (NCT01239134).

6.5.5 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 [318]. 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 [319]. In other tissues, CD40 appears to primarily play a role in modulating local inflammation [319]. It is bound primarily by CD40 ligand (*CD40L*); however, binding by mycobacterial heat shock protein 70 and C4b binding protein has also been identified [320, 321]. *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 [322]. By playing a role in APC maturation, CD40 is also integrally important to lymphocyte priming and activation [323, 324]. Activated $CD4^+$ lymphocytes express *CD40L* which bind to CD40 on APCs, allowing the APCs to mature and effectively cross prime $CD8^+$ lymphocytes [325–327]. 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 [328].

Interest in the CD40 pathway in cancer has come from observations that CD40 ligation is necessary for immune-mediated destruction of cancer cells, that CD40 is expressed on a variety of malignant tissues, and from preclinical trials with CD40 mAbs [329–331]. 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 [330, 332–336]. 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 [337]. 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 [338, 339]. The second mechanism of tumor inhibition, which is independent of CD40 expression on tumor cells, is through the immunostimulatory effects of CD40 ligation.

Multiple strategies have been investigated to therapeutically target CD40 in human malignancy. The first human trials involved treating advanced solid tumors and non-Hodgkin 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-Hodgkin lymphoma [340]. More recent efforts have focused on targeted mAb blockade of CD40, with four drugs currently under investigation in clinical trials.

CP870,893 (Pfizer, New York, NY) is a fully humanized anti-CD40 IgG2 mAb with strong agonistic properties that have been tested in several clinical trials [337]. 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 [341]. 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 [342]. 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 [343, 344]. 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, NCT01008527, NCT01103635).

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 [345]. Similarly, it was tested in a phase I dose escalation trial in 12 patients with chronic lymphocytic leukemia and, again, no objective responses was seen [346]. 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) [347]. In this trial, the combination generated six (20 %) complete responses and eight (27 %) partial responses [348]. However, a randomized phase II trial comparing this combination with chemotherapy alone in DLBCL was terminated early based on perceived futility [337]. 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.

A third agonistic anti-CD40 mAb being tested is Chi Lob 7/4. This chimeric IgG1 mAb is currently undergoing phase I testing in patients with $CD40^+$ advanced solid malignancies or DLBCL (NCT01561911) [349].

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 [338, 339, 350]. 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 [351, 352]. No further studies are currently registered.

6.5.6 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 [353]. This example underscores the care that is necessary when designing and conducting clinical trials in order to maximize patient safety.

6.6 Concluding Remarks

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, CTLA-4 blockade with ipilimumab has added another weapon to our arsenal in the battle against cancer. While its current indications are limited and the impact in most patients is modest, 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.

Perhapms 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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Gene Therapy and Virus-Based Cancer Vaccines

7

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7.1 Introduction

The initial apparently limitless possibility of gene therapy to correct genetic defects, including oncogenic or tumor suppressor mutations in cancers, was quickly found to be a false promise and radical reassessment of the potential of this approach were made. In particular, efficient transfection of all cells within a homogeneous population in culture was found to be extremely difficult, and selective transfection of all cells of a single population in the context of a living organism is currently impossible. Even when reasonable rates of transfection are achieved loss of genetic alterations often quickly occur. In clinical

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studies, even when successful treatment appeared to have been achieved, secondary mutations sometimes led to the development of cancers.

However, researchers have learned valuable lessons from these early studies, and a more realistic and carefully planned approach to implementing gene therapy has led to a succession of promising therapies for a variety of gene defects. In the context of cancer gene therapy, it was quickly appreciated that correcting a genetic defect in every cell in a cancer was unrealistic; therefore the use of targeted delivery vehicles to carry genes with cytotoxic properties and capable of bystander effects in surrounding cells has become the predominant approach. In particular, the expression of genes whose products help to activate and target the immune response against the tumor have displayed considerable promise.

Further, while the application of recombinant cytokine therapies have demonstrated some notable clinical success, it is clear that if cytokines could be continuously produced exclusively from within the tumor itself then a less toxic, more potent and better targeted therapeutic response could be produced. Similarly for more traditional vaccine approaches, co-expression of cytokines, chemokines or other immunomodulatory factors in conjunction with selected antigens can create a more potent or specific immune response.

Here we will primarily focus on viral and non-viral based gene therapy approaches, although recently the use of modified cells, especially genetically engineered T-cell populations and some stem cells have become an attractive alternative that allows targeted delivery of a therapeutic gene to the tumor.

7.2 Viral Vectors Used in Cancer Gene Therapy

Gene therapy introduces foreign genetic material *in vivo* or *ex vivo* into cells of an affected organism in order to destroy a defective cell, replace a defective gene, manipulate a disease-related gene, or introduce an additional gene copy for overexpression of the desired protein to generate a therapeutic effect [1]. The efficient delivery of therapeutic genes and subsequent gene expression is crucial.

An ideal gene delivery vector for cancer therapy should be able to: (i) transduce cells with high efficiency; (ii) mediate high level, long-term expression of the therapeutic transgene; (iii) stimulate a small/negligible immune response *in vivo* (against the delivery vector); (iv) incorporate sufficient lengths of DNA to allow for cloning of transgene(s) of interest and (v) facilitate expression that can be regulated. These properties are not found in one single vector system, and therefore, different viral gene delivery systems are being developed, each with its own advantages and disadvantages [2].

Viruses, in particular, possess various traits that make them ideal for engineering vector systems used in the delivery of therapeutic genes. Not only are viruses naturally evolved vehicles which efficiently internalize their genetic material into host cells [3], many viruses can mediate long term gene expression, while some are also capable of infecting both dividing and non-dividing cells [2, 4]. For these reasons, viruses are transformed into viral vectors capable of therapeutic gene delivery by substituting key genetic components of the viral genome [5–7] with a transgene of interest [8]; essential viral genes can then be provided *in trans* to generate recombinant viral particles [5, 6]. In essence, viral vectors are rendered replication-defective by genetic engineering such that they mainly serve as gene delivery vehicles and do not replicate outside of specialized packaging cell lines [6, 8]. It is important to remember that, given the broadly differing capabilities of various viral vectors, the functionality of the virus meets the requirements of the specific treatment [3].

Some commonly used viral vectors are discussed below (Table 7.1 and Fig. 7.1).

7.2.1 Retrovirus Vectors (RVVs)

Retroviruses (RVs) infect all vertebrates and those that infect humans are associated with various types of cancer, inflammatory diseases and human immune deficiency syndrome [12, 13].

Table 7.1 Summary of viral vectors used for gene therapy [2, 9–11]

	Gammaretrovirus vectors	Lentivirus vectors	Adenovirus vectors	Adeno-associated virus vectors	Herpes simplex virus-1 vectors
Cloning capacity	7–7.5 kb	18 kb	37 Kb	4.9 kb	Up to 152 kb (dependent on type of vector)
Transduction of non-diving cells	No	Yes	Yes	Yes	Yes
Integration into host chromosome	Yes	Yes	No	Yes	No
Immunological challenges	Few	Few	Triggers strong immune response	Few	Can induce an immune response (dependent on type of vector)
Pre-existing host immunity	Unlikely	Unlikely	Yes	Yes	Yes
Safety concerns	Insertional mutagenesis	Insertional mutagenesis	Inflammatory response/ cytotoxicity	Inflammatory response, possible insertional mutagenesis	Cytopathic effects (dependent on type of vector)
Duration of transgene expression	Long term	Long term	Transient	Transient	Long term
Mode of entry into host cell	Fusion	Fusion	Receptor	Receptor	Fusion

This large family is classified into six genera that include alpha-, beta-, delta- and gammaretroviruses, lentiviruses and spumaviruses [14], which are based on genomic structure and sequence relationships. RVs are enveloped RNA viruses that consist of two identical copies of highly condensed positive-sense, single-stranded (ss) RNA enclosed by a capsid. The genome contains three essential genes, namely *gag*, *pol* and *env* that encode for the viral core proteins (matrix, capsid and nucleocapsid), the viral enzymes (protease, reverse transcriptase and integrase) and the viral envelope glycoproteins (surface and transmembrane proteins), respectively [9, 15–17]. RVVs efficiently transfer genes *in vitro* to a broad range of targeted cells and, since they have the capacity to integrate into the host genome, achieving long expression [2, 13, 18, 19]. For these reasons, RVs were among the first viruses engineered for gene therapy and have become the most commonly used RNA virus vectors [2]. Although an advantage, the integration of the vector into the host genome is also of major concern as random insertion into a pre-oncogenic site or a site responsible for the inactivation of tumor suppressor genes increases the risk of subsequent tumor development [10, 13, 20].

7.2.1.1 Gammaretrovirus Vectors (GRVVs)

Early retroviral systems were based on the gammaretrovirus Moloney-murine leukemia virus (Mo-MLV) [6, 21]. Mo-MLV vectors are integrative vectors that offer stable, long-term transgene expression, but a major limitation of using these vectors is that they function only in cells undergoing mitosis [2, 6]. Historically, MLV and other gammaretrovirus vectors were used to restore mutated gene functions in diseased cells [22] and introduce toxic [23] or suicide genes [24, 25] for therapeutic purposes. In the simplest examples of these vectors, the entire virus genome is deleted and replaced by the therapeutic transgene(s), with the exception of *gag*, *pol* and *env* flanked by the two viral LTRs; transcription of the transgene is directly controlled by the viral 5′ LTR [8].

7.2.1.2 Lentivirus Vectors (LVVs)

Lentivirus (LV) is a genus of the retrovirus family. Their genomes are more complicated than other

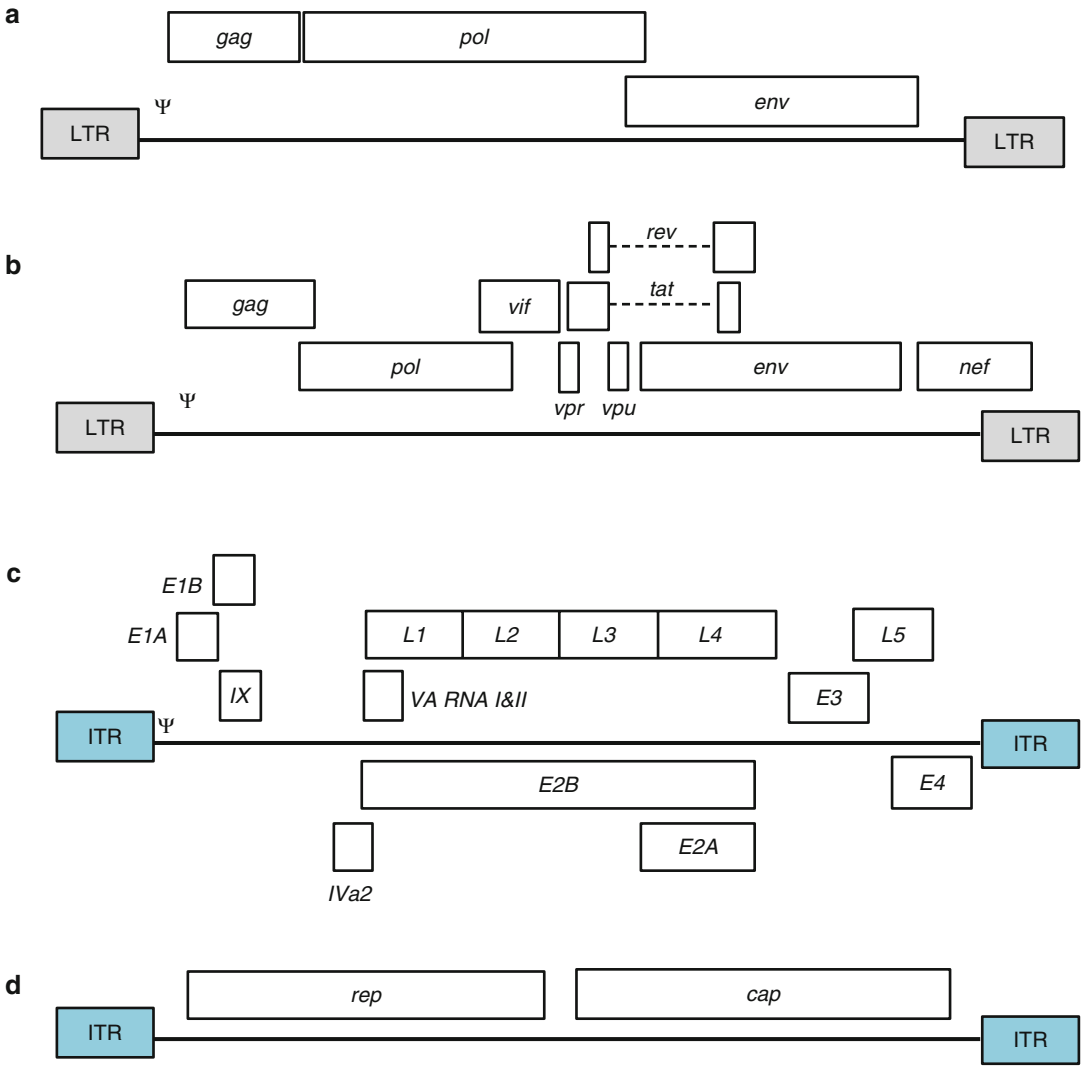

Fig. 7.1 Schematic of the genome organisation of the viruses commonly used to engineer viral vectors. Only the (**a**) Gammaretrovirus, (**b**) Lentivirus, (**c**) Adenovirus and (**d**) Adeno-associated virus genomes are shown and are not drawn to scale (Adapted from Giacca and Zacchigna [8], Verma and Weitzman [9]). Only the major genetic elements for each virus is shown: the long terminal repeats (LTRs) and inverted terminal repeats (ITRs) are boxed and shaded *grey* and *blue*, respectively. The packaging signal (Ψ) is also shown. See text for additional information

retrovirus genomes, containing three to six additional accessory genes that regulate various stages of viral replication and contribute to the persistence of infection [2, 15, 16]. Since late 1990s, the use of LVVs for *in vivo* and *ex vivo* gene transfer applications has been studied extensively. Even though LVVs are mostly based on HIV-1, other LVs have also been used as vectors for gene therapy [26–31]. LVs have various properties that make them ideally suited as gene therapy vectors including their capacity to accommodate large or multiple transgenes [32]; the ability to maintain persistent gene expression by integrating the transgenes into the host cell genome; the absence of pre-existing anti-vector immunity in the host [33]; low anti-vector immunity *in vivo* [34, 35]; and limited potential for genotoxicity due to insertional mutagenesis [36, 37]. Also, due to the capacity of the LV pre-inte-

gration complex to actively cross the nuclear membrane [8], LVs do not require the breakdown of the nuclear membrane in order to integrate [38], thereby allowing efficient transfection of non-dividing cells [2, 26, 39, 40]. To date three generations of LVVs have been produced by deleting genes from the lentivirus genome, consistently improving efficiency and safety at each generation [2, 15, 41]. The current third generation replication-deficient HIV-1-based vectors contain only three (*gag*, *pol*, *rev*) of the nine *HIV-1* genes [42], as well as the LTRs and the packaging signal [9]. This ensures that the stable integration of the provirus into the host genome does not result in the expression of any viral proteins, but only the expression of the transgene [43]. Furthermore, the development of self-inactivating LVVs improves their biosafety, minimizing mobilization of the LVV following infection with HIV [32]. Production of these vectors typically requires three or four plasmids, which include vector and packaging constructs [8, 15].

7.2.2 Poxvirus Vectors (PVVs)

The most extensively studied viral vectors are from the poxvirus (PV) family, a family of viruses that can infect both vertebrates and invertebrates [44, 45]. This family includes variants of vaccinia virus (from the *Orthopoxvirus* genus) as well as fowlpox and canarypox viruses (from the *Avipoxvirus genus*) [45]. PVs contain large linear dsDNA genomes. In contrast to other DNA viruses, poxviruses encode their own transcription machinery, a viral DNA-dependent RNA polymerase and post-transcriptional modifying enzymes, allowing self-sufficient virus replication in the host cell [44, 46]. Since poxvirus DNA is replicated in the cytoplasm, random insertion of the viral genome into the host chromosome is not a concern [18, 45, 47]. Additionally, PVVs have a wide host tropism, form stable recombinants, have accurate replication and efficient post-translational modification of transgenes and can accommodate large inserts of foreign DNA [44, 45]. Interestingly, some strains have a natural tropism for tumor tissue, with a 10^3–10^4-fold higher expression than in other organs [6]. PVVs have long been used in safe and successful vaccination programs, with the vaccinia vector used to vaccinate more than one billion people during the eradication of smallpox [44, 48–50]. To overcome safety concerns, attenuated vaccinia viruses (such as modified vaccinia virus Ankara or MVA) [51, 52], which can infect mammalian cells and express transgenes, but cannot produce infective virus particles, were developed [45, 53]. Unfortunately, vaccinia and MVA vectors can only be administered once or twice to vaccinia-immune or vaccinia-naïve patients due to the development of neutralizing antibodies to the vector [54].

7.2.3 Adenoviral Vectors (AdVVs)

Adenoviruses (AdVs) are non-enveloped DNA viruses than can infect and replicate in a wide range of organs, including the respiratory tract, the eye, bladder, gastrointestinal tract and liver [9]. Generally, these viruses are known to cause benign upper respiratory tract illness and epidemic gastroenteritis and conjunctivitis in humans. Human AdVs are classified into six species (A–F), which are further subdivided into more than 50 serotypes [1–10, 12–52] based on their ability to agglutinate red blood cells, oncogenic potential, genomic organization and DNA homology [2, 3, 55–57]. These viruses are produced and purified to high titres (up to 10^{13} virus particles/ml) and can infect both dividing and post-mitotic cells [6, 55], replicating very efficiently in permissive cells [5]. The AdV genome is easily manipulated and inserted transgenes are maintained throughout successive rounds of replication [5, 6]. To date, the vast majority of recombinant AdV vectors are derived from the human AdV serotypes 2 and 5 of species C [55, 58]. AdV vectors can be genetically engineered to remain replication-sufficient, conditionally replication-sufficient or replication-deficient in the host [58]. First generation vectors are generated by substituting the early gene 1 (*E1*), or the *E1* and early gene 3 (*E3*), with an expression cassette [8, 58–61], rendering the AdV replication-deficient and capable of replication only in specifically designed complementing cell lines

[55]. Second generation vectors have had the *E1*, *E3*, as well as early gene 2 (*E2*), deleted to minimize the host inflammatory response and potential toxicity [62], while third generation vectors (also known as gutted, gutless, high capacity or helper-dependent vectors) are generated by deleting the entire AdV genome with the exception of the ITRs (inverted terminal repeats) and Ψ (packaging signal) regions required *in cis* for viral DNA replication and packaging (see Fig. 7.1) [63, 64]. For this reason, a replication incompetent helper virus is required to provide all the necessary AdV functions *in trans*, which has to be later separated from the AdVVs [65]. Deletion of a large amount of genome increases the cloning capacity of the vector and the adaptive immune response of the host is also decreased [2].

7.2.4 Adeno-Associated Virus Vector (AAVVs)

Adeno-associated viruses (AAVs) belong to the *Parvoviridae* family containing viruses that infect numerous species of mammals, including humans [2, 7]. Even though more than 100 AAV serotypes have been identified, only 12 of these have been shown to infect humans, with AVV-2 the prototype [10]. AVVs are small, non-enveloped ssDNA viruses and the genome could consist of either the sense or anti-sense strand. The genome contains two genes (*rep* and *cap* which encode for seven proteins Rep40, Rep52, Rep68, Rep78, VP1, VP2, VP3), flanked by 5′ and 3′ palindromic sequences (ITRs). The Rep regulatory proteins are required for replication and packaging, whereas VIP1-3 are structural proteins that form the capsid; the ITRs, on the other hand, are indispensable for viral replication, packaging and integration. AAV requires co-infection with another helper virus (either HSV or an adenovirus) or a stressed-cell environment (e.g. when cells are irradiated or treated with genotoxic compounds) to mediate its replication; in the absence of these it establishes latency by integrating site-specifically into host chromosome 19 [2, 66–69]. The different AAV serotypes each use unique mechanisms for cell entry, which results in different host tropisms. A safe, non-rescueable helper plasmid is used to complement the AAV coding sequences for *cap* and *rep in trans*. Following infection of a permissible cell line by either a wild-type AdV or Herpes simplex virus (HSV), the two plasmids are co-transfected into the cells, allowing for the formation of recombinant AAV [9, 10, 70]. Alternatively, a helper virus-free procedure has been developed, in which a mini-adenovirus helper plasmid is co-transfected with the vector and a packaging plasmid into an AdV E1-expressing cell line [2, 9, 10], resulting in the absence of production of infectious AdV particles.

7.2.5 Herpes Simplex Virus Type 1 Vectors (HSVVs)

Wild-type herpes simplex virus-1 (HSV-1) is an enveloped, dsDNA virus that is spread by direct contact and infects and replicates in the skin and mucosal membranes, before infecting cells of the central nervous system [2, 10]. The large complex viral genome encodes for more than 80 proteins that can be classified as either essential or non-essential for virus replication [71, 72]. HSVVs mimic the latent state of HSV-1, producing a highly infectious, efficient vehicle for delivery of foreign genetic material to both neural and non-neural tissue cells [2]. Vectors engineered from HSV-1 have the capacity to deliver large pieces of foreign DNA (up to 150 kbs) to the nucleus of most dividing and non-dividing cells. HSV has several other advantages, including the fact that it can infect many different host cell types (including cells of the nervous system), the viral DNA will not integrate into the host genetic material, and the complex nature of the virus genome, which contains about 40 genes that are not essential for virus replication and can be deleted without interfering with virus production *in vitro*, while the latent behavior of HSV can be used for stable, long-term expression of therapeutic transgenes [11, 71, 73]. High titers of pure, non-pathogenic HSV-1 vectors can be produced by introducing null mutations into viral immediate early genes. This disrupts the capacity for viral

replication, but allows production of the vectors by *in vitro* complementation of these genes *in trans* [2]. HSV-1 is currently genetically engineered to generate three different types of vectors i.e. (i) recombinant attenuated virus vector, (ii) defective, replication-incompetent non-pathogenic recombinant vector, and (iii) amplicon vectors. Of the HSVVs, amplicons have been used in most anti-cancer applications [11, 74, 75].

7.3 Non-viral Methods of Gene Delivery

The use of non-viral vectors (NVVs, natural or synthetic compounds) in which complexes of oligonucleotides, proteins, polymers or lipids are formed in particles capable of efficient transfer of genetic material into cells, precedes the development of viral vectors [10]. In the 1970s, the calcium phosphate-mediated transfection of cells was already widely accepted as an effective non-viral transfection tool [76]. In the same decade, Avery and colleagues reported phenotypic cellular changes following the non-viral delivery of exogenous DNA into pneumococcia [77]. Since those pioneering days, NVVs have been used to deliver synthetic oligonucleotide-based therapeutics that closely resemble traditional pharmaceuticals, including drugs, antibodies, RNA, as well as therapeutic genes to 'diseased' cells. Importantly, these vectors (i) are capable of being administered repeatedly to the host with little or no immune or inflammatory response, (ii) can be produced relatively easily in large quantities with high reproducibility and acceptable cost, (iii) are stable when stored at room temperature, (iv) can carry large transgene inserts, and (v) are easy to administer to patients [78–81]. In addition, non-viral vectors circumvent some of the problems associated with viral vectors including endogenous recombination, unexpected immune responses and oncogenicity [80, 81]. However, regardless of all the advantages of NVVs, they are generally rather inefficient at transfecting cells *in vivo* [6, 82, 83].

Plasmid DNA (pDNA) vaccines are synthesized from bacterial plasmids that have been engineered to express the therapeutic gene using promoter elements recognized by mammalian cells. The plasmids also contain a mammalian transcriptional terminator and a selectable marker to facilitate production of the plasmid in a bacterial system [84, 85]. Vaccination with pDNA mimics the natural intracellular pathogen gene expression pathways, activating both cellular and humoral responses. However, naked pDNA is generally difficult to deliver to the intended diseased site due to rapid clearance, degradation by cellular nucleases, the lack of organ-specific distribution and low efficiency in uptake after systemic delivery [86–89]. Even though nucleic acid modifications can overcome these shortcomings, specialized gene delivery vehicles that improve the delivery efficiency and cell-specificity, whilst protecting the pDNA against immune recognition are needed. Ideally, these gene delivery vehicles should also be able to enhance the therapeutic value of the transgene by providing complementary benefits such as the co-delivery of inflammatory suppressors to reduce potential cytokine production triggered by the naked pDNA [89].

7.3.1 Delivery of Plasmid DNA

7.3.1.1 Needle Injection

Naked pDNA encoding various proteins and peptides can be injected through a needle directly into the tumor, but often results in poor transfection efficiency [6, 90]. Also, only cells in the needle track are transfected [10] making treatment of the entire tumor unfeasible.

7.3.1.2 Liposome-Based Vectors

When a film of lipids is hydrated in an aqueous solution, it spontaneously forms microscopic particles consisting of one or more concentric lipid bilayers surrounding a watery compartment collectively called a liposome. These lipids consist of mono- or multi-cationic heads and a hydrophobic anchor held together by a linker [10, 91]. Cationic liposomes interact spontaneously with DNA to form lipoplexes [81, 92, 93]. Liposomes are the most effective non-viral vectors developed and offer several advantages for

gene delivery to cells including, (i) low cost of synthesis and no resulting disease; (ii) DNA protection from degradation by cellular nucleases; (iii) transfer of large pieces of DNA; and (iv) being targeted to specific cells or tissues [10]. For these reasons, they are often the vehicle of choice for many applications [10, 89]. Even so, inflammatory toxicity, low transduction efficiency (compared to viral vectors) and immune recognition are problems still often associated with the use of liposomes as gene transfer tools [81, 89].

7.3.1.3 Polymer-Based Vectors

They are relatively easy to produce and flexible to modifications. These cationic polymers condense DNA into small particles, thereby preventing DNA degradation, ultimately improving gene expression. The DNA/polymer complex (or polyplex) is transferred into the cell via receptor-mediated endocytosis [81, 94]. Biodegradable polymers (known for their low toxicity and high biocompatibility) have been used to achieve controlled-release of DNA, further enhancing and prolonging gene expression. Controlled-release technology has been reported to increase and prolong the concentration of DNA around an injection site [80, 81], making it a very promising technology.

7.4 Cancer Gene Therapy

Since the first clinical gene therapy trial in 1990s, great attention has been paid to cancer as a potential candidate for gene therapy applications. Today, among a total of 1,902 gene therapy clinical trials worldwide, those for cancer gene therapy constitute about 64 %. This number not only represents the high enthusiasm in this field but also indicates the multiple pathways that can be targeted to stop cancer growth. However, the ongoing failure of any of these clinical trials to yield an efficacious gene therapy product, with the exception of the Chinese approved Gendicine, denotes the multiple challenges that are yet to be overcome. Several strategies have been attempted to stop cancer growth, for example through re- or over-expression of tumor suppressor genes [95], introduction of a suicide gene followed by pro-drug administration [96], knocking down oncogenes or enhancing radio-and chemo-sensitivity of the cancer cells [97]. Another strategy is aimed at targeting the host non-cancerous tissue, primarily to enhance the anti-tumor immune response. This can be achieved by expression of transgenes possessing direct anti-tumor activity (e.g. cytokines) or by indirectly activating the host immune response (e.g., GM-CSF) [98]. This last strategy can also convey chemo-protection to highly vulnerable cells in the bone marrow to protect them against frequent high doses of chemotherapy [99]. The inability to target all cancer cells, especially after tumor dissemination means that systemic cancer gene therapy aimed at developing anti-cancer immunity appear most promising. Specific targeting of cancer cells is also crucial as non-selective expression of toxic transgenes in normal cells can lead to severe toxicity. Fortunately, using promoters specifically upregulated in cancer cells to drive expression of suicide genes can be used, such as epidermal growth factor receptor promoter, transferrin promoter, telomerase promoter or Prostate Specific Antigen promoters [100, 101].

Some of the therapeutic targets used in cancer gene therapy are summarized below;

7.4.1 Oncogene and Tumor Suppressor Gene Targeted Gene Therapy

Normally cell cycle is controlled by two discrete types of signal; proto-oncogenes that promote survival and division as well as tumor suppressor genes that arrest the cell-cycle and initiate apoptotic programs. The interaction between these sets of genes determine the cell's fate and any imbalance in gene expression can lead to malignant transformation.

Proto-oncogenes are genes that encode proteins responsible for stimulation of cell division, inhibition of cell differentiation and prevention of cell death. These genes are important for development and maintenance of human tissues and organs. However, their mutation is associated with neoplastic transformation [102]. These mutant proto-oncogenes are called oncogenes. Proto-oncogenes comprise three main classes; (i) growth

factors and growth factor receptors; (ii) genes encoding proteins that work in the cellular cytoplasm such as; tyrosine and serine/ threonine kinases and (iii) nuclear transcription factors such as the NF-κB family [103]. Identification of these oncogenes provides a therapeutic platform for cancer treatment (Table 7.2). It is believed that silencing or knocking down these oncogenes can dampen cancer associated biological consequences at the gene level and thus treat or maintain the disease. Suppression of gene expression (gene silencing) can be achieved, to a variable degree, either at the transcriptional level by triplex-forming oligonucleotide or at the translational level using antisense RNA, siRNA, ribo-zymes and DNAzyme. Although targeting oncogenes with antisense RNA showed encouraging antitumor effects *in vitro* [104–106], human translation has not been easy. Many factors have to be considered including the gene expression profile of any tumor to determine which oncogenes are involved, as a single target will normally not be sufficient.

Tumor suppressor genes control cell growth in a dominant negative manner and their abnormal function relates to tumor development. These genes possess different functions to protect the host cell from cancer initiation and propagation. They are divided into three main groups; (i) caretaker genes such as the ataxia telangiectasia mutated (*ATM*) gene, which are responsible for maintaining genomic stability by ensuring the fidelity of DNA repair [107]; (ii) landscaper genes which act by maintaining or controlling the microenvironment harboring the growing cells [108];

Table 7.2 Summary of ongoing gene therapy clinical trials in different types of cancer

Gene	Vector	Cancer type	Phase	Status	Trial ID
Anti-oncogenes					
TGF-β2 antisense	Plasmid DNA	Solid tumors	Phase I	Open	US-0908
			Phase II		US-0393
		NSCLC	Phase III		US-0819
k-rasp53 antisense	Retrovirus	NSCLC	Phase I		US-0031
c-fos or c-myc antisense	Retrovirus	MBC			US-0084
(EGFR) antisense	Lipofection	HNC			US-0285
	Plasmid DNA				US-0576
Tumor suppressor genes					
P53	Adenovirus	SCCHN	Phase I	Closed	BE-0003
		NSCLC	Phase II		BE-0004
		HNC	Phase II		BE-0007
		Ovarian tumor	Phase II/III		BE-0008
		HNC	Phase II		CH-0012
		NSCLC	Phase II		CH-0013
		NPC	Phase II/III		CN-0003
			Phase III	Open	CN-0009
P53	Adenovirus	Cervical cancer	Phase III	Open	CN-0010
		Thyroid cancer	Phase IV		CN-0013
		HNC	Phase III	Closed	DE-0037
Suicide genes					
HSV-TK	Adenovirus	HCC	Phase II	Closed	CN-0011
	Adenovirus	Recurrent HNC	Phase II		CN-0021
	Retrovirus	Newly diagnosed previously untreated glioblastome	Phase III		BE-0002
	Adenovirus	Prostate cancer	Phase III	Open	US-0842

NSCLC Non-small cell lung cancer, *MBC* Metastatic breast cancer, *HNC* head and neck cancer, *SCCHN* Squamous cell carcinoma of the head and neck, *NPC* Nasopharyngeal carcinoma, *HCC* Hepatocellular carcinoma

and (iii) gatekeeper genes which provide a monitoring system to maintain the balance between cell division and death. Dysfunctional mutations of these genes lead to altered growth regulation and differentiation and predispose cells to tumor initiation and progression or metastasis [109].

Among the tumor suppressor genes, *P53* gained the greatest attention because of its ability to monitor cellular stress, which could be induced by oncogene activation or DNA damage, and govern whether the cell will survive or enter the apoptosis pathway. The diversity of genes involved in cancer initiation often limits the effectiveness of gene therapy approaches. Fortunately, restoration or overexpression of single genes like *P53* may be enough to induce tumor cell death. Various preclinical studies have demonstrated the efficacy of *p53* in inducing apoptosis and suppressing tumor growth [110–112]. *P53* based-gene therapy was also intensively tested in clinical trials (Table 7.2) with demonstrated safety and efficacy in some cancer types [113–116]. Moreover, *P53* mediated delivery by adenovirus serotype 5 (Gendicine) has become the first approved gene therapy product, to treat carcinoma of the head and neck [117]. Conformational change mutants of *P53* have also been reported in cancer cells. These changes lead to prolongation of the P53 life-span and augment its expression levels compared to normal cells, indicating that P53 could act as a tumor antigen [118], and targeting it with vaccine approaches may overcome the limitations associated with an inability to transfect all cancer cells. However clinical trials using virus-based vaccines encoding mutant *P53* showed limited immunogenicity, attributed to the competition between the antigenicity of the vector and the P53 protein [119, 120].

7.4.2 Enhancing Pro-Drug Cytotoxicity in the Tumor Cells Through Gene Therapy

Confining the toxic effect of antitumor agents (such as chemotherapy and radiotherapy) to malignant cells remains a critical issue in the development of novel-cancer therapeutics. Alternatively, making cancer cells more sensitive to drug toxicity is possible through suicide gene therapy-based approaches. This involves enzymatically modifying cancer cells such that they convert an inactive pro-drug to an active toxic drug, hence increasing local concentrations of the toxic drug exclusively in the tumor and sparing normal tissues and organs. This can be achieved with several suicide gene therapy approaches.

7.4.3 Thymidine Kinase (TK)

Thymidine kinase (TK) is an ATP-thymidine 5′-phosphotransferase naturally present in all living cells. It is also present in viruses including herpes simplex virus (HSV), varicella virus, and Epstein-Barr virus. Unlike human TK, HSV-TK has the ability to phosphorylate some dNTP analogs such as Ganciclovir (GCV), a synthetic analogue of 2′-deoxy-guanosine. This integrates into newly synthesized DNA, terminating DNA synthesis and triggering the apoptotic signalling cascade. In the suicide gene therapy approach *HSV-TK* is delivered and expressed in cancer cells followed by systemic administration of GCV. Limitations in transduction efficiency are partially overcome by the bystander effect produced by the HSV-TK action (due to diffusion of the toxic metabolites to adjacent non-transduced cells) meaning there is no longer a necessity to achieve 100 % transduction efficiency within the tumor cells.

To date, several clinical studies utilizing *HSV-TK* suicide gene delivery systems (Table 7.2) to treat cancer have been reported, but with limited results [121–123]. The ability to transduce glioma cells in the brain with *HSV-TK* after single or multiple injections was demonstrated, however therapeutic effects were limited and associated with various degrees of toxicity [122]. A recombinant version of the suicide gene, *HSV/TK-007* has been shown to be more effective than the wild-type gene product [124, 125]. Also co-administration of valproic acid, an inhibitor of histone deacetylases, enhances HSV-TK/GCV efficacy [126].

7.4.4 Cytosine Deaminase (CD)

A second gene that has been used to trigger selective destruction of tumor cells is the bacterial cytosine deaminase (CD), which encodes a protein capable of converting cytosine into uracil. This protein converts the nontoxic antifungal agent 5-fluorocytosine (5FC) into the wildly used toxic chemotherapeutic drug, 5-fluorouracil (5FU), which inhibits RNA and DNA synthesis during the S phase of the cell cycle [127]. This gene is not expressed in eukaryotic cells, therefore expression of *CD* gene in tumor cells leads to selective conversion of non-active 5FC to the toxic 5FU producing tumor-targeted chemotherapy. Therefore, the systemic side effects of 5FU are reduced and a local bystander effect is produced [128]. Unlike GCV, 5FC is a small, uncharged molecule that can pass freely in and out of the cell by diffusion meaning cell-cell contact is not required for a bystander effect [129]. Moreover it also produces a distant bystander effect through sensitization of the immune system [130, 131]. Initial clinical trials with the CD gene driven by a tumor specific promoter and injected into breast cancer patients using plasmid DNA showed high selectivity of expression in tumor cells, however minimal reduction in the tumor size were reported [96].

7.5 Anti-angiogenesis Gene Therapy

As tumors grow they require a supply of nutrients and oxygen designated as angiogenesis. No matter how unregulated, it is crucial for neoplastic growth, expansion and metastasis [132]. Several growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), interleukins (ILs) and matrix proteins are mandatory for angiogenesis [133]. Moreover, proteolytic enzymes such as cathepsin, urokinase-type plasminogen activator, gelatinases A/B, and stromelysin are critical to the neovascularization process [134]. Anti-angiogenic modulators include natural inhibitors such as endostatin, thrombospondin and angiostatin or inhibitors of the pro-angiogenic factors (antibodies, antisense RNA and soluble receptors for FGF, VEGF). Therefore, anti-angiogenesis-based therapies targeting ECs instead of tumor cells are a potentially powerful therapeutic approach to fight cancer. They possesses several advantages including; reduced toxicity and less capacity to develop resistance. In addition, the therapy is independent on tumor type [135]. In addition, ECs in the tumor are more sensitive to anti-angiogenic therapy because they proliferate more rapidly and express several unique surface markers [136].

Nevertheless, most of the antitumor benefits reported in the clinic after targeting of angiogenesis were subtle and only achievable after intratumoral administration with very high doses of the agents. This could be explained by the presence of large numbers of factors that control tumor angiogenesis, as well as the malleability of cancer cells to compensate for any one missing factor. Therefore, as in most of the gene therapy approaches, combination of antitumor therapies is the most likely way to accomplish effective anticancer results with minimal side effects.

7.6 Cancer Vaccines and Vaccine Production

In recent years, substantial progress has been made in vaccine development for malignant diseases. New technologies have been developed for the identification of a large number of tumor antigens that can be utilized to stimulate the patient's immune system in order to specifically recognize and destroy tumor cells. Cancer vaccination encompasses therapies that involve the administration of some form of antigen to induce a specific antitumor immune response. The vast majority of vaccine studies today employ measures designed to activate tumor-specific T cells. In addition to tumor antigens, cytokine genes (such as *GM-CSF*) or co-stimulatory genes (such as *B7*) are often included to improve the effectiveness of the vaccine. While the success of vaccine strategies are dependent on the route of antigen delivery and adjuvant, it is important to

keep in mind that their success often depends as much on the particular antigen being used. This can be illustrated with the human mucin tumor antigen, MUC-1, which in early vaccine studies caused cellular immune responses in mice, but humoral responses in humans.

7.6.1 Virus-Based Vaccines

The association of highly immunogenic viral proteins with the otherwise weakly immunogenic tumor antigens can allow for the generation of a tumor-specific immune response. Early clinical trials employing this technique, while partially successful, were plagued by inconsistency. The choice of viral vaccine vector is complicated by several factors, including pre-existing immunity to the virus, the immunogenicity of the virus, transduction efficiency, the cloning capacity of the virus and the viral tropism [45]. Importantly, using a virus with high immunogenicity may result in the inability of viral vectors to be expressed efficiently and consequently may evoke a vector-specific rather than tumor-specific immune response. Perhaps the greatest clinical success to date using viral vaccination was seen in the PROSTVAC multi-center randomized trial for prostate cancer, which compared empty vector to one encoding prostate-specific antigen (PSA) and three T cell co-stimulatory genes (*B71*, *ICAM-1*, and *LFA-3*, designated *TRICOM*). While no change in progression-free survival was seen, overall survival was improved from 17 % to 30 % [137]. A related viral vector termed PANVAC, which expresses TRICOM and the tumor antigens MUC1 and CEA, have also been shown to extend survival in colorectal cancer patients [45].

Viral vaccines are also employed as prophylactic agents against cancer. For instance, cervical cancer is causally linked to human papillomavirus (HPV) infection and constitutes a major health problem for women. This type of cancer accounts for about 10 % of all cancers in women worldwide. Recently, two pharmaceutical companies, Merck (Gardasil) and GlaxoSmithKline (Cervarix), have reported a remarkable degree of protection by candidate prophylactic HPV vaccines. These vaccines are based on utilizing a subunit virus-like particle composed of a single viral protein, L1, which is the major structural (capsid) protein of the virus and contains the immunodominant neutralization epitopes of the virus [138]. These vaccines are now FDA-approved for the prevention of cervical cancer.

7.7 Oncolytic Viruses

An oncolytic virus is a replication-selective viral vector used as a cancer treatment [139, 140]. The capacity for these agents to preferentially replicate in those cells with a malignant phenotype can be either natural or engineered; for example, several small viruses that do not naturally replicate in human cells and have no known human pathogenicity have been found to retain a capability to replicate in cancer cells, typically due to the loss of part or all of the anti-viral IFN response that is a feature of many solid tumors [141]. Alternatively, some viruses that naturally replicate in human cells and that may even be disease causing have been engineered to target different phenotypic features of cancers, resulting in attenuation in normal tissues. One such approach involves alteration of viral tropism through modification of viral coat proteins so that the virus only recognizes cell surface receptors upregulated or expressed uniquely on cancer cells or in the tumor microenvironment [142, 143]. Alternatively, several large viruses have instead been modified for selectivity at steps after initial cell entry. In one scenario, essential viral genes can be placed under the control of tumor or tissue specific promoters to restrict viral replication [144, 145]. However, perhaps the most successful approach has been to directly target the 'hallmarks of cancer' [146, 147]. This is possible as many of the adaptations a virus induces within an infected cell to optimize its own replication potential are very similar to the classic hallmarks of cancer as described by Hanahan and Weinberg [147]. These include the induction of uncontrolled proliferation in the host cell, avoidance of host cell immune response and evasion of apoptosis. Many viral virulence genes have been

described in larger viruses whose role is to induce these cellular adaptations in non-tumor cells. However, because they are redundant for viral replication in cancer cells, deletion of these viral virulence genes produces vectors that are highly attenuated in normal cells, but retain replication capacity in tumor cells.

7.7.1 Mechanism of Action

The initial hypothesis behind the development of oncolytic viruses was that the process of viral replication within the tumor would result in both direct viral-mediated lysis of the infected cell and selective amplification and release of the therapeutic within the target environment, resulting in rapid spread throughout the tumor and extensive destruction of tumor tissue. However, many of the earliest vectors tested in the clinic were based on Adenoviruses [148–150], which despite clearly demonstrating an excellent safety profile produced only limited responses, typically requiring combination with other chemotherapies to demonstrate any therapeutic benefit [151, 152]. This appeared to be primarily due to their slow replication cycle, meaning that the vector was cleared by the host immune response before complete tumor eradication could occur. Subsequent trials with more rapidly replicating and spreading viral backbones such as those based on vaccinia, Reovirus or HSV [153–159] resulted in improved therapeutic benefit.

Even though the rate of spread is clearly one determinant of oncolytic virus activity, it has become increasingly clear that the immune response induced by these vectors is also a critical factor in their therapeutic activity. It is apparent that the process of viral replication within the tumor acts to overcome localized immune suppression and the destruction of tumor cells results in the release of relevant tumor antigens in addition to multiple other danger signals. As a result, production of an in situ vaccination effect has been clearly demonstrated in both pre-clinical models and clinical studies, leading to an adaptive immune response targeting both viral and tumor antigens [160–162]. The most advanced oncolytic viruses in clinical testing typically express a cytokine to enhance these immunotherapeutic mechanisms [157, 163].

It is also evident that optimizing this immunotherapeutic mechanism of action requires further refinement as most approaches that enhance or increase stimulation of the immune response (such as *cytokine transgene* expression) also result in more rapid clearance of the virus and a reduction in their directly oncolytic potential (even in cases where enhanced overall therapeutic activity is observed) [164]. Conversely, in several other reports, combination of oncolytic viruses with suppressors of particular components of the innate immune response have been shown to result in enhanced therapeutic activity [165].

Since mechanisms of killing produced by oncolytic viruses are typically different from traditional chemotherapy and radiotherapy, most oncolytic viruses appear to combine well with these other therapies. In addition there is considerable evidence to support combining these viral therapies with targeted therapies (such as TKI) or immunotherapies, as long as some consideration for the potential interactions and timing of addition of the therapies is explored.

Although the primary mechanisms of tumor cell killing appear to be through direct lysis of infected malignant cells and induction of an immune response within and against the tumor, other additional mechanisms have been reported. In particular several oncolytic viruses have been reported to induce a rapid and profound vascular collapse within the tumor soon after delivery [166, 167]. This appears to be mediated both by selective infection of tumor-associated endothelial cells and attraction of neutrophils leading to localized thrombosis. In addition, because oncolytic viruses selectively replicate in the tumor they can act as idealized gene delivery vehicles, expressing therapeutic transgenes selectively from within the tumor environment and amplifying these genes within the tumor. As a result, multiple mechanisms of cell killing can be incorporated through expression of therapeutic transgenes beyond expression of cytokines or immunostimulatory molecules. However, because infected cells will be killed by the virus,

transgenes that mediate a bystander killing effect are most effective. As such, a variety of pro-drug converting enzymes, anti-angiogenic enzymes and receptors for uptake of radionucleotide labeled probes (both for imaging and for therapeutic effect) have been shown to enhance the therapeutic effectiveness of oncolytic agents.

7.7.2 Viral Agents Used as Oncolytic Agents

A variety of viral backbones have been used as the basis for oncolytic agents and several recent reviews have attempted to cover the ever-expanding list of vectors and genetic alterations that have been reported; therefore, a complete list will not be covered here.

The first description of a logically designed viral strain displaying cancer-selective replication was based on an HSV model with mutation of the IFN-γ targeting virulence gene. However, the first serious clinical development of an oncolytic virus was that of dl1520 (ONYX-015, now H-101) [150], an Adenovirus serotype 5 with a deletion of the *E1B-55KDa* gene which has multiple functions including targeting p53 and transport of mRNA. This vector underwent clinical testing up to phase III in the US before being subsequently approved for use in the Chinese market. In the clinic, its therapeutic benefits were generally seen only in combination with chemotherapies and tended to be only partial. In addition, local or regional delivery was also found to be needed. However, this virus demonstrated the safety of this approach and provided a proof of concept for the field. These distinct disadvantages of Adenoviruses, including their slow spread and lack of systemic delivery potential have become a focus of ongoing research for many investigators and mechanisms have been developed to overcome many of these. Meanwhile, many other researchers looked for alternative viral backbones, including some non-engineered small viruses, including Newcastle Disease Virus, NDV [168] and Reovirus [169]; some small viruses with minor alterations (such as VSV [170] and retroviruses [171]); some vaccine strains of larger viruses (including measles Edmonton vaccine strain [172] and vaccinia [140], the smallpox vaccine) and some larger disease causing viruses with more extensive engineering (including poliovirus [173] and HSV [174]).

All of these backbones, often with multiple combinations of gene deletions and transgene expression have demonstrated tumor targeting and activity in pre-clinical models and a significant number have advanced into clinical testing. Among these, some highly promising phase II results have been reported with an oncolytic vaccinia expressing GM-CSF (JX-594, Jennerex) in the treatment of HCC and an HSV strain also expressing GM-CSF (T-Vec, Amgen), in the treatment of melanoma.

7.8 Concluding Remarks

There has been a recent resurgence in the use of viral and gene therapy based vectors in the treatment of cancer, and it is likely that the success of several ongoing clinical trials will lead to the approval of novel therapies in the Western markets. This validation of approach and process will lead to further interest and research of novel targets. Despite the ongoing limitations in the efficiency of gene delivery, a better understanding of the limits of cancer gene therapy and the use of cancer vaccines will allow for the logical development of new therapies which will alter the way cancer patients are treated.

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Cancer Stem Cells: Biology and Potential Therapeutic Applications

8

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8.1 Introduction

The cancer stem cell (CSC) model states that, similarly to adult regenerating tissues, tumors are organized in a hierarchy of heterogeneous cell populations and are sustained from a subset of tumor cells, which possess the same functional hallmarks of stem cells: unlimited self-renewal, multilineage differentiation potential, and reversible transition to a quiescent state [1, 2]. Recent studies have reported evidences of CSC involvement in numerous solid neoplasms, including prostate [3], colon [4, 5], head and neck [6], melanoma [7], lung [8], liver [9], breast [10], brain [11], pancreas [12–14], ovary, [15] and mesenchymal malignancies [16]. The cancer stem cell theory underwent a significant evolution to a more comprehensive model throughout the last 40 years, integrating data from clinical observation and biomolecular approach. Albeit appealing, this concept still lacks a consistent unifying model. The definition and identification of CSCs in most tumor types remain elusive; their role in tumor progression and resistance to conventional

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antiblastic therapies has not been fully elucidated, and even whether or not they exist in all human tumors is still an open question. In this chapter, we will introduce the state-of-the-art and clinical implications of this area of investigation.

8.2 Identification and Characterization of Cancer Stem Cells

Experimental observations on hematopoietical malignancies revealed that a small subset of cancer cells are capable of extensive proliferation [17], which led to the emergence of the CSCs hypothesis. It has been subsequently shown in solid cancers that tumor cells are phenotypically heterogeneous and only a small proportion of cells are clonogenic in culture and *in vivo* models [18]: these cells could be considered as CSCs as they are the pool of cells within the tumor which sustain malignant growth, similar to stem cells within normal tissues sustaining growth. Noteworthy, CSCs do not forcibly correspond to the "cell of origin" (the normal cell that undergoes initial transformation): In most cases, the "prime" cell presents a phenotype different from CSCs [19].

According to this classical concept, CSCs would share peculiar functional features with physiological stem cells:

- Unlimited self-renewal: CSC can undergo indefinite division.
- Differentiation potential: CSCs can generate a differentiated progeny that would constitute the bulk population of tumor cells and contribute to tumor heterogeneity.
- Transition to a quiescent state: CSCs can switch from a dormant, slow-cycling condition to an activated state. This could account for resistance to standard therapies that target cycling cells and also explain relapses occurring years after completion of conventional therapies.

Nevertheless, this classical concept is still controversial. The landmark studies of Lapidot et al. [20] demonstrated that only a small fraction of human leukemia cells could generate leukemia in severe combined immune-deficient (SCID) mice, whereas the majority of tumor cells failed to engraft. However, these studies can be criticized because xenotransplantation assays could be selecting for cells more fit to grow in a foreign environment [21, 22]. Although these arguments were partially invalidated by recent experiences of genetic tracing in mouse models which spontaneously developed tumors [23–25], there is a need to find a further correlation with evidences from experimental results, in order to establish solid criteria to isolate and identify CSCs.

8.2.1 Surface Markers

Regarding solid tumors, a significant step forward has been the identification of immunophenotypical cell surface markers: Al-Hajj et al. [10] reported that $CD44^+/CD24^{-/low}$ fractions from metastatic pleural effusions of primary invasive breast tumor had significantly higher tumorigenic potential compared to $CD44^+/CD24^+$ cell fractions in a NOD/SCID mouse model. Following this work, candidate CSC biomarkers were extensively described in a wide range of tumors: examples include the $CD34^+CD38^{-/low}$ phenotype of many human acute myeloid leukemia stem cells and the $CD133^+$ or $CD15^+$ phenotype of human brain tumor CSCs [26, 27]. Combinations of different antigens in a panel of molecular surface markers have been tested, in order to improve their reliability, in each histological subtype.

Since the isolation of $CD133^+$ stem cell-like cancer cells from brain tumors, CD133, also known as prominin-1, has been one of the most popular biomarkers in CSC-related research [11]. Its expression as CSC marker has been shown in several tumors, such as colon [5], lung [28], gallbladder [29], hepatocellular [30], and prostate cancer [31].

CD44, a multifunctional class I transmembrane glycoprotein, has been suggested as a candidate marker of stemness in a number of cancers including breast [10], colorectal [32], prostate [33, 34], head and neck [35], bladder [36], ovarian [37], cervix uteri [38], gastric [39], and gallbladder cancers [29] as well as hepatocellular carcinoma [30].

CD24 positivity has been reported, in association with CD44, as a potential phenotype for CSCs in breast [40] and pancreatic cancers [13]. As an exclusive marker, interest has been drawn to CD24 in ovarian [41] and colorectal cancer CSCs [42] and, more interestingly, in hepatocarcinoma CSCs [43].

CD90 has been described as a surface label for CSCs in hepatocarcinoma [9], high-grade gliomas [44], and lung cancer [45]. Other surface markers encompass EpCAM [46], CD166 [47], and CD117 [48].

Nonetheless, this list of markers for CSCs identification has several limitations. In fact, several CSC clones may coexist within primary tumors (intra-tumor heterogeneity) and different tumors might express different sets of CSCs surface markers (inter-tumor heterogeneity) [49]. Moreover, a marker valid for the identification of CSCs by one method may not be specific when using different approaches [50, 51]. In addition, the limited specificity of the markers used to identify CSCs is a major issue: none of the known markers solely mark CSCs and they may need to be used in combination with other markers [4, 5]. It has also been shown that antigen positivity is strongly dependent on the employed technique [52] and is widely influenced by epigenetic phenomena implied in the regulation of their expression [53]. Surface markers are frequently expressed in a broad variety of malignant and nonmalignant cells at different steps of their differentiation, resulting in a lack of specificity [5]: implementation of a subset of antigenic variants which selectively targets CSCs such as CD44v6 splice variant is currently under evaluation [54].

In addition, correlation between the expression of biomarkers and content in CSCs was not found in melanoma, possibly representing an interesting exception to the CSC model. In this malignancy, a highly enriched $CD271^+$ population was able to develop tumors in Rag22/2cc2/2 mice, while $CD271^-$ cells did not [55], suggesting their stem cell-like properties. Nevertheless, further experiences on nude mice invalidated this result, implying that melanomas could follow a stochastic model (where tumorigenicity is a random feature distributed among all tumor cells) rather than a hierarchical model (with a cancer stem cell compartment) of local tumor growth and distant spread [56]. Thereby, in order to overcome the poorly specific surface markers and to replace them with more direct functional markers, investigators focused on enzymes or signaling pathways, involved in the maintenance of CSC properties.

8.2.2 Side Population

Early CSC isolation protocols relied on the peculiar ability of a subpopulation of cells, the so-called side population (SP), to exclude the fluorescent dye Hoechst 33342 [57] on fluorescence-activated flow cytometric analysis. When dye emission is analyzed on a flow cytometer equipped with a 405 nm laser in a two-parameter display of red and blue emission wavelengths, a tailing population (side population) exhibiting dim fluorescence is observed, as compared to the majority of cells with bright fluorescence [58]. This specific SP cell feature is related to the expression of the adenosine triphosphate-binding cassette (ABC) transporter of enzymes, especially ABCG2 [59, 60]. Moreover, depletion of ABCB5 in CSCs was related to reduced tumorigenic activity, suggesting a functional role for this transporter in CSCs [61].

8.2.3 ALDEFLUOR Assay

ALDEFLUOR assay detects expression of cytosolic aldehyde dehydrogenase 1 (ALDH1) [62], and it has also been proposed as a method to identify CSCs. Cytosolic aldehyde dehydrogenases (ALDHs) are a group of enzymes involved in oxidation of aldehydes into carboxylic acids: the expression of ALDH1 has been proposed as a putative marker of stemness in normal mammary tissue as well as in breast cancer cells and seems to correlate with the outcome in breast cancer patients [63]. However, ALDH1 does not appear to be a reliable CSC marker in all tumor types [64]. Moreover, it has been suggested that the stem cell population identified by the

ALDEFLUOR assay is heterogeneous and must be dissected using additional surface markers [2]. Recent studies have also shown that ALDH1 inhibition enhances expression of a stem cell-like phenotype, suggesting a possible role of ALDH1 in regulating differentiation – likely related to its involvement in retinoic acid synthesis [65] – rather than in maintaining stemness.

8.2.4 Sphere-Forming Assay

CSCs have the ability to generate nonadherent, three-dimensional (3D) tumor spheres under serum-free conditions in a clonogenicity assay called "sphere-forming" assay, which measures the frequency with which these prospectively isolated cells form colonies when plated at clonal density in nonadherent culture [66, 67]. Originally used for isolation of normal neural stem cells [68], the sphere-forming assay was then adapted to estimate the CSC fraction in various tumors [69–74].

However, this technique has several limitations, such as the possible formation of artifacts due to cell aggregation if cells are plated at a too high density or the likely selection of CSC phenotype made by the sphere assay culture conditions, that can alter the sphere counts and thus confound the interpretation of the obtained results [75].

8.2.5 Signaling Pathways

Multiple regulatory networks are suggested to be involved in CSC self-renewal and differentiation (Fig. 8.1): the constitutional activity of these

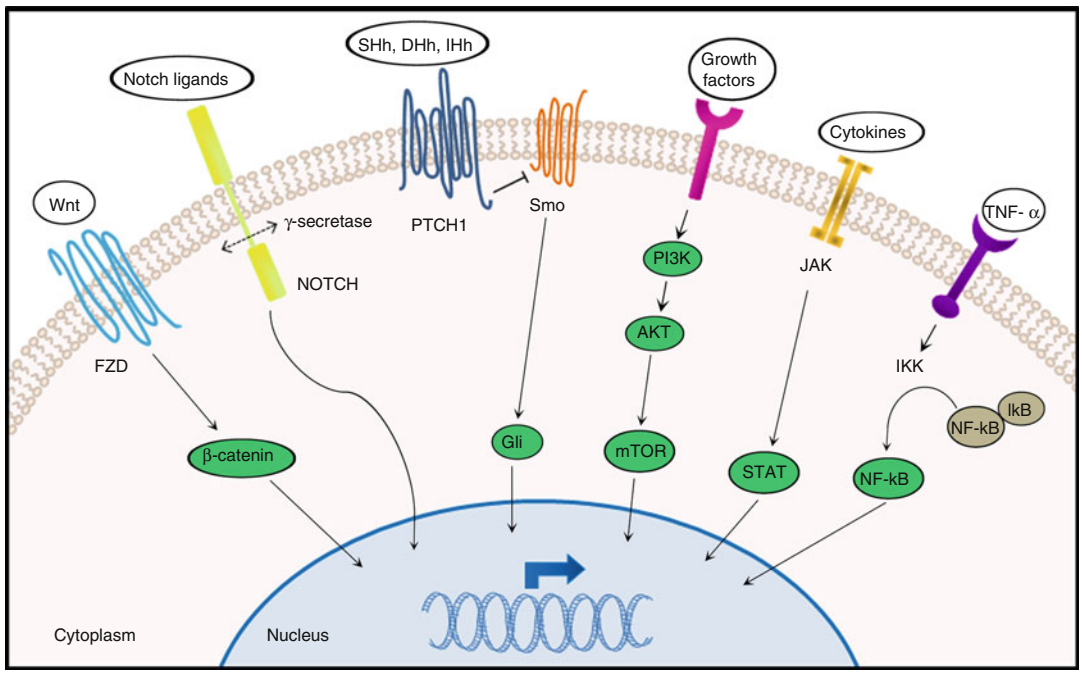

Fig. 8.1 Main signaling pathways involved in the molecular mechanisms underlying CSC control. The activation of these pathways following different stimuli results in the enhanced transcription of several genes (i.e., *cyclins*, *c-Myc*, *EGF*, *VEGF*) involved in physiological cell processes, including cell proliferation, growth, and survival. *FZD* Frizzled (Wnt receptor), *γ-secretase* enzyme responsible of the release of the Notch intracellular domain, *SHh* Sonic Hedgehog Homolog, *DHh* Desert Hedgehog Homolog, *IHh* Indian Hedgehog Homolog, *PTCH1* Patched 1 (Hedgehog receptor), *Smo* smoothened, *Gli* Gli transcription factors, *PI3K* phosphatidylinositol 3-kinase, *Akt* also known as Protein Kinase B (PKB), *mTOR* mammalian target of rapamycin, *JAK* Janus kinase, *STAT* signal transducer and activator of transcription, *NF-kB* nuclear factor-kappa-light-chain-enhancer of activated B cells, *IkB* inhibitor of kB, *IKK* IkB kinase (responsible of IkB degradation)

intracellular signaling pathways can be additionally enhanced by interaction with external stimuli from the cancer cell microenvironment, as discussed in the following section.

8.2.5.1 Wnt/β-Catenin

The Wnt/β-catenin signaling pathway is a pivotal developmental pathway [76], reported to control proliferation *vs*. differentiation in normal stem cell maintenance and growth [2, 77–80]. Aberrant Wnt activation is a key factor for the initiation and progression of various tumors [81–83]. Furthermore, increasing evidence suggests the involvement of Wnt signaling in the molecular mechanisms underlying CSC control. It has been reported that Wnt pathway triggers a response to DNA damage [84] and that genomic instability may drive the malignant transformation of nontumorigenic stem cells to CSCs [85–87].

Wnt pathway-related genes such as *FZD6* and *WNT7B* are highly expressed in undifferentiated mouse mammary tumor cells that are grown in mammosphere to enrich for progenitor-like cells, compared with the differentiated population [88].

The importance of Wnt signaling pathway in CSC control has been strengthened by Vermeulen et al. [89], who demonstrated that Wnt signaling activation was a marker for colon CSCs. In addition, a role for Wnt pathway in cutaneous CSCs has been highlighted which appeared enriched for Wnt signaling: ablation of the *β-catenin* gene resulted in the loss of CSCs and complete tumor regression in a model of squamous cell carcinoma [90].

Connections between Wnt signaling and epithelial–mesenchymal transition (EMT)—the process by which cells acquire a mesenchymal identity, losing cell–cell adhesion properties and polarity [91]—have also been suggested in numerous studies, but the exact role of Wnt pathway in promotion or reversal of EMT is still unclear.

8.2.5.2 Notch

The Notch family regroups four single-pass transmembrane protein receptors involved in cell development [92]: aberrant expression and dysregulation of Notch proteins, ligands, and targets has been described in hematological malignancies [93] and in a multitude of solid tumors [93, 94].

Notch is activated via γ-secretase-mediated cleavage, which releases the intracellular domain from the membrane, allowing it to translocate into the nucleus, where it forms a short-lived transcription complex.

Crosstalk between Notch and other oncogenic pathways has been described to exert a mutual regulation on TGF-α [95, 96], VEGF [97], Wnt [98], and PEA3 [99] pathways. In recent years, Notch activity has been reported to be implicated in the maintenance of CSCs in various cancers [100–108]. The Notch pathway is an important factor in the linkage between angiogenesis and CSC self-renewal; thus, Notch pathway targeting is increasingly considered a therapeutic strategy for cancer treatment, by eliminating CSCs [109]. Accordingly, selective blockage of Notch reduced self-replication and tumor formation capacity of leukemic CSCs [110] and impaired mammosphere formation *in vitro* [111]. Moreover, the inhibition of Notch pathway in association with trastuzumab has been proven effective in preventing tumor relapse in ErbB2-positive xenograft murine model of breast cancer [112]. Additionally, accumulated evidence has demonstrated that Notch signaling might contribute to cancer metastasis [113].

8.2.5.3 Hedgehog

The hedgehog (Hh) signaling pathway coordinates development of tissue progenitors and expansion of stem cells [114]. Pathway activation is initiated by binding of one of the three ligands, Sonic (SHh), Desert (DHh), and Indian Hedgehog (IHh), to the patched receptor (PTCH1), disabling its constitutive repression of smoothened (Smo), that leads to activation of the Gli transcription factors [115] and, hence, to the enhanced transcription of several genes involved in cell proliferation, such as *cyclins*, *c-Myc*, epidermal growth factor (*EGF*), and vascular endothelial growth factor (*VEGF*) [116].

Aberrant Hh signaling has been described both in individuals affected by predisposing genetic syndromes [117] and in tumors displaying sporadic mutations involving PTCH loss [118]. Emerging data from many human tumors suggest that Hh is required to maintain self-renewal, proliferation, and tumorigenic potential

of CSCs in a complex fashion involving both intracellular signalization and interaction with differentiated tumor cells and with the microenvironment [119–121].

Hh signaling has been found to be preferentially activated in clinical specimen-derived colon [122] and breast [116] CSCs, as evidenced by the increased expression of GLI1, GLI2, and PTCH1.

Accordingly, inhibition of GLI1 reduced proliferation of breast CSCs [26], and loss of Smo led to depletion of chronic myeloid CSCs [2]. Furthermore, inhibition of Hh pathway has been proven effective in a pancreatic tumor xenograft model [2].

8.2.5.4 mTOR

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway is a key regulator of physiological cell processes, including growth, cell proliferation, and survival [123–125]. mTOR signaling aberrant activation is frequently observed in human cancers [126]. Recent studies showed that mTOR pathway may be involved in the regulation of CSC biology, notably cell cycle progression and survival [123, 127].

Furthermore, Akt1/2 proteins were more expressed in mammosphere cells than in more differentiated cells [116]. Akt downregulates glycogen synthase kinase-3β (GSK-3β), thus enhancing β-catenin-induced CSC self-renewal. Hence, inhibition of the Akt signaling pathway could be an effective tool to reduce CSCs [128].

8.2.5.5 STAT and NF-kB

Several studies have demonstrated that chronic inflammation is a key factor in initiation and progression of various cancers [129–131].

Pivotal molecular links between inflammation and cancer are the signaling pathways of signal transducer activator of transcription (STAT) and nuclear factor (NF)-κB.

STAT and NF-κB are crucial transcriptional regulators of activation of genes associated with cell proliferation, angiogenesis, metastasis, and suppression of apoptosis [132–134].

TNF-α activates NF-κB by phosphorylation of the inhibitor IkB by IKK. After the dissociation of the inhibitor IkB, free NF-κB migrates into the nucleus and activates the expression of downstream genes, some of which are antiapoptotic.

Recent studies demonstrated that IL-6/JAK2/STAT3 pathway is required for the maintenance of $CD44^{+}CD24^{-}$ CSCs in breast cancer [135] and that ovarian CSCs are characterized by constitutive activation of NF-κB [136]. Therefore, these pathways have been suggested as potential therapeutic targets in cancer stromal cells, in tumor cells, and also in CSCs [137–139].

Accordingly, it has been reported that STAT3 inhibition disrupts proliferation and maintenance of glioblastoma stem cells [140, 141], reduces $CD133^{+}ALDH^{+}$ colonic CSCs—thus affecting colonosphere formation [142]—and reduces the frequency of $ALDH^{+}$ CSCs in prostate cancer [143]. Similarly, inhibition of the NF-κB pathway results in CSC apoptosis and induces cell death in the chemoresistant ovarian CSCs [139].

Actually, the identification of signaling pathways is an active area of investigation, but at present, none of these pathways showed a CSC-specific activity.

Although combinations of these different markers have improved reliability to some extent, their limited specificity is a major obstacle to a definitive validation: advances in transcriptional and proteomic profiling could be helpful to provide more reliable tools to identify CSCs [144].

8.3 A Dynamic Cancer Stem Cell Concept

At present, CSCs are suggested to be those cells, usually sorted by flow cytometry on the basis of the expression of a particular cell surface marker, that have the tumorigenic ability to form a new tumor in an *in vivo* xenograft assay and/or ability to form cell spheres when plated at low density in nonadherent culture [145]. Despite this broad definition, it is still hard to define CSCs as a single universal entity, suggesting that the CSC phenotype may vary substantially across different tumors and, more interestingly, could not fit to a steady definition across the natural history of cancer. Experimental observations led to the

hypothesis that "stemness" may not be a fixed "immutable" property, but rather a more dynamic condition, with cells evolving to more or less differentiated states [146–148]. Indeed, it has been suggested that CSCs can undergo reversible fluctuations in their stem cell status [149–151]. These findings account for the possibility that cancer stemness can be acquired by modification in the gene expression programs. Although the molecular pathways underlying these effects are largely unknown, the concept of phenotypic reversion suggests the presence of a balance between CSCs and differentiated cells. Moreover, in recent years, multiple studies confirmed the presence of an important degree of intra-tumor heterogeneity, with several clones coexisting in the same tumor mass [152], resulting from genetic instability. Two major, not mutually exclusive, mechanisms explain intratumoral heterogeneity [19]:

1 Different genetic or epigenetic mutations occurring within the same target
2. Different tumor subtypes arising from distinct clones within the same tissue [153]

These observations turn our traditional view of the CSC model from a "hierarchical" structure (a cancer stem cell on the top of a differentiated tumor cell population) to an "oligarchic" structure with multiple genetically different cell lineages competing with each other [27], in particular in advanced stage disease where cancer therapy exerts a powerful selective pressure on cancer cells.

8.4 The CSCs Niche

Normal stem cells (SCs) are located in a specialized microenvironment called "stem cell niche" that provides SCs with molecular signals necessary to maintain them in a stem-like state and to reach the right balance between self-renewal, activation, and differentiation [154–156].

Emerging evidences suggest that CSCs are also regulated by a similar microenvironment [145]; therefore, tumorigenicity likely depends not only on the biology of tumor cells but also on the cross talk between tumor cells and the neighboring nonmalignant cells [157]. Such an intimate connection between CSCs and the surrounding microenvironment is supported by the observation that CSCs localize to the tumor–stroma interface in different cancers [158, 159].

The CSC niche is composed of several stromal cell populations including mesenchymal, vascular, and immune cells and myofibroblasts, as well as extracellular components [160–162].

Actually, the presence of three different niches in tumor tissue has been hypothesized: a perivascular niche, a hypoxic niche, and a niche at the invasion front.

The perivascular niche has been described as a microanatomical unit composed of proliferating endothelium and other stromal cells (i.e., pericytes and macrophages) and of extracellular components [163–166], closely interacting with cancer cells.

The existence of a hypoxic niche has been suggested since recent studies showed that stem cells are also located in the necrotic areas of the tumor [167] and that they are regulated by local oxygen concentrations [168]. Lastly, the observation that cancer cells undergoing EMT at the invasion front exhibit some of the phenotypic characteristics typical of CSCs (i.e., marker expression or microRNA) [169, 170] has raised the possibility of an invasive niche.

8.4.1 Functions

It is now well accepted that niche cells are not malignant per se but that the CSC niche supports cancer growth and has an essential role for tumor survival [171].

As already mentioned, the microenvironment is a crucial component that controls CSC functional features, such as stemness, proliferation, and apoptosis resistance [156, 172, 173]. In fact, it has been observed in several tumors that CSCs rely on niche signals to remain in their stem-like state, while maintaining their self-renewal properties and their capacity to originate differentiated cells [165, 172].

The importance of the interaction between CSCs and their microenvironment is confirmed by the observation that the loss of a niche usually leads to the loss of the CSC pool [156].

Several factors are involved in the cross talk between CSCs and niche microenvironment. For instance, the key role of CD44 [174, 175], CXCR4 (expressed by tumor cells), and CXCL12 (SDF-1, expressed by niche stromal cells) [76, 145] for the homing and engraftment of CSCs to the niche and the importance of CD133 for the CSC selective adhesion to the endothelial cells of perivascular niche [165] have been demonstrated. Moreover, endothelial cells of the vascular niche have been shown to stimulate CSC stemness in part through Notch signaling [109, 165, 176].

However, the observation that CSCs promote blood vessel formation by secreting VEGF has raised the hypothesis that the cross talk between CSCs and tumor vasculature could probably be a bidirectional process [177].

It has also been observed that in colon CSCs, Wnt signaling activity is increased by stromal fibroblasts, through secretion of hepatocyte growth factor (HGF) [89]. Furthermore, the niche microenvironment has been reported to support CSCs in different tumors through the release of inflammatory molecules, such as interleukin (IL)-6 [178, 179]. Also physiologic conditions existing within niches, such as hypoxia and low pH, are critical determinants in the maintenance of the CSC pool [180, 181]. Indeed, the hypoxia-inducible factor 2-α (*HIF2α*), the main mediator of hypoxia effects, promotes self-renewal and tumorigenicity of glioma CSCs [180]. It has yet to be fully elucidated whether these factors are really essential for the maintenance of CSC homeostasis; however, recent data seem to demonstrate that their loss reduces the clonogenicity of CSCs *in vivo* [165, 182]. Although a major role of the niche in CSC self-renewal has been suggested, clues from microenvironment may be sometimes negative for CSCs. Indeed, tumor growth can be inhibited by immune-mediated signals [50] or by differentiated tumor cells after xenotransplantation [10]; the latter observation leads to the interesting hypothesis that differentiated tumor cells control CSC spread.

Despite the similarities between normal SC and CSC niche, the microenvironment seems to regulate CSC behavior in a way more favorable to cell proliferation and growth as compared to normal SCs. In fact, while normal SC proliferation and differentiation are usually inhibited by the niche and tissue regeneration can be activated only after a transient proliferating signal, it is believed that internal mutations and niche signals can lead CSCs to proliferate without control [183].

Interestingly, the niche not only plays a pivotal role in the preservation of the CSC population and tumor growth enhancement but also has the ability to induce a CSC phenotype in more differentiated tumor cells [89, 156].

Moreover, the CSC niche is also able to protect CSCs from genotoxic injuries, increasing their resistance to therapeutic treatments [109, 184].

8.4.2 CSC Niche as Therapeutic Target

Given the fundamental role of microenvironment in supporting tumor growth and in affecting CSC response to therapy, the CSC niche, together with the factors involved in niche–CSC interaction, has become an attractive therapeutic target [185]. This proposition is encouraged by the observation that microenvironment deletion leads to tumor's drug sensitivity restoration [186, 187]. However, even if physiological stem cell niches are known to play important roles in the maintenance of CSC quiescence and resistance to stress-inducing treatments [188], the mechanisms by which modulations in the local milieu regulate CSC behavior have yet to be fully clarified [189].

8.5 CSCs in Tumor Invasion and Metastasis

Metastasis, the path through which a tumor cell exits the primary tumor and colonizes in distant organs, is a complex process with many questions still unanswered [190].

This multistep process [191] can be summarized in two major phases:

(a) First, the translocation of a cancer cell from the primary tumor to a metastatic site has to occur: cancer cells have to escape from the primary tumor mass and enter the microvasculature of

the lymphatic and blood systems. Cancer cells moving through the circulation exhibit anchorage-independent survival features and are called circulating tumor cells (CTCs) [192]. CTCs usually head to organs where an environment suitable for their survival has been previously primed; they can be attracted by chemoattractive signals coming from certain tissues and also adhere to the wall of blood vessels through specific surface adhesion molecules. Sometimes they even settle in the bed of the capillaries of target organs, with a diameter too small to let them pass through [192]. Then, cancer cells have to exit the circulation and survive in the new microenvironment of the metastatic site.

(b) Colonization of the target tissue by translocated cells: cells have to adapt to the different environmental conditions and proliferate, in order to engraft and form a secondary tumor [193, 194].

Mechanisms controlling colonization still remain largely unknown; nonetheless, experimental and clinical data support the hypothesis that disseminated cancer cells adapting to the new microenvironment can be found as solitary viable cells in a dormant, nonproliferative state. These dormant cells can then become more responsive to proliferative signals arising from the primed microenvironment, thus forming micrometastases—whose size is maintained small by a balance between proliferation, apoptosis, and phagocytosis by the target tissue immune system—or even proliferating macrometastasis, after recruitment of an adequate blood supply [192].

8.5.1 CSCs, EMT, and Metastasis

It is now largely believed that only a small fraction of cancer cells have the capability to form metastases [157].

The definition of CSCs as the only self-renewing tumor cells capable of initiating a new tumor implies that CSCs likely have the major responsibility in invasion and metastasis [192]. This hypothesis is supported by the observations that CSCs are the only cells capable of giving rise to distant metastases and to the growth of new tumors following tail vein injection in mice [194, 195] and that cancer cells that had spread to the bone marrow display a CSC marker profile [196, 197]. The notion that CSCs are responsible for initiation of metastasis is then strengthened by the established association between CSCs and EMT [146, 147, 198]. It has been recently shown that EMT plays a key role in tumor progression and metastasis [199, 200] and that cancer cells have to activate the EMT pathway and acquire a migrating CSC phenotype in order to disseminate and metastasize [156, 171]. The acquirement of this mesenchymal-like phenotype requires cues from the tumor stromal components that secrete EMT-inducing factors (such as TGF-β) [171]. Indeed, EMT can be induced by autocrine or paracrine secretion of mediators such as cytokines and growth factors, due to the cross talk between tumor cells and the microenvironment [156, 185, 199, 200].

When they arrive at the metastatic site, cancer cells have then to undergo the reverse process of mesenchymal–epithelial transition allowing them to initiate the growth of a new tumor [201, 202].

In addition to transforming differentiated cancer cells in migrating cells with self-renewing features and enhanced proliferative capacity, EMT induces proliferation and spread of the existing CSC population, thus further increasing the chances for seeding at distant sites and forming metastases [146, 203, 204]. Moreover, cells generated by EMT show an enhanced resistance to apoptosis that certainly potentiates their capability to survive to the adverse conditions met during the translocation from the primary tumor to metastatic sites [205]. In line with these results, EMT has been associated with poor prognosis in several tumors [156, 206]. However, the role of EMT in facilitating the metastatic spread still remains to be fully demonstrated, especially since there are technical difficulties in detecting this transitory process in human cancer patients [192].

8.5.2 Signaling Pathways Involved in Metastasis

CSCs are believed to share physiological SC trafficking mechanisms; thus, migration of CSCs is

probably regulated by several redundant and overlapping pathways, similarly to SC homing [194].

One of the critical regulators of metastatic spread is hypoxia that has been shown to increase the expression of Snail—a pivotal EMT-inducing transcription factor [155] shown to be expressed at the tumor–stroma interface in several cancers [156]—via the Wnt signaling activation. The Wnt/β-catenin pathway is thought to be a critical factor in the regulation of metastatic process [185, 198, 207]. Accordingly, colorectal cancer cells, residing at the host–tumor interface and thus suggested to be CSCs on the verge of metastasizing [158], exhibit a high nuclear β-catenin expression [208].

In addition, hypoxia is able to increase the expression of c-MYC, OCT4, and NANOG, important stem cell factors, in differentiated cancer cells [185, 199, 200].

Furthermore, expression of *HIF2α* facilitates the metastatic spread both by enhancing the tumorigenic potential in differentiated cells and by inducing proliferation and dissemination of the preexisting CSCs [146, 203, 204].

Interestingly, HGF, which has been recently shown to induce CSC properties and high tumorigenic potential in differentiated colon cancer cells [89], was used to induce cell scattering of MDCK cells in the initial studies on EMT [209].

In addition, HGF enhances migration and metastasizing capability of bone marrow hematopoietic cells, by activating the receptor tyrosine kinase MET [210], involved in metastases establishment. MET expression has been shown to be induced in marrow cells from highly metastatic melanomas by the tumor-derived factors exosomes that thus may stimulate the formation of metastases [210].

Other factors involved in CSC metastatic and invasive behavior include growth factors, VEGF receptor1 signaling, as well as cytokines and chemokines, such as the SDF-1/CXCR4 migration axis [194, 211].

The observation that SDF-1 is often highly expressed in typical sites of metastasis as lung, liver, bone marrow, and lymph nodes suggests that it is associated with a metastatic process [212]. CSCs, that express the SDF-1-specific receptor CXCR4, can migrate along a gradient of SDF-1, thus facilitating metastasis [145]. The involvement of SDF-1/CXCR4 axis in metastasis has been, indeed, described in various tumor models, such as lung, breast, colorectal, and pancreatic cancers [12, 213–217]. Several studies reported that SDF-1/CXCR4 signaling increases cancer invasion and metastasis also by enhancing the expression of metalloproteinases (i.e., MMP-2 and MMP-9) and integrins (i.e., α5-, β1-, β3-integrins), enzymes known to promote tumor dissemination by extracellular matrix degradation [218]. More recently, an alternative receptor for SDF-1 has been identified and named CXCR7, that shows a high affinity for SDF-1 and for another chemokine, I-TAC [219]. The correlation between CXCR7 expression and tumor aggressiveness, adhesion, invasion, and survival increase was observed in both *in vivo* and *in vitro* studies [220].

Another cytokine appearing to be connected to metastasis is IL-8, which, together with its receptor CXCR1, has been demonstrated to be related to $ALDH^+$ CSCs invasion in breast cancer cell lines [221]. However, the involvement of this chemokine in the metastatic process has not yet been completely clarified. Furthermore, a recent work [222] suggests that the capability of CSCs to express the matrix protein tenascin C (TNC) demonstrates a direct relationship between CSCs and the metastatic process, since TNC expression by CSCs seems to be required to form metastases in the lungs of an animal model. TNC is described to regulate two key pathways for metastatic spread, Notch and Wnt [83, 195, 223–225], through the increase of the expression of musashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), respectively [222]. The matrix protein osteopontin (Arg-Gly-Asp (RGD)-containing sialoprotein) was also reported to be involved in metastasis [226], since it interacts with CD44, α4-integrins, and α5β1-integrins, markers of cell adhesion typically expressed by stem cells [227–229].

CD44 is considered to support the recruitment of cancer cells into secondary tumor sites in lymph nodes, lungs, and bone marrow [230], and $CD44^+$ breast cancer cells (displaying a breast CSC phenotype) appeared to have enhanced metastatic properties in xenograft models [10, 231].

However, data on CD44 are contradictory: in colorectal cancer, tumor progression has shown to be associated to a loss rather than a gain of membranous CD44 [91].

8.5.3 Premetastatic Niche

In addition to its role in controlling CSC stemness and proliferation and saving CSCs from depletion [232–234], microenvironment is suggested to be of crucial importance in metastatic spread as well. Growing evidences suggest that disseminated cancer cells are able to initiate a secondary tumor only if they migrate in a favorable microenvironment ("seed and soil" hypothesis). Thus, metastasis is likely not a random process, but it selectively takes place in specific organs, such as lungs, liver, brain, and bones, whose microenvironment appears to be more responsive to migrating cancer cells, as compared to other organs [156]. Accordingly, the engraftment of cancer cells in different organs seems to be enhanced by the establishment of a so-called pre-metastatic niche that allows secondary tumors' initiation and growth. The cells of this pre-metastatic niche have shown to release factors (i.e., SDF-1, S1000A8, S100A9) that can attract disseminating cancer cells, thus enabling their successful homing to distant organs and metastasis development [156, 192]. It has been hypothesized that these pre-metastatic niche cells can be educated within primary tumor and then migrate to form distant metastases or activated locally or in the circulation by tumor-secreted factors [192]. The capability of the primary tumor to prime pre-metastatic niches, in preparation for and before the disseminating tumor cell arrival, by secreting systemic factors (i.e., cytokines, VEGF-A, PlGF, PSAP), has been observed in several experimental models [235–238]. The presence of bone marrow-derived cell clusters in pre-metastatic sites before the arrival of green fluorescent protein (GFP)-labeled cancer cells has been proved by flow cytometry and immunofluorescence studies [239]. These bone marrow-derived hematopoietic progenitor cells (HPC) have been demonstrated to express VEGFR1 and other hematopoietic markers, such as CD34, CD11b, c-kit, and Sca-1 [236, 240–242], thus making pre-metastatic microenvironment more hospitable for metastases. Moreover, the VEGFR1 agonist, placental growth factor (PlGF), secreted by the primary tumor, is reported to enhance the production of fibronectin by tumor stroma in pre-metastatic niches [156]; since fibronectin appears to bind to VLA-4 ($\alpha4\beta1$), a fibronectin receptor expressed on HPC, its increased expression likely primes HPC to these sites, to establish the clusters in preparation for metastasis [210, 236]. Furthermore, an experimental model of melanoma showed that tumor fibroblasts participated in pre-metastatic niche formation by inducing the stroma remodeling that was necessary for the establishment of liver metastases [243].

Despite the increasing studies on CSC metastatic process, it is still debated whether the pre-metastatic niche only keeps metastasized CSC properties or it is also able to induce a CSC identity in differentiated cells [156]. As already mentioned, the central role of the CSC microenvironment in tumor growth and progression and metastasis formation, combined with its CSC protection against genotoxic damages, strongly highlighted CSC niche and mediators of the cross talk between CSCs and CSC niche as important therapeutic targets [156]. Thus, a better knowledge of CSCs is necessary, in order to develop improved therapies without the risk of tumor recurrence.

8.6 Tracking CSCs

Advances in imaging technology could allow to track the subpopulation of CSCs in a noninvasive manner in order to monitor migration, engraftment, and morphological differentiation and to assess their response to treatment.

Cell tracking can be performed with two molecular imaging approaches:

1. Direct stem cell labeling by tracers, such as magnetic particles, luminescent nanoparticles, or radionuclides to directly mark cells [244].
2. Indirect labeling by reporter-gene imaging: cells are transfected with a reporter gene that encodes for molecules that can be detected by imaging after administration of a reporter probe [245].

Both techniques can be applied to integrate existing radiodiagnostics, in order to implement their ability to provide functional information about biodistribution and activity of CSCs. Initially intended for cancer research, these tools have been most extensively tested in baseline research on human physiological stem cells and immune cells, and their use in CSCs tracking is still under investigation at an initial stage.

Bioluminescence imaging (BLI) requires incorporation of a reporter gene, such as firefly luciferase (Fluc), in the stem cells: light emission is triggered by interaction with an intravenously administered optical probe, D-luciferin. In a recent experience, breast CSCs were labeled through the expression of optical bifusion reporter genes, to facilitate their visualization in a human xenograft model on NOD/SCID mice [231]. Ethical issues concerning genetic manipulation *in vivo* represent a severe limitation to this technique.

Fluorescence imaging employs administration of organic fluorophores (such as fluorescein or rhodamine) that emit specific wavelengths following exposition to visible light; another valuable approach that resulted in improved stability of the compound is indirect labeling with genetically encoded fluorescent proteins. Such color-coding of cancer cells growing *in vivo* could allow the monitoring of cell–stroma interactions, subcellular processes, and distinction of different cell types with single-cell resolution [246]. Whole-body imaging with fluorescent proteins could represent a powerful technology to follow the dynamics of cancer development and metastasis [247], but low resolution and technical limitations linked to light penetration in depth call for further advancement in technology, like fluorescence-mediated molecular tomography [248].

Quantum dots (QDs) are inorganic fluorescent semiconductor nanoparticles with superior optical properties as compared with organic dyes: QDs have been used to study extravasation of intravenously injected, QD-labeled tumor cells in preclinical research [249]. It is still controversial whether their use could affect CD133 expression [250].

In order to increase accuracy, superparamagnetic iron oxide (SPIO, 50–500 nm) nanoparticles, ultrasmall superparamagnetic iron oxide nanoparticles (USPIO, 5 nm) [251], and manganese oxide (MnO) nanoparticles [252] have been tested as contrast agents for MRI [253]. Gadolinium–rhodamine nanoparticles, which provide a stronger positive signal, have been also tested for labeling and tracking cancer cells *in vivo* in rodents [254].

However, metal nanoparticles are not able to discriminate viable cells. This limitation can be overcome by radionuclides, such as fluorine-19 [255]: it was found that $CD34^+CD133^+CD31^+$ stem/progenitor cells readily internalized these agent nanoparticles, without the aid of adjunctive labeling techniques, and remained functional *in vivo*.

Radionuclide imaging can follow the distribution and concentration of radioactive-labeled molecular tracers introduced into a subject. There are two main modalities for radionuclide imaging: positron emission tomography (PET) and single-photon emission computed tomography (SPECT). SPECT tracers directly emit a gamma ray in one direction, while PET tracers send two gamma rays in opposite directions, providing higher spatial resolution. Longitudinal tracking is dependent on the specific half-life of decay of the chosen isotope. Disadvantages include leakage of radiotracers from labeled cells and nonspecific uptake by normal tissues. Targeting stem cell surface markers with radiolabeled antibodies could provide information; longitudinal tracking could be performed with appropriate choice of radioisotope, according to its half-life of decay. For example, ^{64}Cu-diacetyl-bis (*N4-methylthiosemicarbazone*) (^{64}Cu- ATSM), a PET imaging agent, selectively accumulated in regions of $CD133^+$ high expression in a preclinical model [256].

MicroCT is similar to the conventional CT systems but is capable of achieving a spatial resolution about three orders of magnitude lower (0.3 μm) [257]. Radio-opaque contrast agents, like gold nanoparticles attached to specific ligands, could be useful to target cell population, in order to acquire information on cell topography and behavior [258].

The imaging modalities reviewed in this chapter are characterized by different sensitivity, tissue penetration, and spatial resolution: integration of multiple diagnostic tools in a single imaging

session would allow to combine the advantages of each technique.

In summary, CSC-based clinical imaging is a promising goal in the improvement of diagnostic and prognostic tools in cancer therapy. However, current developments do not allow immediate application in the clinical setting; moreover, it is debated whether large-scale use of such techniques would raise ethical issues related to genetic manipulation in patients.

8.7 CSC Resistance: Clinical Implications

Cancer stem cells account for a minor fraction of a tumor population; nevertheless, they could play a central role in treatment failure and relapse. At present, neoadjuvant treatment targets the proliferative potential of the tumors by killing rapidly dividing cells within the bulk of the tumor. However, even in the event of a considerable shrinkage of the tumor burden following a highly effective therapy that successfully affects the vast majority of tumor cells, CSCs could be unaffected. In glioblastoma, $CD133^+$ CSC population was found to be enriched after radiation and exhibited lower rates of apoptosis in response to chemotherapy [259] in comparison with $CD133^-$. In breast cancer, significantly increased levels of cells expressing CSC markers have been reported in residual tumor cell populations of patients after conventional chemotherapy [260, 261]. Moreover, in pancreatic cancer, $CD133^+$ cells showed increased resistance to chemotherapeutic agents [12]. Indeed, CSCs display enhanced resistance to conventional cytotoxic agents (i.e., chemotherapy and ionizing radiation, inducing cell death mostly by DNA damage) due to numerous strategies: quiescence propensity, enhanced DNA repair, upregulated cell cycle control mechanisms, free-radical scavenging mechanisms, and specific interaction with stromal microenvironment. Development of targeted therapies based on inhibition of these features could allow to overwhelm treatment resistance, in order to eradicate CSCs and achieve long-lasting tumor remission.

8.7.1 Enhanced DNA Repair

Cancer cells improperly activate DNA repair pathways, implied in preservation of genome integrity, in order to overcome standard anticancer treatments. DNA repair pathways include [262]:

- Nucleotide excision repair (NER) that corrects massive helix-distorting lesions
- Base excision repair (BER) that targets point base modifications
- Mismatch repair (MMR), removing mispaired nucleotides in the event of replication errors
- Monoenzymatic direct repair, involving O6-methylguanine methyltransferase (MGMT), that performs a one-step methyl transfer reaction
- Double-strand break (DSB) recombinant repair, a complex and cycle-dependent mechanism that encompasses homologous recombination repair (HRR), prevalent in cycling phases, and nonhomologous end-joining (NHEJ) that is eminent in G1 phase. This pathway can consequently lead to activation of secondary effectors, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia/Rad3-related kinase (ATR), and checkpoint kinases (Chk1 and Chk2)

The connection between DNA repair signals and CSC chemoresistance has been highlighted in glioma cell lines, showing that enhanced activation of ATM and Chk1 in $CD133^+$ resulted in increased survival after irradiation, while radiosensitivity was restored with pharmacological inhibition [263]. Enhanced DNA repair ability has been also observed in breast CSCs [10]. Early clinical trials with DNA repair inhibitors, such as the MGMT-depleting agents O6-benzylguanine, in association with chemotherapy showed disappointing results [264, 265]. Attention has been drawn on PARP inhibitors, in reason of PARP-1 and PARP-2 involvement in single-strand repair via the BER pathway: these molecules have been particularly tested in breast cancers harboring *BRCA* mutation that relies on BER due to impaired HRR [266, 267].

A phase II multicenter study conducted in patients with advanced, refractory, *BRCA*-mutation carrier breast cancer evaluated two different schedules of the oral PARP inhibitor olaparib (AZD2281)

in two sequential cohorts: overall response rates were 41 % (11 patients) and 22 % (6 patients) with olaparib at 400 and 100 mg, respectively, with an acceptable toxicity [268]. Iniparib (BSI-201) has been tested in a randomized, phase II study trial in association with carboplatin/gemcitabine doublet for treating metastatic triple negative breast cancers, showing a significant clinical benefit in the experimental arm [269]. However, the subsequent phase III trial failed to confirm any efficacy [270]. Chk1 inhibitors (AZD7762, PF-477736, SCH900776, LY2606368) are undergoing early phases of clinical development.

Research on the contribution of polycomb group proteins, such as BM1, to the DNA damage response pathways is another promising area of investigation [271].

8.7.2 Free-Radical Scavenging

Hypoxia is involved in radioresistance, as cells located in areas of low-oxygen tensions are less exposed to reactive oxygen species (ROS)-mediated damage [272]. It has been reported that lower levels of oxidative radicals are detected after irradiation in CSC-enriched MCF-7 breast cancer cell mammospheres in comparison with monolayer cultures [273], which could be explained by improved free-radical scavenging pathways. For example, overexpression of glutathione-related genes (*Gclm* and *Gss*) has been observed in CSCs: selective inhibition by buthionine sulfoximine (BSO), a glutamate-cysteine ligase inhibitor, induced a decrease in the colony-forming ability and restored radiation sensitivity in CSC models [274]. As previously stated, also *HIF* expression, in its two isoforms *HIF1α* and *HIF2α*, is implicated in CSC promotion and maintenance [275]. In some hematological malignancies, CSCs are sustained by high levels of *HIF1α* (under normoxia) that promotes gene expression of the stem cell transcription factor *Hes1*, via the stimulation of the Notch pathway. Echinomycin, an *HIF1α* inhibitor, was selectively effective on these CSCs [276]. Recent experiences by Lee et al. suggest that sorafenib, in combination with radiotherapy, could enhance the efficacy of irradiation on CSCs, by inhibition of *HIF-1α* in an *in vitro* breast cancer model [277].

8.7.3 Quiescence

Experimental evidences indicate that, both *in vitro* and *in vivo*, subpopulations of slow-cycling tumor cells are mostly spared by DNA-damage-induced death as compared to the bulk of tumor cells [278]. It is known that cells change in their sensitivity to DNA-damaging agents all along the division cycle, ranging from extreme sensitivity in the mitotic phase and increased resistance in late S-phase [279]. Specific therapies might induce CSCs to differentiate into more mature tumor cells, thus limiting their tumorigenic and invasive potential. Salinomycin has been described as the first "quiescence-disrupting" compound that is able to decrease the proportion of CSC phenotypic breast cancer cells and to selectively eradicate the tumor, by inducing terminal epithelial differentiation [280]. It has been also suggested that histone deacetylase (HDAC) causes a lysin residues epigenetic modification and is responsible of chromatin condensation of CSCs [281]. A novel class of therapeutic agents, the epigenetic-acting histone deacetylase inhibitors (HDACi), is currently under investigation in this setting [282].

8.7.4 Signaling Pathways

Notch-targeting agents like gamma secretase inhibitors (GSIs), a category of compounds blocking the release of the Notch intracellular domain, showed promising activity in preclinical studies and are currently undergoing clinical evaluation [283]. RO4929097, a new Notch inhibitor [284], has been extensively studied for toxicity in a number of settings, both as a single agent [285] and in combination with standard chemotherapy [286]; phase II trials are ongoing for patients with recurrent or progressive glioblastoma (http://clinicaltrials.gov: NCT01122901); nevertheless, disappointing results were observed when used as a single agent in metastatic colorectal cancer [285]. Another approach exploiting antibodies against delta-like 4 ligand

(DLL4), a component of Notch signaling pathway, achieved inhibition of the expression of Notch target genes and reduced proliferation of tumor cells in a mouse model of human colon cancer, either alone or in combination with irinotecan [287].

- Hedgehog signaling could be pharmacologically inhibited by targeting Smo. Preclinical studies with pancreatic cancer models showed that pharmacological Smo inhibition was effective against $CD133^+$ pancreatic CSCs, with enhanced apoptosis, probably associated with Fas and death receptor (DR) overexpression [288], while combined treatment with Sonic Hedgehog and mTOR inhibitors, together with standard chemotherapy, has proved to be capable of eliminating pancreatic CSCs in *in vitro* and *in vivo* models [289]. Preliminary experience with Smo antagonist GDC-0449 (vismodegib) showed significant efficacy, with mild toxicity, in patients affected by basal cell carcinoma, leading to FDA approval in this setting on January 2012 [290]; nevertheless, clinical activity in other solid tumors was controversial [291] and rapid onset of Smo acquired mutation was observed in one patient treated for medulloblastoma that progressed after initial stabilization of disease [292]. Other strategies to achieve Hedgehog inhibition encompass Gli antagonists [293] or, indirectly, modulation of other signaling pathways, like EGFR and TGFβ [294, 295].
- WNT: antibodies directed toward Wnt receptor Frizzled7 (FZD7) reduce clonogenicity and tumorigenicity in preclinical models of Wilms' tumor; a synergistic gain in efficacy could be obtained with the addition of the analogues of Dickkopf1 (Dkk1), a secretase related to the differentiation of $CD44^+CD24$ low breast CSCs [296] that prevents the formation of the Frizzled-Wnt-LRP6 complex [297]. Another strategy could imply direct inhibition of LRP6 or FZD7, which proved effective in suppression of tumor growth in *in vitro* models of triple negative breast cancer [298]. Kendizorra et al. described enhanced radioresistance in rectal cancer cell lines overexpressing Wnt transcription factor T cell factor (TCF-4), while sensitivity was restored by silencing TCF-4 [299]. RO4929097, an investigational Wnt pathway inhibitor, is currently being evaluated in a phase I trial recruiting breast cancer patients (http://clinicaltrials.gov: NCT01351103). Further advances in the knowledge of the Hippo pathway that intersects both the Wnt and Notch pathways could allow the development of new generation targeted therapies [300].
- mTOR/AKT: inhibition of the PI3K/AKT/mTOR signaling pathway could be effective in restoring sensitivity to chemotherapy and radiation in CSCs that aberrantly activate this pathway [301]. Strategies to achieve this goal encompass pharmacological abrogation of AKT, mTOR inhibitors, and PI3K antagonists. Novel AKT inhibitors have shown to be promising in *in vitro*/*in vivo* antitumor activity, in combination with chemotherapeutic agents [302], and are currently undergoing phase I/II trials. mTOR inhibitors have been extensively studied in association with standard chemotherapy, but their activity on CSCs has not been specifically investigated yet. On stem cells from HER2-overexpressing primary breast cancer cells and on BT474 breast cancer cell line, it has been recently highlighted that everolimus, in combination with trastuzumab, provides a rationale for strategies that overcome resistance to HER2-directed agents [303].

8.8 Perspectives in Radiation Oncology

Advances in CSC biology would not only prove useful in the development of targeted drugs but represent a major challenge in the creation of new paradigms of treatment in radiotherapy.

Definition of total tumor volume and prognostic stratifications could be implemented with integration of data upon total number of CSCs, spatial distribution of CSCs, and detection of CSC niches: this information could be integrated in the treatment planning and dose prescription process. Mapping distribution of neural stem cells in human brain led to the observation—in a retrospective cohort of 55 patients affected by

glioblastoma—that patients receiving higher doses to the subventricular zone (SVZ) and the subgranular layer (that could act as a reservoir for brain CSCs) experienced a benefit in progression-free survival [304].

Histopathologic reports, or imaging with radiolabeled antibodies directed to specific CSC markers, could be useful to forecast the likeliness of metastatic spread and, according the ratio of CSCs on total tumor volume, to predict radioresistance: these information could be subsequently applied to determine the correct pattern of care, for example, schedule and timing of associated chemotherapy. Biomolecular profiling on CSCs from pretherapeutic biopsy or postoperative specimens could further drive the choice for altered fractionation schedules or appropriate target therapy, to restore radiosensitivity by pharmacological abrogation of aberrant signaling pathways, concomitant with or prior to radiotherapy. Real-time CSC-specific imaging [305] could be useful to improve feasibility and effectiveness of adaptive radiotherapy.

Moreover, assessment of therapeutic response in neoadjuvant treatments adding CSC-specific imaging could significantly improve the accuracy of restaging [306]. Ion therapy could yield a major benefit in this setting: preclinical data suggest that, in *in vitro* NSCLC models, protons may be more effective than photons, at the same biologically effective dose, to eradicate CSCs, despite producing equivalent effects in normal bronchial epithelial cells [307]. In a recent publication, carbon ions showed superior biological efficacy, in terms of lower CSC fraction, as compared to classical photon beam therapy [308].

8.9 Concluding Remarks

According to the CSCs theory, cancer is sustained by a small subgroup of self-renewing cells that exhibit stem cell-like properties such as asymmetrical division and ability to shuttle to a quiescent state. Despite their elusive nature, there is a growing body of data that accounts for their role in cancer initiation, progression, and resistance to conventional cancer therapies such as chemotherapy and radiation therapy. Efforts are ongoing to deeply understand CSCs biology, to refine detection and to elucidate the complex network of interactions CSCs establish with their microenvironment.

A better knowledge of the mechanisms underlying CSCs peculiar behavior could help to target this cell population, their eradication representing a valuable strategy to overcome resistance to cancer treatments and to prevent relapse.

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Immunologic Approaches to Targeting Cancer Stem Cells

9

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9.1 Introduction

Evidence supporting the existence of cancer stem cells (CSCs) can be traced back to more than 70 years. In 1937, Furth and Kahn reported that a single cell from a mouse leukemia could initiate leukemia in a recipient mouse [1]. Since that time, numerous studies in different types of cancer have showed that like their normal tissue counterparts, tumors comprise heterogeneous populations of cells that differ in multiple states of differentiation and proliferation [2]. Researchers have demonstrated that only a small subset of tumor cells is capable of extensive self-renewal and differentiation which generate tumor cells [3–6] incapable of tumor initiation. These tumor cells with stem cell properties have been identified by their expression of various stem cell markers and by their self-renewal capacity both *in vitro* and *in vivo*. These observations have led to the hypothesis that only a small proportion of cancer cells are tumorigenic and these are considered to be cancer stem cells [3].

The definition of cancer stem cells by American Association for Cancer Research (AACR) is "cells within a tumor that possess the capacity for self-renewal and that can generate the heterogeneous cancer cell populations that constitute the tumor bulk" [7]. Cancer stem cells are proposed to persist in tumors as a distinct population responsible for relapse and metastasis. Conventional chemotherapy and radiation therapy preferentially kill differentiated tumor cells, which form the bulk of the tumor, but are unable to eradicate cancer stem cells. Cancer stem cells are hypothesized to be resistant to toxic environmental agents due to expression of ATP-binding cassette (ABC) transporters, increased DNA-repair capacity, and interactions with their microenvironment which promote cell survival [8].

Clinical responses in cancer immunotherapy involving tumor-reactive T cells and dendritic cell (DC)-based vaccines have been confined to a limited number of patients. The inability to target cancer stem cells with current immune approaches contribute to treatment failures with these immunotherapies. Novel immunotherapeutic strategies which specifically target cancer-initiating cells may increase their efficacy. Development of immunologic approaches targeting cancer stem cells may prevent local disease recurrence and distant metastasis, resulting in prolonged patient survival.

9.2 ALDEFLUOR^{+}/ALDH^{high} Serves as a Specific Marker for Cancer Stem Cells in Multiple Tumor Types

In 1997, Bonnet and Dick first isolated a subpopulation of leukemic cells possessing differentiation capacity and the potential for self-renewal. The isolated subpopulation of leukemic cells was characterized as CD34^{++}CD38^{-} and was shown to be capable of initiating tumors in NOD/SCID mice [9]. This represented the first report of cancer stem cell identification and isolation. The finding of leukemic stem cells prompted further research into other types of cancer. In the following decade, numerous studies have indicated the existence of cancer stem cells in both nonsolid and solid tumors.

Al Hajj et al. identified and characterized CD44^{+} CD24$^{low/-}$ cancer stem cells in human breast cancer [10], and Singh et al. identified human brain cancer stem cells using the surface antigen CD133 [11]. Cancer stem cells have been isolated from many other cancers, such as melanoma, head and neck, lung, gastric, liver, pancreas, ovarian, bladder, colon, leukemia, and prostate (Table 9.1).

Different markers, such as CD44, CD24, CD90, CD133, and ESA, have been used to identify cancer stem cells in different tumor types. In addition, in many tumor types cancer stem cells have been demonstrated to express increased levels of aldehyde dehydrogenase (ALDH) (Table 9.1). ALDH1, a detoxifying enzyme, is responsible for the oxidation of aldehydes to carboxylic acids and thus might serve to prevent cells from oxidative insult facilitating their survival. It has been shown that hematopoietic and neural stem and progenitor cells have high ALDH1 activity [25, 40–42]. Increased ALDH1 activity has been found in stem cell populations in many cancers including bladder, breast, colon, gastric, head and neck lung, pancreatic, prostate,

Table 9.1 Cell surface phenotype of cancer stem cell identified

Tumor type	Markers	Reference
Bladder	$ABCG2^{high}$ SP (side population, Hoechst dye)	[12]
	$ALDH1^{high}$	[13]
Breast	$CD44^{+}CD24^{-/low}$	[10]
	$ALDH1^{high}$	[14]
Colon	$CD133^{+}$	[15]
	$EpCAM^{high}$ $CD44^{+}$	[16]
	$ALDH1^{high}$	[17]
Gastric	$CD44^{+}$	[18]
	$ALDH1^{high}$	[19]
Glioma	$CD133^{+}$	[11]
	$A2B5^{+}$	[20]
	$CD15^{+}$	[21]
Head and neck	$CD44^{+}$	[22]
	$ALDH1^{high}$	[23]
Leukemia	$CD34^{++}CD38^{-}$	[9]
	$CD44^{+}$	[24]
	$ALDH1^{high}$	[25]
Lung	$CD133^{+}$	[26]
	$CD133^{+}$epithelial-specific antigen $(ESA)^{+}$	[27]
	$ALDH1^{high}$	[28]
Liver	$CD90^{+}$	[29]
	$CD133^{+}$	[30]
Melanoma	$ABCB5^{+}$	[31]
	$CD133^{+}$ $ABCG2^{+}$	[32]
Ovarian	$CD44^{+}$ $CD117^{+}$	[33]
	$ALDH1^{high}$	[34]
Pancreas	$CD133^{+}$	[35]
	$CD44^{+}CD24^{+}ESA^{+}$	[36]
	$ALDH1^{high}$	[37]
Prostate	Side population, Hoechst dye	[38]
	$ALDH1^{high}$	[39]

and so on [13, 14, 17, 19, 23, 28, 37, 39], suggesting that ALDH1 may serve as a reliable cancer stem cell marker for many types of tumor.

9.3 Cancer Stem Cells Are Resistant to Conventional Tumor Therapies

Cancer stem cells share many properties of their normal tissue counterparts. It has been demonstrated that normal stem cells demonstrate relative resistance to drugs and toxins through the expression of ATP-binding cassette (ABC) transporters and an active DNA-repair capacity. It follows that cancer stem cells might also possess these resistance mechanisms [8]. Indeed, resistance has been seen in both *in vitro* and *in vivo* models of cancer stem cells in response to chemotherapy or radiation therapy [43].

Hermann et al. isolated the human pancreatic cancer stem cells defined by CD133 expression. They found that the $CD133^{+}$ cells showed increased resistance to standard chemotherapy (gemcitabine) compared with the $CD133^{-}$ cells derived from the same tumor [35]. Dylla et al. described epithelial-specific antigen $(ESA)^{+}$ $CD44^{+}$ phenotypes in human colorectal cancer stem cells. When these cells were xenotransplanted into NOD/SCID mouse and treated with cyclophosphamide (CPA) or irinotecan, residual tumors were enriched for cells with the cancer stem cell phenotype [44]. They found that a large subpopulation of ESA^{+} $CD44^{+}$ cells has high ALDH1 activity. Using shot hairpin RNA against ALDH1, they demonstrated that knockdown of ALDH1 expression sensitized these cells to CPA [44]. Since ALDH oxidizes and inactivates the bioactive metabolic by product of CPA, aldophosphamide/4-hydroxycyclophosphamide (4-HC), they speculated that ALDH1 may play a major role in CPA resistance. Therefore, ALDH1 may not only serve as a marker for cancer stem cells but also play a key role in cancer stem cell resistant to chemotherapy.

Although the mechanisms underlying drug resistance are poorly understood, recent studies show that cancer stem cells resistance to chemotherapy and radiotherapy may also involve increased expression of drug efflux pumps, DNA repair, as well as interactions of cancer stem cells with their microenvironment. Cancer stem cells, like normal stem cells, express high levels of ABC transporters [8, 45]. ABC transporters are transmembrane proteins that utilize the energy of adenosine triphosphate (ATP) hydrolysis to transport a wide variety of substrates across extra- and intracellular membranes. These efflux transporters allow cancer stem cells to preserve genome integrity by pumping DNA damaging drugs out of the

cell. Downregulating the expression of ABC transporters in cancer stem cells results in the increased death of cancer stem cells due to acute cytotoxic injury and the induction of apoptosis by chemotherapy agents *in vitro* [45]. In addition, cancer stem cells are able to rapidly repair DNA damage caused by DNA-targeting agents and radiation therapy [46]. Checkpoint kinases 1/2 (Chk1/2 kinases), which become activated after genotoxic stress induces cell cycle arrest allowing for DNA repair, may be one of the potential modulators of cancer stem cell resistance to DNA-targeting agents. Chk1/2 inhibitors partially reverse the resistance of glioblastoma cancer stem cells to radiation-induced cell death [46, 47]. In another report, Woodward et al. found that side-population cells with stem cell characteristics enriched after radiation in human breast cancer cell line MCF-7. They demonstrated differential expression of activated β-catenin and γH2AX in mammospheres derived from the cancer stem cells *vs*. non-cancer stem cells. These results suggested an important role of Wnt/β-catenin signaling in mediating more effective DNA repair in cancer stem cells promoting their resistance to radiation [48]. Furthermore, interactions of cancer stem cells with their microenvironment (niche) may also contribute to drug resistance. For example, CXCR4, a receptor for stromal cell-derived factor 1 (SDF1, also known as CXCL12) is expressed on many cancer cells. SDF1 is a niche-derived chemoattractant for $CXCR4^{+}$ cells, thereby enhancing their entry into the bone marrow [49]. CXCR4-SDF1 inhibition sensitized cancer stem cells to chemotherapy [50, 51]. Altogether, these results suggest that alternative therapeutic strategies are needed to specifically target cancer stem cells. To this end, cancer stem cell-targeted immunotherapy has shown promise.

9.4 Innate Immune Response to Cancer Stem Cells

The role of innate immune effector cells in tumor immunosurveillance remains controversial. On one hand, these cells allow for the recognition and destruction of malignant cells before they generate a tumor mass. On the other hand, innate cells may be suppressed and therefore mediate immune tolerance to tumors. Recent advances in understanding NK cell function in antitumor immune responses have revealed a complex dynamic interaction between NK cells and tumor cells. Decreased peripheral blood NK cell function has been reported in many cancer patients [52, 53]. Accumulating evidence also indicates that NK cell cytotoxicity and INF-γ secretion are suppressed, which facilitates a minor group of stem cells to survive in the tumor microenvironment [54, 55]. It was proposed that cytotoxic NK cells ($CD16^{+}CD56^{+/dim}CD69^{-}$) can be conditioned to differentiate into noncytotoxic cells ($CD16^{-/dim}CD56^{-/dim}CD69^{+}$) through the interaction of cancer stem cells or primary stem cells with monocytes in situ [56, 57]. These anergic $CD16^{-/dim}CD56^{-/dim}CD69^{+}$ NK cells have increased ability to secrete inflammatory cytokines and growth factors. Supporting this concept, tumor associated macrophages (TAMs) have been shown to medicate cytokine-dependent inhibition of NK cell function [58].

In contrast, other studies have demonstrated that cancer stem cells, e.g., glioblastoma stem cells and primary oral squamous carcinoma stem cells (OSCSCs), are significantly more susceptible to NK cell-mediated cytotoxicity than their differentiated counterparts [59, 60]. Castriconi et al. reported that human glioblastoma cells with stem cell-like properties display markers typical of neural stem cells [59]. These cells, despite their resistance to freshly isolated NK cells, are highly susceptible to lysis mediated by both allogeneic and autologous IL-2 (or IL-15)-activated NK cells. These stem cell-like glioblastoma cells do not express protective levels of HLA class I molecules, but express high levels of CD155 and CD112, the ligands of DNAM-1-activating NK receptor which trigger optimal NK cell cytotoxicity [59]. Increased NK cell cytotoxicity and augmented secretion of IFN-γ were also observed by Tseng et al. [60]. In their study, oral squamous cancer stem cells (OSCSCs) were identified by expression of CD133 and $CD44^{bright}$ markers. These OSCSCs release significantly lower levels of GM-CSF, IL-6, and IL-8; have decreased expression of phospho-Stat3, B7H1, and epidermal growth factor receptor

(EGFR); and have much lower constitutive NF-κB activity than differentiated oral squamous carcinoma cells (OSCCs). When these OSCSCs were cocultured with IL-2-treated NK cells, the NK cells lysed OSCSCs significantly more than differentiated OSCCs. An increase in IFN-γ secretion and decrease in IL-6, GM-CSF, and IL-8 secretion were also detected in the supernatants of NK cells cocultured with OSCSCs compared with cytokine secretion in the supernatants of NK cells cocultured with differentiated OSCCs. In addition, the authors demonstrated that normal primary stem cells, like human embryonic stem cells (hESCs), mesenchymal stem cells (hMSCs), dental pulp stem cells (hDPSCs), and induced pluripotent stem cells (hiPSCs), are also susceptible to NK cell-mediated cytotoxicity. Collectively these studies suggest that undifferentiated cells are susceptible targets of NK cell cytotoxicity. Stem cells may become resistant to NK cell-mediated cytotoxicity once they differentiate.

Another kind of innate immune cell, γδ T cells represent a small part of the lymphocyte population that expresses a TCR complex where CD3 is associated with γ and δ chains. These lymphocytes were originally characterized as strong IFN-γ-producing cells which exhibit MHC-unrestricted lytic activity, thus making them potential anticancer stem cell mediators [61, 62]. The majority of γδ T cells in human peripheral blood are of the Vγ9Vδ2 phenotype and constitute 1–5 % of circulating lymphocytes [63, 64]. Many *in vitro* and *in vivo* studies have demonstrated antitumor activity of the Vγ9Vδ2 T cells. The possible mechanism for this γδ T cell-mediated cytotoxicity includes MHC nonrestricted direct killing of tumor cells, antibody-dependent cell-mediated cytotoxicity (ADCC), and activation of other immune effectors. Vγ9Vδ2 T cells have a unique capacity to recognize and be activated and expanded by non-peptide phosphoantigens, such as zoledronate and pamidronate. In 2009, Todaro et al. combined human Vγ9Vδ2 T cells with bisphosphonate zoledronate and found that Vγ9Vδ2 T cells efficiently killed human colon cancer stem cells [65]. In this setting, production of cytokines (TNF-α and IFN-γ) and cytotoxic and apoptotic molecules (TRAIL and granzymes) were induced after exposure of Vγ9Vδ2 T cells to sensitized human colon cancer stem cells. According to their report, γδ-TCR predominantly mediates cancer stem cell recognition and killing. The granule exocytosis pathway mediates Vγ9Vδ2 T cell cytotoxicity, which is highly dependent on isoprenoid production by tumor cells. In a clinical study, activated Vγ9Vδ2 T cells in combination with zoledronate show increased CD69 expression, indicating an activated phenotype. These Vγ9Vδ2 T cells displayed upregulated expression of peripheral tissue-homing chemokine receptors, CCR5 and CXCR3. In contrast, expression of lymphoid homing receptors, CCR7 and CXCR5, decrease [66]. More importantly, these zoledronate-activated Vγ9Vδ2 T cells are cytotoxic *in vitro* against tumor targets, and adoptively transferred Vγ9Vδ2 T cells traffic predominantly to the lungs, liver, and spleen and, in some patients, to metastatic tumor sites outside these organs, suggesting that therapy with activated Vγ9Vδ2 T cells is feasible [66]. Taken together, these results indicate that *in vitro* expansion of autologous γδT cells in combination with other antitumor agents, like zoledronate, pamidronate, and cytokines, may benefit cancer treatment via cancer stem cell elimination.

9.5 Cancer Stem Cell-Primed T Cells Specifically Targeting Cancer Stem Cells

Cell-mediated immunity plays a major role in the rejection of tumors and apparently has an advantage over innate immune cells. In conventional cancer immunotherapy, bulk tumors comprising heterogeneous cancer cell populations have been used as antigen either to prime pre-effector T cells or to generate DC-based vaccines. However, tumor-specific antigens may be selectively expressed on differentiated tumor cells and are not expressed on cancer stem cells [43, 67]. Cancer stem cells express cancer stem cell-specific antigens which may be different from either differentiated tumor cell antigens or normal stem cell antigens [68].

In support of this hypothesis, recent studies have demonstrated that cancer stem cell-specific $CD8^+$ T cells can be generated *in vitro* for injection into NOD/SCID mice to mediate tumor regression [69–72]. In 1999, Bonnet et al. identified putative human acute myeloid leukemia (AML) stem cells and cancer stem cell-specific $CD8^+$ T cells [71]. When NOD/SCID mice were transplanted with the mixture of human AML cells and a CTL clone specific for minor histocompatibility (H) antigens, these human leukemic cells were completely eliminated from the inocula. Based on the data, they proposed that AML stem cells can be eliminated by minor H antigen-specific CTL clones. Consistent with Bonnet's report, Brown and colleagues identified tumor spheres (TSs), which were expanded from glioma explants, displayed consistently high CD133 expression, and exhibited stem cell-like characteristics [70]. These $CD133^+$ TSs express significant and comparable levels of MHC I and ICAM-1/CD54, which are required for T-cell/tumor immunologic synapse formation. These brain tumor stem/initiating cells (BTSCs) were susceptible to perforin-dependent CTL-mediated cytolysis. To assess whether the protein processing machinery is sufficiently intact for the BTSCs population to process and present antigen for $CD8^+$ CTL recognition, the authors engineered glioma TSs to endogenously express the cytomegalovirus (CMV) pp65 antigen by reconstructed pp65-lentiviral transduction. They found that CMV-specific CTLs mediate the CMV-transducted glioma TSs cytotoxicity. To test whether CTL can eliminate all tumor-initiating activity of the BTSCs *in vivo*, CMV pp65-expressing TSs and pp65-specific CTLs were co-injected into NOD/SCID mice. It was found that all pp65 antigen-positive tumor cells were ablated, while $pp65^-$ tumor cells were resistant to the pp65-specific CTL and efficiently engrafted. This result established that direct recognition of antigen-expressing TS cells by CTLs is required to ablate tumor initiation. In another study, Visus and colleagues reported that cancer stem cell-specific $CD8^+$ T cells can be generated by using antigenic peptide from aldehyde dehydrogenase 1 family member A1 (ALDH1A1) expressed by $ALDHA1^{bright}$ cancer stem cells from squamous cell carcinoma of the head and neck (SCCHN) [69, 73]. They demonstrated that transfer of ALDH1A1-specific $CD8^+$ T cells eliminated $ALDH^{bright}$ cells, inhibited tumor growth and metastases, and prolonged survival of xenograft-bearing immunodeficient mice [69]. CD133 is a common marker of cancer stem cells. Huang et al. generated an anti-CD3/anti-CD133 bispecific antibody (BsAb) and bound it to the cytokine-induced killer (CIK) cells as effector cells (BsAb-CIK) to target $CD133^{high}$ CSCs. The killing of $CD133^{high}$ pancreatic (SW1990) and hepatic (Hep3B) cancer cells by the BsAb-CIK cells was significantly ($p<0.05$) higher than the killing by the parental CIK or by CIK cells bound with anti-CD3 (CD3-CIK) without CD133 targeting. In nude mice, the BsAb-CIK cells inhibited $CD133^{high}$ tumor growth significantly ($p<0.05$) more than that by CIK or CD3-CIK cells or by the BsAb alone. Treatment with the BsAb-CIK cells significantly downregulated the expression of S100P and IL-18 bp, but upregulated STAT1. The findings may facilitate the development of novel immunotherapies for patients with cancer containing $CD133^{high}$ CSCs by selectively targeting this cell population [74]. Together, these results support the conclusion that potential cancer stem cell-specific T cells can be generated *in vitro* for subsequent adoptive transfer into tumor-bearing hosts to target cancer stem cells and lay the foundation for the development of new immunotherapeutic approaches to eradicate tumors *in vivo*.

9.6 Development of Cancer Stem Cell-Specific Vaccine in Immunocompetent Host

In contrast to the generation of cancer stem cell-specific T cells *in vitro*, for their *in vivo* use, the development of a cancer stem cell-specific vaccine depends on the integrated host cellular and humoral immunity. However, most cancer stem cell studies have been performed with human tumor-derived cancer stem cells in immunocompromised mice [69–72]. These xenotransplantation assays have

shown a great deal of variability in the frequency of cells identified with tumorigenic potential, depending on the degree of host immunodeficiency [75]. Most importantly, due to the lack of cellular and humoral immunity in the NOD/SCID mice, these hosts are not suitable for the immunological evaluation of cancer stem cell vaccines. The efficacy of cancer stem cell-based vaccination against tumors needs to be assessed in immunocompetent hosts.

Given the fact that dendritic cell (DC) vaccines have significant potential in cancer immunotherapy, cancer stem cell-primed DC vaccines have been proposed. Glioblastoma-derived cancer stem cells express MHC I [72]. After coculturing human immature, autologous DCs with these irradiated brain tumor stem cells, the cancer stem cell-primed mature DCs express costimulatory molecules CD80, CD86, and CD40 and stimulate significant Th1 (IFN-γ) response *in vitro* [72]. Our group at the University of Michigan assessed the feasibility of cancer stem cell-primed DC vaccine *in vivo* and demonstrated that cancer stem cell-primed DC vaccination confers significant antitumor immunity in immunocompetent hosts [76]. In this study, the tumorigenicity of murine $ALDH^{+/high}$ cancer stem cells were characterized in two histologically different tumors (D5 melanoma and SCC7 squamous cell carcinoma) from two genetically distinct immunocompetent hosts (B6 and C3H mice). Using purified cancer stem cells as an antigen source to prime DCs, we evaluated the protective effects of cancer stem cell-primed DC vaccines in syngeneic mouse tumor models. The study demonstrated that cancer stem cell-primed DC vaccination significantly prevented lung metastasis formation in murine D5 melanoma model and subcutaneous (s.c.) tumor growth in murine SCC7 squamous cell carcinoma model compared with the positive control group using DCs pulsed with the unsorted heterogeneous tumor cells [76]. Mechanistically, this study observed high IgG production by splenocytes obtained from the host subjected to the cancer stem cell-DC vaccine, efficient binding of these antibodies to the cancer stem cells, and significant cancer stem cell lysis mediated by these antibodies in the presence of complements. In addition, CTLs generated from the PBMCs and splenocytes obtained from cancer stem cell-DC-vaccinated hosts selectively killed cancer stem cells. This study revealed direct targeting of cancer stem cells by cancer stem cell-primed antibody and CTLs. Collectively, these data indicate that enriched cancer stem cells are immunogenic and more effective as an antigen source than unselected tumor cells in inducing protective antitumor immunity [76]. In line with the above mentioned studies, Phuc and colleagues used cancer stem cell-primed DC vaccine in murine breast cancer models [77]. They found that breast cancer stem cell extract-loaded DCs migrate to the spleen, activate $CD8^+$ and $CD45^+$ T cells, and induce CTL responses [77].

The mechanisms responsible for mediating cancer stem cell-DC responses remain to be elucidated. Experimental evidence has demonstrated that cancer stem cell-DC vaccines confer animal host antitumor immunity by direct targeting of cancer stem cells by antibody and CTLs [77], but the molecule(s) responsible for such cancer stem cell-conferred antitumor activity remain unknown. In this regard, Duarte et al. used mass spectrometry to compare protein expression difference between cancer stem cells and non-cancer stem cells in colon carcinoma and identified four proteins specifically expressed in the cancer stem cells [78]. Among these proteins, two of them (heat shock protein 27-kDa and aldose reductase) are already known to be associated with treatment resistance and poor prognosis in colon cancer. They then inoculated rats with the cancer stem cell lysate (not purified antigens) as vaccine and found that the cancer stem cell-based vaccine reduced tumor volume and occurrence and inhibited experimental liver metastasis in half of the animals [78]. This report provides evidence for the existence of cancer stem cell-associated antigens in the lysate used to prime DCs.

Different signals induce distinct DC phenotypes (subsets) and yield distinct immune responses, e.g., Th1 response, Th2 response, Th17 response, or the generation of T_{reg} cells. To circumvent potential unfavorable outcomes, it is important to understand how cancer stem cell antigens interact with distinct subsets of DCs.

This will help elucidate the molecular pathways of DC maturation, cancer stem cell antigen presentation as well as the discovery of novel adjuvants for cancer stem cell-DC-based vaccines.

In protective studies, normal animals are inoculated with cancer stem cell-based vaccines before tumor cell injection. If a cancer stem cell vaccine is to be clinically relevant, it needs to be evaluated in a therapeutic setting. Although cancer stem cell-based vaccines have shown vigorous protective antitumor activity in several animal models, it is important to determine the therapeutic efficacy of cancer stem cell-based vaccines in established tumors, a more clinically relevant setting.

9.7 Targeting the Tumor Microenvironment as a Strategy to Enhance Immunological Targeting of Cancer Stem Cells

Normal stem cells reside in a distinct environment called the "stem cell niche." The niche regulates stemness, proliferation, and apoptosis resistance of stem cells. Cancer stem cells also reside in a niche within the tumor. The local tissue environment contributes to the self-renewal and differentiation of cancer stem cells. Growth factors, cytokines, and diverse stromal cells, such as mesenchymal stem cells and immune cells in the cellular microenvironment, are essential for cell nutrition, intercellular communication, signal transduction, and cell fate [79]. Therefore, these components in cancer stem cell niche may provide additional therapeutic targets.

Inflammatory cytokines including IL-1, IL-6, and IL-8 in cancer stem cell niche are involved in driving cancer stem cell self-renewal [80]. These cytokines activate Stat3/NF-κB pathways in both tumor and stromal cells and in turn stimulate further cytokine production, generating positive feedback loops contributing to cancer stem cell self-renewal. Inhibitors of these cytokines and their receptors have been developed [80]. Using siRNA to knock down testicular nuclear receptor 4 (TR4) in the prostate cancer (PCa) stem/progenitor cells led to downregulation of octamer-binding transcription factor 4 (Oct4) expression, which, in turn, downregulated the IL-1 receptor antagonist (IL1Ra) expression. This approach resulted in increased drug sensitivity of cancer stem cells to the two commonly used chemotherapeutic drugs, docetaxel and etoposide [81]. In addition, blockade of the IL-8 receptor CXCR1 using antibody or repertaxin (a small-molecule CXCR1 inhibitor) selectively depleted the cancer stem cell population in human breast cancer cell lines *in vitro*, followed by the induction of massive apoptosis in the bulk tumor population via FASL/FAS signaling [82]. Another cytokine IL-6 has been shown to be a direct regulator for cancer stem cell self-renewal [83, 84]. Kim and colleagues observed that part of the non-stem cell population converted to cancer stem cell-like cells by promoting *OCT-4* gene expression. Anti-IL-6 antibody inhibited the JAK1 and STAT3 activation as well as *OCT-4* gene expression [85]. These studies indicate that IL-6 and its receptor may serve as attractive therapeutic targets in attempt to immunologically target cancer stem cells.

Tumor associate macrophages (TAMs) have been shown to modulate the tumorigenic and angiogenic potential of cancer stem cells within tumor-transplanted mouse model [86]. Thus, inhibiting TAM function may lead to cancer eradication via diminishing the cancer stem cells inside the tumor microenvironment. It has been reported that inhibition of TAM by targeting either the myeloid cell receptors colony-stimulating factor-1 receptor (CSF1R) or chemokine (C-C motif) receptor 2 (CCR2) decreased the number of cancer stem cells in pancreatic tumors [87].

Finally, little is known about the interactions between myeloid-derived suppressor cells (MDSCs) and cancer stem cells. Since MDSCs are pivotal for the generation and maintenance of an aggressive cancer microenvironment, it has been recently hypothesized that these cells may also act as a distinct tumor niche whose main function is the maintenance of self-renewal ability of niche itself [88]. MDSCs can directly incorporate into tumor endothelium and secrete many proangiogenic factors. They also induce the production of matrix metalloproteinases (MMPs) and chemoattractants and create a premetastatic environment [89]. Therefore, immunologically direct targeting of MDSCs may be a useful strategy to prevent tumor angiogenesis and cancer

stem cell-conferred disease recurrence, invasion, and metastasis.

9.8 Concluding Remarks

Cancer immunotherapy represents an important addition to our cancer therapy armamentarium. However, until recently these approaches have had limited clinical utility. The fact that current immunotherapeutic approaches failed to adequately target cancer stem cells may have limited their effectiveness. These cells are also relatively resistant to chemotherapy and radiation therapy; hence, it is important to develop immunotherapies capable of targeting this cell population. The use of cancer stem cell markers such as aldehyde dehydrogenase has facilitated isolation of these cells. Innate immune responses mediated by NK cells, NKTs, or γδT cells capable of targeting cancer stem cells have been described. Cancer stem cell-primed T cells generated *in vitro* have been shown to target cancer stem cells in human xenographs. To induce adaptive immunity, cancer stem cell vaccines have demonstrated the capability of specifically targeting cancer stem cells by cancer stem cell-primed T cells and antibodies in the immunocompetent host. To enhance immunological targeting of cancer stem cells, significant attempts have been made to target the tumor microenvironment in order to interrupt the interaction between cancer stem cells and its niche. Collectively, these efforts may help the development of novel immunologic approaches to target cancer stem cells.

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Hematopoietic Stem Cell Transplantation and Lymphodepletion for the Treatment of Cancer

10

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Contents

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10.1 Introduction

The complete elimination of hematopoietic cells in the bone marrow (called myeloablation) occurs when intensive chemotherapy or radiation is administered in an effort to eliminate cancer cells. Following myeloablative therapy, previously harvested hematopoietic progenitor cells, which have the potential to differentiate into all blood cell subsets and reconstitute the hematopoietic space, are infused. This latter process is called hematopoietic stem cell transplantation, or HSCT. 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, 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 2003 he was awarded the Nobel Prize for his pioneering work in the field of allogeneic HSCT [2]. Myeloablative conditioning followed by 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]. Worldwide, more than 45,000 HSCTs are performed annually [4]. This chapter explores current methods of myeloablation and HSCT for the treatment of cancers.

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10.2 Hematopoietic Stem Cell Transplantation (HSCT)

HSCT is the intravenous infusion of hematopoietic stem cells into a myeloablated 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.

10.2.1 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 can be aspirated from large bones such as the pelvis, and progenitors may be further enriched based on CD34 expression. For harvest of HSCs from peripheral blood, the donor is treated with an agent, such as the cytokine granulocyte colony-stimulating factor (G-CSF), that "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. There is a controversy regarding the best source of HSCs for transplant (Table 10.1). Some studies suggest that peripheral blood is superior to bone marrow as the source of HSCs [12, 13], while others have demonstrated that there is no significant difference in outcomes based upon the source of stem cells [14].

Table 10.1 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

Cells collected from the umbilical cord and placenta after childbirth can also be used as a source of HSCs [15–20]. 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 [15, 17, 21]. 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 active area of research [22–24].

10.2.2 Autologous and Allogeneic HSCT

Autologous HSCT refers to the infusion of hematopoietic stem cells that were harvested from oneself. 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. 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].

Allogeneic HSCT refers to donor-derived cells that were obtained from a genetically non-identical individual. Cancers that are often treated with allogeneic transplantation include AML, acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CLL), NHL, HL, chronic lymphocytic leukemia (CLL), MM, and juvenile chronic myeloid leukemia (CML) [4]. In addition to cancer, myelodysplastic syndromes and myeloproliferative disorders are also treated with allogeneic transplantation. 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).

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

Mismatches in major histocompatibility proteins as well as 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 [25]. As a consequence, the host-reactive donor T cells expand and release pro-inflammatory cytokines that support recruitment of other immune effector cells. Together, the activated immune cells can eventually destroy host tissues [26].

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 occurs within the first 100 days post-transplant. 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 occurs after 100 days post-transplant, 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 (Table 10.2).

There is an estimated 30 % lower life expectancy in cancer patients that receive an allogeneic transplant as compared to the general cancer population [27–30]. The leading causes for this increase in mortality include recurrent malignancies, infection, secondary cancers, respiratory disease, and chronic GVHD [29]. 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 HSC transplant. Autologous GVHD appears to occur as a result of immune dysregulation by autoreactive T cells [31].

Despite the devastating consequences of GVHD, low levels of alloreactivity can be beneficial for generating a graft-versus-tumor (GVT) effect [32]. 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 correlated with a decrease in leukemia-free survival [33]. 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 [34]. GVT effects have been identified in MM, NHL, HL, CLL, and acute leukemia (ALL and AML) [35]. GVHD and GVT both include three interlinked phases: (I) induced pro-inflammatory environment, (II) donor T cell activation and proliferation, and (III) migration of immune effector cells to target tissues [36]. Although the mechanisms of GVHD and GVT both involve the

Table 10.2 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	

(continued)

Table 10.2 (continued)

Name	Type	Details	Use
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

activation of donor T cells against host alloantigens, it appears that these outcomes can occur independent of each other [37, 38]. Approaches which 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 [36]. Mechanisms which allow for separation of GVHD from GVT are still not fully understood.

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 [39]. NK cells eliminate tumor cells in a MHC-unrestricted manner either by direct cytotoxicity or by the production of inflammatory cytokines [39]. Clinical trials using NK cells as part of transplant immunotherapy have demonstrated that NK cells have potent antitumor effects [39].

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 [32, 33].

10.2.4 Myeloablative Effects That Promote the Elimination of Hematologic Malignancies

Depletion of host bone marrow via myeloablative conditioning is a critical prerequisite for the successful engraftment of transplanted HSCs. Bone marrow destruction that occurs from myeloablative conditioning results in the elimination of malignant hematopoietic cells, normal hematopoietic cells including lymphocytes (lymphodepletion), and bone marrow progenitor cells. Myeloablative 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 10.2 are chemotherapeutic drugs that are commonly used for myeloablative 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 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 myeloablative 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 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.

10.2.5 Non-myeloablative Conditioning

Non-myeloablative conditioning results in transient depletion of lymphocytes and other leukocytes without completely ablating the host HSC compartment. Therefore, HSCT is not required following non-myeloablative conditioning, although HSC transplant may still be given in an effort to generate a state of mixed donor-host chimerism. The goal of non-myeloablative conditioning is to eradicate hematologic malignant cells while preserving the HSC compartment and some normal mature hematopoietic cells including immune cells. Non-myeloablative 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 non-myeloablative conditioning are often similar to those used for myeloablative conditioning (see Table 10.3); however, these drugs are administered at lower doses. Chemotherapeutic drugs used specifically for non-myeloablative conditioning include fludarabine, cladribine, and pentostatin. Non-chemotherapeutic agents, such as alemtuzumab, can also be used for non-myeloablative 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 10.3 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 Purine analog	Inhibits DNA synthesis 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 Purine analog	Binds CD52 protein on mature lymphocytes Results in depletion of lymphocytes only

Total lymphoid irradiation (TLI) is a type of non-myeloablative 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 non-lymphoid 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 [40, 41]. The major advantage of TLI versus non-myeloablative TBI is an observed reduction in organ toxicity and decreased severity of GVHD [41, 42].

Both myeloablative and non-myeloablative 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 [43–46]. For these reasons, both myeloablative and non-myeloablative conditioning regimens have been incorporated into treatment protocols for a variety of hematologic malignancies and solid tumors.

10.3 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). Reduction in hematopoietic cells, including lymphocytes (T, B, and NK cells) creates "space" in hematopoietic tissues which is necessary for transplanted stem cells to divide and expand. 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) [47]. The 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. 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. Overall, lymphodepletion favors the maturation of APC necessary for efficient presentation of tumor antigens to tumor reactive T cells, thereby facilitating antitumor immunity [48].

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

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 [47]. 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. T cell reconstitution after non-myeloablative conditioning results from thymopoiesis, the homeostatic proliferation of host T cells that have survived the conditioning, or from the adoptive transfer of allogeneic or autologous T cells. 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.

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 [49, 50]. Thymopoiesis is influenced by cytokines, growth factors, and hormones. Interleukin-7 is important for the survival of developing thymocytes [51]. As a result, IL-7 administration after transplant enhances donor-derived thymopoiesis [52]. 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 [51]. Keratinocyte growth factor (KGF) boosts thymic productivity by expanding thymic epithelial cell populations, and KGF-deficient mice are more susceptible to thymic damage [53]. Growth hormones, such as insulin-like growth factor-1 (IGF-1), 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 [47]. T cell reconstitution in children is relatively quick and results in generation of a normal CD4:CD8 T cell ratio of 2:1 [54]. 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 [54]. 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) [54, 55].

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

T cell homeostatic proliferation is the spontaneous proliferation of existing peripheral T cells that expand to fill “empty space” in the T cell compartment. Homeostatic proliferation 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 by self-MHC/peptide complexes 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 [47]. Naïve cells with a memory phenotype revert back to a naive phenotype after proliferation ceases and homeostasis is restored [56]. HP in the absence of primary antigen stimulation can mediate a secondary response to antigen, suggesting that lymphopenia can promote polyclonal T cell differentiation [57].

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 [58]. T cells in IL-7-deficient mice do not undergo HP, demonstrating that IL-7 is required for stimulating naïve T cell HP and sustaining survival of these cells [59, 60]. Administration of IL-7 drives proliferation of naïve T cells and restricts T cell expansion following the recovery of T cell numbers [59, 60]. IL-7 also restricts T cell expansion following T cell recovery to prevent an overabundance of naïve T cells [47]. IL-15 and IL-21 both promote the expansion and survival of memory $CD8^+$ T cells [61, 62]. Increased concentrations of IL-7 and IL-15 are produced during whole body irradiation [63], and increased IL-7 and IL-15 signaling causes T cells to undergo HP [63–65]. Naïve T cells also require TCR activation with self-peptide/MHC complexes to undergo HP [63], 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-7 and IL-15 signaling, but does not require interaction with antigen or MHC molecules [63]. T cell repopulation is also influenced by other growth factors and hormones [47].

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 [66]. 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 [67–69], and (3) ex vivo manipulation of T cells to promote expansion of tumor-reactive T cells for adoptive transfer. 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 [70–72].

10.3.3 Use of Animal Models to Address Immunological Effects of Lymphodepletion

Mouse models have provided excellent systems for determining the underlying mechanisms responsible for the immunologic 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 [47, 59, 60]. 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 [55]. Information gathered from these models can provide further insights to new cancer therapies that involve lymphodepletion and HSCT.

10.4 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/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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Combination of Chemotherapy and Cytokine Therapy in Treatment of Cancers

11

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11.1 Introduction

Classical approaches to treat cancer include the use of chemotherapeutic cocktails and radiotherapy [1, 2]. On the other hand, emerging novel strategies include molecular targeted therapies, anti-angiogenic molecules, monoclonal antibodies (mAbs) or immunotherapy. However, despite the wide range of therapeutic options, their impact on patient's overall survival has been rather limited. Therefore, there is an urgent need for new therapeutic options mainly in patients with advanced disease. There is a limited clinical experience regarding the application of immunotherapy in cancer; however, increasing 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 [3]. During the last two decades, a growing area of research has been focused on the combination between classical chemotherapy and novel strategies such as the use of cytokines, which can act not only at the induction but also at the effector phase of the immune system [4]. The new studies indicate that reducing the dose of conventional chemotherapy could act in synergy to generate immunity against many tumors [5]. In this chapter, we will discuss how these combinations can be exploited to treat cancer.

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11.2 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, cross talk exists among all components of tumor microenvironment and immune cells (macrophages, natural killer cells, lymphocytes, dendritic and mast cells, among others) which may result in the stimulation of cancer [6]. In solid tumors, for example, colorectal carcinoma or liver cancer, immune cells could infiltrate tumors playing a key role in the control of cancer aggressiveness [7, 8].

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 [9]. On the other hand, chronic inflammatory diseases such as ulcerative colitis predispose to colorectal carcinoma [10]. 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 proangiogenic molecules into avascular areas, resulting in angiogenesis stimulation [11]. Macrophages may promote tumor invasion by secreting proteases and cytokines such as IL-1 and IL-6 [12]. In addition, macrophages could suppress both arms of the immune system by blocking dendritic cell maturation and inhibiting cytotoxic T-cell responses [13]. On the contrary, experimental and clinical data support that macrophages might exert antitumoral effects [14]. 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 [14].

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 [15, 16]. The density of tumor-infiltrating T lymphocytes with cytotoxic and memory phenotypes is highly predictive of favorable clinical outcome in melanoma, non-Hodgkin lymphoma (NHL), and breast, ovarian, head and neck, non-small-cell lung, and esophageal cancer [16, 17]. 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. As a result, certain suppressive cells, molecules such as cyclooxigenase-2 (COX-2), indoleamine 2,3-dioxygenase enzyme (IDO) or arginase, and cytokines (IL-6,-10, transforming growth factor beta (TGF-β), macrophage colony-stimulating factor (M-CSF)) might promote tumor growth, whereas other components, on the contrary, have a protective role.

11.2.1 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 [18] to the recent immunoediting concept developed by Schreiber and colleagues [19]. As a result, we know that the immune system is able to recognize and eliminate cancer cells, but also a parallel part of the relationship between immune cells and cancer cells shows that inducing some selective pressure on tumor 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 [19]. Cancer immunoediting theory proposes three subsequent phases: (i) *elimination*, in which the immune system can recognize and eliminate nascent tumor cells (immunosurveillance); (ii) *equilibrium*, between host and cancer cells; and (iii) *escape* of cancer cells from the immune attack (immunoediting) [20].

Multiple mechanisms are used by cancer cells to escape from the immune recognition and tumor elimination: (i) impairment of appropriate

antigen presentation mechanisms, (ii) production of immunosuppressive factors, (iii) inactivation of co-stimulatory signals, (iv) promotion of suppressor cells such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and immature dendritic cells (DCs) [21].

11.2.2 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 [21]. Accordingly, removal of these immature 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.

11.2.2.1 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* [22]. It has been observed that 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) [23]. In addition, two $CD4^+$ $CD25^+$ Treg 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-1cells that are characterized by their ability to inhibit the effector T-cell response by the secretion of IL-10 and TGF-β [22]. Tregs block antitumor immunity through several mechanisms such as (i) inhibiting $CD8^+$ and $CD4^+$ T-cell activation and cytotoxic activity, (ii) impairing NK cells cytokine production, (iii) inducing tolerogenic DCs, (iv) stimulating the activity of the immunoregulatory molecule CTLA-4, and (v) increasing the activity of IDO which is responsible for the degradation of tryptophan resulting in the apoptosis of $CD4^+$ and $CD8^+$ T cells [24].

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 [25] and is considered potential therapeutic targets. In this sense, monoclonal antibodies (mAbs) directed against specific epitopes located on the cell surface of Tregs such as CD25 and CTLA-4 have been recently developed [24]. Together with this, 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 anti-metastatic effects in several experimental models [26, 27]. 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 [28].

11.2.2.2 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 [29]. 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 [30]. 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 [30]. MDSCs can take up antigens *in vivo* and process and present to T cells resulting in anergy;

moreover, they can release nitric oxide (NO) and peroxynitrite inhibiting T-cell activation and may induce expansion of regulatory T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ cells, *in vivo* [31]. In summary, there is ground for improvement in cancer immunotherapy by inhibiting MDSCs activity, and the use of blocking antibodies against cell surface molecules [32] or drugs affecting the number and activity of these cells are areas of great interest [33]. For example, a recent study demonstrated that gemcitabine or 5-fluorouracil can promote antitumor immune response by selectively removing MDSCs in mice [34].

11.3 Immunotherapy of Cancer

Cancer immunotherapy aims to control the growth and dissemination of malignant tumors by the activation of a specific immune response [35]. To this end, a variety of strategies are being investigated in preclinical and clinical settings including (i) nonspecific activation of the immune system by cytokines, (ii) cancer vaccination using autologous/allogeneic tumor cells modified to produce cytokines or antigen-preloaded DCs, (iii) adoptive T-cell therapy, and (iv) immunostimulatory mAbs and others.

Although several immunotherapeutic strategies have demonstrated to be potent in animal models, it was not until a few years ago with the use of DCs in hormone refractory prostate cancer or with immunostimulatory mAbs (ipilimumab, tremelimumab, daclizumab) that clinical results were more satisfactory [36, 37]. A partial explanation for the frustrating clinical results is based on the presence of immunosuppressive mechanisms used by tumors 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 [38]. However, these mAbs lack specificity and may also recognize the same epitopes on other cell types, including effector T cells. On the other hand, some drugs have been investigated to inhibit MDSCs activity such as retinoic acid, vitamin D, the COX-2 inhibitor celecoxib, and others with dissimilar results [39].

In the design of a therapeutic strategy, the need to implement multiple approaches to block immunosuppressive mechanisms has to be taken into account. Protocols of combined therapy consisting of a chemotherapeutic agent such as cyclophosphamide, gemcitabine, paclitaxel, or doxorubicin associated with immunostimulatory cytokines might act in synergy [40].

11.3.1 Enhancing Antitumor Immunity Using Cytokines

Cytokines are secreted by different immune cells in response to pathogens and other antigens acting not only at the induction but also at the effector phase of the immune system regulating the innate and the adaptive immunity in an autocrine or paracrine fashion. In clinical practice, some cytokines (e.g. IFN-α or IL-2) are used routinely in patients with metastatic RCC or melanoma [41, 42]. However, in spite of some objective responses, the clinical development of IL-2 has been proved unsuitable because in parallel to their efficacy, the results involved severe toxicity, including systemic vascular leak syndrome.

Cytokines can act as:

(i) 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) and IL-6; and IL-15, IL-18, IL-23, and IL-27.

(ii) 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.

(iii) 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 leukocytes 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 [43]. Unfortunately, its toxicity and low potency make it unsuccessful as 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 [44].

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 and 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 [45]. However, IL-12 was shown to eventually induce severe toxicity when administered systemically as a recombinant protein (in a phase II clinical trial) [46]. 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 [47]. 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 [48].

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 anti-angiogenesis [49]. 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 [50]. On the other hand, intratumoral 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 [51]. 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 [52].

11.4 Overcoming Tumor Resistance and the Use of Chemotherapeutic Agents

Chemotherapy was introduced in the 1940s and conceived as a single antineoplastic drug which could stop cell division by inhibiting its DNA synthesis leading to cell death. DNA alkylating agents and antimetabolites were the first chemotherapeutic agents used. Later other drugs with different mechanism of action were developed such as the taxanes which promote microtubular assembly and stability, antitumor antibiotics which intercalate DNA, and topoisomerase inhibitors. 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 [53].

According to World Health Organization (WHO), cancer death rates increased from 1950 to 1980 and remained stable from 1980 to 1990; since then a steady decline has been observed affecting all four major cancer sources (lung, colon, breast, and prostate). Over the past 10 years, the largest annual declines in death rates were for chronic myeloid leukemia (8.4 %), cancers of the stomach (3.1 %), colorectum (3.0 %), and NHL (3.0 %). Reduction in overall cancer death rates is due, mainly, to early diagnosis and improvements in the treatment of advanced disease.

11.4.1 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 or to reduce tumor-cell resistance to specific cytotoxic T lymphocytes [54]. Chemotherapy and immunotherapy have been considered antagonistic forms of cancer therapy because, for example, chemotherapeutic agents kill target cells by the induction of apoptosis that is considered a nonimmunogenic cell death. In addition, lymphopenia is frequently induced after chemotherapy with the subsequent impact on immune system [55]. However, some of these combinations have been found to generate synergistic rather than additive effects.

11.4.2 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 [56]. DNA damage and subsequent apoptosis is the mechanism of cancer destruction by drugs such as doxorubicin, cyclophosphamide, gemcitabine, cisplatin, and others [57]. Some other drugs induce non-apoptotic cell death; for example, paclitaxel modulates the activity of small Rho GTPase family members [55]. Apoptosis has been considered as a nonimmunogenic 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 the Toll-like Receptor 4 (TLR4) present in antigen-presenting cells [58]. Doxorubicin and methotrexate also promote apoptosis by inducing upregulation of FAS-L in some cancer cells [59].

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 DCs 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 [59].

Toxicity induced by chemotherapy is extremely frequent in clinical practice. However, there is experimental evidence that 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 [60]. 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 [34]. 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 demon-

strated 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 [61–63]. 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.

11.5 Combined Therapies

11.5.1 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 [64]. 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) [65], and $CD8^+$ T-cell depletion abolished the effect of combined therapy [65]. More recently, 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 [66].

TNF-α is a cytokine also used in combination with chemotherapy in a number of murine models. This cytokine is produced by activated macrophages, $CD4^+$ T lymphocytes, and NK cells. Studies describe that the combination of TNF-α and doxorubicin leads to complete tumor regression in C57BL/6 mice inoculated with EL4 lymphoma. Moreover, the combination showed a synergistic effect, since complete regression could not be elicited in tumor-bearing mice treated with single agents [67]. TNF-α combined with doxorubicin could also induce complete regression and long-term tumor-free survival in C57BL/6 mice inoculated with EO77l mammary tumor cells [68]. In addition, Regenass et al. have demonstrated that TNF-α 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 [69]. TNF-α in combination with cyclophosphamide was also explored in this model, showing that a low dose of cyclophosphamide combined with TNF-α resulted in 80 % of complete tumor eradication. Furthermore, a higher dose of cyclophosphamide was less effective [69].

In multiple 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 [70]. The optimal schedule and mechanisms of action of a novel vaccination with irradiated tumor cells engineered to secrete GM-CSF in combination with chemotherapy have been studied in a variety of tumor models [70]. For example, the antitumor efficiency of paclitaxel in combination with the vaccine was examined in a mouse model of RM-1 prostate cancer [71]. 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 [71]. 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 [72].

As mentioned above, IL-12 is a cytokine that acts as a link between the innate and the specific immune response [73]. 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 [74]. 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 [45].

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 [63, 75]. Synergistic effects were also observed in squamous cell spontaneous tumors in C3H mice combining cyclophosphamide with a plasmid carrying IL-12 genes [76].

11.5.2 What Have We Learned from the Clinical Practice?

The high efficacy of different immunotherapy strategies at eliminating tumors in animal models contradicts the very limited results achieved in patients. There are many explanations to why immunotherapy strategies fail or have little impact on patient survival. In general, immunotherapeutic protocols involve patients with advanced cancer disease that precludes, or at least decreases, the possibility of success. In addition, the immune system of the majority of treated patients is deteriorated or unable to recognize tumor antigens. In this context, cytokines were used in combination with chemotherapy in order to improve its efficacy. The most widely used cytokines are INF-α and/or IL-2-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.

INF-α is commonly used in this kind of combined strategy in the treatment of patients with advanced renal cell carcinoma. In a phase II clinical trial, the combination of INF-α and vinblastine improved patient response rate but did not impact on overall survival [77]. Similar results in terms of survival were achieved in a phase III trial combining INF-α with cis-retinoic acid [78]. In contrast, in a randomized phase III trial which included patients with similar characteristics, the addition of cis-retinoic acid to INF-α significantly increased progression-free and overall survival [79]. Another promising combination was 5-FU with IFN-α which has produced response rates of 23 % [80] and 30 % [81] 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 % [82, 83]; nonetheless, their efficacy remains a matter of controversy [84]. IFN-α was tested in patients with advanced hepatocellular carcinoma (HCC). A randomized, phase II trial, compared INF-α 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 [85]. Another study evaluated the efficacy of combined 5-FU and pegylated interferon (PEG-IFN) α-2b in patients with advanced HCC with similar results [86]. In contrast, a recent publication describes an open-label, multicenter, randomized phase III trial where 5-FU, cisplatin,

Table 11.1 Cytokine plus chemotherapy combination in clinical trials

Cytokine	Condition	Chemotherapy	Phase	State	Reference	Outcome
IL-2	Melanoma	Dacarbazine	II	Ongoing	NCT00553618	
	Melanoma	+Cy	II	Ongoing	NCT01833767	
	Breast cancer	+Paclitaxel	II	Ongoing	NCT01134250	
	Breast cancer	+Doxorubicin	II	Ongoing	NCT01131364	
	Pancreatic cancer	+Gemcitabine	I	Ongoing	NCT01198522	
IL-2+ IFN-α	RCC	5FU + gemcitabine	II	Completed	NCT00003664	
	Melanoma	Cisplatin + dacarbazine + vinblastine	III	Completed	NCT00002882	
IL-15	Metastatic melanoma	+Cy + TILs	I	Ongoing	NCT01369888	
	Skin cancer	+Flu + TILs	II	Ongoing	NCT01369888	
IFN-α	RCC	+Vinblastine	III	Completed	72	Increased RR[a] similar OS[a]
	RCC	+Cis-retinoic acid	III	Completed	73	Similar RR[a] Similar OS[a]
	RCC	+Cis-retinoic acid	II/III	Completed	74	Increased OS[a]
	RCC	+5FU	II	Completed	76	No additional side effects
	HCC	+5FU + cisplatin	II	Completed	80	No additional side effects similar OS[a]
	Ovarian carcinoma	+Carboplatin/paclitaxel	III	Completed	NCT00047632	
	Glioma	+Temozolamide	III	Ongoing	NCT01765088	
	GI, renal, and lung cancer	+5FU	II	Ongoing	NCT01658813	
GM-CSF	Breast cancer	+FLAC	I	Completed	NCT00001269	

5FU 5-fluorouracil, *Cy* cyclophosphamide, *FLAC* 5-fluorouracil, leucovorin, doxorubicin, cytoxan, *Flu* fludarabine, *GI* gastrointestinal carcinoma, *GM-CSF* granyulocyte macrophage colony-stimulating factor, *HCC* hepatocelullar carcinoma, *NCT* national clinical trial code, progression-free survival, *RCC* renal cell carcinoma, *RR* response rate, *OS* overall survival, *TILs* tumor-infiltrating lymphocytes

[a]Combined chemoimmunotherapy *vs*. chemotherapy alone

and IFN-α2b combined with radiotherapy did not improve the survival rate compared with 5-FU monotherapy in patients with advanced pancreatic adenocarcinoma [87].

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 [88]. G-CSF was 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 (for details, please see Table 11.1) [89].

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

11.6 Concluding Remarks

Combined immunotherapy clinical trials in cancer patients are challenging, and several strategies have been opened for clinical applications. However, the high efficacy of different immunotherapeutic strategies at eliminating tumors in animal models is in contrast with the very limited results achieved in patients. There are many explanations to why immunotherapeutic strategies fail or have little impact on patient survival. In general, for all solid tumors, the common scenario chosen to test immunotherapeutic protocols almost always involves patients with advanced diseases that precludes, or at least decreases, the possibility of success. Then, due to the advanced status of the cancer, the immune system of the majority of treated patients is deteriorated and unable to recognize tumor antigens. Thus, conventional chemotherapy 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 improvement in survival.

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T Cell Immunotherapy: From Synthetic Biology to Clinical Practice

12

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Contents

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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 stemlike precursors, we are about to enter an era of unprecedented innovation in the application of biological therapy for cancer. This hope is based on decades of research that sought to define the fundamental mechanisms of immune cell function, much of it in animal model systems. From the first Nobel Prize in Medicine or Physiology granted to Emil von Behring for the presence of what came to be known as immunoglobulin in immune serum in 1901 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 recognition that the same cytotoxic activity elicited against virus-infected cells by T effector cells could also be directed towards cancer cells has been the driving force behind the development of both cancer vaccines and the adoptive transfer of T cell populations for therapeutic effect. The early history of effective T cell therapy was demonstrated in the antitumor activity seen during bone marrow transplantation (the graft-versus-leukemia effect) and in the presence of tumor infiltrating cells in melanoma, that could be isolated, expanded and re-infused into the patient. Given the ability to understand a cell type that could lyse cancer cells, and our ability to

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culture and expand these cells, the technological innovation of conferring antibody-like specificity to cytolytic T cells by genetically engineering these cells to express a chimeric antigen receptor (CAR) has brought a sea change of expectation to the effective application of cell-based immunotherapy to human disease. For the first time, we can now 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 these cells has engendered caution, and the future of applying CAR-based therapy to human disease will depend of 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 often been a point of controversy. Some have argued that cancers arise quite often and that the immune system often does recognize aberrant cellular proliferation that threatens the host and eliminates it. In this view, cancer immunity is part and parcel of healthy somatic homeostasis. The alternative argument is that we see cancer in the clinic because immunity often, or usually, fails. In this scenario, with respect to clinical disease, the immune system has become at best, irrelevant. How does one approach this question quantitatively?

In a transgenic mouse cancer model, where the SV40 virus oncoprotein, large T antigen, is placed under the control of the insulin promoter, pancreatic tumors are induced. However, the combined action of interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) was shown to induce tumor cell senescence in a p16INK4a-dependent manner [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 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]. Meaning, 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 does prevent tumor outgrowth even in this model. These basic observations highlight our current understanding of tumor immunosurveillance in which both antigenic and tolerogenic signals need to be modulated for disease to be recognized and eliminated. Recently, important clinical breakthroughs have demonstrated the ability of T cells to mediate antitumor immunity once tolerogenic signals are inhibited. Using either anti-CTLA4 antibody, or anti-PD-1 antibody, clinical antitumor responses have been demonstrated [3, 4]. This indicates that even while an autochthonous tumor may be actively inducing tolerance in T cells, T cells are present in the host that has the potential to respond. Once these negative signals are blocked, antitumor immunity does indeed result. These important findings have only increased the drive to develop 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 in to remission by the reinfusion of lymphocytes from the bone marrow donor. Logistically, this is complex as the donor has to be recontacted and leukocytes harvested or the donor harvested and consented ahead of time. 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 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 in to 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 donor leukocyte infusion (DLI) is graft-versus-host disease (GVHD), which is related to the overall dose of infused T cells [5]. 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 [6]. In ongoing researches, 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 then focus on increasing the frequency of these cells and decreasing the number of cells causing GVHD. The collective term for these leukemia-specific antigens is minor histocompatibility antigens (mHags), and some have already been defined.

The first class I-MHC-restricted mHAgs identified were HA-1 and HA-2 [7]. 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 [8]. 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 [9]. The hope expressed in this study was that class II expressed antigens may be less broadly presented throughout normal tissues and thereby the polyclonal CD4 population that recognizes this antigen might be less prone to GVHD induction. 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, antileukemia 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 posttransplant lymphoproliferative disease (PTLD) in the posttransplant period was a devastating complication [10]. 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 [11]. As in DLI, continued description of the antigens associated with EBV-driven disease, the discovery of other nonviral tumor-associated epitopes, and the refinement of techniques to expand reactive T cells, has led to the continued expansion of adoptive immunotherapy approaches to human cancers [12].

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 (TIL). The ability to culture and expand TIL from patient tumor biopsy material remains the primary therapeutic bottleneck, but when TIL can be cultured and expanded, stunning results are seen. When the infusion of TIL is combined with lymphodepletion of the host, transferred TIL persist long term and complete cures are seen [13]. The preparative regimens developed for HSCT to deplete the host immune system 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 full resolved and likely all of these factors may be true. The combination of host preparation and experience in the generation of effector T cell populations has opened the door to a whole 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 T cell receptor (TCR) specific for the MART-1 antigen was used for the adoptive immunotherapy of melanoma by T cells [14]. 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 1989, Gross et al. demonstrated 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 [15]. 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 chain molecule combined an extracellular antigen binding motif with an intracellular T cell signaling motif in a single transcript [16]. The specific lysis of tumor cell lines by T cells engineered to express CARs was greeted with interest, but in hindsight, it is clearly a watershed moment in the history of adoptive immunotherapy. In CAR-expressing T cells, the chimeric molecule binds antigen on the surface of target cells through an antibody-like binding moiety (scFv) and activates the lytic pathway of transduced T cells through the intracellular signaling sequences encoded by the TCR-associated zeta chain molecule (part of the CD3 antigen complex). 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 [16–22].

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 ones. Potentially, any cell surface tumor-restricted antigen could be used as target. However, current CARs are limited to recognition of cell surface antigen. The exception is in a newer generation of CARs wherein the scFv used to create them is derived from an antibody specific for a peptide-MHC molecule [23].

12.4.2 Inclusion of T Cell Signaling 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 T cell receptor (TCR) signaling complex member CD3ζ in their cytoplasmic domain. 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 [24, 25]. 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 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 [26–28]. 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 [29]. 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 endodomain had a strikingly enhanced expansion and persistence compared with CAR-T cells lacking this endodomain [29]. 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* [27]. However, in a mesothelioma tumor model, equal antitumor efficacy for CD28 and 4-1BB containing second-generation CARs was seen [30]. 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. The optimal signaling endodomains 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 immunosurveillance.

12.4.3 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 [31–33]. 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 particles 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 [34, 35]. Nevertheless, retroviral gene transfer has shown acceptable safety and efficiency for the expression of CAR genes in human lymphocytes [36]. To date, there has been no report of insertional oncogenesis or even clonal overrepresentation in gene-modified mature lymphocytes harvested from peripheral blood using gamma-retrovirus-based vectors [37]. Lentiviral vectors offer certain advantages over gamma-retroviral vectors. Lentiviral vectors can transduce nondividing 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 insertion mutagenesis of the cellular target genome [38].

Transposon-based nonviral gene delivery systems, such as *sleeping beauty* and *PiggyBac* vectors [39–41], 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 [42]. 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 do eliminate the safety concerns of CAR therapy caused by genomic vector integration, might limit toxicity due to transient transcript expression, and also can avoid extensive *ex vivo* activation and expansion, allowing for better persistence of CAR-T cell *in vivo*.

Two of the major concerns with CAR-T cell therapy can be addressed by including control elements in the vector backbone. Concerns associated with CAR therapy include "on target/off tumor" toxicity and the cytokine storm related to immune response associated with a large tumor burden. One vector-based option is to use a suicide gene to allow the elimination of CAR-T cells *in vivo*. The most 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 [43]. However, the depletion is not always complete, and the foreign TK protein displays significant immunogenicity [44]. 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 successfully control GVHD in recipients of haplo-identical HSCT [45]. Since the caspase 9 is of human origin, it may be a less immunogenic 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 vector-encoded CD20. CD20 expression on transduced cells provides for a means of elimination by anti-CD20 antibody. Preclinical data has demonstrated that CD20-transduced T cells could be killed specifically and rapidly by exposure to rituximab. However, unwanted depletion of $CD20^+$ B cell and unexpected depletion of gene-modified T cells when treating $CD20^+$ EBV tumors with the antibody are limitations to be considered [46, 47]. Other suicide gene strategies include human thymidylate kinase and a modified Fas gene and have been evaluated *in vitro* and in several mouse models [48, 49].

In the allogeneic hematopoietic stem cell transplantation setting (HSCT), donor-derived T cells could be redirected by CAR vectors to achieve clinical response independent of MHC restriction manner. However, continued cell surface expression of TCRs from an HLA-disparate donor can cause GVHD upon adoptive immunotherapy. In order to generate universal allogeneic CAR-T cells for multiple recipients, Torikai et al., designed zinc finger nuclease (ZEF) strategy to irreversibly knock out the endogenous TCRα αvδ TCRβ chains [50]. Their data showed that disrupting endogenous TCR expression in CD19 CAR-T cells did not alter killing of cells expressing the CAR target antigen. If successful, this strategy provides insight into a potential means to generate a "universal" CAR-T cell to treat multiple patients with one cell product.

The use of a "biotin CAR" is another approach, making CAR therapy more 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)) [51]. 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 [51].

12.4.4 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 are then expanded 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 [52–54]. 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 [55]. Alternatively, evidence from other studies demonstrated enhanced efficacy when T central memory (Tcm) cells were redirected by CARs [56, 57]. 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 [58, 59]. 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 [60] and the GSK3b inhibitor TWS119 [61]. 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 also 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 [62, 63]. 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 [64, 65]. 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 [66–69]. 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 [70]. Currently, this inducible IL-12 vector is under evaluation in clinical trials at the NCI featuring melanoma-specific TIL (NCT01236573, clinical trial.gov).

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 [71]. 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 PSCA with a suboptimal activation profile and a chimeric co-stimulatory receptor (CCR) targeting a second antigen, PSMA, resulted in regression of tumor where both antigens are expressed [72]. This combinational antigen recognition strategy is one means to enforce stricter tumor specificity. 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 [73]. In this way, bioinformatics will continue to identify target antigens which must subsequently be analyzed for actual protein expression in tissue arrays.

12.4.5 Clinical Advances with CAR Therapy

When Wacław Szybalski used the term "synthetic biology," he was referring to the creation of whole genomes [74]. 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 impact 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 watershed moment, 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 developmental 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 (+) ve 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 with a set of side effects that is seen with some consistency in all CAR trials reported (hypotension, fever, and fatigue) [75, 76]. 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. At essentially the same time 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 [77, 78]. 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 it has had a lasting impact on the field. Anti-CD19 CAR approaches are now being implemented in pediatric patients with chemorefractory B cell precursor ALL as well. 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 $1.4 \times 10^6–1.2 \times 10^7$ 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 [79]. In the subsequent cytokine storm that followed T cell infusions, the onset of severe fever was abated by the administration of anti-IL-6 antibody. This informative result demonstrates that 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, CD79, and TSLPR [80]. Identifying expendable self-antigens for the treatment of solid tumors remains a serious challenge. Investigators have begun to formulate bioinformatic 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 [73]. A recent string of on-target but off-tumor (that is reacting to the intended antigen – but finding that its expression on normal tissue, as opposed to cancerous tissue, is problematic) 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 [81–83]. 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 % [84]. 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 [85]. Interestingly, the transduced T cells were generated from EBV-specific polyclonal T cell lines and thus did not strictly fall in either the Tscm or the Tcm category. The reported long-term persistence of these CAR-modified T cells may be due to the fact that endogenous EBV antigen recognition of the transduced T cells is sufficient to provide co-stimulatory signals not encoded by the first-generation CAR vector. The primary side-effect common to various trials with anti-GD2 antibody is peripheral nerve pain, indicating an off-tumor on-target antibody effect [86]. Carrying out clinical trials with more effective third generation anti-GD2 CAR constructs will reveal whether or not this side effect is unique to antibody-based therapy or if CARs amplify this effect.

Clinical trials administering CAR-modified T-cells to patients are increasing rapidly in number, and some have shown promising results. In a recent review of open clinical trials, CAR-T cells specific for the following tumor-associated antigens were reported: CD19 and CD20 (B cell malignancies), alpha-folate receptor (ovarian cancer), CAIX (renal cell carcinoma), EGFVIII (glioblastoma), GD2 (neuroblastoma), HER2 (glioblastoma), and L1-CAM (neuroblastoma), along with over 30 other targets in various stages of development [87]. 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

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
I. T lymphocyte population selection and culture
(a) Mechanism of T cell activation (OKT3, CD3-CD28 beads)
(b) Cytokines or small molecules included in culture and expansion protocol
(c) Selection of optimal T cell phenotype (Tcm, Tem, Tscm)
II. Gene vector design
(a) Selection of target antigen (both at the epitope and tissue expression levels)
(b) Creation of scFv 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) Inclusion of other immunomodulatory transcripts in the vector (co-stim, cytokine)

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 in to the patient

development of the clinical science of adoptive immunotherapy should prove as rich areas of investigation providing new benefits for cancer patients for many years to come.

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 NCT02030847 and NCT01626495 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 entirely suitable, especially if the patient goes on to HSCT (see NCT01593696, NCT00586391, and NCT00924326). The combination of CAR-based therapy with lymphodepletion or immune checkpoint 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 would be the ability to more rapidly define or create T cell populations that could be infused at lower doses (thus requiring less laboratory effort) while retaining high anti-leukemic 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 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 the tumor microenvironment that prohibits clinical antitumor effectiveness. The recent opening of a 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.

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Role of γδ T Lymphocytes in Cancer Immunosurveillance and Immunotherapy

13

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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

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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 contrary, there are 20–30 chicken Vγ chain gene segments and more than six 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).

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 Vγ7Vδ5 Vγ7Vδ6	Intermediate	Not applicable (extra-thymic development)	Gut epithelia
Human	Vδ1		High	Unknown	Spleen, liver, epithelia, dermis
	Vδ2	Vγ9Vδ2	Intermediate	Unknown	Peripheral blood
	Vδ3		High	Unknown	Liver, gut epithelia

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 remodelling 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 IELs (intestinal epithelial lymphocytes) 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 (such as 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, e.g., heat-shock proteins [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 tularensis* [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, Bonneville, and coworkers 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 dimethylallyl pyrophosphate (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 1-deoxy-d-xylulose-5-phosphate (MEP pathway or DOXP pathway) [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 1,000-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 (like *Mycobacterium tuberculosis* and *E. 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 HMGR level [48]. High levels of farnesyl pyrophosphate (FPP), sterols, or phenylalanine inhibit HMGR activity. In mammalian cells, the HMGR activity is inhib-

ited 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 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 EBV-infected cells as well as on colon, ovarian, and liver cancer cells, suggesting a role in anti-infection and anti-tumor 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]. 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 (HSP)

Because of their role as sensors during cell stress or transformation, HSP (heat-shock proteins) were initially proposed as antigenic targets for γδ T cells. Some members of HSP 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 signalling 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 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 signalling has been shown to produce both qualitative and quantitative changes leading to lower activation thresholds and enhanced αβ T-cell functions. CD28 signalling 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 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 ITAMs (immunoreceptor tyrosine-based activation motifs). 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 signalling that paralleled enhanced CD16-mediated T-cell activation [90]. The mechanisms underlying the balanced contribution of TCR *vs*. CD16 signalling 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 signalling 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 signalling 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, 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,

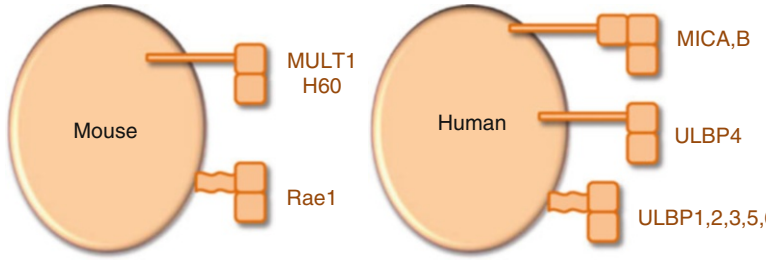

Fig. 13.1 Mouse and human NKG2D ligands. All NKG2D ligands have α1 and α2 domains with structural homology to MHC class I, and MICA and MICB have also a α3 domain. By contrast with MHC class I, none of the NKG2D ligands associates with β2-microglobulin or binds peptides. MULT1, H60, MICA/B, and ULBP4 are transmembrane-anchored type I glycoproteins, whereas Rae1 and ULBP1, ULBP2, ULBP3, ULBP5, and ULBP6 bind to cell membrane by a GPI anchor

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 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 nonredundant 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 ULBP1mRNA 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, and 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 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].

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.

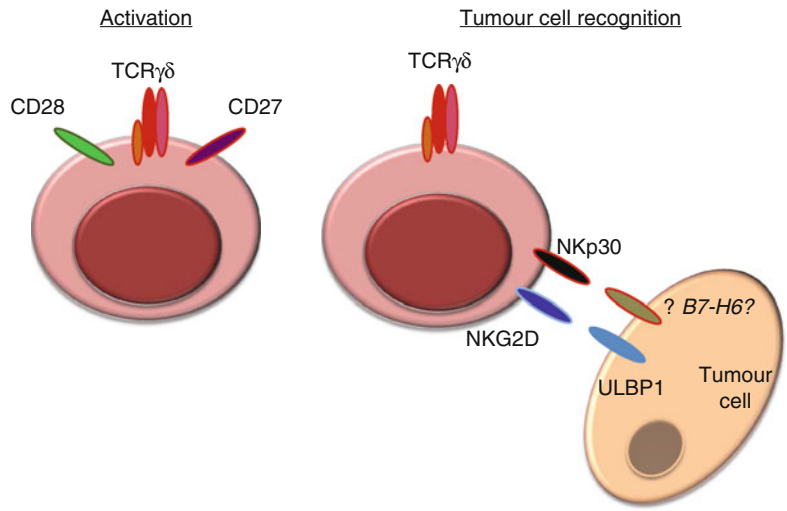

Fig. 13.2 Tumor cell targeting by γδ T cells is two-step process of activation and subsequent tumor cell recognition. γδ T-cell activation requires TCRγδ signalling plus costimulation (CD27 and CD28), whereas tumor cell recognition involves natural killer receptors such as NKG2D and NKp30 (and DNAM-1) that bind counter-ligands (over)expressed on tumor cells

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-mediated 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 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

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+		Squamous cell carcinoma	[176]
	TPA			
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

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 TRAMP (transgenic adenocarcinoma mouse prostate cancer) 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 Vγ9Vδ2^{+} 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], 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 VEGF (vascular endothelial growth factor) and IFN-γ was found in cancer patients treated with amino-bisphosphonates [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 lymphocyte (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 EPCR (endothelial protein C receptor) 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, e.g., 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 [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 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 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

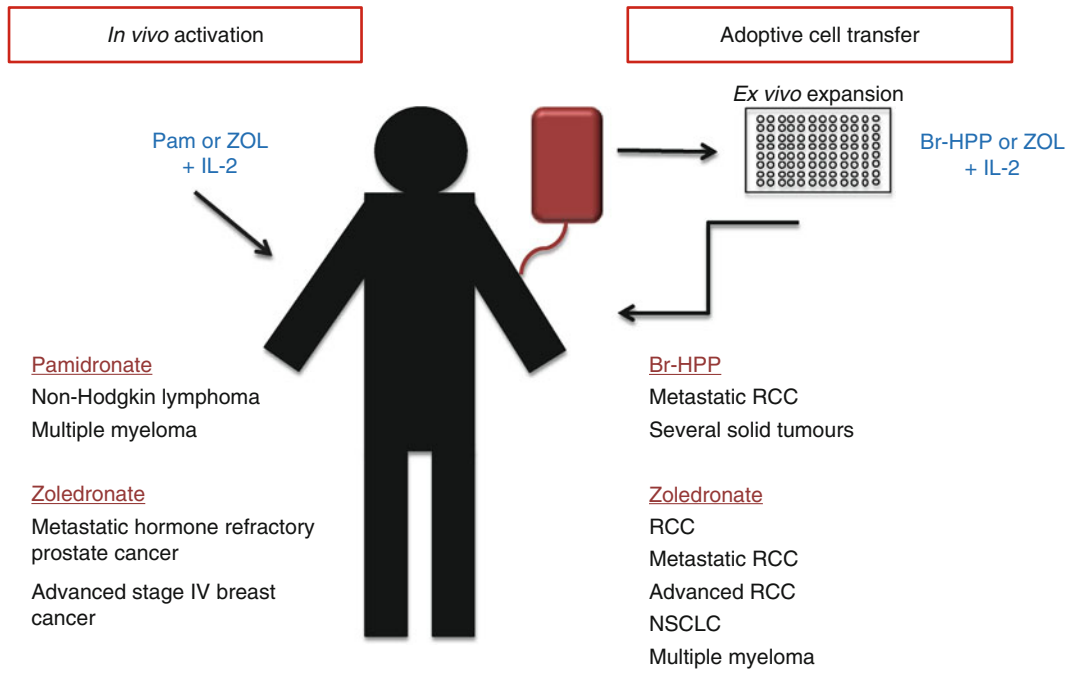

Fig. 13.3 Vγ9Vδ2 T-cell-based clinical trials. Strategies used in clinical trials include *in vivo* activation or adoptive transfer of *ex vivo* expanded γδ T cells with aminobisphosphonates (pamidronate or zoledronate) or phosphoantigens (BrH-PP), in combination with IL-2. *RCC* renal cell carcinoma, *NSCLC* non-small cell lung cancer, *ZOL* zoledronate, *BrH-PP* bromohydrin pyrophosphate

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 suggests 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.

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

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

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 signalling cascades induced upon sequential or concomitant receptor engagement [79].

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,

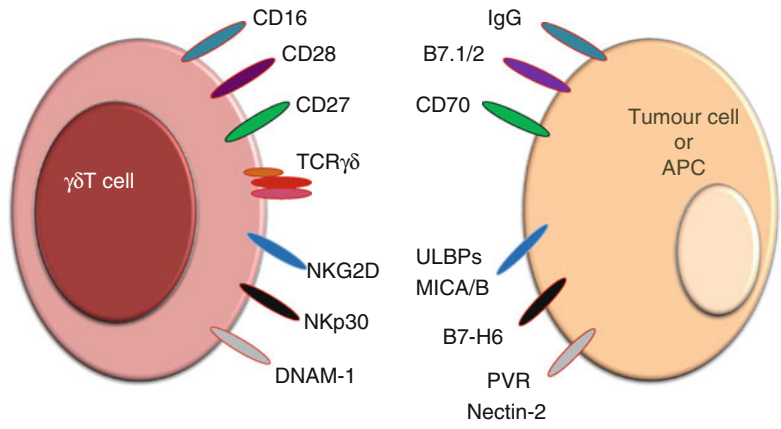

Fig. 13.4 Receptors involved in γδ T-cell activation and tumor cell recognition. T cells use their signature TCR to recognize antigens and cellular immune responses whose magnitude depends on the integrated engagement of a series of other surface receptors, including CD27, CD28, CD16, and natural killer receptors such as NKG2D and DNAM-1

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" *vs.* "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 signalling) 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 *vs*. antitumor γδ T-cell subsets? Do these differentially infiltrate tumor types? Does the tumor microenvironment manipulate the balance between pro-tumor *vs*. 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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Contents

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14.1 Introduction

Cancer is a major public health problem in the USA and many other parts of the world. Currently, one in three women and one in two men in the USA will develop cancer in his or her lifetime [1]. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as increase in the adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and "Westernized" diets. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56 % of the cases and 64 % of the deaths occurred in the economically developing world [2].

Among the standard methods of treatment for cancer (surgery, chemotherapy, and radiotherapy), immunotherapy is increasingly of growing interest. Cancer immunotherapy is the use of the immune system to reject cancer. The mode of action is stimulating the patient's immune system to attack the malignant tumor cells that are responsible for the disease. During the last two decades, several approaches for the activation of immune system against cancer have been developed [3–5]. These include administration of immunostimulatory agents, highly specific monoclonal antibodies (mAbs), cancer vaccines, and cell-based therapies. Cancer immunotherapy

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has now been broadly divided into three major branches: (A) immunostimulatory interventions, (B) anticancer vaccines (including protein, peptide, and cell-based vaccines), and (C) adoptive cell transfer (ACT)-based therapy [6].

Immunostimulatory interventions include systemic administration of lymphocyte targeting growth factors such as interleukin-2 (IL-2), proimmunogenic cytokines such as interferon alpha (IFN-α), or compounds that block immunosuppressive mechanisms, including mAbs that are specific for the cytotoxic T lymphocyte antigen 4 (CTLA4) 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 improvement on overall survival compared with chemotherapy alone [8]. Preliminary results using ipilimumab (anti-CTLA4 antibody), to block an inhibitory receptor on lymphocytes, indicate that durable responses can also be seen in some patients. Immunostimulatory agents given as monotherapy have been associated with tumor regression in cancers like melanoma and renal carcinoma, perhaps because these cancers are able to elicit elevated levels of antitumor lymphocytes [7, 9–11]. Several anticancer agents that are currently used in the clinic also mediate immunostimulatory effects, either by actively triggering immune effector mechanisms or 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 cytotoxic/cytostatic effect on tumor cells and vessel network, as well as stimulatory effect on the immune system [12, 13].

Vaccines constitute an appealing approach to cancer immunotherapy. The advantage lies in many factors such as their relatively easy administration, being cheap (especially in the case of peptide vaccines), and their being virtually devoid of side effects [14]. However, cancer vaccines comprising both peptide vaccines [15] and dendritic cell (DC)-based approaches [16] so far have failed to meet the high expectations that they had raised, being associated with modest and often non-reproducible clinical benefits [6]. This can perhaps be attributed to the fact that end-stage cancer patients often exhibit immune defects that can compromise their ability to mount a vaccine driven antitumor response. One notable exception is provided by sipuleucel-T (Provenge), a DC-based vaccine that has been granted FDA approval for the treatment of asymptomatic or minimally symptomatic, metastatic castration-resistant (hormone refractory) prostate cancer [17–19]. In addition, promising results have been observed in prostate cancer patients receiving prostate specific antigen (PSA)-targeted poxviral vaccines (PROSTVAC-FS) [20], as well as in melanoma patients treated with a peptidic vaccine combined with high-dose IL-2 [21]. The presumed goal of vaccinations, cytokine treatments, and antibody therapies such as ipilimumab is to stimulate an endogenous antitumor immune response of sufficient magnitude and intensity to cause tumor rejection.

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 % [22, 23]. Notably, ACT must be conceptually differentiated from other cell-based immunotherapies, including the reinfusion of autologous DCs 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 curative graft-versus-disease effect) [24, 25]. Immunotherapy using autologous T cells has emerged to be a powerful treatment option for patients with metastatic melanoma. These include 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 progress of recent advancement and studies evaluating various aspects of T-cell adoptive transfer in current oncological settings.

14.2 History of Adoptive Immunotherapy of Malignancy

The idea that immunocompetent cells are capable of mediating an antitumor effect was first validated experimentally in 1957 by Barnes and Loutit [26] who showed that leukemic animals that were lethally irradiated and reconstituted with allogeneic bone marrow had a lower tumor burden following transplantation than animals that were reconstituted with syngeneic marrow. In 1973, Bortin and colleagues [27–29] attempted to quantify the immunologic antitumor effect, which they called "graft versus leukemia, GvL effect," of donor lymphocytes. These observations in animal models led Mathé and colleagues to speculate that leukocyte transfusions could mediate antitumor effects in cancer-bearing recipients. To test this hypothesis, pooled white cell products were transfused into non-transplanted patients with end-stage acute leukemia, which resulted in responses [30]. Meanwhile, a series of clinical observations provided evidence for GvL activity in humans. These included a higher relapse rate in recipients of syngeneic compared with allogeneic transplants [31], a reduction in relapse rates in patients with graft-versus-host disease (GVHD) compared with those free of GVHD [32], induction of remission in patients after withdrawal of immunosuppression [33], and higher relapse rates in recipients receiving T-cell depleted grafts compared with unmanipulated grafts [34]. The first patient to receive donor lymphocyte infusions (DLIs) for a hematologic malignancy in relapse after bone marrow transplantation (BMT) was a boy with acute lymphocytic leukemia (ALL) who was resistant to chemotherapy and cytokines [35]. He ultimately obtained a sustained complete remission of his disease by receiving multiple transfusions of lymphocytes from his sister, the original bone marrow donor. Then, Kolb and colleagues [36] reported on three patients with relapsed chronic myeloid leukemia (CML) who failed to respond to treatment with interferon *alpha* (IFN-α), but obtained complete remissions with the combination of IFN-α plus DLI. Thus, the era of adoptive immunotherapy to treat posttransplant relapse of hematologic malignancies was born [37].

14.3 T-Cell Infiltration Correlates with Prognosis

T cells move through tissues, scan for MHC peptide complexes that activate their receptors (TCRs), and are capable of sensing a variety of signals that can alert them against potentially threatening pathogens and 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 [38]. Tumors contain variable numbers of tumor-infiltrating lymphocytes (TILs) and the importance is highlighted by their prognostic value in human cancer [39]. It may seem surprising that there is no consensus in the literature from available preclinical and clinical data regarding the complex cellular mechanisms that mediate tumor killing or rejection. T cells traffic to areas where their target antigens are expressed and can produce cytokines, chemokines, and antiangiogenic 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 [39]. 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 antigen-presenting cells (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 [40]. On the contrary in other tumors, like melanoma, the protective role of TIL is compromised by the high proportion of T regulatory cells (Tregs) that downregulate the activation and expansion of tumor reactive lymphocytes [41].

It has been shown using genetic and histological analysis of a large cohort of colorectal cancer patient biopsies that both the type and 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 [42]. Other studies also show that in some tumors, particularly in colon carcinoma, the presence of TIL 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 [43]. Many studies examining other cancers reached similar conclusions, consequently defining a better picture in which immune infiltrates correlate with improved prognosis or protumorigenic potential [44]. Indeed, increased antitumor response has been shown to correlate with increased leukocyte infiltrate in mice and humans [45–49] and aiming to increase the trafficking of T cells to tumors may 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 tissue, and clonal expansion of antigen-specific T cells [50, 51]. After their generation in lymphoid tissue, antigen reactive T cells need to traffic 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 [52] and the interaction between chemokines, secreted by tissues, with chemokine receptors expressed on the surface of T cells [53–55].

14.4 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 mass such as TIL expanded with T-cell growth factor interleukin-2 (IL-2) *ex vivo*, or genetically engineered T cells has a rich history dating back to several decades ago [56–58]. 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 GVHD. Adoptive T-cell therapy is proven to be an effective treatment for viral infections and has induced regression of cancer in early-stage clinical trials. 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 cells, and Tregs [59, 60].

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. Adoptive transfer of TILs for the treatment of human metastatic melanoma was initially described in 1988 [61] and has since yielded dramatic results since these early days with greater than 50 % clinical responses [62], many of which are lasting for years in recent clinical trials [22, 63–67]. Although ACT with TIL has delivered promising results in phase 1 and 2 trials at the Surgery Branch, NCI, USA [65, 66], 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. Other investigative protocols have 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 alternative for those patients without tumors amenable to surgical resection or patients in whom viable TIL cannot grow in their tumors [68–74].

More recently, other forms of ACT using engineered T cells are being tested clinically. 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) [73, 75], or expressing chimeric antigen receptors (CARs) composed of immunoglobulin variable regions recognizing tumor antigens fused to signaling domains of the TCR and co-stimulatory molecules, such as CD28 and CD137/4-1BB [76, 77]. The pace of research in autologous T-cell-based therapies for melanoma has increased dramatically over the last decade with new target antigens and increased numbers of clinical trials testing both TILs and TCR- or CAR-transduced T cells [78]. 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, 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.

14.5 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 the minority of patients. In addition, although some ACT patients achieve long-term disease free survival, most patients still recur with disease [79]. Furthermore, the requirement of large numbers of laboratory expanded T cells ($>1\times10^{10}$) makes ACT a costly and labor-intensive treatment [80]. 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 [48]. However, this analysis also showed that the trafficking efficiency of transferred T cells was extremely low [81]. 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. Homing of effector T cells to inflamed tissues is thought to depend on various adhesion molecules such as LFA-1 and VLA-4 [49, 82] and also on the activity of specific chemokines [83]. 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 [84, 85]. 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, which 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 major hurdle for tumor-specific T cells to get in direct contact with the target by crossing the abnormal tumor vessel barrier and interstitium [86]. 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 [87]. Thus, leukocyte-vessel wall interactions are diminished in tumors and effector T cells, regardless of being induced *in vivo* by vaccination or adoptively transferred, and are impaired in their deployment at tumor sites where they get in direct contact with target tumor cells. Strategies have been employed in the past to improve immunotherapy by reducing the endothelial barrier that might favor the penetration of both drugs and

improve T-cell infiltration [88] based on the use of angiogenesis inhibitors like anginex, endostatin, and angiostatin [89] or anti-VEGF reagents like soluble chimeric VEGF receptor (VEGFR) [90] and anti-VEGF [91] or VEGFR antibodies [92]. These drugs transiently normalize the tumor vasculature, pruning away immature and permeable vessels and remodeling the remaining vasculature. In the tumor microenvironment, these drugs [87] can also overcome the endothelial barrier by preventing VCAM and ICAM downregulation, therefore promoting leukocyte infiltration in tumors.

14.6 Chemokines

Chemokines were first recognized as a family of small protein molecules, induced by inflammation and capable of attracting inflammatory leukocytes (such as monocytes, activated T cells, and neutrophils) [93]. 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 [83, 94]. 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 [83]. Two functional types have been defined, including the "inflammatory" or inducible chemokines, recruiting effector cells in inflamed tissues and the "homeostatic" chemokines, constitutively produced by lymphoid or nonlymphoid tissues which control leukocyte traffic under physiologic conditions [54, 95–98]. 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 receptor that bind chemokines but do not transmit signals [99, 100]. Though originally identified in the control of leukocyte chemotaxis, especially during infection and inflammation, it is now known that virtually all cells, including tumors, 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 [84, 85, 101, 102]. Recent characterization of various chemokines and chemokine receptors in the immune system has increased our knowledge of the regulatory mechanisms of the immune response and tolerance based on immune cell localization. Tumor cells and the microenvironment constitutively express a variety of chemokines which play a key role in orchestrating the recruitment and positioning of leukocytes, including effector cells with potential antitumor functions. The immune cell recruitment and cell-based systems that can potentially control leukocyte trafficking and their use in cancer immunotherapy are some of the potential areas of focus 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 [85, 96, 103]. CCL2 and related chemokines contribute to polarizing tumor-associated macrophages (TAMs) in a tissue repair/remodeling, promoting tumor growth [104, 105]. 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 [106, 107], as well as CCL5 and CXCL12 [108]. Furthermore, chemokines produced by cancer-associated fibroblasts (CAFs) recruit suppressor cells such as T regulatory cells (Tregs), inducing metastatic progression [109].

14.7 The 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 to boost

immune cells and recruit effectors into the tumor microenvironment and lymphoid tissues [110]. Some of the most promising chemokine networks for cancer immunotherapy are CCL21-CCL19-CCR7 and CCL2, CCL3, CCL5, CCL16, and their cognate receptors. The chemokine receptor CCR7 and its ligand 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 for antigen presentation to naive T cells, which also use CCR7-mediated mechanisms to enter the T-cell zone [111]. 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 [112]. 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 [113]. 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 through its angiostatic effect and by its activation of $CD8^+$ T and possibly NK cell-mediated mechanisms leading to reduced implantation of CCL21 transduced CT26 colon carcinoma cells [114]. Furthermore, CCL19 transduction of murine breast carcinoma cells led to the rejection of tumors in a NK and $CD4^+$ T-cell-mediated manner [115]. 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 [116, 117]. Tumor growth inhibition was significantly better with CCL21-expressing DCs as compared with control DCs or CCL21 alone. Furthermore, CCL21-expressing DCs injected into growing tumors were able to recruit and prime naive T cells by creating a lymph node-like structure within the tumor microenvironment. Curiously, a recent study by Shields et al. found that CCL21 expression by murine B16-F10 melanoma tumors contributed to tumor immune tolerance, while CCL21 negative tumors were found to induce antigen-specific immunity [118]. Dubinett et al. have suggested that this discordant result may be attributed to multiple modifications introduced into the tumor model in addition to overexpression of CCL21 [119].

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 [120–122]. 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 subsequent tumor challenge in multiple preclinical models, as described later by Homey et al. [112]. The role of chemokine receptor CCR5 is studied in T-cell migration post IL-12 treatment that shows upregulation of mRNA expression of CCR5 in splenic T cells as well as ligand for CCR5 such as 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. Furthermore, anti-CCR5 antibody was found to inhibit T-cell migration in the lymphoid cell migration assay. These results indicate a critical role for CCR5 in the induction of T-cell migration to tumor sites after IL-12 treatment [123].

Parker et al. showed enhanced tumor growth inhibition and greater levels of $CD4^+$ and $CD8^+$ T-cell infiltrates in murine flank neuroblastoma treated with sequential treatments of HSV-1 expressing IL-12 and HSV-1 expressing CCL2 when compared with either treatment alone [124]. Furthermore, Nagai et al. demonstrated constitutively secreting

CCL2 human malignant glioma vaccinations in nude mice induced tumor infiltration by NK cells and monocytes [125]. 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 [126]. 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 [127]. 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 [128]. 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 [129]. 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 [130]. 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 JC mammary carcinoma murine models [120].

The CXC chemokines CXCL9 and CXCL10 are considered the main attracting stimuli for TIL, which express high levels of the cognate receptor CXCR3. Increased expression of these chemokines can elicit antitumoral responses correlated with increased infiltration of CD4 and CD8 lymphocytes [131]. The importance of CXCL9 and CXCL10 in the recruitment of TIL at tumor site is also supported by observations in human tumors characterized by the abundance of TIL, such as gastric and colorectal carcinoma [132, 133]. In these tumors, TIL predominantly expresses CXCR3, and significant levels of CXCL9 and CXCL10 are produced by stromal cells, mainly macrophages. TIL can be recruited through the production of CX3CL1. CX3CL1-overexpressing neuroblastoma cells are capable of inducing migration, adhesion, and IFN-γ secretion by immune effector cells [134]. High expression of CX3CL1 was positively correlated with good prognosis and the number of TIL in colorectal carcinoma [135]. CXCL16 can also contribute to the recruitment of TIL in carcinomas. CXCL16 was found overexpressed by reactive astrocytes and glioma cells [136], neuroblastoma, pancreatic ductal adenocarcinoma [137], and breast carcinoma [138]. It has been reported that ionizing radiation therapy markedly enhanced CXCL16 secretion by mouse and human breast cancer cells, which recruited $CXCR6^{+}$ve effector cells [139]. CXCL16 has been described as a positive prognostic marker in renal [140] 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 [141]. On the contrary in prostate cancer CXCL16 expression has been correlated with poor prognosis [142].

However, chemokines attracting different types of cells to tumor microenvironment 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 to facilitate tumor growth and metastasis, suppressing antitumor immune responses, regulating angiogenesis, and influencing the formation and spread of metastases [85, 97]. Expression of chemokines by tumors may also have immunomodulatory effects resulting in decreased immunogenicity of tumor [143, 144] or desensitization of chemokine receptors on T cells [145]. 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 [146]. 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 [147]. In the tumor microenvironment, CXCL12 functions as an antiinflammatory 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 [148, 149]. CCL2 and CCL3 have been shown to increase the infiltration of Tregs, myeloid-derived suppressor cells (MDSCs), and TAM [150–153]. Furthermore, Foxp3$^+$ regulatory T cells migrate to the paracortical areas of peripheral lymph nodes in a CCR7-dependent manner [154].

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 prove especially effective.

14.8 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 dramatic results in some cancers. However, T cells need to traffic properly into tumors to adequately exert therapeutic effects. One approach to improving 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/or 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 [155].

Furthermore, after T-cell activation, the chemokine receptor expression can be transiently modulated, thus acquiring new migratory capacities [95, 156]. Engineering T cells by methods such as introduction of chimeric antigen receptor or introduction of co-stimulatory signal gene has yielded dramatic results in adoptive T-cell-based cancer immunotherapy. Likewise, introduction of chemokine receptor gene into T-cell engineering is also 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 which produce CXCL12, the ligand for CXCR4 [157]. 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 [158, 159].

The published data regarding overexpression of chemokine receptors on T cells directing antitumor effector T cells to tumor sites are scarce. It was found, for example, that CCL2 and CCR4 play a role in T-cell chemoattraction by melanoma *in vitro* [160] and that tumor infiltration of T cells is strongly associated with high CXCL9 and CXCL10 expression in melanoma in in situ hybridization studies [161]. CXCL12 is shown to enhance T-cell migration toward melanoma *in vitro* [162], but also cause chemorepulsion in other systems [163]. 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

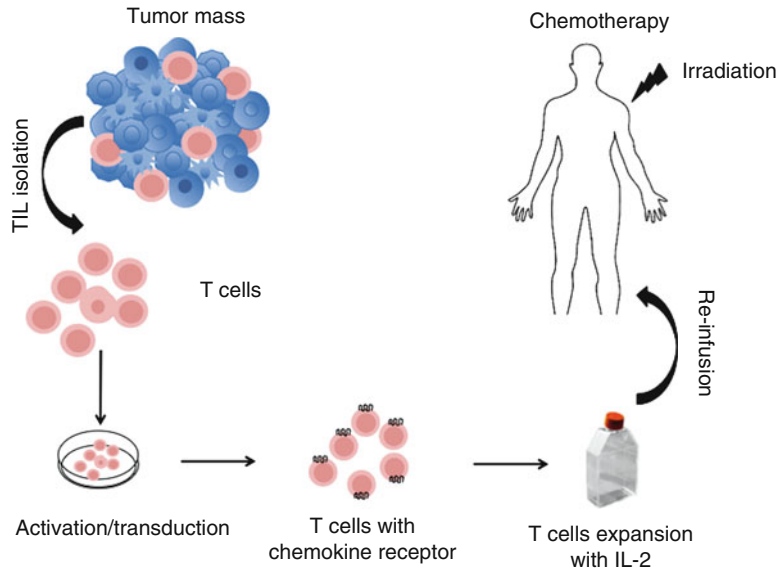

Fig. 14.1 Schematic representation of adoptive T cell transfer therapy using T cells genetically modified with chemokine receptor. Tumor mass is excised from the patient and TILs (tumor-infiltrating lymphocytes) are isolated from the tumor. TILs are transduced with the chemokine receptor matching with the ligand abundantly produced by tumor cells. Chemokine receptor positive T cells are expanded in cell culture using medium enriched with IL-2. Expanded modified T cells are infused back into patient to have better homing potential and effective tumor cell killing

CCL19, are produced [164]. The expression of chemokine receptors by T cells and chemokines at sites of antigenic challenge determine the specific traffic of lymphocytes. For example, the ligands for CXCR3, CXCL10, and CXCL9 [165], which can be expressed by activated monocytes, fibroblasts, keratinocytes, and endothelial cells [166], may enable cells bearing CXCR3 to traffic preferentially to IFN-γ producing inflammatory sites. Though, 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* 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 [167], but its receptor, CXCR2, is expressed only in a small subset of T cells [168]. 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, and therefore, 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 [159].

Several approaches have been applied to find out the mechanism of unsuccessful migration and homing of effector T cells into 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 more effectively recruited human $CD8^+$ effector T cells 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 [169]. The majority of tumors, including neuroblastoma, produce the chemokine CCL2. In one recent study, it has been shown that forced coexpression of chemokine receptor CCR2b, along with chimeric antigen receptor specific for the tumor-associated antigen GD2, enhanced the tumor trafficking of activated T cells [170]. 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 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) proves 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 tumor-infiltrating lymphocytes (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 is validated by the enhanced migration of CXCR1 engineered TIL cells toward melanoma or recombinant CXCL8. Cytotoxicity and interferon secretion activity are unaltered by CXCR1 expression profile. Taken together, these results mark CXCR1 as a candidate for genetic manipulations to enhance the trafficking of adoptively transferred T cells [171]. 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 chemokine CXCL1 and CXCL8 [80]. 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 effector T cells in breast tumor tissue and impaired tumor regression [139]. 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 [172].

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* [173]. Identifying the mechanisms of trafficking as well as suitable chemokine and chemokine receptor pair that can enhance the tumor trafficking and migration of adoptively transferred T cells with antitumor effect is therefore of the utmost importance in optimizing therapeutic benefits.

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 $^{+}$ve T cells [174]. 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 which produces 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.

One potentially interesting chemokine 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 [134]. The role of this chemokine/receptor pair CX3CL1/CX3CR1 has been well established in glioblastoma multiforme, an aggressive tumor of the central nervous system, and in the adenocarcinoma of the pancreas [175, 176] and is now being investigated extensively in colorectal cancer. Recent studies by our group show the overexpression of Fractalkine in colorectal cancer assessed in human clinical samples [177]. 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 [178]. High expression of CX3CL1 was positively correlated with good prognosis and the number of TIL in colorectal carcinoma [135]. 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 [179]. The choice of the chemokine receptor CX3CR1 to enhance the homing potential of adoptively transferred T cells is currently being studied in mouse tumor models.

14.9 Concluding Remarks

Several strategies were introduced to enhance the efficacy of ACT [180]. The development of targeted small molecules, mAbs, and biological therapies that demonstrate greater efficacy and lower toxicity remains highly desirable in hematology and oncology in general. In the context of biological therapies, T lymphocyte-based treatments have enormous potential. 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 [181]. To exert a therapeutic effect, adoptively transferred tumor-specific cytotoxic T lymphocytes must traffic to sites of tumor burden, exit the circulation, and infiltrate the tumor microenvironment. This can be addressed with the idea that chemokines play a major role in antitumor immune responses. As such, they hold great potential in cancer immunotherapy for increasing immune cell infiltration of the tumor microenvironment to facilitate productive immune interactions. Studies in experimental tumor models and cancer patients clearly demonstrate the potential of chemokine immunotherapy and suggest that future trials should seek to incorporate chemokines into therapy protocols. The possibility of developing novel strategies aimed at improving T-cell homing to tumors used alone or in combination with current regime of adoptive T-cell therapies against cancer, such as introduction of antigenic receptor or signaling molecules, may prove to be more efficient

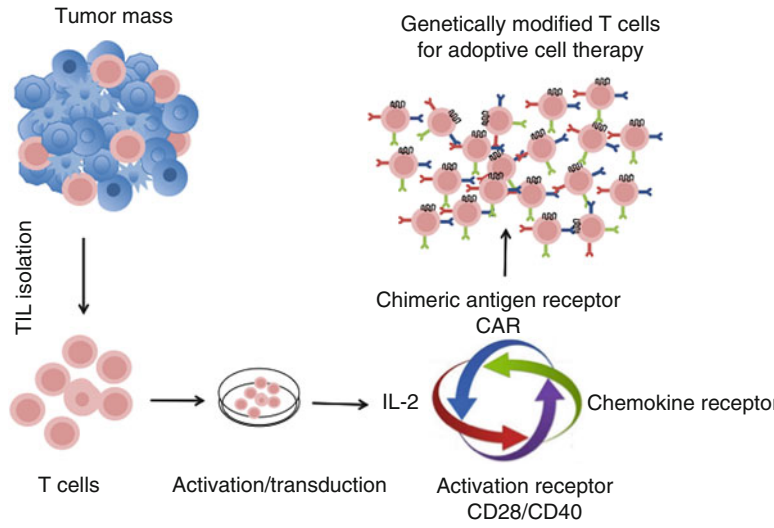

Fig. 14.2 Optimizing adoptive T cell transfer therapy using T cells genetically modified with multiple factors. Tumor mass is excised from the patient and TILs (tumor-infiltrating lymphocytes) are isolated from the tumor. TILs are transduced with the chemokine receptor matching with the ligand abundantly 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 expanded in cell culture using medium enriched with IL-2. Expanded modified multifunctional T cells are accessed further for their antitumor activity in ACT

and holds great promises in several other oncological settings (Fig. 14.2).

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B Cell Regulation of Antitumor Response

15

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15.1 Introduction

Infiltration of human tumors with B cells has been well described, and significant B cell infiltration is often seen in tumors with ovarian, breast, and, more recently, lung origin [1–3]. In addition, antibody development against a variety of neoantigens expressed in tumors such as HER2/NEU, p53, and others has been well described, and these B cell-mediated antibody responses have not generally been associated with a meaningful immune response [4].

Our laboratory, as well as others, has described a variety of murine tumors, which grow well in wild-type mice but poorly in B cell-deficient mice (BCDM) [5–9]. B cells were observed to dampen the antitumor immune response in the setting of spontaneous mammary adenocarcinoma (TS/A) as first reported by Qin and Blankenstein [5], and the enhanced response in BCDM was attributed to B cell suppression of antitumor immunity. The authors have first described B cell-mediated inhibition of antitumor responses using the MC38 colon carcinoma model. In this model, MC38 tumors could be successfully implanted in wild-type mice but were observed to consistently regress in BCDM. Subsequent investigation demonstrated that the reason for regression was an augmented T cell response to MC38 in BCDM and that partial restoration of the B cell population by adoptive transfer would restore tumor growth [6]. Impaired growth of tumors in BCDM was

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accompanied by significant increases in CD8⁺ T cell and NK cell infiltration into tumors, as well as enhanced adaptive tumor response manifested as increased cytotoxic T lymphocytes (CTL) [6–8]. Other investigators demonstrated similar findings for a variety of murine tumors such as the EL-4 thymoma and the D-5 mouse melanoma [9]. We demonstrated qualitatively similar results in the murine mammary tumor model EMT-6 (Fig. 15.1, [7]).

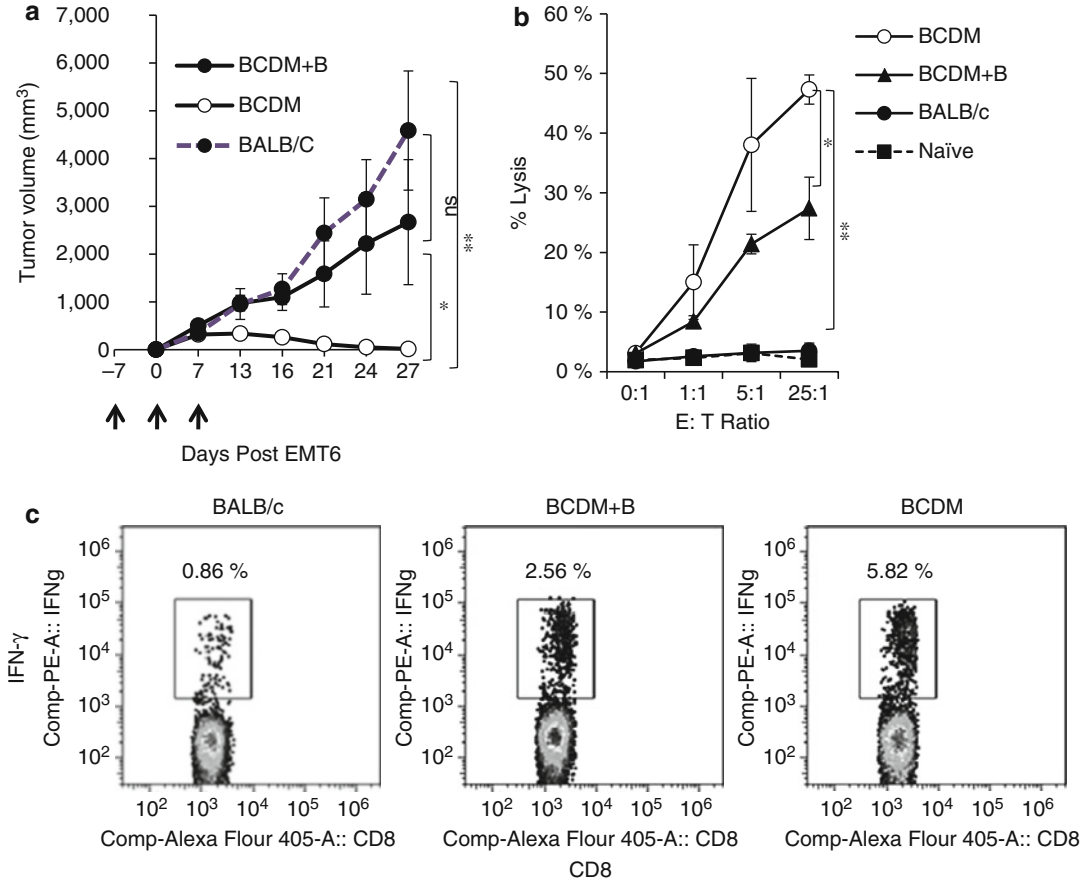

Fig. 15.1 Tumor growth, CTL activity, IFN-γ expression; a comparison in B cell-depleted mice (*BCDM*), B cell-reconstituted BCDM, and immunocompetent wild-type (WT) mice. Mice were subcutaneously injected with 10^6 EMT-6 mammary tumor cells. For B cell-reconstituted BCDM, adoptive B cell transfers were done at day −7, 0, and +7. Spleens were harvested and processed 30 days post implantation. (**a**) Tumor growth following EMT-6 injection: five mice/group, mean tumor volume ± SEM. *$p<0.05$; **$p<0.01$. (**b**) CTL assay: EMT-6 tumor cells were treated with mitomycin C then cocultured for 7 days with splenocytes at an 8:1 splenocyte:tumor ratio. Using Lympholyte-M (Cedarlane), splenocyte effector cells were harvested then cocultured with mitomycin-C-treated Cr^{51}-labeled EMT-6 at indicated E:T ratios for 4 h. Four mice/group, mean ± SD and ^{51} chromium release measured and percent lysis calculated. (**c**) Expression of IFN-γ in CD8⁺ T cells from representative mice in each group. CD8⁺ T cells were purified from splenocytes 30 days post tumor implantation then treated with PMA/ION for 4 h in the presence of GolgiStop (BD Pharmingen) and stained for CD8 followed by intracellular IFN-γ staining and analyzed by flow cytometry. (**d**) CD45+ tumor-infiltrating lymphocytes in tumor tissue from B cell-depleted mice (*BCDM*), B cell-reconstituted BCDM, and immunocompetent wild-type (WT) BALB/c mice with or without anti-CD25 antibody (PC61) treatment (PC61 administered on day −7 and day 0 relative to tumor implantation in treated group) were analyzed for CD4, CD8, CD49b, CD19, and Foxp3 expression. After 30 days post EMT-6 tumor implantation in each group with or without PC61 treatment, tumor was digested using Collagenase D/DNase. Dead cells, tumor cells, and red blood cells were removed using Histopaque-1077 (Sigma-Aldrich). Representative flow cytometry data for mice in each treatment group is shown using antibodies for CD8⁺ T cells, NK cells (CD49b), and/or CD19 + T cells as indicated [7] (Adapted from Zhang et al. [7] with kind permission from Springer Science and Business Media)

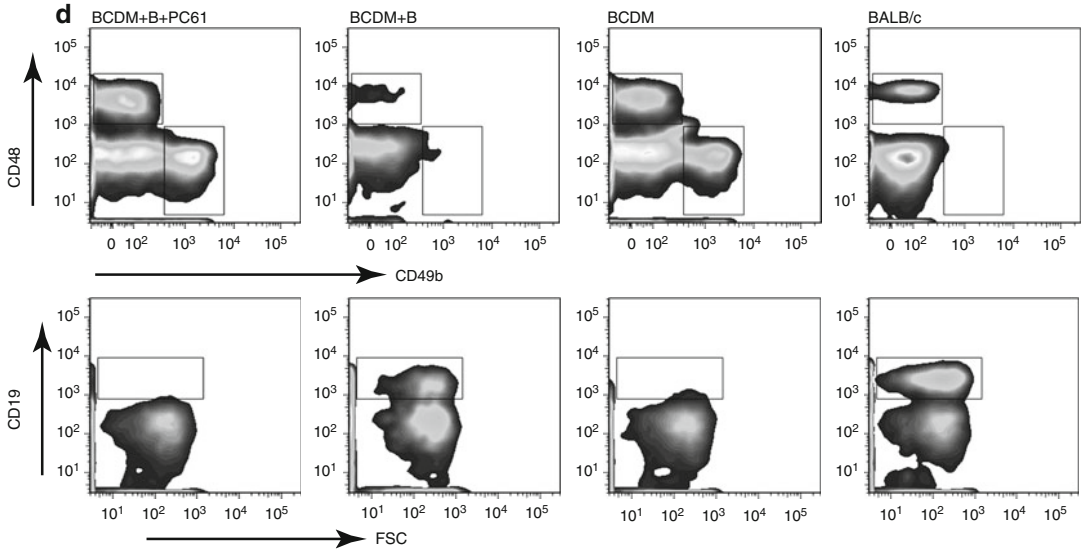

Fig. 15.1 (continued)

B cells can also modulate responses to vaccination in several murine tumor models. Using a secreted gp96-Ig heat shock protein-based vaccine in the LLC-OVA tumor model, Podack and coworkers demonstrated that rejection of established tumors required frequent and repeated vaccination in the presence of B cells, but in the absence of B cells, a single vaccine administration could elicit antitumor response resulting in rejection [10, 11]. Similarly, in a model for immunization with melanoma-associated antigens, using adenovirus encoding gp100 or murine TRP-2, single immunizations were incapable of inhibiting tumor growth in wild-type mice but could prevent the growth of B16 tumors in BCDM [12]. B cells may also serve to modulate and suppress antitumor effects of cytokines such IL-15. BCDM showed complete recovery in response to a combination treatment with cyclophosphamide and IL-15, whereas wild-type mice showed partial responses [13]. Collectively, these observations suggest a qualitative difference between BCDM and wild-type mice, which results in an impaired antitumor immune response in wild-type mice relative to BCDM, due to the suppressive effects of B cells.

In other studies, B cells were observed to play a critical role in the pathogenesis and development of tumors in response to chemical carcinogenesis. In a study using 7,12 dimethylbenz(a)anthracene (DMBA) and tetradecanoyl phorbol acetate (TPA) as a means of inducing papillomas in the skin of mice, it was found that both B cells and TNF-α were required for full-blown development and induction of papillomas. Transfer of normal B cells from DMBA-TPA-treated wild-type mice to TNF-α (−/−) mice would restore papilloma development to levels seen in the absence of B cells. B cell transfer from TNF-α (−/−) mice did not mediate this effect. In this model, resistance to papilloma development in TNF-α (−/−) mice was associated with an increase in interferon gamma (IFN-γ)-producing $CD8^+$ T cells, as well as a reduction in IL-10-producing B-regulatory cells [14]. Hence, in that model B cells appear to be critical for tumor development and carcinogenesis, and the effects of B cells in modulating antitumor immunity appear to be complex. The relationship between B cell-mediated secretion of TNF-α and carcinogenesis is unclear; nonetheless, it is interesting to speculate that B cells fostered both an inflammatory environment and immunosuppressive aspect conducive to the development of papillomas. Collectively, it would appear that B cells play an important role in modulating antitumor responses in several murine systems.

The role of B cells in carcinogenesis may be complex. In a model of inflammation-associated epithelial carcinogenesis, in K14-HPV16 mice, the progression of malignancy appears to be dependent upon B lymphocytes [15]. Adoptive

transfer of B lymphocytes and/or serum from HPV16 mice into B cell-deficient HPV16 mice restored progression from premalignancy to frank malignancy. The role of B cells in this case was somewhat unclear; however, more extensive innate immune cell infiltration was noted in premalignant tissue in the presence of B cells, which appears to enhance the establishment of a chronic inflammatory state, required for initiation of carcinogenesis. The fact that carcinogenesis was impeded by the absence of B cells suggests that in this model, B cells may either have been required for the establishment of chronic inflammatory state or, alternatively, the presence of B cells allowed the progression of carcinoma, perhaps due to its inhibitory effects on an adaptive immune response. Hence, the role of B cells may be complex and context dependent relating to specific mechanisms of carcinogenesis and immunogenicity of resultant tumors [16].

15.2 Mechanisms Underlying B Cell Modulation of Antitumor Immune Response

A variety of mechanistic explanations have been provided to explain observations of decreased antitumor immunity in the presence of B cells. One explanation that has been considered is that the presentation of tumor antigens by B cells may favor Th2-type T cell responses while diminishing Th1 response. Conversely, in the absence of B cell antigen presentation, T cell responses may tend toward Th1 response [17, 18]. Why B cell presentation of antigen may favor Th2-type responses is still a matter of speculation.

B cells may also differentiate along pathways in which they preferentially secrete Th1- or Th2-type cytokines. B cells which preferentially secrete IFN-α and IL-12 tend to augment Th1 response and have been labeled as Be1 cells, while B cells secreting IL-4 and IL-5 have been designated Be2 cells [19] and tend to support Th2 differentiation. Differentiation along Be1 and Be2 pathways has been well demonstrated following infection with pathogens that preferentially induce Th1- or Th2-type immune responses [20]. IL-4 and IL-4Rα receptor expression on B cells may result in preferential Be2 differentiation [21]. Be2 cells may skew differentiation along with Th2 pathways through elaboration of Th2-type cytokines. Whether tumor-infiltrating B cells are differentiated along Be1 or Be2 pathways in murine systems remains to be elucidated; nonetheless, it is conceivable that Be1 or Be2 differentiation could serve as a means of modulating antitumor immune responses by B cells, as has been observed in relation to several pathogens.

15.3 B Cells and the Role of Tregs

$CD4^+CD25^+FoxP3^+$ Tregs have increasingly been identified as a means of suppressing $CD8^+$ T cell responses. We and other laboratories have demonstrated that B cells may partially regulate the expansion of $CD4^+CD25^+FoxP3^+$ T cells in both autoimmune and tumor settings [8, 22–28]. For example, in response to an antigenic challenge with ovalbumin coupled to a cholera toxin B subunit, the combination of B cell antigenic presentation and B cell elaboration of TGF-β and IL-10 appeared to induce Treg expansion and proliferation [23]. In another model using myelin oligodendrocyte glycoprotein (MOG) peptide as an antigen coupled with cholera toxin B, a similar expansion of Tregs was noted and served to suppress the development of experimental autoimmune encephalomyelitis [29].

In the EMT6 murine mammary tumor model in our laboratory, increased numbers of Tregs were noted following tumor inoculation in both BCDM and wild-type mice, and Tregs obtained from wild-type mice appear to have enhanced inhibitory function relative to Tregs isolated from BCDM suggesting conditioning of Treg function by B cells. Adoptive transfer of B cells into BCDM resulted in a marked increase of $CD4^+CD25^+FoxP3^+$ Tregs compared to BCDM. The increase in Tregs correlated with enhanced tumor growth following B cell reconstitution [7, 8]. Interestingly, depletion of Tregs using an

anti-CD25 antibody abrogated the growth of tumors despite the adoptive transfer of B cells into BCDM. Increased tumor growth was associated with diminished $CD8^+$ T cell cytolytic response, as well as a decrease in $CD8^+$ T cell and NK cell infiltration into tumors (Fig. 15.1). As previously mentioned, reconstitution with IL-10(−/−) B cells also facilitated EMT6 growth; hence, this Treg expansion and function did not appear to be contingent on B cell elaboration of IL-10 [7]. It would therefore appear that a variety of B cell regulatory subsets may affect response depending upon the tumor context and the overall nature of the immune response.

Other molecules that have been implicated in playing an important role in the development of Tregs are GITR and GITR ligand (GITR-L), costimulatory molecules belonging to the TNF superfamily. A variety of immune effector cells including B cells, NK cells, and $CD8^+$ T cells, as well as Tregs express GITR following activation. GITR-L is expressed on a variety of cells including B cells and dendritic cells, endothelial cells, and others in a mouse [30]. In a mouse model of experimental autoimmune encephalomyelitis, B cells appear to regulate the number of $CD4^+CD25^+FoxP3^+$ Tregs in the central nervous system through GITR-GITR-L interactions [31]. It is not known whether GITR-L expressing B cells also play an important role in the regulation of antitumor immunity.

A variety of investigators have demonstrated important regulatory role for B cells in autoimmune diseases including rheumatoid arthritis, systemic lupus erythematous (SLE), and inflammatory bowel disease [32–35]. In many of these systems, B-regulatory cell function appears to be mediated by IL-10. IL-10-producing B-regulatory cells (B10) were initially described in mice by Tedder and his group [36]. A variety of immunophenotypic markers serve to distinguish the $IL\text{-}10^+$ subset. The so-called B10 cells appear to be important negative regulators of autoimmunity in mouse disease models. In a murine model for EAE, adoptive transfer of B10 cells prevents onset of disease [36]. B10-like cells have also been implicated in SLE in man [37]. The B10 subset appears to be able to skew T cell differentiation away from the Th1 pathway and has been implicated in the pathogenesis of SLE. However, in several murine tumor models, IL-10 appears to be less important for B cell-mediated immune suppression, and whether B10 cells have played a role in immune suppression in human tumors remains speculative.

A variety of other B cell subsets with regulatory function have been described including $CD1d^+$ marginal B cells, transitional marginal and precursor B cells as well as $CD5^+$ $CD1d^+$ cells [32]. Gallipeau and coworkers also described the induction of regulatory B cell subset capable of attenuating autoimmune encephalitis in a murine model through administration of a GM-CSF-IL-15-fused cytokine or fusokine [38].

In addition to IL-10, TGF-β has been implicated in Breg function. B cells have also been implicated in the suppression of allergic airway disease and induction of inhalational tolerance through elaboration of TGF-β [39]. As in our EMT6 murine tumor model, IL-10 did not appear to figure prominently in induction of inhalational tolerance. Another mechanism of inhibition of immune response has also been described involving secretion of IgG linked to LAP-TGF-β. This unique fused form appears to reduce CTL response [40]. This mechanism, only recently described, has not yet been observed in the context of malignancy.

In another tumor model, metastatic disease of the lung following orthotopic implantation of mammary carcinoma cells also appeared to be dependent on TGF-β elaborated by B-regulatory cells. The B-regulatory cell population appears to be evoked by tumor implantation and resembled a subset of B cells called B2 cells ($CD19^+CD25^+CD69^+$) [41]. These tumor-evoked Bregs were able to support conversion of $CD4^+$ T cells into Tregs, which were $FoxP3^+$. Interestingly, in the absence of tumor-evoked Breg, these investigators did not see metastatic disease into the lung in part due to lack of Tregs [41]. Recently, other investigators have demonstrated significant Treg and B cell infiltration into human squamous cell

carcinomas of the lung [1]. Whether similar mechanisms are operative relative to those encountered in the aforementioned murine model is not clear. In the EMT6 model, a very small percentage of tumor-infiltrating B cells express CD25, suggesting that B cells may differentiate differently or that an alternative B cell subset is involved in attenuating the immune response.

15.4 B-Regulatory Cell Infiltration into Human Tumors

In contrast to extensive data associating Treg infiltration with immune suppression and prognosis in human tumors, the prevalence and extent of B cell infiltration in human tumors have been poorly characterized to date. Extensive B cell infiltration has been described in ovarian carcinoma and lung cancer, as well as in squamous cell carcinomas of the oropharynx, and correlated to poor outcome [1–3]. Interestingly B cell infiltration into tongue lesions appears to correlate with progression from early hyperkeratosis to frank carcinoma of the oropharynx [2]. In advanced renal cell carcinoma (RCC), B cell infiltrates have been described which appear to be out of proportion to the relative number of B cells circulating in the peripheral blood [42]. B cell infiltration is thought to potentiate a Th2 response, in the case of RCC.

Despite the immune suppressive attributes of tumor-infiltrating B cells, other human tumors such as medullary carcinomas of the breast may carry a good prognosis in relation to B cell infiltration [43, 44]. Reasons behind extensive B cell infiltration in medullary breast carcinoma have not been elucidated, and whether the presence of B cells is critical to ongoing growth of the tumor regardless of prognosis has also not been established.

Recently, Ganesan et al. have described significant infiltration of human non-small cell lung carcinoma (NSLSC) with $CD4^+CD25^+FOXP3^+$ Tregs [1]. The investigators also described significant infiltration with $CD20^+CD19^+HLA\text{-}DR^+$ B cells. Although no immune suppressive benefit has been attributed to B cell infiltrates, it is tempting to speculate that they may play an immune suppressive role, either directly as seen in murine tumors or through the support and expansion of Tregs.

15.5 Breg Function in Non-Hodgkin Lymphoma

While the role of Bregs has been extensively characterized in autoimmune diseases, the role in malignant disorders is less clear. Since malignant B cells retain many of the underlying characteristics of normal B cells, it is reasonable to assume that they may invoke B-regulatory cell pathways to suppress T cell responses. Malignant B cells may be involved in multiple pathways that inhibit antitumor immune response. To facilitate their own development and avoid immune elimination, lymphoma B cells may target the balance of T cells, recruiting and expanding Tregs while inhibiting killer $CD8^+$ T cells and helper T cells [45, 46]. It is known that antitumor response in some B cell non-Hodgkin lymphoma (NHL) is profoundly suppressed by the presence of large numbers of intratumoral Tregs [47]. Malignant B cells may alter the overall balance of Tregs and so-called Th17 cells [45, 48]. Skewing the balance toward Treg activity may facilitate tumor survival. It appears that a reciprocal regulatory relationship may exist between Tregs and Th17 cell numbers. $B7\text{-}H1^+$ Tregs have been known to infiltrate B cell-derived NHL, thereby inhibiting the proliferation of T cells in a B7-H1 (also known as PD-L1 or CD274)-dependent mechanism [48]. Samples from NHL patients were observed to have significantly lower $CD4^+$ IL-17-producing T cells compared with samples from patients with benign hyperplastic lymph nodes. In the absence of lymphoma B cells, treatment with IL1-β/IL-6 or lipopolysaccharide (LPS) enhanced IL-17 expression in $CD4^+$ T cells; nevertheless, this enhancement was attenuated when $CD4^+$ T cells were cocultured with lymphoma B cells [45]. In the presence of lymphoma B cells, Th17 cell generation was inhibited. Conversely, depletion of lymphoma B cells using anti-CD19 antibody resulted in the enhanced generation of

IL-17-producing T cells by IL1-β/IL-6. Both IL-1-β and IL-6 delivered alone or in combination increased the number of $CD4^+$ IL-17-producing cells [45].

Lymphoma B cells may also contribute to the expansion of Tregs, thereby leading to the attenuation of $CD8^+$ response [46]. When intratumoral Tregs were cocultured with infiltrating activated $CD8^+$ T cells, $CD8^+$ cytotoxic activity against lymphoma B cells was significantly inhibited when compared to infiltrating activated $CD8^+$ T cells alone. Conversely, cocultures of infiltrating activated $CD8^+$ T cells with $CD4^+CD25^-$ T effector cells did not have a significant effect on cytotoxic activity when compared to infiltrating activated $CD8^+$ T cells alone [46]. Hence, B cells may skew responses due to their effects on Th17 and Treg generation. This, in turn, may affect levels of $CD8^+$-mediated cytotoxic response. The prognostic significance of Th17/Treg balance remains to be established.

A mechanism by which lymphoma B cells induce Treg expression has recently been described. Stimulated by TGF-β and IL2, $CD4^+CD25^-$ cells can convert into $CD4^+CD25^+$ Tregs and express Foxp3 [49–52]. Non-Hodgkin lymphoma B cells were also found to induce the expression of Foxp3 in $CD4^+CD25^-$ cells [53]. Additionally, follicular lymphoma B cells have been shown to produce IL-12, which in turn can promote T cell immunoglobulin and mucin protein 3 (TIM-3) expression on intratumoral T cells. TIM-3 has been characterized as a marker of T cell exhaustion and functional impairment. The observation of high levels of serum IL-12 and increased numbers of $TIM\text{-}3^+CD4$ and $TIM3^+CD8^+$ T cells has been correlated to worse outcome in patients with follicular B cell NHL [54].

In conclusion, malignant lymphoma B cells may actively support the development of Tregs and may also inhibit Th17 generation. Lymphoma B cells have also been demonstrated to facilitate the production of IL-12, which can in turn lead to T cell exhaustion as identified by TIM-3 expression. These results require confirmation but clearly suggest a direct role for malignant B cells in suppression of antitumor response in man.

15.6 Effects of Depletion of B Cells on Antitumor Immunity

Selective depletion of B cells has been employed as a therapeutic maneuver in the context of both autoimmune disease and lymphoma. The chimeric anti-CD20 monoclonal antibody (mAb) rituximab was the first of several antibodies directed at CD20 antigen associated with B cells approved for human use [55]. Rituximab appears to mediate depletion of normal and memory B cells in patients with autoimmune disease, as well as malignant B cells in the context of lymphoma. The mechanism of action of rituximab is thought to depend primarily upon antibody-dependent cellular cytotoxicity (ADCC); however, under conditions of high antibody density, rituximab can fix complements as well and mediate complement-dependent cytotoxicity (CDC). Rituximab is also thought to engender delayed T cell responses, and this property is felt to relate to Fc receptor-mediated immunization [56]. B cell depletion using rituximab has been successfully employed in a variety of autoimmune diseases. Interestingly in some cases, depletion of pathogenic B cell populations has been shown to be effective in the setting of autoimmune disease such as SLE or rheumatoid arthritis [57–59]. To understand this observation it is important to note that some $CD20^-$ B cell subsets have been implicated as possessing B-regulatory cell activity and may be enriched following rituximab depletion of $CD20^+$ B cells [60]. It is also conceivable that various B cell subsets may be affected differently by rituximab-mediated B cell depletion and that this might result in variable effects. In animal models, B cell depletion using anti-CD20 antibodies is only partially effective, with less than complete depletion of B cells observed in the spleen and bone marrow compared to blood and lymph nodes [61].

Response to B cell depletion is by no means uniform. In a B16 melanoma model depletion of mature B cells with anti-CD20 actually exacerbated tumor progression [62]. Our laboratory has observed little or no effect of murine $CD20^+$ B cell depletion in either the MC38 or EMT6 models despite the fact that neither tumor grows well

in BCDM and that growth is restored by adoptive B cell transfer. It is unclear whether CD20-based B cell depletion may actually deplete immunosuppressive Breg subsets or may spare such subsets. Additional strategies for depletion of B cells including other anti-CD20 antibodies such as ofatumumab or anti-CD22 and/or anti-CD19 antibodies may be useful. More effective strategies will likely require more selective phenotypic characterization of Bregs.

15.7 Concluding Remarks

A variety of immune suppressor cells have now been identified which may play a role in the suppression of immunity in both murine and human setting. Examples of such cells include myeloid suppressor cells, tumor-associated macrophages, and $CD4^+$ Tregs. However, the potential role of immunosuppressive B cells is not well appreciated. A variety of mouse tumor models demonstrate a role for B cells in modulating both innate and Th1 responses in a manner that facilitates tumor escape from immunosurveillance. Examples from both autoimmune and malignant disease suggest that B cell subsets may provide immunosuppressive co-stimulatory ligands such as PDL-1 (also known as B7-H1) or ICOS-L and/or cytokines such as IL-10 and TGF-β which may serve to downregulate the adaptive immune response. Significant B cell infiltration has been noted in a variety of human tumors including breast, lung, and ovarian cancers often accompanied by large numbers of so-called Tregs. Evidence from animal models suggests that B-regulatory cells may play an important supportive role in the expansion of the Treg population, thereby leading to immune suppression and tumor growth. Further characterization of B cell subsets with suppressor regulatory properties may allow improved design of immune strategies incorporating B cell depletion to augment antitumor responses in men.

Finally, an increasing body of data supports the notion that many human B cell malignancies may also directly suppress antitumor immunity by virtue of expansion of the Treg population or through direct effects on $CD8^+$ T cells leading to T cell exhaustion. Additional evidence suggests that malignant lymphoma cells may skew immune responses toward Treg generation due to the reciprocal regulatory relationship between Treg and Th17 responses. Altering the balance of Tregs and Th17 cells may play an important role in the genesis of immune tolerance to malignant B cells and in dampening beneficial immune responses. Recent evidence supports a direct role for B cells in the suppression of antitumor responses. Better characterization of Breg function in lymphoid malignancies and solid tumors may yield new means of augmenting antitumor immune response in the clinic.

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Monoclonal Antibodies for Cancer Immunotherapy

16

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16.1 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. Apart from the continuously growing number of 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.

16.2 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) held together by covalent (disulfide) and non-covalent bonds. 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 (*VH1-CH1*) associated with a complete light chain (V_L–C_L), and accounts for specific binding of Ab 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. 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 [1].

16.3 Natural Antibodies in Cancer

There are currently many mAbs that have been approved for treatment of various tumor types [2]. 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 [3]. 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 [1].

Numerous tumor-specific monoclonal natural Abs have been isolated from either normal individuals or cancer patients [4–6]. An intriguing feature of these Abs is their preferential binding to post-translationally modified carbohydrate Ags that are unique to transformed cells [4, 7, 8]. In fact, by modifying certain carbohydrate structures on their surface, tumor cells try to hide from humoral immune responses [9, 10]. However, this modification renders tumor cells easy targets for naturally occurring Abs.

Heat shock proteins (HSPs) are an example of membrane-associated molecules with glycosylation patterns that are modified in cancer cells. Heat shock proteins serve to preserve the perfect folding of cellular proteins in normal cells [11, 12], and their overexpression or modification functions in favor of tumors causing higher drug resistance and malignancy level [13, 14]. 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 [15], lung [16] and breast [17] cancers. An anti-GRP78 natural Ab, called SAM-6, was isolated from a patient with gastric cancer [18]. 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 [18, 19]. 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 [20].

Post-translational modification in glycosylation patterns has also been reported for decay acceleration factor (DAF or CD55) which serves to protect host cells from complement-associated lysis [21, 22]. 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 [23, 24]. 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 [7, 23, 25–27]. 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 [26, 28, 29].

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 [8, 30, 31]. 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 [8, 30, 31].

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 [32–35]. Injection of certain human cancers with intravenous immunoglobulin (IVIg) has led to favorable antimetastatic results [36–38]. Interestingly, one study reported the existence of anti-NGF natural Abs in IVIg commercial batches. These Abs were able to hinder growth and differentiation of PC-12, a prostate cancer cell line [39]. 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 [40]. 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 [4, 6, 7, 31]. Additionally, tumor Ag-specific natural Abs isolated from normal individuals and cancer patients can be used to indentify 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 [20].

16.4 Finding an Appropriate Antibody Target for Cancer Therapy

16.4.1 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 [41, 42]. 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 [2]. Furthermore, a perfect target should be highly immunogenic [43], 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 [2].

16.4.2 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 16.1) [44, 45].

16.4.3 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.

Table 16.1 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, NSCLC, metastatic breast cancer, glioblastoma, metastatic renal cell carcinoma
	αVβ3	Melanoma and prostate cancer

16.4.3.1 Genomics

Cancer-related alterations in genome include silent mutations (e.g. deletions and insertions) [46, 47], gene amplification [48] and larger scale defects such as chromosomal translocations [49]. Today, gene amplifications or deletions as well as chromosomal translocations are detected using several techniques such as comparative genomic hybridization (CGH) [50, 51] and spectral karyotyping (SKY) [52–54]. Amplification of *HER2* gene is known as the first solid tumor-associated genomic aberration, which led to the successful development of trastuzumab [55].

16.4.3.2 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 [56, 57]. Complementary DNA (cDNA) microarray has been used to identify the frequency of elevated tumor Ag expression, for instance, in acute myeloid leukemia (AML) [58]. In 1995, Velculescu et al. [59] 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-indentified sequences. In contrast, SAGE does not require prior knowledge, and represents an unbiased, comprehensive representation of transcripts [60]. Furthermore, SAGE can quantitatively identify low-abundance transcripts and detect relatively small differences in their expression [61]. Nonetheless, it is expensive and time-consuming [62] and requires relatively high amounts of RNA samples [63].

16.4.3.3 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 [64–67]. 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) [68] 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 [69–74]. 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) [75]; 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) [76]; liquid chromatography combined with tandem MS (LC–MS–MS) (used for phosphoproteomic analysis of HeLa cells at various stages in the cell cycle) [77]; 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) [78]; and isotope tags for relative and absolute quantification (iTRAQe) (utilized for identification of serum biomarkers in metastatic prostate cancer) [79]. 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.

16.4.3.4 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: (i) forward-phase arrays (FPAs) in which Abs are arrayed and probed with cell lysates, and (ii) reverse-phase arrays (RPAs), where cell lysates are arrayed and probed with Abs [80, 81]. 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 [82] or to indentify biomarkers of bladder cancer [83].

Serological expression cloning (SEREX) was developed to combine serological analysis with Ag cloning techniques to identify human tumor Ags that elicit high-titer IgG [84]. SEREX is now being used for screening the sera of patients to

detect a large range of different solid [85–88] and hematological malignancies [89, 90]. Moreover, SEREX in combination with two dimensional polyacrylamide gel electrophoresis (2D-PAGE) technology created a serological proteome analysis (SERPA) technique [91] through which investigators were able to identify melanoma [92], breast [93] and colorectal cancer Ags [94].

16.5 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 [2].

16.5.1 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 [95]. 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 [96]. 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 [97]. Moreover, Pertuzumab, another anti-HER2 mAb, has been shown to bind to a site different from that of trastuzumab and inhibit receptor dimerization [98]. Notably, a combination of trastuzumab and pertuzumab has shown promising antitumor results in preclinical models [99]. Cetuximab, a chimeric EGFR-specific mAb, could inhibit ligand binding and prevent receptor dimerization [100]. Further efforts are underway to target similar molecules such as HER3 and HER4 [101, 102].

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) [42, 103], lung cancer, adenocarcinoma [104] and breast cancer [105]. Ab targeting of this transmembrane receptor by several studies has culminated in tumor cell elimination through the induction of apoptosis and necrosis [106–108]. 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 [109].

16.5.2 Harnessing the Potential Capacity of Immune System to Eliminate Tumors

Due to their indispensible 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. 16.1).

16.5.2.1 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 [110]. 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 [111]. 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 [112].

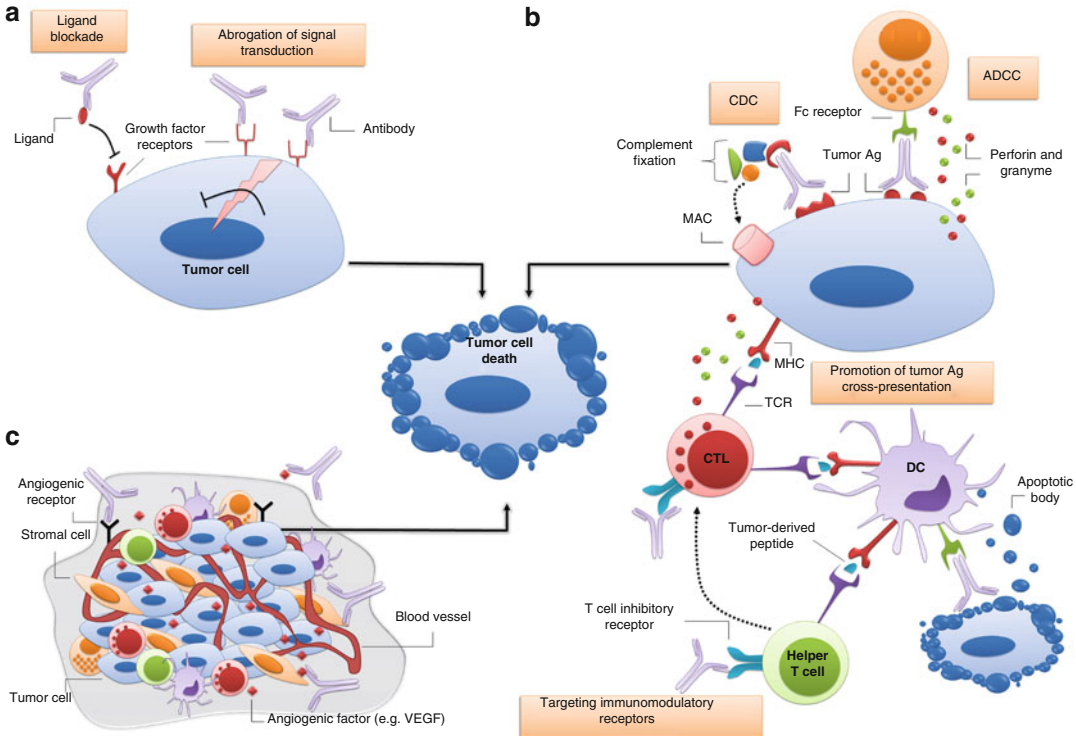

Fig. 16.1 Major mechanisms of tumor cell elimination by monoclonal antibodies. (**a**) Direct elimination of tumor cells is often elicited by abrogation of signal transduction via growth factor receptors (e.g. members of the epithelial growth factor receptor family) and/or blockade of ligand-receptor 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 histocompability complex, *NK* natural killer

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 [113].

16.5.2.2 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. A preclinical therapy model showed that the antitumor impact of anti-CD20 mAb (rituximab) was thoroughly abrogated in C1q-deficient mice [114]. Consistently, complement depletion culminated in decreased protective effect of rituximab in a murine model of human B cell lymphoma [115]. The majority of so far clinically-approved antitumor mAbs have been shown to activate ADCC and the complement pathway.

16.5.2.3 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 [116, 117]. 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 [118], and that cross-presentation was enhanced following the blockade of FcγRIIB, an inhibitory receptor [119].

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 [120]. Secondly, Ab-coated apoptotic tumor cells can be phagocytosed, through FcγRs, and sent to the cross-presentation pathway ending in effective tumor-specific T cell responses [118, 120]. However, one should bear in mind that DCs can mediate both immunostimulatory and immunomodulatory responses depending on the tumor microenvironment [121]. Thus, it is recommended to employ Ab-based antitumor strategies in combination with approaches that target suppressive agents of tumor microenvironment.

16.5.2.4 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 [2]. Therefore, Ab-mediated targeting of receptors on T cells or the ligands of these receptors has received widespread attention by several therapeutic studies.

There is a great deal of evidence confirming the antitumor potency of mAbs blocking T cell inhibitory receptors [122]. Among these receptors, cytotoxic T lymphocyte antigen-4 (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 [123].

Data obtained from preclinical studies has provided the foundation for production of two clinically-approved anti-CTLA-4 mAbs (ipilimumab and tremelimumab). Ipilimumab owes its clinical approval to a pivotal study indicating that treatment with this mAb results in improved overall survival of patients with metastatic melanoma [124]. 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) [124, 125]. Likewise, blockade of another T cell inhibitory receptor (programmed death-1; PD-1) using an anti-programmed death-1 mAb (MDX-1106) has led to favorable antitumor responses [126] and additional PD-1 targeting Abs are being investigated [127, 128].

Antibodies with agonistic effects on immunostimulatory receptors have also been tested in immunotherapeutic settings. A fully human Ab specific to CD137, a T cell activating receptor, has shown encouraging antitumor efficacy in phase I clinical trials [120]. On a cautionary note, high doses of this Ab can result in toxic effects, and studies with lower less toxic doses are currently underway [2]. Encouraging results upon employing Abs with agonistic impacts on CD40 have also been noted in the literature [127].

16.5.3 Targeting Tumor Stroma and Vasculature

Factors that support angiogenesis as well as those that form the extracellular matrix play an indispensible role in tumor survival [129–131]. Therefore, targeting tumor microenvironment has been shown to be of great therapeutic value in preclinical and clinical settings [132].

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) [131]. 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 [133]. Consistently, targeting of VEGFR-1 by a fully human mAb showed favorable preclinical results [134].

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) [135]. 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 treatment of bevacizumab-resistant tumors [136].

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 [137]. 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 [130].

16.6 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, rapid renal clearance and ease of production. Notably, advances in molecular biology has made it possible to go beyond optimization and in fact has created entirely new Ig domain-based structures, not found in nature, which can be tailored to achieve favorable results. This section describes Ab engineering (Fig. 16.2) as a way of generating optimized therapeutic Abs with improved effector functions.

16.6.1 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 [138–142]. However, injection of humans with murine Abs induces the generation of human anti-mouse Abs (HAMA). Not only can these HAMA remove murine Abs upon repeated administrations, but also the formation of antibody-HAMA-complexes has shown to end in mild to severe allergic reactions [143]. Therefore, major shortcomings of intact murine Abs have limited their clinical applications [144].

Although the first mAb approved for clinical applications was a murine IgG2a Ab (OKT3, or muromonab; 1986) [145], 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 has 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 [146–148].

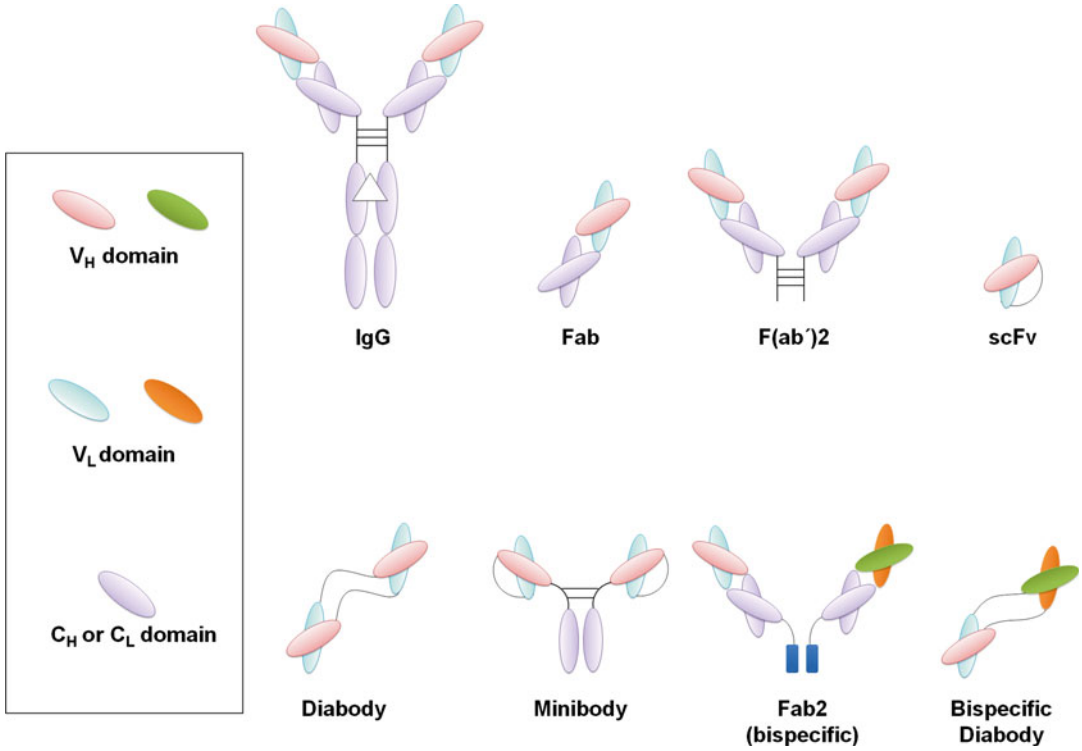

Fig. 16.2 Schematic representation of different antibody fragments with therapeutic applications. Fragment antigen-binding (Fab) and F(ab')2 may be generated by papain or pepsin digestion of intact IgG, respectively. Other types of antibody fragments can be produced using antibody engineering methods. 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

16.6.2 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 chimeric, and humanized mAbs [44, 149, 150]. Chimeric mAbs are produced through grafting the murine variable regions onto human constant regions. 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 [151]. Humanized mAbs, on the other hand, are constructed via engrafting of murine hypervariable Ag binding regions (also named complementarity determining regions (CDRs)) onto human Abs rendering them 85–90 % human, with less immunogenicity than chimeric Abs [151]. 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 in the CDRs of Abs [152]. In fact, the majority of currently approved Abs used in oncological applications, and those used in advanced clinical trials are of humanized construct.

16.6.3 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 [153, 154]. Replacement of mouse Ig variable and constant domains with those of the human effectively reduces the incidence of anti-antibody response (AAR) hypersensivity reaction [155].

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.

16.6.3.1 Human Monoclonal Antibodies from Transgenic Mice

A new approach for 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 [156]. 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 [157]. 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. Panitumumab, ofatumumab and ipilimumab are three fully human anticancer mAbs that have been produced by this technology and approved for use in the clinic (Table 16.2).

16.6.3.2 Human Monoclonal Antibodies Created Through Phage Display Technology

Phage display was first described by George P. Smith [158] in 1985, when he demonstrated that a foreign DNA fragment can be fused to the 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 [146] 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 [159]. Today, this is a well-established technology for 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. 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 [160]. To create fully synthetic libraries, germ line Ab gene segments, VH, DH, and JH or *Vκ/λ* and *Jκ/λ* are cloned and arranged combinatorially *in vitro* to reconstitute genes encoding complete VH and VL chains [157]. Although, currently, there is no FDA-approved anticancer therapeutic mAb produced by phage display technology, several of such mAbs are in clinical development [161].

16.6.4 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 [162]. In fact, tissue penetration is known as a vital parameter in therapeutic settings, and often severely restricts the complete efficiency of the treatment [41, 163]. 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 [164]. 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 [151]. In fact, better renal clearance and improved tumor penetration made such fragments attractive alternatives to the whole Ab molecule for radiotherapy and/or imaging applications [165]. The biodistribution of intact radiolabeled chimeric mAb U36 (125I-cMAB U36)

Table 16.2 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
Tostitumomab 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)

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 [164]. 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 [166].

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 [167]. 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 [151]. Interestingly, the intracellular expression of anti-Ras neutralizing scFv induced cell death in tumor cells expressing oncogenic Ras [168]. In a preclinical *in vitro* study, scFv-PEG-lipid conjugate, as an anti-HER2 liposome-inserting agent, was applied to HER2-overexpressing cancer cells [169].

Diabodies are homodimers of scFvs, covalently linked by a short peptide linker of four amino acids [170]. 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 [171]. Larger fragments such as minibody (scFv-CH3; 80 kDa) [172] and scFv-Fc (110 kDa) [173] 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 [174].

16.6.5 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 simultaneously target two different Ags on the cell surface [175].

These hybrid proteins can be produced using different approaches such as chemical cross-linking, hybrid of hybridoma (also termed as quadromas) [176], and genetic manipulation (the holes and knob technique) [177]. 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 [178].

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 [179, 180]. 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 [181], HER2 [182], CA-125 [183] or CD20 [184]. Such BsAbs have been administrated in the immunotherapy of NHL, breast, ovarian, and prostate cancers. 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 [185, 186]. 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 needs to interact with co-stimulatory molecules such as B7 on APCs,

and this is considered a major drawback to T cell-based modalities [151].

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 [187–189]. These immunotoxins were directed against CD19 and CD22 and showed improved efficacy against murine xenograft models of B cell malignancies and metastases [187–189].

16.6.6 Antibody Fusion Constructs

Antibody molecules in the fusion constructs are generally used to direct therapeutic agents such as toxins [190], cytokines [191], drugs [192], and radioisotopes [193] 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 [194]. In fact, several potent drugs such as auristatins [195] and maytansinoids [196] (inhibitors of microtubulie assembly) or emtansin [197] (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 [198]. Trastuzumab-DM1 has recently been shown to inhibit tumor growth via induction of apoptosis, ADCC and mitotic catastrophe in a trastuzumab/lapaninib (a kinase inhibitor used in breast cancer therapy) resistant murine model, [199].

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 [200–202]. One therapeutic approach has combined a humanized Ab recognizing ED-B (extra-domain B of fibronectin) with IL-12 [203]. This conjugated Ab has been evaluated in a phase I study in malignant melanoma and renal cell carcinoma (RCC) patients [203]. Moreover, Ab-IL-2 fusion proteins have been used in several phase I clinical trials to treat melanoma and neuroblastoma [204–206].

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 (90Y)-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 [207].

16.6.7 Improvement of 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 [208–210]. 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 [211, 212].

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 [213]. 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 [214].

Monoclonal Abs elicit effector functions following interactions of their Fc portion with various Fc receptors [2]. 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 [215]. 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 [215].

16.7 Evaluation of Antibody Efficacy

16.7.1 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 [216, 217]), inhibition of metastasis or angiogenesis (e.g. bevacizumab [218, 219]), induction of apoptosis (e.g. rituximab [220, 221]), and induction of secondary immune functions such as ADCC (e.g. trastuzumab [216, 217] or CDC (e.g. rituximab) [220].

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 [222, 223], and sensitization to chemotherapy [224] or radiotherapy [225].

Choosing the most relevant animal model is a critical step for successful preclinical safety evaluation of a mAb [226–228]. 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 [222]. 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) [229].

16.7.2 Clinical Evaluations

Valuable information on the whole procedure of clinical safety evaluation of mAbs have 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 [223].

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 [130, 230, 231]. This step is essential for predicting Ab toxicity [231, 232], 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 [230] to the first-in-human clinical trials of several anticancer Abs [130, 230, 231, 233]. 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 [234].

16.8 Clinically-Approved Monoclonal Antibodies

At the beginning of the twentieth century, Paul Ehrlich postulated "magic bullet" as a tool for specific targeting of diseases [235]. His hypothesis became practical with the development of an

efficient method for generation of mAbs, in 1975, by Kohler and Milstein [138]. Since then, these molecules have been known as ideal tools for therapy and imaging applications [151]. 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 [236] 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 [153].

In 1982, for the first time, a therapeutic mAb was successfully used to treat B-cell lymphoma patients [237]. Consequently, Ehrlich's magic bullet hit the target by introducing rituximab (1997) and trastuzumab (1998) as the first chimeric and humanized FDA-approved mAbs for cancer therapy, respectively [235]. Since 1997, 13 mAbs including seven mAbs specific to solid tumors and six mAbs specific to hematological malignancies have received FDA approval (Table 16.1). Here, we provide an overview of trastuzumab, and bevacizumab (applied for solid tumors) and rituximab (applied for hematological malignancies) as instances of the most successful therapeutic mAbs in clinical oncology [2].

16.8.1 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 [238–240]. 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 [241]. 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 %), and a longer duration of response (median, 9.1 *vs*. 6.1 months) and survival (median, 25.1 *vs*. 20.3 months) [242]. 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 [243].

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 [238–240]. 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 [244, 245]. Moreover, acquired trastuzumab resistance is a serious concern ending in disease progression [245, 246]. Notably, due to HER2 expression on cardiomyocytes, cardiac toxicity issues such as symptomatic congestive heart failure has been observed in some of the patients receiving trastuzumab therapies [247, 248]. In general, these shortcomings call for creation of novel and improved Ab-mediated therapies for MBC. Pertuzumab has recently been approved by FDA as a new humanized mAb that blocks HER2 dimerization [244]. This mAb in combination with trastuzumab, and docetaxel is a standard of care for patients with previously untreated MBC [249].

16.8.2 Bevacizumab

As mentioned earlier, vascular endothelial growth factor (VEGF) is a proangiogenic molecule with a critical role in tumor metastasis [250]. Bevacizumab is a humanized mAb that inhibits VEGF activity and is mainly used in combination with chemotherapy for treatment of many types of advanced cancers such as colorectal cancer, RCC, NCLCs, ovarian cancer and glioblastoma [251–257]. The addition of bevacizumab to cytotoxic chemotherapy has improved response rates and survival of patients with metastatic colorectal cancer (mCRC) [258]. 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 [259, 260]. 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 [256].

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. 16.11. 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.

16.8.3 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 [261]. 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 [261–263].

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 [264]. In untreated FL patients, utilization of rituximab as the first-line therapy along with maintenance therapies led to the improvement of the overall response rate from 47 % (7 % CR) after initial treatment to 73 % (37 % CR) following maintenance treatment [265]. 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 90Y-ibritumomab *vs*. 3.0 years for control [266].

Furthermore, utilization of rituximab in combination with fludarabine and cyclophosphamide led to a significant improvement of 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 [267]. 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 [268].

16.8.4 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) [269, 270]. 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 [271]. 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 [269, 270, 272].

16.9 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. At present, there are around 350 mAbs with potential applications for a wide range of diseases. Historically, about 50 % of these Abs recognize tumor Ags [273]. Although most of these mAbs are in initial development stages, more than one hundred anticancer mAbs are being evaluated in different phases of clinical trials [274]. 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 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 [275, 276].

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 [277–279]. 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 [280, 281]. 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 [281–283].

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 [284]. 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 [285]. 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 [185, 286].

Finally, immunotoxins 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) [287]. Furthermore, this mAb is being evaluated in phase I trials in patients with CLL, B-cell lymphomas, and childhood ALL [288].

16.10 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 [120, 289, 290]. This section describes the studies that have examined the effectiveness of combining Ab-targeting with additional common antitumor strategies.

16.10.1 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 [291, 292]. 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 [293]. 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 [294].

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 [293, 295].

16.10.2 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. In fact, current evidence suggests that ionizing radiation per se can successfully induce immunogenic cell death leading to effective activation of antitumor immune responses [296, 297]. 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 [298, 299].

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 [300–302]. Interleukin 1β and TNF-α are examples of proinflammatory cytokines induced by radiation [300, 303, 304]. Moreover, sublethal doses of radiation has 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 [297]. 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 [305, 306]. Moreover, radiotherapy has been indicated to induce tolerogenic properties in macrophages [307, 308]. Furthermore, an increase in the number of Tregs has been reported in some patients receiving radiation as an antitumor modality [309, 310].

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 [297].

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 [311]. 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 [312]. Antibody targeting of 4-1BB (CD137), a critical receptor on T cell surface, combined with ionizing radiation has resulted in several other beneficial antitumor effects [313–315]. Interestingly, the combination of radiotherapy and anti-CTLA-4 Ab has also led to promising results in clinical trials [316]. In a case report of

melanoma, treatment of the patient with ipilimumab (anti-CTLA-4 Ab) following radiation [317] could mimic the successful results previously observed in murine models [311, 312].

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 [297, 312, 318].

16.10.3 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 anti-tumor function of transferred T cells and resulted in persistence of infiltrated effector T cells [319]. 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 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 [320]. 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* [321]. These examples substantiate the advantage of employing alternative immunotherapeutic approaches along with Ab-based modalities to obtain more potent and less toxic antitumor responses.

16.10.4 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 [322]. 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 [323].

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.

16.11 Current Limitations in Monoclonal Antibody-Based Therapies

16.11.1 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 [324]. 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 [325]. Protectin (CD59) inhibits homologous CDC by preventing formation of the membrane attack complex, thereby inhibiting cell lysis [326]. 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 [327, 328].

Tumor cells might circumvent ADCC via expression of NK cell inhibitory molecules such as HLA-G, a non-classical HLA class I [329], which is known to be expressed on melanoma and other malignancies [330–332]. Interestingly, rituximab-mediated NK cell lysis depends on the HLA class I expression level on B-lymphoma cells [329].

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 downregualtion following Ab therapy, a proper response may not always be achieved [2]. Similarly, acquired rituximab resistance in B-cell lymphomas following exposure to rituximab has been associated with reduced levels of CD20 [333–335].

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 [336–338].

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 [324, 339]. Furthermore, resistance to cetuximab treatment in colorectal cancers is often related to point mutations of *KRAS* and its downstream signaling molecules (e.g. BRAF) [340–343].

16.11.2 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. 16.10).

16.11.3 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 [344].

Using mAbs with high affinity can further diminish tumor penetration of Abs, a factor called "binding site barrier effect" [345]. In fact, there are several reports verifying that very high affinities can lead to suboptimal antitumor responses [346, 347]. 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.

16.11.4 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 express low affinity variants of the Fc receptor [112]. 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 [348, 349], and this replacement is shown to correlate with improved responses to rituximab therapy [112, 350].

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 [212].

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 [351].

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 [352]. 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 [151].

16.11.5 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 of alternative culture systems (e.g. microorganisms or plants) might lead to substantial reduction of production cost in the near future [353, 354].

16.12 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. 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

17

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17.1 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].

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Table 17.1 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	TLR2, 3, 4, 7, 8, and 9
Melanoma	TLR2, 3, 4, and 7
Ovarian cancer	TLR2, 3, 4, and 5
Oral squamous cell carcinoma	TLR2 and 4
Pancreatic carcinoma	TLR4 and 7
Prostate cancer	TLR3, 4, and 9

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 17.1). Later, it has been discovered that TLRs promote proinflammatory cytokines, leading to tumor growth and chemoresistance. However, various differential pro- and antitumor effects have been recognized for TLRs [12].

17.2 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) [13]. 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 [14]. 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 [15]. TLR7 plays important roles in pancreatic carcinogenesis by upregulation of intrapancreatic Notch, MAPK, and NF-κB signaling pathways [15, 16]. It has been discovered that Notch signaling pathway exacerbates inflammation and therefore regulates human pancreatic cancer initiation and maintenance [17]. The NF-κB and MAPK signaling pathways also have proinflammatory effects, mediating TLR7-stimulated pancreatic carcinogenesis [15]. In contrast to TLR7 effects on the pancreas, the expression of TLR4 has been shown to suppress lung carcinogenesis [18], whereas TLR2 expression leads to lung tumor cell progression [19]. 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 [20]. 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 [15]. 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 [15].

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 [21]. It has been suggested that TLR4 activation is crucial for dysplasia [22]. 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 [21]. Altogether, TLR7 agonists have been discovered as novel therapeutic approaches for the treatment of BCC and melanoma [23]. However, TLR7 ligation plays opposite roles in pancreatic cancer, indicating the importance of TLR7 signaling blockade in the prevention and treatment of malignancy [18]. Also, targeting of TLR4 signaling pathway in CRC may prevent tumor initiation [12].

17.3 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 17.1). 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 [24]. It has been postulated that TLR4 expression is responsible for immune suppression (Fig. 17.1). LPS-stimulated TLR4 expression inhibits T cell proliferation. Also, TLR4-mediated NO suppresses T cell activation [25]. In addition, TLR4-induced IL-6 promotes impairment of Dendritic cells (DCs) maturation, activation of natural killer (NK) T cells, and can also influence NK cell anergy [26]. Furthermore, IL-12 inhibits the generation of allogenic or tumor-specific CTL, contributing to the immune suppression [27].

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 [24]. Therefore, TLR4 plays an important role in the protection of tumor cells from the immune system response (Fig. 17.1); nonetheless, it has been suggested that TLR4 function is necessary for DC maturation and $CD4^+$ $CD24^+$ regulatory T cell blockage [28].

TLR4 is highly expressed in both cell membrane and cytoplasm of human oral squamous cell carcinoma (OSCC) [29]. 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.

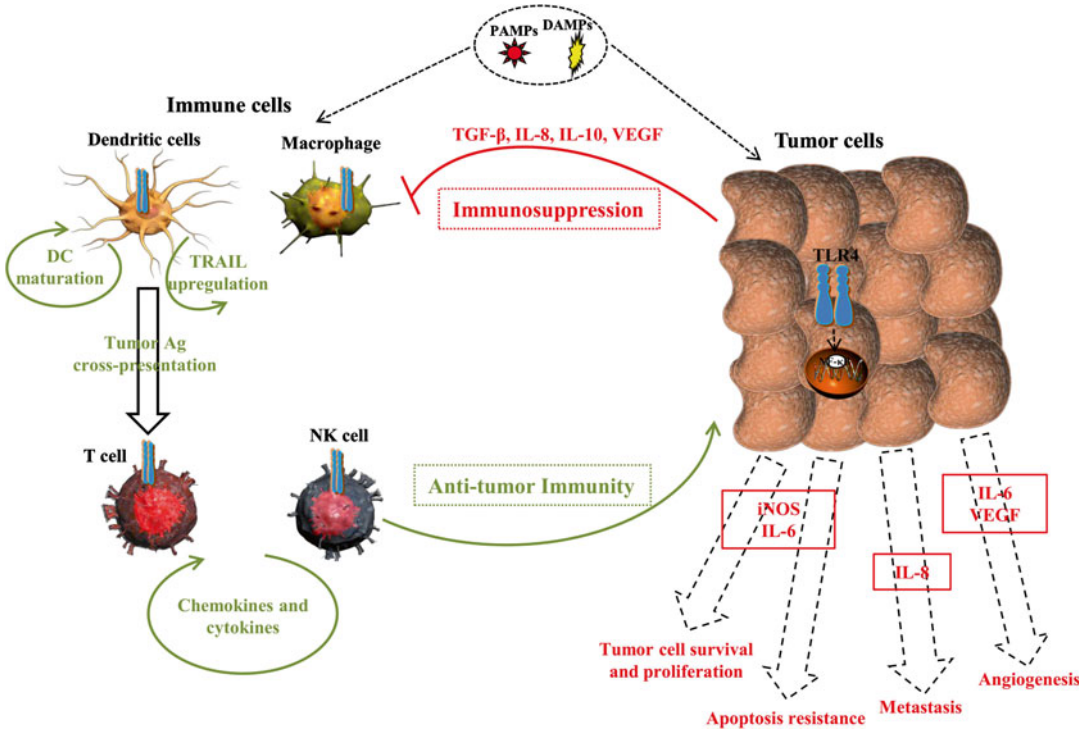

Fig. 17.1 Role of TLR4 signaling in cancer. TLR4 is widely expressed on both immune and tumor cells. TLR4 signaling in cancer is considered a double-edged sword with both pro- and antitumor consequences. TLR4 signaling on immune cells (depicted on the left-hand side in *green color*) enhances antitumor 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

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 principle biomarker of poor prognosis in several human cancers [29]. Higher levels of IL-6 can lead to tumor progression, resistance to apoptosis, chemoresistance [30], tumor angiogenesis, and tumor invasion [31]. IL-8 plays antiapoptotic roles and promotes tumor metastasis [32]. VEGF is involved in angiogenesis and immunosuppression and also suppresses DC number and differentiations [33]. 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 [34]. 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 [35]. 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) [34]. Higher levels of TNF-α induces IL-6 and CCL2 synthesis, leading to the tumor progression. Also, TNF-α regulates infiltration of leukocytes in cancers by chemokine modulation [36]. Besides, CCL2 and CXCL10 promote escalating inflammation and

immunity in melanoma cancer [37]. Additionally, TLR3 triggers NF-κB-mediated upregulation of inflammatory molecules and recruits leukocytes, promoting anticancer immune responses [38]. 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 [39]. 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) [40]. MDSC can promote chronic inflammation and also immune suppression by stimulation of regulatory T cell function [41].

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 [42]. TLR5-induced infiltration of immune cells, including neutrophils, suppress proliferation marker PCNA, promoting strong antitumor response through tumor necrosis and inhibition of tumor growth [42]. Thus, flagellin-induced TLR5 expression can be used as a novel therapeutic approach for human breast cancer.

17.4 TLR Targeting May Inhibit Cancer Cell Proliferation

TLR7 expression suppresses phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [15]. Suppressed levels of PTEN lead to PI3K/Akt pathway activation and increased level of TGF-β, mediating phosphorylation and activation of STAT3 [43]. STAT3 acts as a proinflammatory marker and central to neoplastic progression in pancreatic tumor [44]. TGF-β promotes cancer invasion [45], and PI3K/Akt signaling pathway stimulates tumor cell proliferation, thus leading to tumor progression [46]. 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 [40]. 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 [40].

17.5 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 [47]. 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 cytokines (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 [42].

17.6 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 [48]. Also, Akt inhibits GSK-3b phosphorylation and therefore suppresses β-catenin nuclear translocation [49]. In addition, Akt regulates cell death through decreasing levels of pro-apoptotic molecules, such as caspase-9, p53, NOXA, and PUMA [50]; however, it inversely regulates increasing anti-apoptotic molecule levels including XIAP, Bcl-xL, and Mcl-1 [51]. Moreover, Akt functionally suppresses both p21Wsf1/Cip1 and p27Kip1 that are negative regulators of the cell cycle [52]. Furthermore, the presence of phosphorylated Akt has been reported to be associated with advanced stages of tumor and poor clinical prognosis [53].

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 [54]. Also, poly(I:C) increases β-catenin translocation into the nucleus [49, 54]. 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 [55]. However, TLR4 pathway targeting seems to be a novel valuable therapeutic approach for the prevention of CRC progression and metastasis.

17.7 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 [56], but, in normoxic situations, oxygen-sensing prolyl hydroxylases degrade HIF-1α and keep its level low [57]. 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 [58]. Higher levels of HIF-1α have been detected in prostate cancer bone metastasis indicating the importance of HIF-1α in prostate tumor prognosis [59]. 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 [60]. HIF-1α complex upregulates anti-apoptotic genes including Bcl-xL, survivin, and MCL-1 [61]. 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.

17.8 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 [62]. 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 [63]. 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 [64]. Imiquimod-induced TLR7 also increases MyD88 and NF-κB signaling pathways, leading to caspase pathway activation and therefore resulting in tumor cell death [62].

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 [65]. Poly(I:C)-stimulated TLR3 leads to the production of TNF-α and, therefore, CD54 expression [66]. 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 [62]; 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 [15]. 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 [46]. 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 [15].

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 [40]. During the targeting of the TLR4 signaling pathway, beneficial effects of TLR4 stimulation should be harnessed while eliminating the possible negative ones (Fig. 17.1). 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 [54].

17.9 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 [67]. 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 [68]. It has been speculated that higher levels of TLR3 in breast malignancy and HNC is strongly associated with tumor invasion and metastasis [69]. The administration of bafilomycin A1 (BA1) which antagonizes TLR3 leads to decreased levels of CCL5 and IL-6, therefore controlling tumor aggressive behavior [69]. Also, TLR4 activation has been found to be responsible for apoptosis resistance in ovarian cancer cell [70]. 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 [34]. 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 [55]. 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 [71]. It has been found that surgical resection can induce local recurrence or distant metastasis [72]. Recently, it has been suggested that systemic inflammation and postoperative infection are associated with CRC recurrence [73]. TLR4 has been found to be highly expressed in patients with liver metastasis and poor clinical outcome [74]. 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 [75]. Additionally, TLR4-mediated signaling promotes hepatic involvement and liver metastasis [76]. Although few studies have found that TLR4-induced cascade plays proliferative and anti-apoptotic roles in cancer cells, leading to cancer metastasis [77], the same results were not obtained in other studies [55]. 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 [52]. Thus, targeting of TLR4 signaling pathway can be beneficial for patients both with and without postoperative infection.

Even though TLR3 upregulation have 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 [78]. Thus, manipulation of TLR pathways should be performed meticulously in order to prevent improper interactions.

17.10 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

18

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Contents

18.1 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, we will introduce the important facts about NK cells that are required to understand their function in the tumor microenvironment and will 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.

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18.2 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.

18.2.1 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 well known to activate NK cells including IL-2 and IL-12 [8]. 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 [9]. 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 [10]. 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 [11]. Unlike what was initially thought, 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 [12]. This type of NK cell activation is known as "stress-induced self" activation [11]. 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 [11]. 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 is 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 [11].

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 [13]. 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 [10]. The direct mechanism of IFN-γ on cancer cells still remains to be determined.

18.2.2 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 [14]. 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 [15]. The importance of NK cells in early tumorigenesis was also shown in a Her2/neu transgenic mouse model generated on a perforin-deficient background [16]. 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 [8]. 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 [17]. In addition, the presence of NK cells within several difference cancers, including squamous cell lung cancer, gastric cancer, and colorectal cancer, has been shown to be a positive prognostic factor for these patients [18–20]. 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 [21]. 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.

18.3 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.

18.3.1 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 [22]. 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 [23, 24]. 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 decrease 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 [25]. In

addition to overall low NK cell numbers, distant tumor growth has been found to have significant effects on NK cell maturation [26]. 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 [27]. 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 [28]. 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 [13]. 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 [13]. The goal of any NK cell cancer immunotherapy should involve two points: 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.

18.3.2 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 responses. These include the co-activation of various activating receptors present on NK cells with their corresponding ligands on the surface of tumor cells [13]. 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 [22]. 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 [29]. 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 [30]. Defective expression of activating receptors has also been a hallmark of metastatic melanoma [31] and acute myeloid leukemia (AML) [32] 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 [33]. 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 [34]. 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 [35]. It was previously found that an inverse correlation exists between NK cell activation and T-regulatory cell expansion in tumor-bearing patients [36]. 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. This data suggests 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 [37]. 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 [38].

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 [39]. 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 [39]. 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 [40]. 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 [41]. Flow cytometric analysis of NK cells from these tumors revealed that these cells produced vascular endothelial growth factors (VEGF), placental growth factor (PGF), 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 PIGF in NK cells from healthy subjects. Further research into the pro-angiogenic phenotype of NK cells and the impact they have on tumorigenesis are needed in other cancer types.

18.4 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 them. 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.

18.5 Adoptive NK Cell Transfer

18.5.1 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, 42, 43]. Thus, they were a natural starting point for this technology. Klingemann and Martinson [44] published an early study in which lymphocytes were isolated from PBMCs and underwent CD56 positive selection via magnetic bead technology [44]. 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 [44]. Another group performed a similar protocol, in which $CD3^+$ cells were removed and the remaining cells were cultured overnight with IL-2 [45]. 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 [46, 47]. 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 [46, 47]. 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 [47]. Patient NK cells incubated with healthy donor feeder cells were able to expand more and had increased purity (93.8 % $CD56^+CD3^-$) [47]. 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 as feeder cells to expand NK cells (with the addition of IL-2) [48]. After 28 days of culture, the

NK cells expanded 300–1,000-fold and had high cytotoxicity [48].

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) [49–51]. 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 [50]. 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 [52]. 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 [52]. Another breakthrough came recently 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 [53]. Researchers engineered the K562 cell line to express FcγRI, B7-2, and 41BBL and added either mbIL-15, mbIL-21, or both [53]. IL-21 is another gamma chain cytokine involved in NK cell proliferation [54]. When the irradiated K562 cell line that included mbIL-21 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) [53]. 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 [53]. Not only were these cells highly cytotoxic they also had an increased ability to perform ADCC [53]. Others have also used these aAPCs to produce NK cells from human embryonic stem cells and induced pluripotent stem cells [55].

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 recent study attempted to expand NK cells in all three of these conditions and compare the resultant products [56]. 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 [56]. 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 [48]. Others found that NK cells could be successfully expanded from frozen $CD34^+$ umbilical cord blood samples [57]. 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 and still function well when thawed [51, 58]. 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.

18.6 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 [59]. 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 [60–64]. 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 [60]. These trials took place in patients with advanced colon, breast, lung, ovarian, pancreatic, renal cell, and melanoma cancers and overall had very disappointing results [61–64]. In addition, some reported treatment-related deaths due to high-dose IL-2 [62]. 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 [65, 66]. In a more recent trial, patients with metastatic melanoma and renal cell carcinoma (RCC) received autologous transfer of IL-2-activated NK cells after lymphodepletion [67]. 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 [67]. 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-isolated 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. 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 [68, 69]. In fact, in an animal model of lung cancer, depletion of T-regulatory cells improved the outcome of NK cell adoptive transfer [70]. 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 recently published which utilized the K562-mbIL21 aAPC previously described [53, 58]. Researchers were able to expand NK cells from children with neuroblastoma by 2,363+/−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 [58]. If results could 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.

18.7 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 [71]. 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 [71]. For instance, 112 leukemia patients received a hematopoietic transplant with either KIR ligand incompatibility or not (from an HLA haplotype-mismatched family donor) [71]. 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 [71]. It also simultaneously prevented graft-versus-host disease (GVHD) and decreased rejection [71]. 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 [45]. These were then infused into 19 poor prognosis AML patients after they underwent a high-dose immunosuppressive regime [45]. Remission was achieved in 5 of 19 patients and the NK cells expanded *in vivo* [45]. Success in these early studies led to a plethora of similar clinical trials both in hematological cancers [72–75] and solid tumors [73, 75–77]. While some early studies found success with enriched but not expanded alloreactive NK cells [72, 74], others at the phase II level proved non-beneficial [75]. There have been several preclinical studies using the newest methods of NK cell expansion (feeder cells lines – irradiated allogeneic PBMCs, K562-mbIL15-41BBL, the additive OKT3) and the testing of their efficacy in various solid tumor xenograft models [78–82]. 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) [79]. This study indicates that NK cells can persist in the host and remain active. Collectively, the results indicate that generating large numbers of activated NK cells with the latest techniques may be very useful and efficacious in future allogeneic NK cell adoptive transfers.

18.8 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 [83]. This cell line is dependent on IL-2 for growth and is highly cytotoxic against tumor cell lines, primary tumor cells, and xenograft tumor models [83, 84]. The high cytotoxicity can be attributed to the lack of inhibitory KIRs on these NK cells [85]. 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 [86, 85]. In a phase I trial conducted on 12 patients with refractory RCC and melanoma, escalating doses of NK cells from 1×10^8 to $3\times10^9/m^2$ were administered [87]. There was only mild toxicity at the highest dose and some responses (one mixed response, one partial response, one survived) [87]. New cell lines are also being established that have even higher levels of cytotoxicity than NK-92 to improve results in clinical trials [88]. 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 [89]. It is able to efficiently kill $CD20^+$ targets normally resistant to NK killing [89]. Another NK-92 variant expresses a CAR that targets an antigen overexpressed in neuroblastoma called disialoganglioside [90]. 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 from PBMCs can also be manipulated to express CARs specific to HER-2 (overexpressed on many epithelial tumors) or to express chemokine receptors such as CCR7 to promote migration of the NK cells to the lymph node [91, 92]. Strategies targeting chemokine receptors on NK cells may be able to overcome inefficient homing of NK cells to tumors in certain cancer types. As these advances improve results in preclinical models, genetic manipulation may prove to be a powerful tool for NK cell therapies.

18.9 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 [93]. Herceptin (trastuzumab-TZB) was unable to protect from $Her2^+$ breast cancer cells in a xenograft model when Fc receptor γ was knocked out [93]. 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 [94, 95]. 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 [96, 97]. 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 [98]. 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 [99, 100]. This was also related to an increased ability of their PBMCs to kill tumor cell lines via ADCC [100]. 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 [101]. 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, 23, 25, 31, 32]. 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 [95, 97]. 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 [95]. There is currently a clinical trial (NIH-NCT00941928) in progress that will combine donor NK cells with a mAb epratuzumab (targets CD22 antigen on B cells) and IL-2 in acute lymphocytic leukemia (ALL) patients. 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 a really interesting ongoing trial (NCT01221571), researchers have 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 [102]. In their *in vitro* studies, this antibody was able to restore NK cell cytotoxicity to patient NK cells that were previously nonfunctional [102]. The phase I trial has been started, and early results show that after one dose, patients with Hodgkin lymphoma have cytotoxic peripheral blood NK cells, and three of the six patients have stable disease [102]. 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) [103]. *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 [103]. In an autologous setting, patients with NK cells were capable of killing their own tumor cells when this MOR 208 was utilized [103]. 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 [104]. This combination decreased tumor growth in a xenotransplant model using patient breast tumors by increasing ADCC of tumor cells [104].

Lastly, cytokines may play a role in enhancing NK cell activation/numbers and increase 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 [105]. 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, 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.

18.10 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 [106–108]. In addition, it has come to light that IL-2 increases T-regulatory cells, which are highly undesirable in any anticancer therapy [68]. 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, 109, 110]. 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 [111–113]. In many animal models, IL-15 has been shown to have strong antitumor effects [114–116]. Unlike IL-2, IL-15 does not increase T-regulatory cells [117]. IL-15 appears to have low toxicity in primate studies and is effective at increasing NK cells [117–119]. The wait for these results to be translated into clinical trials has been quite long due to the difficulties encountered in generating large amounts of GMP quality IL-15. Currently, there are many ongoing phase I/phase II trials with recombinant IL-15 as a treatment (NCT01727076, NCT01021059, NCT01572593) or in combination with NK/lymphocyte cell infusions (NCT01385423, NCT01369888, NCT01337544). This cytokine, alone or in combination, may be a great candidate for enhancing NK cell tumor killing.

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 [54]. In contrast to IL-2, IL-21 inhibits the differentiation of T-regulatory cells and does not promote vascular leak syndrome [108, 120]. It has been safely used in multiple phase I and phase II studies with metastatic melanoma or renal cell carcinoma [121–123]. It has been shown to have antitumor activity and is able to boost antitumor NK cell responses [121–123]. 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 [124, 125]. 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 [126]. 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.

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 of 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) [127]. 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 [127]. This has proceeded to phase I trials in MM and AML and has proven to be safe and tolerable [128, 129]. 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 [130]. Lastly, TGF-β is frequently produced in the tumor microenvironment and can negatively regulate NK cell activity [34, 35]. 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) [131]. In 29 malignant melanoma and RCC patients, this antibody was well tolerated [131]. This trial is still in the early phases of testing, but for certain tumor types that express high levels of TGF-β, this may be an important additional therapy when considering NK cell immunotherapy.

18.11 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. The knowledge we have gained in learning how mAbs work to kill tumors has led to revolutionary ideas in regards 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 are in phase I trials currently, so their efficacy is unknown, but they may be able to subvert the effect of the tumor on NK cell deactivation. 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 specialists and produce 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 [132]. 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 to produce the most important 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 immu-

notherapies 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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Dendritic Cell Vaccines for Cancer Therapy: Fundamentals and Clinical Trials

19

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Contents

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19.1 Introduction

Mobilization of the immune system for the generation of an effective lymphocyte response against tumor tissue is one of the main 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 autoimmune response. In this aspect, dendritic cells (DCs) perform a fundamental role in linking the innate defenses to the specific responsiveness by lymphocytes.

The very first report on DCs 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, and functional identification and classification of DCs 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 DC, a myeloid-derived cell lineage, and the plasmacytoid DC (pDC), a lymphoid-derived lineage [8]. Although these two popula-

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tions can be differentiated by morphological and surface markers, each DC type shows a wide phenotypical variation and multifunctional role in the immunosurveillance and regulation of the immune system [9, 10]. Thus, conventional human DC express CD4, CD11c, and CD1a or CD83 and the MHC class I [11, 12]. Maturation/activation of these cells is characterized by the expression of CD80, CD86, CD40, and CCR7 [8]. Differently, lymphoid pDC are featured as $CD4^+/CD1a^-/CD11c^-/CD123^+$ cells [13].

DCs are the main professional antigen-presenting cells (APCs) and perform a continuous surveillance and recognition of the microenvironment of tissues and organs where they are found as immature cells (iDCs). In this condition, they have high capacity for capturing soluble and particulate antigens by endocytosis, phagocytosis, and micropinocytocis [3, 11, 14, 15]. The intakes of opsonized and non-opsonized antigens can be mediated by several surface receptors such as FcγR [11], mannose receptor (MR) [16], DC-SIGN [17], type C lectin receptors (DEC-205) [18], as well as *Toll*-like receptors [12, 19]. 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, 20].

Immature DCs 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 [21, 22], DC maturation is critical for achieving the balance between effector responsiveness and autotolerance [11].

Proinflammatory signals induce not only the migration of iDC to the secondary lymphoid organs but also their maturation and activation. In contrast to iDC, mature DCs 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. Mature DCs express a higher density of CCR7 that drives their chemotactic migration toward the T-cell sites [11, 23].

Maturation is also followed by increased expression of a set of the abovementioned surface markers and by production of several proinflammatory cytokines, such as IL-12, IL-18, TNF-α, IL-23, IL-10, and IFN-α, depending on the stimulating factor [24–26].

Phenotypical and cytokine profile of mature DC contribute to the recruitment, interaction, and activation of lymphocytes for the development of an efficient response against pathogenic microbes, allergens, and allogeneic tissues [27, 28] and were also evidenced in antitumor response [8]. In fact, it was reported that tumor mass-infiltrating DCs are usually suppressed or maintained as iDC in situ. These observations have instigated many authors to try to stimulate infiltrating DCs to play a more effective role against tumor cells [29, 30] or to transfer autologous or allogeneic DCs 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, 31–34].

Being the main professional antigen-presenting cells, DC constitutively express both MHC class I and class II antigens on their surface. This feature is closely associated with their effective antigen-presenting function, whereas 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 DCs by transfecting them with MHC class II transactivator genes (*CIITA*). It induces four times more CTL than parental untransfected DC or DC transfected with irrelevant genes [35].

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 [36]. 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 including generation of mature DCs [37–39], sustained production of IL-12 [40–43], and overcoming the suppressive microenvironment provided by regulatory T cells [37, 44–47] and myeloid-derived suppressor cells [48–51]. 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.

19.2 Strategies for Developing Clinical Grade DC Vaccines

One of the main issues for generation of clinical grade antitumor DC vaccines is the choice of 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 to the generation of tumor-DC hybrid cells or their transfection with tumor nucleic acid. However, there is still no definitive agreement on what strategy is the best.

Results with DCs loaded with lysates of tumor cells are controversial since some studies have shown that this approach results in a poor protective role of DCs, whereas other authors have successfully prepared them. Some details can be crucial to the effectiveness of lysate-pulsed DC vaccines. For instance, [52, 53] inhibitory effect of lysate on DC maturation can be reduced when tumor cells are stressed by heating at 42 °C for 25 min prior to the cell lysate preparation. It is hypothesized that the expression of heat shock proteins (HSPs) by tumor cells can avoid the suppressive effect of cell lysate by increasing DC maturation, an observation corroborated by others [54–56]. Induction of HSPs may be a required feature for increasing the immunogenicity of tumor cells by treatment with chemotherapeutic agents. The authors have observed that low nontoxic concentrations of paclitaxel or doxorubicin are able to alter the expression of a number of genes including HSP70, HSP40, and HSP105 mRNA [53].

Aiming to compare different methods for loading DCs with tumor antigens, it was observed that lysate obtained from a homogenate of solid tumor cells exerted a poor effect on the ability of DCs to stimulate antitumor activity [57]. Stressed tumor cells were obtained by freeze-and-thaw cycles or by irradiation at 30 Gy, with the irradiation being more useful than a freeze-and-thaw process. However, the best method for loading DCs in the mentioned study was their fusion with live tumor cells. The authors 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. For clinical purposes, loading DCs with tumor-associated proteins or peptides has been preferred in relation to the total tumor lysates. In a phase I study, patients with advanced melanoma were vaccinated with $CD34^{+}$-derived DC pulsed with melanoma peptides. Some patients showed peptide-specific DTH response, as well as Melan-A- and gp-100-specific CTL in the peripheral blood [45].

One of the limitations of preparing DC vaccines pulsed with tumor lysate is that the available tumor tissue is usally not sufficient for repeated applications for the patient. The use of tumor RNA for encoding tumor antigens was first proposed by Nair and Gilboa's group [58, 59], and there is substantial evidence that RNA transfection is a superior method for loading antigens onto DC [60–62]. 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 in order to drive the antigen presentation toward a more specific immune response. Finally, RNA shows a safety advantage on 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 [63, 64] and human systems [59, 65, 66]. Sakakibara et al.

[67] 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 a maturation cocktail for an additional 48 h. The authors observed that mature fast DCs and standard DCs displayed comparable levels of many markers expressed on DCs, 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 DCs are functional antigen-presenting cells (APCs) capable of inducing primary T-cell responses.

Vaccination with tumor-DC hybridomas using autologous melanoma or renal carcinoma cells and allogeneic DCs is able to change the natural history of the disease, since it may present stabilization [31] or even regression of metastatic lesions with local fibrosis [68]. Whether a patient was unable to fight the tumor development, it is probable that his/her own DCs were 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 DCs with autologous metastatic colon cancer cells was able to activate both $CD4^+$ and $CD8^+$ T cells in just 24 h, in a higher number than controls, while $CD8^+$ cells were significantly able to lyse target cells [69]. It can also solve some practical problems, namely, (a) it is usually possible to generate a limited number of samples of autologous DCs for vaccination, whereas a higher number of DCs could be generated from healthy allogeneic or semi-allogeneic donors; (b) the cellular reactivity triggered by allogeneic or semi-allogeneic DCs 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 DCs.

Evaluation of the efficiency of syngeneic, allogeneic, and semi-allogeneic DCs has shown that hybrid cells prepared with allogeneic or semi-allogeneic DCs were more effective than syngeneic ones and also worked better as therapeutic vaccines, thus protecting hosts against pulmonary metastasis. Actually, allogeneic and semi-allogeneic DCs more effectively induce CTL activity, as well as NK cytotoxicity, and induce higher levels of IFN-γ, as well as the IFN-γ:IL-10 ratio [70].

The use of exosomes for DC loading has also been proposed by some authors [71–74]. Exosomes are defined as constitutive nanovesicles that can be exocyted by both tumor and DCs displaying a sample of all membrane molecules of original cells [75, 76]. It was observed that vaccination with tumor peptides is more effective when carried on exosomes [72, 77]. Dai et al. [54] revealed 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. DCs 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 also be 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 [78].

Cross-priming performed by DC is a phenomenon that can enhance the transference of antigenic peptides through HSP, such as gp96 and HSP70 [79–81]. 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 $CD8^+$ response and subsequent specific attack toward tumor cells [82–85]. Although the use of HSPs seems to represent a good strategy for enhancing the DC loading with tumor antigens [86–88], the clinical application faces some limitations including 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 [89].

19.3 Routes of Administration

Another fundamental aspect of DC-based immunotherapy is the route of choice for administrating ex vivo prepared DCs. Clinical trials have reported various routes of DC administration, aiming to achieve an efficient delivery of cells to the appropriate immune site. Therefore, DCs 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 DCs in multiple myeloma patients revealed their migration to the regional lymph nodes [90]. 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 [91]. Although these routes lack DC migration to the spleen, they appear to be more effective for inducing specific antitumor responses compared to the i.v. method [92, 93]. Tracking studies have also revealed that i.v. inoculation promotes DC distribution to the liver, spleen, lungs, and bone marrow. It was observed that DCs accumulate in the spleen just 3–24 h after inoculation [92]. Since the majority of relapsing diseases result from metastatic tumor cells, it is reasonable to infer that systemic distribution of DCs to the main targets for metastasis (lung, liver, and bone marrow) would be preferred in the protocols developed for preventing them [94–96].

Despite the suppressive microenvironment established at the tumor site, intralesional administration of DC was shown to be feasible, safe, and well tolerated [97–99]. Of course, this choice is limited by the tumor accessibility, while Mirvish et al. [100] 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.

19.4 DC Vaccine for Prostatic Cancer

Prostate cancer is the second most frequent type of neoplasia worldwide, accounting for more than 903,500 new cases each year [101]. Most patients are successfully treated by prostectomy or radiotherapy, but about 30 % of them relapse [102]. In this aspect, immunotherapeutic approaches became an attractive 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 U.S. Food and Drug Administration (FDA) for cancer therapy targets prostate cancer [103–105]. This vaccine, called *sipuleucel-T* (Provenge® – Dendreon, Seattle, WA, USA), was developed for castration-resistant metastasis of PC (for both symptomatic and asymptomatic patients). It is a DC-enriched autologous cell suspension from the own patient 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 [105–107]. The analysis of disease progression and overall survival in two phase III studies (D9901 and D9902A) showed that this vaccine was able to increase the overall survival from 4.5 to 6.7 months [104, 105].

A third phase III trial has shown that *sipuleucel-T* improved patient survival time by 4.1 months, showing a 22 % lower relative risk of death than the placebo group [103]. Another positive result of these trials is that patients have

shown variable reduction of PSA levels (prostatic-specific antigen), the main prognostic marker of this disease [104, 108].

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 [103]. 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 [106, 109].

In another successful approach, prostatectomized patients with biochemical relapse were treated with autologous DCs pulsed with human recombinant PSA (Dendritophage-rPSA) [110, 111]. Nine out of twenty-four 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. [111] have proposed the use of xenogeneic murine PAP for loading autologous DCs. Six out of twenty-one 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 tumor-DC hybrid cells was also tested in prostate cancer. Hybridomas prepared with three different 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 ONYCAP23 based hybridoma have induced specific T-cell response to different tumor targets [112].

A phase I/II study using DCs 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 [113].

19.5 DC Vaccine for Melanoma

The first clinical study on DC vaccines in melanoma patients was published by Nestle et al. [114], who analyzed the efficacy of DCs 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.

The use of allogeneic tumor cell lysate for loading DCs, assessed in a phase I/II study, found 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 [115].

More recently, melanoma patients were treated with DCs 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 of efficacy was observed between DCs loaded with cell lysate and peptides, although no correlation was found between the development of specific immune response and clinical response [116].

The use of autologous tumor RNA for loading autologous DC has promoted increased numbers of IFN-γ-producing CD4$^+$ cells [117]. This result merits attention because the strategy of using RNA aims to stimulate CD8$^+$ response since it implies the generation of tumor peptides at cytoplasm, which would be processed through

the cytosolic machinery. Thus, the expected effect on the activation of $CD4^+$ cells can favor the establishment of memory $CD8^+$ cells (Shedlock and Shen 2003; Janssen et al. 2003). In a phase I/II study, Kyte's group showed that administration of RNA-pulsed DCs was able to induce a specific DTH reaction and *in vitro* lymphoproliferative responsiveness as well as IFN-γ production [118].

Cell fusion technology was also applied to melanoma and kidney cancer patients, by fusing autologous tumor cells with allogeneic DC obtained from healthy donors [31, 119]. The measurable clinical response from these patients demonstrated that the disease had been stabilized for a median of 6 months, with no relevant collateral effects [31].

19.6 DC Vaccine for Colorectal Cancer

DCs are constituent 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 DCs correspond to 2 % of cells isolated from lamina propria [120]. 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 DCs poorly stimulate T lymphocytes in a primary allogeneic culture (MLR) and are not able to induce significant levels of IL-2 or IFN-γ [120].

The C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin) is involved in the recognition of colorectal cancer cells by DCs [121]. Immature DCs within colon tumor tissue expressing DC-SIGN, but not mature DCs, interact with tumor cells by binding to $Lewis^x$ and $Lewis^y$ carbohydrate of CEA in tumor cells. Interestingly, DC-SIGN do not interact with CEA expressed by normal colon epithelium that shows low levels of Lewis epitopes. Therefore, DCs 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 [122].

Immunohistochemical analysis of infiltrating cells showed that mature $CD83^+$ DCs are found in almost all primary colon carcinoma samples and in some metastases. Heterogeneous infiltration patterns vary from diffuse cells to clustered DCs that tend to accumulate around vascular structures and the marginal zone of lymphoid aggregates [123]. Data on maturation markers on DCs 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 [123], whereas others reported that 64–97 % of cells do not express B-7 molecules [124, 125], even after stimulation with TNF-α, IL-4, and GM-CSF [125]. The density of DCs 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 [123].

Immunization of patients with DC vaccine in phase I/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 the phase II study [59]. Messenger RNA of TAT protein transduction domain and calreticulin increase the immunogenicity of CEA and the effectiveness of mRNA-pulsed human DCs. It is interesting that transfection of DCs with calreticulin mRNA seems to be associated with activation of $CD4^+$ T cells, whereas TAT protein mRNA preferentially stimulates $CD8^+$ cells [126]. 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 [65].

Instead of using mRNA for known specific antigens such as CEA and HER2/neu, DCs 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, namely, HCT-116 (colon cancer) and A498 (kidney cancer) [127]. Supporting this strategy, a clinical trial using total RNA extracted from metastasis tumor cells for pulsing autologous DCs, followed by inoculation in the patients (four injections, every 4 weeks), showed an ability to induce specific T response to CEA [128].

Analysis of ten clinical samples of colorectal carcinomas showed that 60 % of them overexpressed the antigen EphA2 [129]. Murine DCs pulsed with human EphA2 were 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 $CD4^+$ cells which play a critical role in the antitumor response.

19.7 DC Vaccine for Nervous Tissue Cancer

As reviewed by Montelli et al. (2009), the potential clinical use of DC vaccines against brain tumors has also been investigated by some groups. The first DC vaccination study in patients with malignant glioma was reported in 2001 by [130], showing increased tumor-specific cytotoxicity in four out of seven patients treated with peptide-pulsed DCs. In a phase I clinical trial conducted by Sampson et al. [131], 13 patients with glioblastoma (GBM) and 3 with WHO grade III glioma were i.d. inoculated with autologous DC vaccine. Peripheral blood monocyte-derived DCs 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 reaction at the administration site. 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 [132]) and carmustine wafers (59.6 weeks [133]).

Parajuli et al. [134] studied *in vitro* the ability of different DC-vaccine strategies to induce T-cell response against malignant astrocytomas. Autologous monocyte-derived DCs were pulsed either with autologous tumor lysate, transfection with total tumor mRNA or by fusion of DCs with tumor cells. The authors concluded that all strategies used for pulsing DCs efficiently induced T-cell cytotoxicity, which was further improved by addition of CD40 ligand [135].

Twelve GBM patients followed in a phase I trial were treated with DC vaccines pulsed with peptides eluted from autologous tumor cells. After 3 doses, 50 % of the patients presented increased immunological response against autologous tumor cells and survival time was higher than historical control data [136].

In a very expressive clinical trial, 56 patients with relapsing GBM were treated with at least 3 doses of autologous DCs loaded with autologous tumor lysate, producing 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 [137]. In a phase II study, patients producing increased levels of IFN-γ showed higher overall survival than nonresponders [138].

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. This product acts on TLR3 [139] to induce the production of IFN-γ, IL-6, TNF-γ, and chemokines including CCL2, CCL5, CCL20, and CXCL10 from astrocyte and microglia [140, 141]. 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 [142]. Antitumor response was associated with activation of 2′5′-oligoadenylate synthetases, which are antiviral proteins induced by type I IFN [143]. 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 [144]. Okada's group is currently analyzing the effect of associating poly-ICLC with DC vaccines generated under INF–α(αDC1), previously shown to be more effective than conventional DCs at inducing an antigen-specific CTL response [145].

19.8 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 DCs of breast cancer patients induce higher levels of regulatory T cells (Treg) than DCs from healthy donors [146], which determines a low immunogenicity of autologous monocyte-derived DCs usually suppressed or induced to tolerance by Treg cytokines.

Reduction of Treg activity by blocking the regulatory molecule CTLA-4, through a monoclonal antibody, 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-Myers 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 [147, 148].

In conclusion, despite these limitations, promising results are stimulating the search for the best pathways toward improving tumor immunogenicity, DCs' antigen-presenting function, 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 [53] can open up new avenues for fighting cancer.

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Tumor-Associated Macrophages and Cancer Development

20

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20.1 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 and hints meaningful to

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further understanding the properties and function of TAMs and helpful to develop tumor therapy.

20.2 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 TAMs 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 TAMs 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

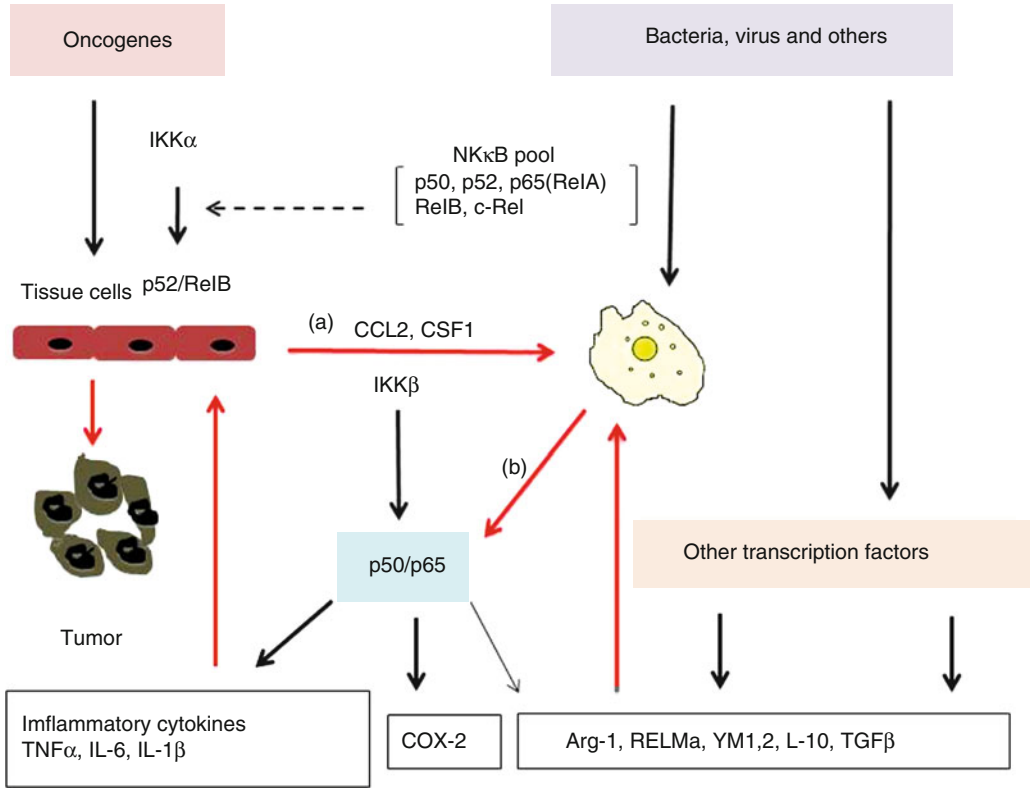

Fig. 20.1 Two mechanisms proposed to explain the association between TAMs and tumorigenesis. (**a**) A large set of chemokines (CCL2 and others) and cytokines (G-CSF and so on) secreted by tumor cells can promote the recruitment of monocytes in local region and then educate these filtrated monocytes 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

heterodimer [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 pro-inflammatory 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. 20.1) [26].

20.3 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 macrophages) 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 are 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 recruit 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].

20.4 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) were 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 $CD11b^+$, $Gr\text{-}1^+$, $IL\text{-}4\alpha^+$, $F4/80^-$.

20.5 "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 acti-

vate 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-baring mice inhibited NFkβ 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].

20.6 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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Photodynamic Therapy and Antitumor Immune Response

21

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21.1 Introduction

Despite major investments in cancer research and cancer prevention and treatment, the current statistics are grim [1–4]. Considering the spiraling cost of cancer care, in particular the cost of cancer therapeutics, what has been thus far achieved in benefits is only marginal [5]. The new generation cancer drugs, under the banner of "patient-tailored medicines," which are narrowly directed against tumor-associated factors (such as ligands, receptors, and signaling pathways) are not only costly but, more importantly, are not applicable to a broad range of cancer patients and, disappointing enough, very often fail to show better results over much more widely used (and cheaper) chemotherapeutic drugs [6, 7]. Not to mention difficulties arising due to the specific factors applying to the tumor mass itself, such as persistency and advanced stage detection [8–15]. To make things worse, some tumors appear to adapt to survive these specialized drugs, and any time a specific pathway gets blocked, tumors circumvent this blockage by developing an alternative route to survive. Owing to all these difficulties, and in spite of novel developments in cancer treatment technologies, the mainstream and conventional treatment package that includes surgery + radiation therapy + chemotherapy remains the most widespread option available for the oncologists. In this chapter, photodynamic therapy and antitumor immune responses would be discussed in more detail.

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21.2 Photodynamic Therapy

Worldwide preclinical and clinical studies for over two decades have shown that photodynamic therapy (PDT) can be a promising ingredient of a multimodality treatment approach to various cancer types and other malignancies [16–18]. PDT has the potential to alleviate many of the problems/drawbacks associated with conventional cancer treatments. It is already a clinically approved therapeutic modality used for the management of nonmalignant and neoplastic diseases.

PDT has three essential components: light, oxygen, and photosensitizer (PS) [19, 20]; individually all these components are nontoxic, but when combined together, they initiate a cascade of photochemical reactions which culminate in the generation of highly reactive oxygen species such as singlet oxygen. Since PDT is highly localized and the lifetime of the singlet oxygen is very short, approximately 10–320 ns, followed by limited cellular diffusion depth, approximately 10–55 nm [21], the photodynamic damage only occurs in the vicinity of the PS molecular location.

The antitumor effect of the PDT arises due to three interrelated and/or inter-dependent mechanisms: (1) direct cytotoxic effects on tumor cells, (2) damage to tumor vasculature, and (3) induction of a robust inflammatory reaction that can lead to systemic immunity development. The interplay between these three mechanisms and the tumor mass is critically dependent on factors such as the type and dose of the used PS, time frame of the PS administration, the light component characteristics (exposure, light dose, fluence rate, etc.), tumor oxygen concentration or gradient, and possibly other (still poorly understood) variables.

The process begins with administering the PS to a patient either topically or parenterally, and depending on the pharmacokinetic and pharmacodynamic properties, it accumulates in the tumor cells and the associated vasculature. Upon illumination with an appropriate wavelength and dose of light, the photons are absorbed thus triggering the chain of reactions through "photoactivation," the activated PS undergoes a cascade of energy conversions and transfers, and in the presence of tissue-molecular oxygen, the process ends up generating a range of reactive oxygen species (ROS) which ultimately destroy tumor cells in close proximity (Fig. 21.1) [22–25].

PDT has several advantages over other cancer treatment modalities currently in use. In addition to its selectivity and multiple application possibility, it is inexpensive with tolerable side effects. Moreover, it is rarely resistant to the observed treatments [26, 27]. More importantly, clinically approved PS does not accumulate in the cell nuclei and thus have limiting DNA damaging effects that can be by nature carcinogenic or can lead to the development of resistant clones. Several classes of inexpensive PS are commercially available and some are already approved to be used on patients. Most of the PS classes in use are of porphyrin or chlorin backbones or their modifications. With the newer PS classes, problems such as prolonged skin sensitization have been virtually eliminated [28]. Moreover, these compounds absorb in the region of visible spectrum, optimal for deep-tissue penetration. The list of benefits can be extended to include absence of the adverse effects of radiation and chemotherapies, no significant change in tissue temperature during illumination, preservation of the connective tissue at the PDT application site, thus minimal fibrosis induction, and improved cosmetic outcome. Clearly this is a very promising treatment modality that needs further translational and clinical studies.

In *in vivo* studies, the observed PDT effects can be attributed to several and interconnected biological and physiological effects. Depending on the PS concentration, location in the organism/tumor site, and applied irradiation dosage, PDT effects can be direct cell killing, occlusion of the tumor-associated vasculature, and modulation of the immune system, and sometimes cumulatively all of these effects can be observed. At the cellular level, both necrosis and apoptosis have been observed as the outcome of the PDT [17, 29–32]. It is a known fact that direct damage of the tumor cells and nearby vasculature initiates several cell-signaling cascades. In

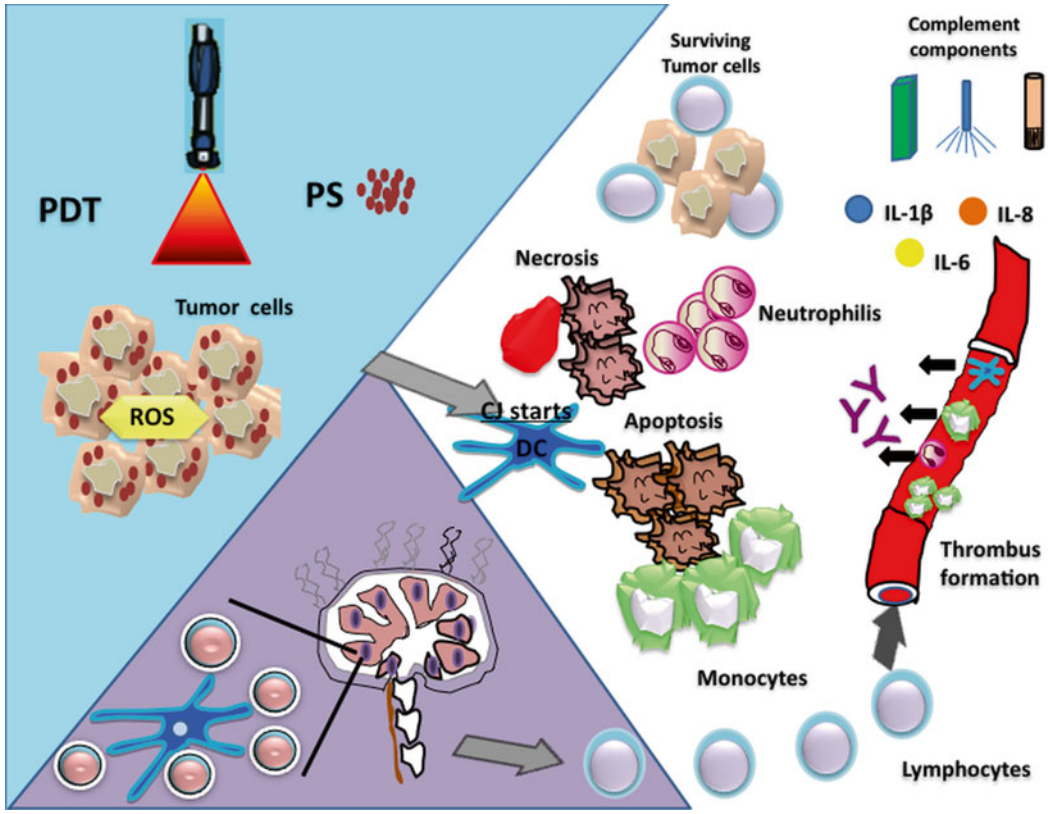

Fig. 21.1 PDT induced effects. In tumors, cells loaded with PS upon excitation generate ROS species which leads to predominantly apoptotic and necrotic cell deaths. Tumor cell death is accompanied with complement cascade activation; proinflammatory cytokine activation; rapid neutrophils, DCs, and macrophages recruitment. Dying tumor cells and their debris are phagocytosed by phagocytic cells and DCs, which then migrate to the local lymph nodes and there differentiate into antigen-presenting cells. Tumor antigen presentation is then followed by clonal expansion of tumor-sensitized lymphocytes that home to tumor site and eliminate residual tumor cells

addition, damaged endothelial cells lead to formation of thromboses and consequently to vascular occlusion. In all these cases, the released cell fragments and cytokines trigger a range of inflammatory mediators which in turn activate the body's defense mechanism, i.e., the immune response, which can be classified as innate or adaptive immunity. In essence, PDT treatment is generating a pronounced systemic effect as well as working in sync with the body's natural defense mechanisms; the success of the PDT lies in the fact that it employs 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 an application to other diseases such as retinal diseases [33, 34] or cardiovascular disorders [35, 36].

21.3 Closer Look Up at the PDT and Triggered Immune Response

In cancer treatment, one of the most important effects of PDT, besides tumor destruction, is that by the virtue of triggering an acute inflammatory reaction, it "activates" body's immune system (Fig. 21.2). In fact, induction of a strong inflammatory reaction is the central paradigm of the antitumor effect of PDT. At the treatment locality

due to PDT-induced oxidative stress, strong acute inflammation reaction and localized edema are generated [19, 37], i.e., PDT ends up producing a chemical (and subsequently a physiological) insult in the tumor tissue which is perceived by the body as a localized trauma. The next step is launching the protective mechanisms to reestablish tissue integrity and homeostasis at the treated/affected site [38]. At the onset, an acute inflammatory response is the principal effector. During this stage, the body is engaged in "containing the damage" – disruption of the homeostasis – which includes removal of damaged cells, and then promoting the healing process at the affected area, in order to restore normal tissue functions [38]. This elicited inflammation is nonspecific for the tumor antigen and is being orchestrated by the innate immune system [38]. The pattern recognition receptors are responsible for detecting the PDT-caused localized insult perceived as "altered self" [38]. PDT is responsible for speedy and prolific generation of "danger" signals, called damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs), at the treatment site that get detected by the innate immunity [39–42]. At the onset of inflammation, the tumor vasculature undergoes significant changes and becomes adhesive for inflammatory cells (via over expressing selections) and permeable/leaky for blood proteins [38]. The inflammatory cells, first the 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/CAMPs by eliminating cellular debris, compromised

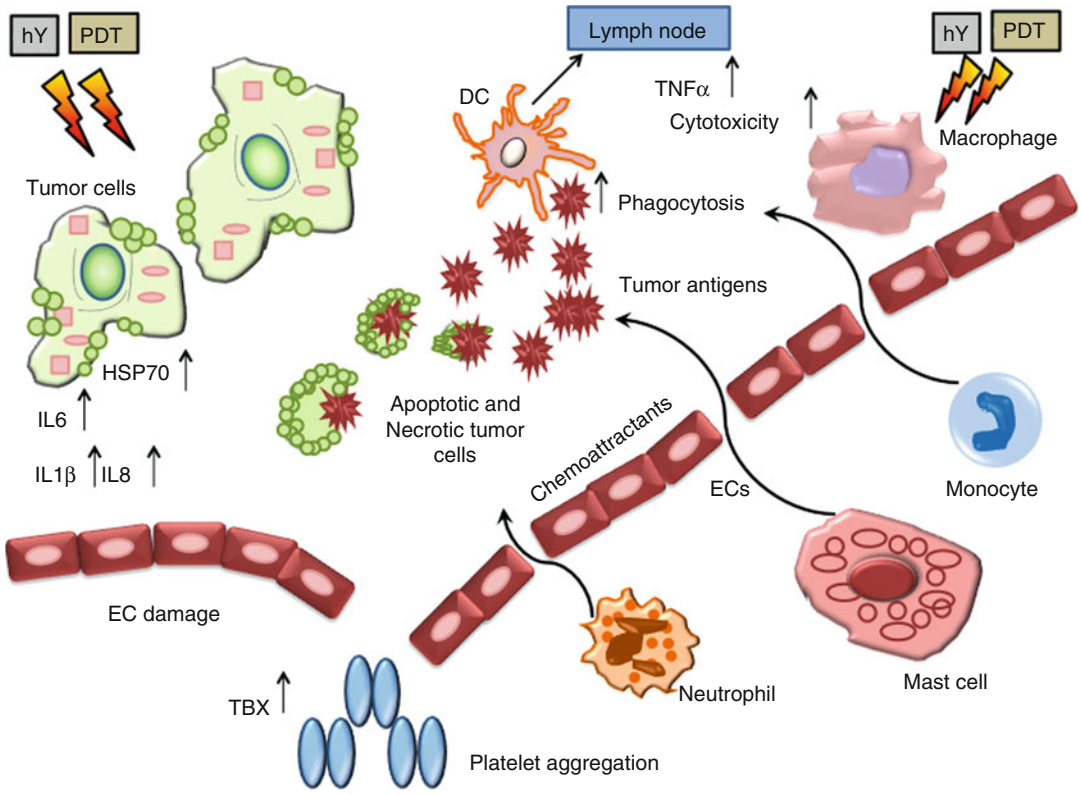

Fig. 21.2 PDT induced inflammation. Damaging the endothelial cells (*ECs*) activates a cascade of events leading to local inflammation, vessel dilation, and platelet aggregation. Much of these effects are caused by the release of thromboxane (*TBX*), cytokines (such as interleukins IL1β, IL6, IL8, tumor necrosis factor-α), and infiltration of immune system cells (necrotic and apoptotic cells provide antigens to the DCs that migrate to lymph nodes)

tissue components, etc. [38]. The vascular occlusion, observed after PDT illumination, effectively "walls off" the damaged area, until it is removed by phagocytosis, thus preventing further spreading of the homeostasis disruption [38]. Studies have shown that depletion of these inflammatory cells or inhibiting their activity diminishes the therapeutic effect of the PDT [44–47]. Moreover, it is elucidated that interleukins IL-1β and IL-6 are amongst the most critical ones in this process [48, 49]. Also 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 improve the PDT effect remarkably [50, 38].

21.4 Significance of PDT and Adaptive Immunity

Both preclinical and clinical studies have shown that PDT influences adaptive immune response in different ways; some regimens potentiate adaptive immunity, whereas others lead to immunosuppression. Although the precise mechanisms leading to the former or later response are not entirely clear, they appear to be PDT-regiment dependent [47, 51, 52]. Interestingly, PDT-induced immune suppression is mostly confined to cutaneous and transdermal treatments that involve larger surface areas [51, 53].

As previously mentioned, the efficacy of the PDT treatment strongly depends on the induction of antitumor immunity; research is showing that long-term tumor response is reduced or absent in immune-compromised mice [44, 54], whereas transfer of bone marrow or T-cells, from immunocompetent mice, results in improved PDT efficacy. 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 resistant to cell-mediated antitumor immune reactions [55, 56]. Case in point, patients with vulval intraepithelial neoplasia (VIN) who lacked the MHC-I molecules did not respond to PDT treatment effectively as did patients expressing MHC-I [57, 58]; patients with positive PDT treatment response had increased $CD8^+$ T-cell infiltration into the treatment site to differ with nonresponders, who lacked that effect.

The PDT effect over the immune system and more specifically induction of immune potentiation was demonstrated for the first time in the seminal study by Canti et al. [59]; the study proved that cells isolated from tumor-draining lymph nodes of PDT treated mice were able to pass on tumor resistance to naïve mice. Even more importantly, Korbelik et al. [60] in an *in vivo* study of murine tumors showed that PDT treatment generated an immune memory effect [60]. Multiple clinical studies support these lab research findings that PDT enhances the antitumor immunity effect. In clinical trials, PDT treatment of multifocal head and neck angiosarcoma showed reduction of untreated metastatic tumors owing to increased immune-cell infiltration into these untreated formations [61]. Further clinical phase I and II trials revealed promising results in proving the effectiveness of the PDT for induction of antitumor immunity effect [62–67].

21.5 Mechanism of PDT Immunologic Effects

Although the exact mechanistic pathways of immunologic activation are not entirely clear, there is a consensus that PDT activates both the humoral and the cell-mediated antitumor immunity systems. It is known by now that PDT efficacy is reduced and even null in the absence of $CD8^+$ T-cell activation or their infiltration to tumorous sites [44, 68, 69]. Thus, it is imperative to have a clear understanding about the mechanisms of the potentiation of $CD8^+$ T-cell activation due to PDT. One thing is clear, however, that PDT treatment induces acute local and/or systemic inflammation which culminates with antitumor immunity induction [52]. During this process, upon inflammation induction, dendritic cells (DCs) get matured and activated as critical components of tumor-specific $CD8^+$ T-cell activation and, subsequently, antitumor immunity generation [70]. This chain reaction starts with DC activation (due to PDT treatment) followed

by migration to the lymph nodes, where they activate the T-cells via presenting their antigens [49, 71]. At this stage, another class of T-cells may also be involved, the $CD4^+$ T-cells, called also helper T-cells; they start dividing rapidly and secreting the cytokines that regulate and/or assist the immune response. The PDT-induced antitumor immune response may or may not depend on $CD4^+$ T-cell presence [69, 72] and that role may be taken by the natural killer cells [69]; these are the cells bridging the adaptive immune system with the innate immune system, to differ from conventional T-cells (which recognize the peptide antigens presented by MHC), and these cells recognize the glycolipid antigens (however, once activated they can perform functions attributed to T-cells). In this cascade of cause-effects, it is believed that DC stimulation (thus increased ability to stimulate T-cells), at least partly, is due to dead and/or dying tumor cells [73]; it is known that PDT causes both cell death and cell stress [19, 74] and the initial activation of DCs at the PDT-treated locale is a result of DAMPs/CDAMPs recognition generated from the dying cells [75–77]. Recent studies have been looking extensively at the release patters of DAMPs after PDT [40, 41], and the most frequently expressed DAMP after PDT treatment seems to be the upregulation and translocation of the heat-shock proteins (HSPs) of the cell membrane [78].

21.6 Case Studies

For over a decade now, the Hamblin laboratory has been involved and has taken a leading role in elucidating mechanistic pathways of PDT-induced inflammation and antitumor immunity with the aim to trace novel immune mediated cancer treatment avenues stemming from PDT effects [79–89]. In the following section, we will discuss some of our findings, including the most recent study results, emphasizing the effects of PDT-generated inflammation and its reflection/implications in cancer therapy modalities.

It is widely accepted now that most deaths from cancer are caused by metastatic tumors; thus, our vision has been to develop methodologies that not only will destroy the primary tumor mass but also will activate the patient's immune system to battle distant (untreated and may be not even detected yet) metastases [89]. It is well known now that removal of primary tumors via surgery and radiotherapy, which has immunosuppressive effect at high doses, renders micro-metastases to grow unchecked. On the other hand, after PDT treatment, there is an induction of an acute inflammatory response causing a massive regulated invasion of neutrophils [49], mast cells, and macrophages [90]. Not only that, but also, it has been shown that depletion of neutrophils in tumor-carrying mice decreased the PDT-mediated tumor treatment effect [54]. As discussed before, acute inflammation is implicated in attracting and activating DCs; as a result, they prime the tumor-specific cytotoxic T-cells (CTLs). In addition, it is well known that CTLs activity is not limited to the PDT treatment area alone and that they have a broader effective range [60]. Other groups have shown that low-dose cyclophosphamide (CY) can potentiate antitumor immunity in murine models. Suggested mechanistic explanations included depletion of suppressor T-cells [91], reduction of immunosuppressive cytokines [92], and anti-angiogenesis [93]; it has been generally accepted now that low-dose CY selectively depletes T-regs in mice, and by doing so, it increases both the priming and effector phases of the antitumor immune response [94]. In this crucial context, the authors reported, for the first time, that a combination of PDT with low-dose CY could cure a highly metastatic mouse tumor and could produce tumor-specific CTLs and potent memory immunity [89]. In this seminal work, we used J774, a highly metastatic reticulum cell sarcoma in BALB/c mouse, a highly aggressive, invasive, metastatic macrophage tumor, and PDT with benzoporphyrin derivative monoacid ring A (BPD). The CY was injected 48 h before light delivery. Our study demonstrated that PDT combined with low-dose CY generates a dramatic improvement in survival and numbers of cures. On the other hand, no cures but only some survival advantage were seen with each one of the components used separately, whereas when PDT was coupled with

high-dose CY (as opposed to low dose), no additional benefit was observed. In comparison, with a combination treatment of BPD-PDT and low-dose CY, a long-term memory immunity generated allowed the cured mice to even reject rechallenging with tumorigenic doses of J774 cells. The observed long-term cures with only low-dose CY-PDT combination treatment suggests that in some tumor models, there is a kind of host factor which is counteracting the immune-stimulating effect of PDT. Judging by our flow cytometry results, this factor could be $CD4^{+}FoxP3^{+}$ T-regs, and the benefit of low dose CY could be due to their particular susceptibility to low-dose cytotoxic drugs. The effect of low-dose CY on the tumor was much more pronounced than the high-dose CY alone (Fig. 21.3). Our overall results are proving that the effects of CY on J774 tumor are due to the immunostimulatory effect rather than the traditional cytotoxic effect of the CY [89].

It is widely accepted now that cancer treatment involving PDT modality is effectively engaging both arms (innate and adaptive) of the immune systems via stimulating the release or expression of various proinflammatory mediators [19, 37, 49, 75, 95, 96]. 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 attacking the cancer cells [37, 43]. The fact is that this cycle is not only a powerful tool in eliciting direct antitumor effects [97–99], but as importantly, it is stimulating the cells to release secondary inflammatory mediators (including the cytokines IL-1β, TNF-α, IL-6, and IL-10 and prostaglandins, histamines, leukotrienes, etc. [100]). The one area 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 antigens expression. Our lab was the first to recognize this effect. The authors designed a study in which a pair of equally lethal BALB/c colon adenocarcinomas were used: first, CT26 wild-type (CT26WT), i.e., antigen negative, and, second, CT26.CL25 transduced with lacZ gene, thus expressing the tumor antigen

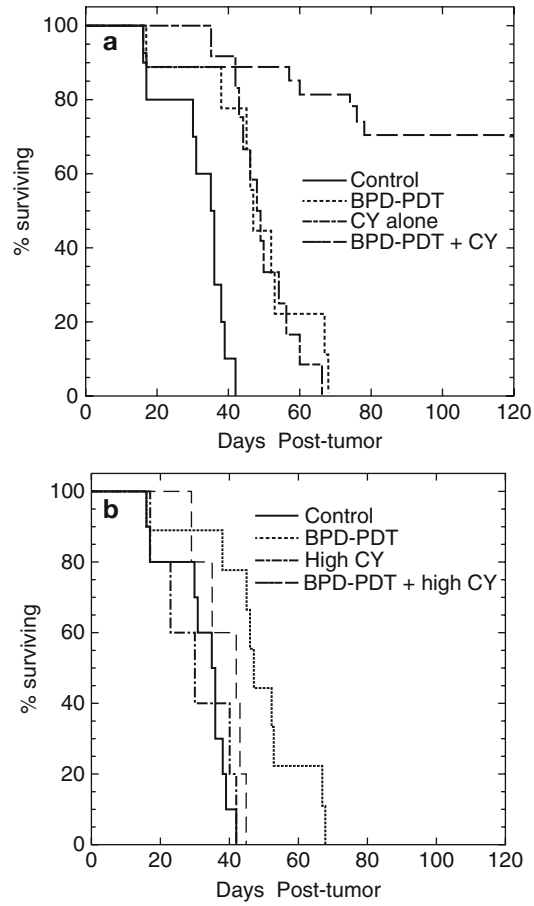

Fig. 21.3 Kaplan-Meier survival curves of mice undertreatment. (**a**) Plots represent no tumor treatment (*as control*), only PDT, low-dose CY, and low-dose CY+PDT. (**b**) Plots represent no tumor treatment (*as control*), only PDT, high-dose CY, and high-dose CY+PDT. Mice were killed in cases when the primary tumor diameter reached 1.5 cm or body weight dropped >15 % (Adapted with permission from Proceedings of National Academy of Science: Castano et al. [89])

β-galactosidase (β-gal). The idea was to study if PDT treatment would elicit a systemic antigen-epitope-specific antitumor immune response in otherwise identical cancer cells [86]. In this study, both used cell lines were equally lethal, and the level of β-gal expression was low enough to allow the tumor to grow without triggering any clinically significant immune response (often seen in cancer patients), thus only PDT application could generate significant differences in the therapeutic outcome and the observed elicitation of immune response.

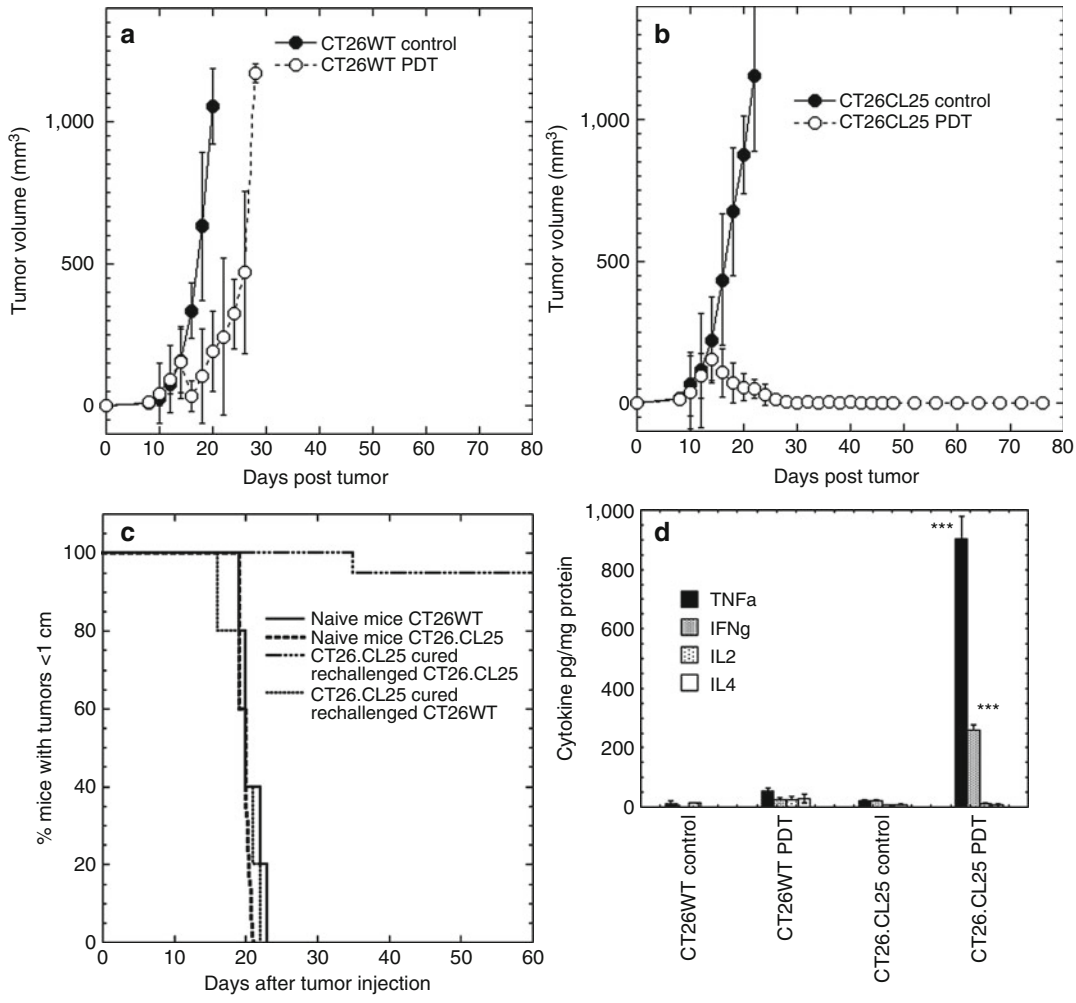

Fig. 21.4 *In vivo* PDT of tumor (1 leg model). (**a**) Mean tumor volumes of CT26WT tumors and (**b**) CT26.CL25 tumors; means of 10–15 tumors. (**c**) Kaplan-Meier survival curves of % of mice cured from CT26.CL25 tumors and rechallenged either with CT26.CL25 or CT26WT tumor cells. (**d**) Mean level 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 reference [86])

The outcome was that PDT induced a local response in all β-gal antigen-negative CT26WT tumors, with clear reduction in size, but only until day 18 (Fig. 21.4) after that the regrowth took hold. The net result was only that the growth was 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 [86]. During the study, it was also observed that the PDT-induced immune response leads to elevated levels of released IFN-γ and TNF-α cytokines. Our study also shows that PDT can induce a very strong antigen-specific immune response, capable of generating memory immunity which allows mice to reject the rechallenge with the same antigen-positive cells. The induced immune response is potent enough to cause regression of a distant well-established antigen-positive tumor outside the treatment area [86] (Fig. 21.5). The presence of the activated antigen-specific effector CTLs was

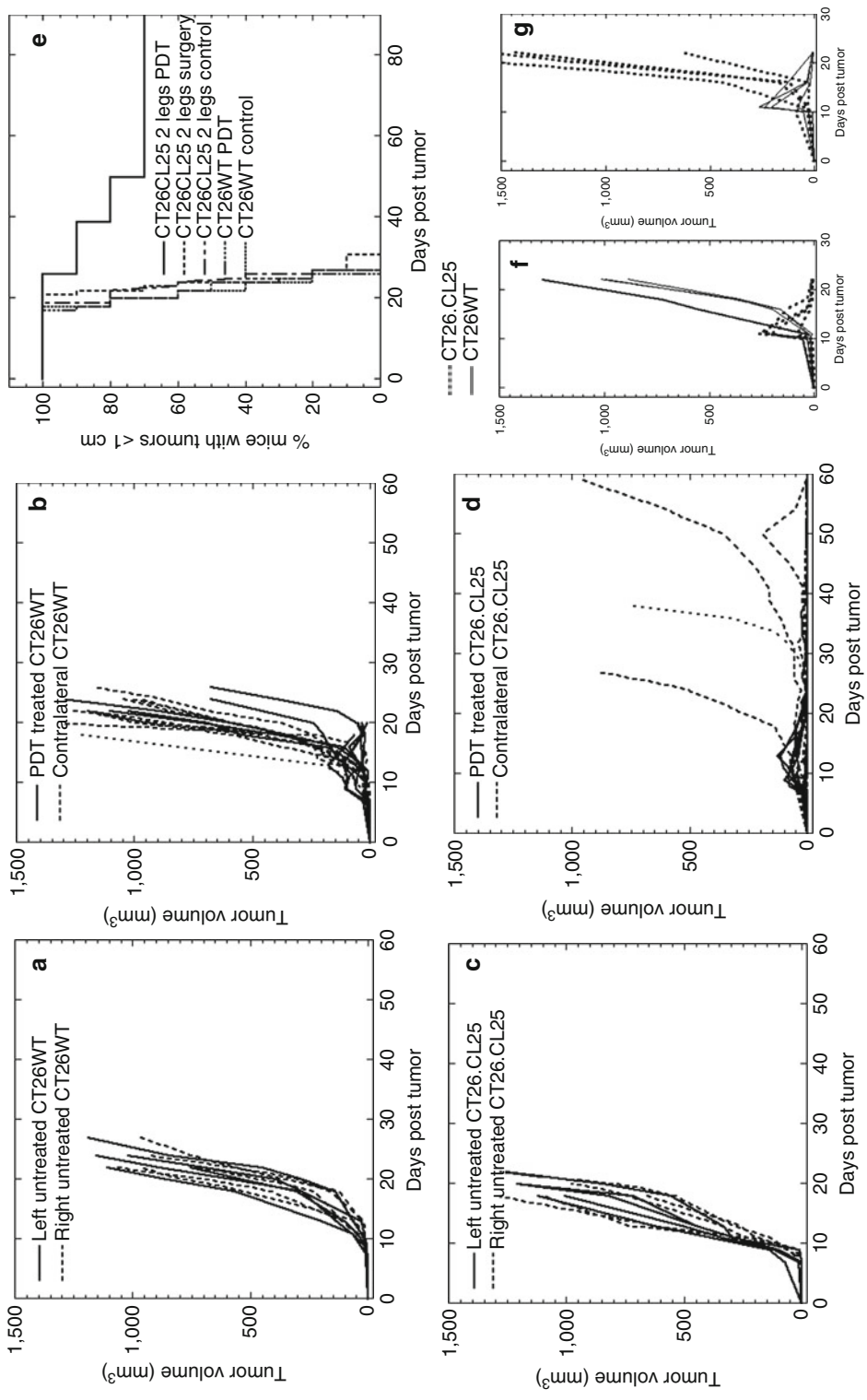

Fig. 21.5 *In vivo* PDT of tumor (2 leg model). Time courses of individual tumor volumes with two similar or mismatched bilateral tumors on the right and left legs. (**a**) Bilateral CT26WT tumors; right leg PDT treated. (**b**) Bilateral CT26WT tumors, untreated. (**c**) Bilateral CT26.CL25 tumors; 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 with permission from Mroz et al. [86])

also confirmed. During the study, it was realized that regression of distant and untreated tumors took place in 70 % of the treated mice.

Moreover, our study demonstrated, for the first time, that tumor cells may escape PDT-induced immunosurveillance due to antigen loss. In clinical settings, it is known that some tumors escape from immune recognition and elimination; only now, we realized that this is happening 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. 21.6). Clearly, effective vascular PDT treatment can not only destroy a local tumor but also induce systemic strong antigen-specific antitumor immune response. And this immunity is so potent that it is capable to induce regression and destruction of distant, antigen-positive tumors outside the irradiation reach. The treatment also proved to be effective in inducing long-term immune memory effect, imprinting 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 immunodominant 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 separate study, where a naturally occurring cancer antigen, the P1A, a mouse homologue of human MAGE-type antigen, was employed [101]. We decided to use this specific cancer-testis antigen, since it is not only a well-established one, but more importantly, it is mostly expressed in testis and cancers and only at very low levels in other tissues [102–105]; P1A antigen-positive mouse 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 [106]; these antigens are not expressed in most mature tissues with the exception of testis and placenta [107]. It is known that P815 can elicit CTL response against at least four distinct antigens: AB, C, D, and E [107–115]. It appears that the main CTL response against P815 tumor is geared towards AB and E antigens [111]. Also, it has been shown that T-cells isolated from DBA/2 mice inflicted with P815 tumor primarily recognize either antigen AB or C-D-E, but not both [116]. Moreover, the two epitopes of the P815AB, P815A, and P815B are recognized by two different CTLs. Another gene code for P815E and a different CTLs recognize its antigen. On the other hand, P815-derived P1.204 cell line is an immune system escape variant [117]; 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 revealed regression upon PDT irradiation and no recurrence during the trial period of 90 days. *In stark contrast*, *mice with P1.204 tumor 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 rechallenging, 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 challenging with a tumorigenic dose of cells.

In the *ex vivo* study, the extent of host antitumor immune response induction, as a result of PDT treatment of P1A antigen-presenting P815 mastocytoma cancer cells, and whether the antigen is activating T-cells before and/or after PDT, was looked into. The answer for that was provided by the cytokines secreted from $CD4^+$ and $CD8^+$ T-cells. Our results showed that PDT of

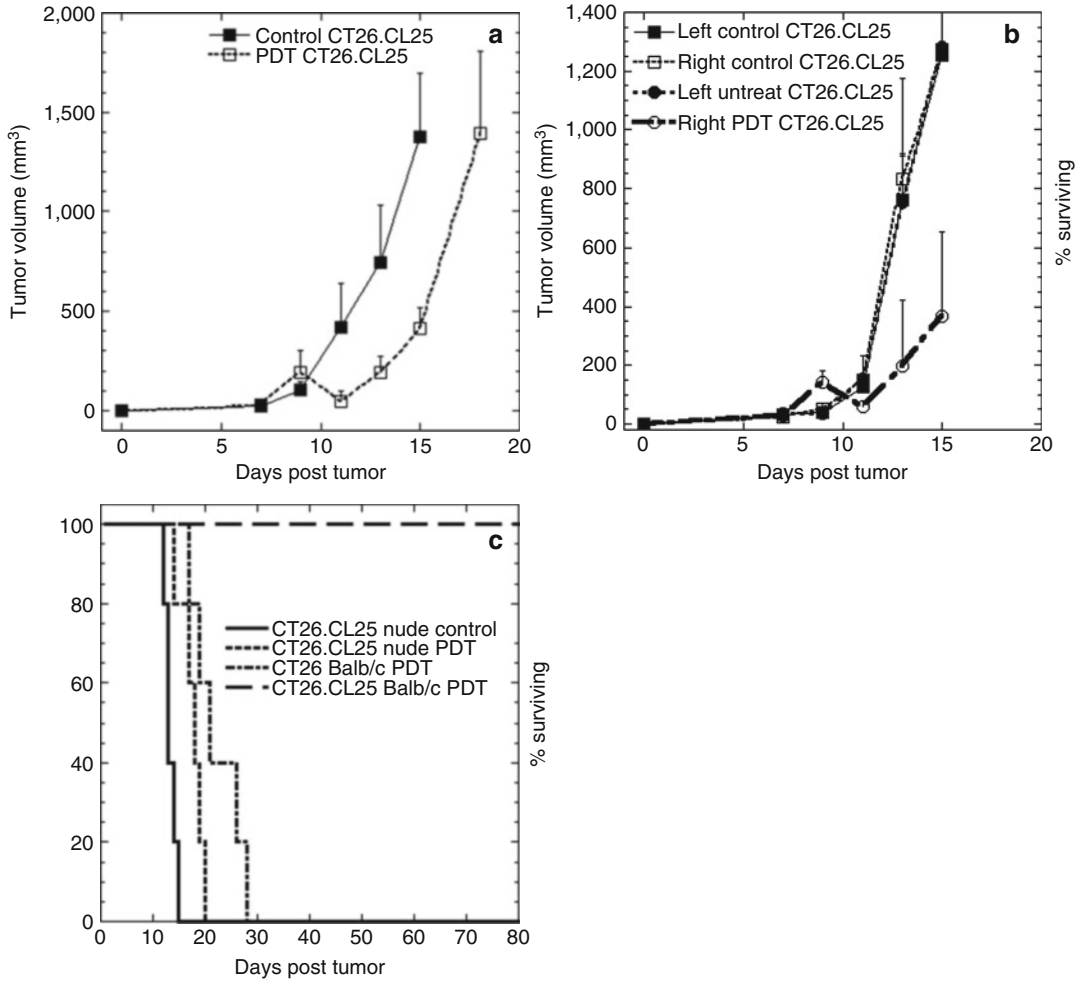

Fig. 21.6 (**a**) Tumor volumes of CT26.CL25 tumors PDT treated and untreated in BALB/c Nu/Nu immunocompromised mice. (**b**) Tumor volumes in bilateral CT26.CL25 tumors PDT treated and untreated in BALB/c Nu/Nu immunocompromised mice. (**c**) Kaplan-Meier survival curves of % surviving BALB/c and BALB/c 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 (Adapted with permission from Mroz et al. [86])

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 LPYLGWLVF epitope of P1A antigen. In addition, in nude mice (lacking an adaptive immune system) bearing the P1A antigen-positive P815 tumors, antitumor effectiveness of PDT is curtailed to nil. Interestingly, their survival can 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 response) is documented to be due to antigenic loss [21, 39, 40]. It has been shown [110] that there are three different escape mechanisms employed by P1A tumors, presenting the peptide antigen LPYLGWLVF (expressed in different tumor models), for avoiding immune response: in P815 tumors, all progressions occur due to antigenic loss, while in J558 tumors, all progressions take place due to antigenic drift (antigen mutation [39]), whereas all progressing methA tumors develop resistance to CTLs.

Our study confirmed that if an antigen is expressed in a tumor tissue, PDT may be more successfully applied in patient population containing tumors positive for a particular antigen. Secondly, even though many solid tumors show heterogeneous expression of tumor antigens, it has been shown that de novo induction of tumor antigens in these tumors may represent a novel means to break tumor escape mechanisms [40]. Thirdly, combination of PDT with various tumor antigen expression enhancement and their presentation via MHC class-I, may have beneficial treatment effects for those cancers that are otherwise untreatable.

Application of PDT for localized microbial infections, especially those caused by multiple-drug-resistant bacteria, is a very promising alternative modality to antibiotics, particularly in intractable microbial infection situations; bone or joint infections caused by multidrug-resistant bacteria are extremely intractable. Moreover, treating orthopedic infectious disease (such as osteomyelitis, arthritis) can be problematic due to the aseptic nature of joints, bones, and cartilages. For such cases, we looked into the induction of protective innate immune response due to PDT treatment and observed that the process germinated through neutrophil accumulation.

It is well known that bacterial phagocytosis by innate immune cells, such as neutrophils, plays a critical role in the elimination of invading bacteria, especially *Staphylococcus aureus* [118–120]. Malfunction of the phagocytic immune system, therefore, renders the host susceptible to bacterial infections [121]. If a treatment impairs the function of phagocytes in combating microbial infection, the efficacy of the antimicrobial treatment might be reduced, resulting in deterioration and prolongation of the infection. We established a murine chronic MRSA arthritis model using a combination of bioluminescent MRSA and resin microparticles, which allowed sequential noninvasive optical evaluation of the course of infection in an individual mouse and enabled us to carry out a detailed examination of the PDT effects in an efficient manner [81]. We established that administration of anti-GR-1 (anti-neutrophil) antibody eliminated the therapeutic effect of PDT, indicating that the therapeutic PDT using methylene blue had a curing effect for bacterial infection via the attraction and accumulation of neutrophils into the infected region [81].

There are other studies showing the curing role played by neutrophils in the therapeutic response at various PDT regiments. DeVree et al. [122] showed that depletion of neutrophils, using a neutralizing antibody, abrogated the tumoricidal effect of PDT, whereas increasing the number of circulating neutrophils, with injection of granulocyte colony-stimulating factor, potentiated the antitumor effect. Cecic et al. [90] found a rapidly developing systemic (as well as local) neutrophilia in tumor-bearing mice after PDT with two different PS that could be abrogated by inhibitors of complement activation. Although the role of PDT-activated/stimulated neutrophils in the therapeutic effects of PDT against cancer is established, the role of neutrophils in the therapeutic effects of antimicrobial PDT had not been previously reported (prior to our studies on murine bacterial arthritis [123–125]).

Our PDT treatment system [81] showed a promising therapeutic effect in murine chronic MRSA arthritis model with neutrophil accumulation and migration. Preventive PDT, used as a preconditioning regimen before bacterial inoculation, suppressed bacterial growth and inhibited the establishment of infection [81]. This is the first demonstration of a protective innate immune response against a microbial pathogen being induced by PDT.

21.7 Concluding Remarks

The proven ability of PDT to trigger inflammation and improve immune response can be successfully used, in tandem with other treatment modalities, to combat cancer and to achieve long-term tumor control. By making this therapeutic treatment more targeted and dose controlled, the arising strong inflammatory response can be confined to the tumor site, and thus, the body's immunoregulatory and immunosuppressive mechanisms can be kept at bay. On the other

hand, PDT-triggered controlled inflammation can be effective for treating distant, untreated/inoperable tumors and may also have a role in controlling microbial infections.

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Polarization of Tumor Milieu: Therapeutic Implications

22

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22.1 Introduction

Tumor progression can be described using terms borrowed from evolutionary biology. Tumorigenesis is a process affected by genetic diversification of cancer cells and their adaptive qualities [1].

Due to genomic instability (one of ten hallmarks of cancer, proposed by Hanahan and Weinberg 2011) [2], variants of cancer cells form with various adaptive properties. During the evolutionary process, cancer cells accumulate features (hallmarks) such as ability of unrestrained replication, evading growth suppressors, production of own growth factors, resistance to apoptosis, and ability of metabolic reprogramming during hypoxia [2]. Due to these highly adaptive ("favorable") features, cancer cells become autonomous and refractory to various control signals produced by hosts. As opposed to normal cells, cancer cells acquire the capability to survive when in underoxygenated and acidified microenvironment [3].

Besides hallmarks warranting autonomy and independence, cancer cells display features such as ability to form own blood vascular supply and escape from immunosurveillance, promotion of inflammation, invasion, and metastasis [2]. These properties allow cancer cells to establish specific relations ("dialogues") with normal cells [2]. These features allow, in fact, to form new microenvironment, a specific ecological niche promoting further tumor growth and progression. Paradoxically, it appears that normal cells become a milieu for cancer cells. Behavior

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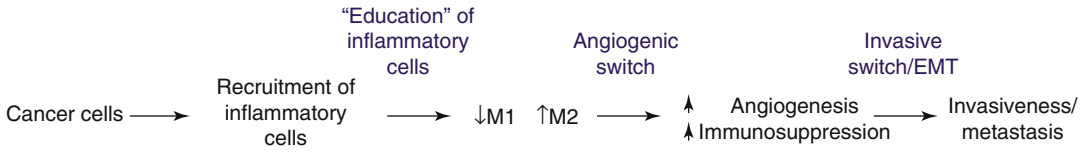

Fig. 22.1 Simplified scheme of tumor progression. During progression, cancer cells recruit inflammatory cells and "educate" their phenotype (in the case of macrophages, from M1 to M2). The phenotype abbreviated as M2 contributes to angiogenic switch and formation of proangiogenic and immunosuppressive tumor microenvironment. The development of abnormal tumor vascular network and the resultant hypoxia lead to invasive tumor phenotype (invasive switch/epithelial-mesenchymal transition/EMT)

of cancer cells is thus determined not only by accumulated mutations and mutation profiles but also by social interactions with other cells.

Tumor microenvironment is shaped by two intertwined processes (Fig. 22.1), both of which are elements of inflammation response [4] (inflammation is an important hallmark of cancer) [2]. The first one consists of recruitment or mobilization of inflammatory cells by cancer cells, while the other involves "education" of the former. Influenced by cancer cells, the recruited immune cells change their phenotype [5]. They become "cancer-specific" cells participating in tumor growth [2]. Modified immune reaction cells form a novel specific microenvironment which is both proangiogenic and immunosuppressive [6]. This milieu is co-formed by emerging blood microvessels and owing to immunosuppressive properties of both cancer and inflammatory cells. Such a milieu shields cancer cells from immunosurveillance [7]. However, tumor microenvironment is dynamic, and it may be reprogrammed to become anti-angiogenic and immunomodulatory. In other words, it can become a milieu that inhibits tumor progression.

The aim of this chapter is to draw attention to the possibility of using such reprogrammed tumor microenvironment in anticancer therapeutic context.

22.2 Recruitment of Inflammatory Cells by Cancer Cells

Mutations of certain genes in cancer cells (e.g., *RET, RAS, Myc,* and *p53*) trigger transcription of genes encoding chemotactic factors, including CC and CXC subgroup chemokines, the main chemoattractants of inflammatory reaction [4].

Chemokines released by cancer cells lead to the recruitment of various inflammatory cells from the bloodstream. For example, monocytes and macrophages are recruited mainly by CCL2 chemokine, whereas dendritc cells (DCs) are recruited by CCL20. The recruitment of regulatory lymphocytes (T_{regs}) is accomplished by CCL22, while CXCL1, CXCL5, CXCL6, and CXCL8 mobilize polymorphonuclear leukocytes (PMNs) [8].

Generally, recruitment of immune reaction cells is stimulated by cytokines and growth factors. For example, tumor-associated macrophages (TAMs) are mobilized (besides CCL2, CCL5, CCL7, CXCL8, and CXCL12 chemokines) by VEGF and PDGF cytokines as well as M-CSF [9].

Mobilization and recruitment processes also involve damage-associated molecular pattern (DAMP) molecules, especially high-mobility group box 1 (HMGB1) protein. HMGB1 is passively released from necrotized cancer cells, whereas actively, it is released from immune cells. HMGB1 stimulates neutrophils and monocytes to release proinflammatory cytokines [7]; it is also proangiogenic [10, 11].

Recruited inflammatory cells are specifically "educated" by cytokines, growth factors, and chemokines released by cancer cells [5]. In essence, such "education" results in the appearance of a specific type of inflammatory cells. Generally, such a reaction leads to the appearance of tumor growth-promoting phenotype. Among the best-studied types of cells affected by appearance of such phenotype are macrophages.

Rise of tumor-promoting phenotype among macrophages is possible due to extraordinary plasticity of these cells [12].

22.3 Macrophage Plasticity: M1 and M2 Phenotypes

Depending on organ localization, macrophages differ in phenotype and transcriptional profiles. As mentioned, macrophages display great plasticity, i.e., phenotype-changing capability [13]. Under the influence of TLR ligands (including LPS) or IFN-γ, macrophages exhibit the so-called M1 phenotype (classical macrophage activation), whereas alternative activation (M2) takes place when macrophages are stimulated by IL-4/IL-13 [14]. Polarization of macrophages into M1 and M2 classes reflects classification of Th1/Th2 immune cells.

Macrophages with M1 phenotype release proinflammatory cytokines (e.g., TNF-α, IL-1, IL-6, IL-12, and IL-23) [14]. Owing to increased expression of class I and II MHC molecules, M1 macrophages are capable of antigen presentation. These cells stimulate arginine metabolism and production of nitric oxide (NO) and citrulline. Due to released NO and reactive oxygen species (ROS), M1 cells are cytotoxic. M1 macrophage phenotype is controlled by signal transducer and activator of transcription 1 (STAT1) and interferon-regulatory factor 5 (IRF5) [15]. Typical M1 phenotype is correlated with elevated levels of released IL-12 and IL-23 as well as low level of IL-10 (IL-12^{high}, IL-23^{high}, IL-10^{low}) [13]. In the course of inflammation, polarization from M1 to M2 macrophages is observed. At present, several M2 cell subtypes have been identified: M2a, M2b, and M2c. M2a phenotype is stimulated by IL-4 or IL-13 cytokines, whereas M2b is stimulated by LPS, TLR, and IL-1ra receptor antagonist, respectively. Finally, M2c phenotype is induced by IL-10, TGF-β, and glucocorticoids [14]. If M1 cells are capable of removing pathogen factors, then cells of M2 phenotype participate in reconstruction of damaged tissues and de novo formation of blood microvessels [13]. M2 macrophages do not release nitric oxide. Instead, they highly express arginase I which metabolizes arginine to ornithine and polyamines, compounds necessary to synthesize collagen and to subsequent fibrosis and damaged tissue remodeling. M2 cells feature high levels of mannose and galactose receptors, as well as scavenger receptors. M2 inhibits the release of CXCL5, CXCL9, and CXCL10 chemokines while stimulating that of CCL24, CCL17, and CCL22, which facilitate the recruitment of eosinophils, basophils, and Th2 cells. Due to released IL-10 cytokine, M2 macrophages become immunosuppressive. M2 phenotype is controlled by STAT6, IRF4, and peroxisome proliferator-activated receptor-γ (PPARγ) [15]. Typical phenotype of M2 cells is linked with low levels of IL-12 and IL-23 and high level of IL-10 (IL-12^{low}, IL-23 ^{low}, IL-10 ^{high}) [13].

22.4 TAM: Cells with M2 Phenotype

In tumors, a specific population of macrophages (tumor-associated macrophages (TAMs)) has been observed. TAM cells make up for over 50 % of the tumor mass [9]. The phenotype of TAMs is similar to that of M2 macrophages [16]. Formation of this phenotype from progenitors requires Th2 lymphocytes (which are the source of IL-4 and IL-13), T_{reg} lymphocytes (which synthesize TGF-β and IL-10), as well as cancer cells and tumor-specific fibroblasts (CAFs).

TAMs play different roles in tumor environment [17]. They strongly affect tumor progression. For example, TAMs synthesize EGF, which stimulates the growth of cancer cells. They release proangiogenic factors (VEGF, PDGF, and TGF-β) and several FGF family factors. TAMs stimulate immunosuppression (IL-10) [6]. Through the release of CCL17 and CCL22 chemokines, TAMs are capable of recruiting T lymphocytes (T_{reg} and Th2). TAM cells also release CCL8, which recruits "naïve" T lymphocytes. These lymphocytes become anergic in the tumor microenvironment.

TAM macrophages have the tendency to accumulate in underoxygenated (hypoxic) tumor regions [17]. Under such conditions, TAMs induce transcription factor HIF-1α, VEGF, and CXCL12 (and its receptor CXCR4), which

modulate TAM migration into avascular regions. HIF-1α controls expression of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1). At low concentrations of IFN-γ, transcription factor HIF-2α induces expression of Arg1, inhibits NO synthesis, and favors 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 [5].

TAM cells release immunosuppressive cytokines (TGF-β and IL-10) and synthesize the immunosuppressive arginase 1 enzyme [14]. These cytokines and arginase exert considerable effects on the growth of cancer cells. TGF-β cytokine stimulates M1 to M2 polarization of macrophages and inhibits cytolytic activity of NK cells, as well as migration and activity of DCs. TGF-β stimulates differentiation of $CD4^+$T cells to Th2 and blocks activity of $CD8^+$ T cells by inhibiting the activity of granzyme A and B as well as IFN-γ. TGF-β also promotes the activity of T_{reg} lymphocytes.

Immunosuppressive interleukin-10 is released by both TAM and $CD8^+$ cells, as well as by cancer cells. IL-10 inhibits the activity of IL-12, maturation of DCs, and release of cytotoxic cytokine IFN-γ, the main cytokine stimulating differentiation of "naïve" T lymphocytes [6, 14].

Arginase 1, a Th2 cell molecular marker, is also active in cancer cells. This enzyme exerts a negative effect on functioning of T cell receptors (TCRs) and inhibits $CD8^+$ T cell response [14].

TAM cells are programmed to release proangiogenic factors and enzymes involved in the formation of blood vasculature [6, 14]. Proangiogenic agents include, among others, VEGF, PDGF, TGF-β, and FGF, whereas enzymes modifying extracellular matrix (ECM) are MMP-2, MMP-7, MMP-9, MMP-12, and "plasmin system". MMP-9 metalloproteinase releases proangiogenic factors sequestered by extracellular matrix proteins [6]. TAM macrophages also participate in the formation of vascular junctions [18] and play a major role in the creation of the so-called angiogenic switch [19]. 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). TAM cells which synthesize VEGF-C and VEGF-D also participate in the formation of lymphatic vessels [6].

TAM cells, as well as other immune cells (e.g., T_{reg} lymphocytes), link immunosuppression and angiogenesis [20–23]. This link between angiogenesis and immunosuppression may be due to pluripotent properties of some proangiogenic agents. VEGF is not only proangiogenic, it also acts as an inhibitor of dendritic cells' maturation [24]. VEGF also stimulates proliferation of T_{reg} lymphocytes [23]. PlGF, VEGFR1 receptor ligand also inhibits differentiation of DCs [25]. HGF factor inhibits antigen presentation by DCs and stimulates the appearance of Th2 lymphocytes [26]. TGF-β is not only proangiogenic but also a cytokine which stimulates polarization of M1 macrophages into M2 [27].

Macrophages possess a dual nature (thus they have been dubbed "a double-edged sword"): 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., TAM (M2) macrophages) [28].

Polarization of macrophages depends on the environmental context of various signals secreted by both cancer and other tumor milieu cells [13]. Depending on certain signals' domination, macrophage cells present either M1 or M2 phenotype. 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 macrophage cells. Under hypoxic conditions, macrophages display their M2 phenotype.

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 [7]. For example, 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) [29]. Milieu lacking TGF-β causes neutrophils to participate in the elimination of cancer cells (type I). Dual nature

is also shown by NKT cells [30], DCs [31], mast cells [32], T_{reg} cells [33], and NK cells [34, 35]. Figure 22.2 shows examples of immune system cells possessing such dual nature.

22.5 M1 → M2 Tumor Microenvironment Reversal: Therapeutic Approach

Is it possible to revert macrophage phenotype from M2 to M1? In other words, is it possible to revert tumor milieu from proangiogenic and immunosuppressive to anti-angiogenic and immunostimulatory one?

If M1 or M2 phenotype is a result of destabilized equilibrium between proangiogenic/immunosuppressive agents and anti-angiogenic/immunostimulatory ones, then shifting this equilibrium in favor of anti-angiogenic and immunostimulatory one (M1 phenotype), we can perhaps revert tumor growth dynamics and create conditions leading to elimination of cancer cells (Fig. 22.2).

One of the first therapeutic attempts of this kind was proposed by Guiducci et al. [39]. In order to revert M2 to M1 phenotype, these authors used an antibody-inhibiting IL-10R receptor and CpG oligonucleotide-activating TLR9 receptor. Combination of these drugs permitted a specific "reeducation" of M2 macrophages and led to a considerable therapeutic benefit. Specific "reeducation" of macrophages can also be accomplished by using other therapeutic agents. For instance, a CD40-activating agent (CD40 is a protein belonging to the TNF-like receptor superfamily) was shown to promote M2 → M1 conversion [40]. Therapeutic effects were also observed in animals burdened with tumors in which genes encoding NF-κB transcription factors were active [41] Also, certain proinflammatory agents (e.g., imiquimod), which stimulate TLR7/8 receptors, induce antitumor reaction. M2 → M1 conversion could also be observed in the case of polyI:C therapeutic agent (dsRNA analog) [42]. Its receptor turned out to be the Toll-like receptor 3 activating TICAM-1 signaling pathway. This pathway is not only important from the standpoint of dendritic cells' maturation; it is also necessary for eliciting an antitumor response. A substantial role in M2 → M1 conversion is played by CpG oligonucleotides activating TLR9 receptors [43]. These factors inhibit immunosuppressive activity of monocytes and stimulate release of Th1 cytokine

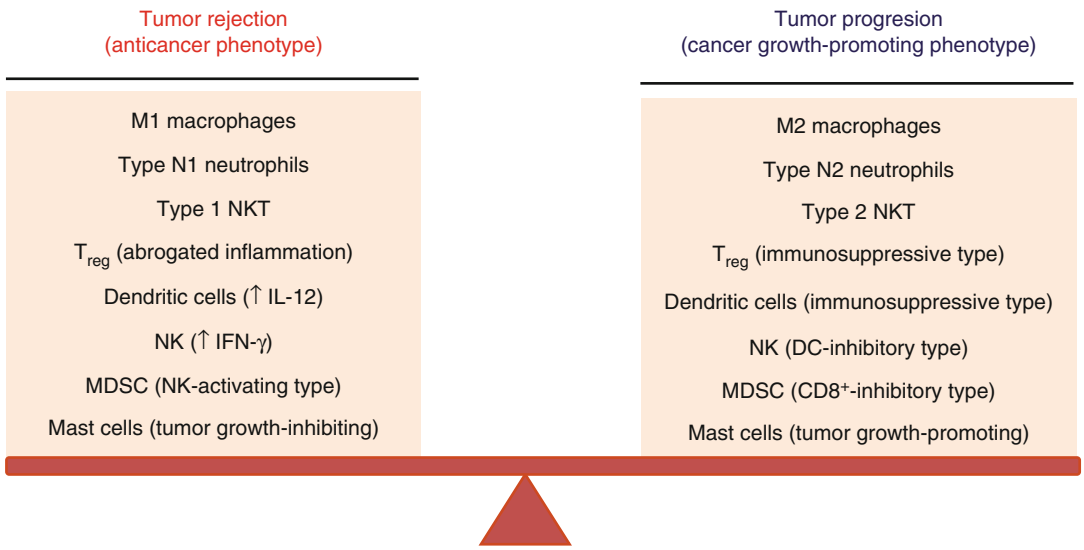

Fig. 22.2 Dual nature of inflammatory cells. Prevalence of either phenotype results in tumor rejection or continued growth and progression (Data from Ostrand-Rosenberg [36], Noonan et al. [37], Grivennikov et al. [38], Shurin et al. [31] and Chow et al. [7])

as well as the appearance of macrophages having cytotoxic properties (M1). Rolny et al. [44] demonstrated the effect of histidine-rich glycoprotein on normalization of blood vasculature and polarization of TAMs.

Different combinations of anti-angiogenics and immunostimulants also possess therapeutic properties [45, 46]. Sunitinib, a specific inhibitor of tyrosine kinase activity (found in many receptors of proangiogenic factors), when combined with IL-12, a cytokine with anti-angiogenic and immunostimulating properties, had a distinct effect on tumor growth [47]. Equally effective were also combinations of anti-TGFβ with agents stimulating immune response [48–50]. Finally, the combination of DNA vaccine directed against endoglin (CD105), a tumor vascular endothelial cell-surface protein, with interleukin-12 (IL-12) showed increased efficacy [51], although it had been shown that IL-12 alone is able to polarize the phenotype (M2 → M1) [52].

22.6 Concluding Remarks

Cancer cells recruit and "educate" immune system cells, i.e., change their phenotype. "Educated" immune cells lose their basic properties, i.e., the ability to present antigens, as well as their cytotoxicity, and become active in forming blood vasculature (angiogenesis) and in immunosuppression. Latest studies point out to the feasibility of reverting such a phenotype (proangiogenic and immunosuppressive) to anti-angiogenic and immunostimulating, which could be important in inhibiting tumor growth. We believe that such therapeutic solutions merit closer scrutiny.

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Immunotherapies Targeting a Tumor-Associated Antigen, 5T4 Oncofetal Glycoprotein

23

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Contents

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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) associated 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

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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 trophoblast syncytio-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 kD 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 cervix [3], cervical precancer [7], colorectal [8–10], gastric [11, 12], ovarian [13], oral [14], prostate [15], lung [16, 17], and renal tumors [18]. For colorectal, gastric, and ovarian cancer, there was evidence of tumor expression levels correlating with poorer clinical outcome.

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 [19] (Fig. 23.1). The latter motif is associated with protein-protein interactions of a functionally diverse set of molecules [20]. 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 functionality relevant to cancer spread. Constitutive expression of human 5T4 cDNA in murine fibroblasts showed 5T4 to be found on the

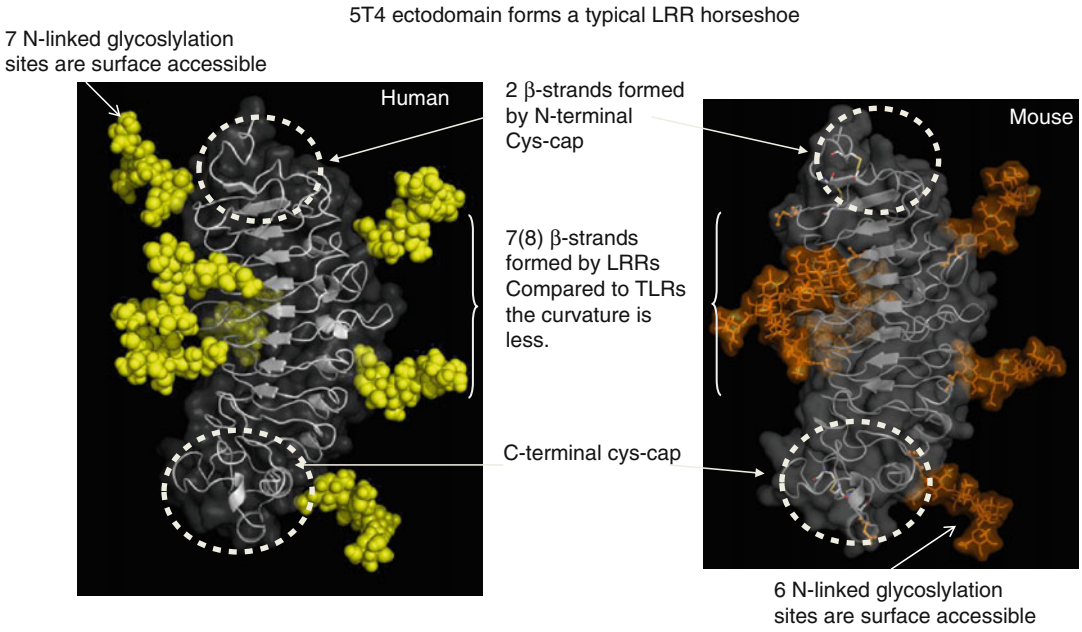

Fig. 23.1 Structure of 5T4 molecules. Human and mouse 5T4 analyzed by a homology modeling approach using the variable lymphocyte receptor A29 (PDB entry 2o6q) and energy minimized to produce RAW structures (Courtesy of Alex Weber and Andriy Kubarenko, DKFZ, Germany)

tips of microvilli and induced a more spindle-shaped morphology, disruption of cell contacts, and a reduction in adherence [21]. 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 [22]. 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 [23]. The isolation of the murine 5T4 gene confirmed its evolutionary conservation and provided additional tools for evaluating 5T4-targeted immunotherapies [24, 25].

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 [26]. The 5T4 oncofetal antigen is an early marker of differentiation of mouse and human embryonic stem (ES) cell [27–29]. 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 [30, 31]. 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 [30, 31]. These data and our previous observations showing 5T4 overexpression in epithelial cells associated with downregulation of E-cadherin [22] 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 –ve) and early differentiating (5T4 +ve) 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 [32]. CXCL12 binds to the widely expressed cell surface seven transmembrane domain G-protein-coupled receptor CXCR4 [33] and to the recently identified receptor CXCR7/RDC1 [34]. Subsequently, 5T4 molecules were shown to be required for functional expression of CXCR4 at the cell surface in some embryonic and tumor cells [17, 35, 36]. Both CXCL12 expression and CXCR4 expression have been associated with tumorigenesis in many cancers including breast, ovarian, renal, prostate, and neuroblastoma [33, 37, 38]. 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 [35]. Further, FRET studies do not support a direct interaction between the molecules, while preliminary proteomic analysis following cross-linking of 5T4 molecules indicates

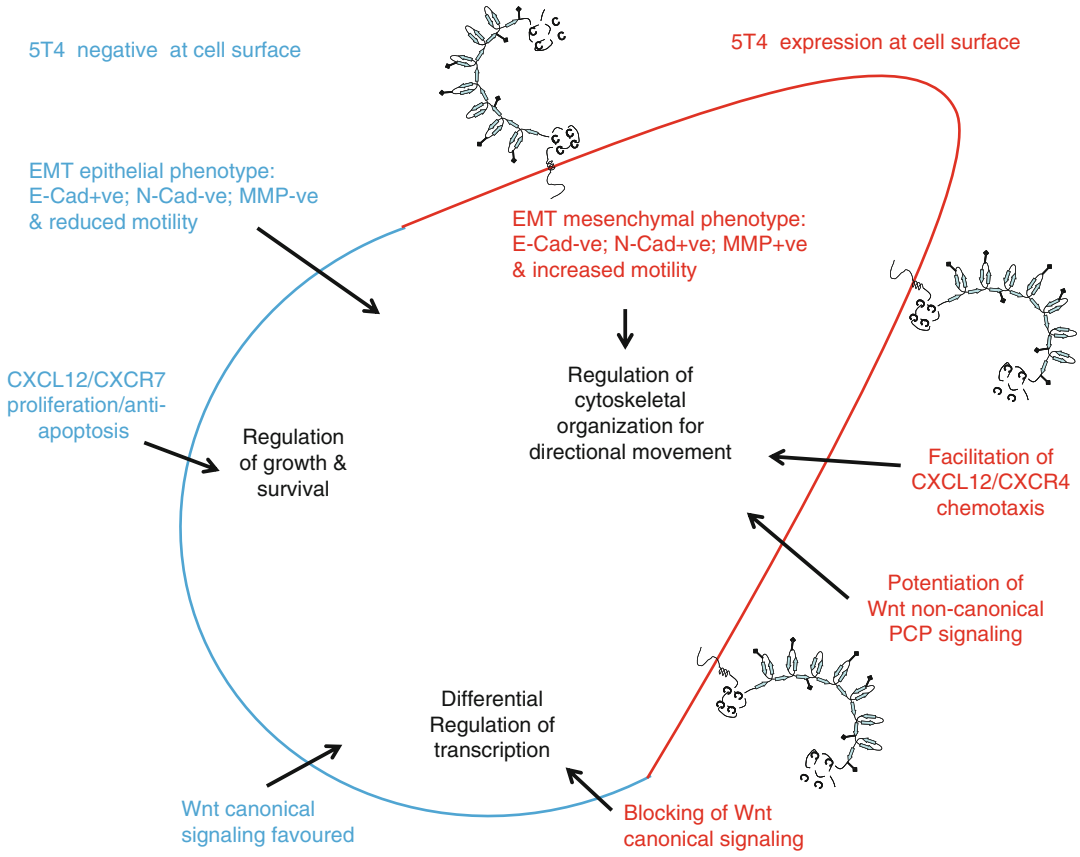

Fig. 23.2 5T4 functional influences on tumor spread. Integrated 5T4 regulation of both the chemokine and Wnt pathways acts to promote cancer spread as well as functional migration in development and cancer

many cytoskeleton-associated interactions [39] (Vaghjani and Stern, 2012). 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. We have recently shown that 5T4 also inhibits Wnt/β-catenin canonical while concomitantly activating the non-canonical Wnt signaling pathway associated with increased motility [40]. 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

The selective tumor expression pattern of 5T4 as well as a putative role in tumor spread established this target as a suitable target for vaccine immunotherapy. 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 the viral vector of choice for expressing 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 [41]. In this xenogeneic-TAA model, it was shown that the likely component of protection was antibody with induction dependent on the $CD4^+$ T cells [42]. 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 [41].

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 [43, 44]. These data are consistent with the influence of Tregs on limiting immune responses to TAA [45]. 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 [46].

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, prostate, 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 [49]. 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

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. [42]
Metastatic colorectal phase II (19)	1st line+5FU/ LV/irinotecan	83	83	92	100	None (12)	Harrop et al. [47]
Metastatic colorectal phase II (17)	1st line+5FU/ LV/ oxaliplatin	91	91	91	100	ELISPOT *vs.* tumor response (11)	Harrop et al. [48]
Metastatic colorectal phase II (20)	Adjuvant to liver metastasis surgery	100	88	53	100	Proliferation *vs.* survival (17)	Elkord et al. [49]
Prostate-hormone refractory phase 11 (27)	2nd line+/– GM-CSF	100	nt	36	100	ELISPOT *vs.* PFS (24)	Amato et al. [48]
Metastatic renal cell carcinoma phase II (11)	1st & 2nd line + IFN-α	100	nt	36	100	None (11)	Hawkins et al. [50]
Metastatic renal cell carcinoma phase II (28)	1st & 2nd line +/–IFN-α	91	nt	30	91	Antibody *vs.* survival (23)	Amato et al. [51]
Metastatic renal cell carcinoma phase II (25)	2nd line low dose Il-2	90	nt	30	90	ELISPOT *vs.* survival (20)	Amato et al. [50]
Metastatic renal cell carcinoma phase II (28)	2nd-line high-dose IL-2	100	nt	36	100	Antibody *vs.* survival (19)	Kaufman et al. [52]

TroVax clinical development overview
nt not tested

relative immune suppression and/or escape, highlighting the complexity of positive and negative factors challenging any simple correlation with clinical outcome [53].

23.4.3 TroVax Phase III Clinical Trial in RCC

Building on the several phase II studies in RCC (Table 2 in ref. [54]), 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 [55]. The primary endpoint 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 endpoint, 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 [56, 57]. 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 [58]. 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 [46, 53].

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 in Kim et al. [54]). 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 [59]. 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 both stimulate regulatory as well as 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 [60]. 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 [61]. Mouse 5T4-specific T-cell epitopes were identified using the 5T4KO mouse, and wild-type (WT) responses were evaluated as a model to refine and improve immunogenicity. Studying the immune response

(INF-γ ELISPOT) of 5T4KO mice to rAdm5T4 vaccination identified only two dominant $H2^b$-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 5T4KO 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 [62], 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 [60]. An alternative to vaccination is the adoptive transfer of tumor-specific lymphocytes. To test the potency of this approach in the m5T4 model, primed 5T4KO 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 [45, 63, 64]. However, a clinical study investigating the adoptive transfer of CD25-depleted (includes Tregs) peripheral blood mononuclear cells in cyclophosphamide/fludarabine pre-conditioned RCC patients showed that this treatment resulted in only a short period of *in vivo* Tregs depletion [65].

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 [66] can potentially improve vaccine immunogenicity by favoring generation of high-avidity $CD8^+$ T cells capable of functioning in an autologous tumor-bearing animals.

Another way to overcome limited immunity to TAA is by modulating costimulatory/inhibitory signals on T cells such as through CTLA-4 [67]. However, while clinical results with the humanized anti-CTLA-4 antibody, ipilimumab, have led to its licensing for the treatment of advanced melanoma; the precise mechanism accounting the effects in patients is not known [68]. The benefits of increased survival of a few months are only seen in a small 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 [69]. 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 [69].

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 [70, 71]. 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 [72].

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 [73]. This agent (ABR-214936) maintained 5T4-specific superantigen antibody-dependent cellular cytotoxicity (SADCC) while toxicity for MHC class II expressing cells was reduced by 1,000-folds *in vitro* (SDCC); therapeutic efficacy was demonstrated in murine xenograft tumor models [74].

23.5.2 Early-Phase Clinical Studies

In a phase I study 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 [75]. 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 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 [76].

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

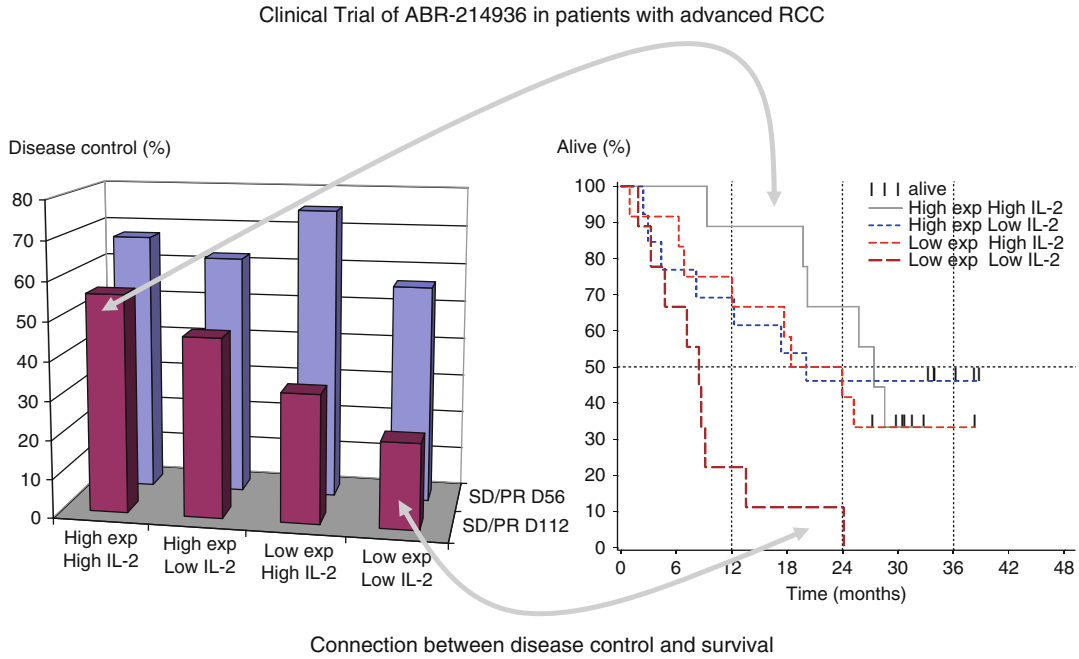

Fig. 23.3 5T4 antibody-directed superantigen therapy. Clinical trial of 5T4 antibody-directed superantigen therapy in patients with advanced RCC. This shows that patients with high IL-2 after the second infusion and high exposure are more likely to have disease control at day 112 and the longest survival (Adapted by permission from *British Journal of Cancer*: Shaw et al. [76])

hybrid SEA/E-120 superantigen sequence with additional point mutations reducing MHC class II binding and antigenicity [77, 78]. 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 [79]. 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, plus selective retention of ANYARA in tumor tissue as demonstrated using PET.

23.5.3 A Phase II/III Clinical Trial in RCC

A multinational (50 sites in Europe: UK, 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 endpoint – 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 ABR214936 in RCC patients [76]. 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 [58] or pazopanib [80]. In a hypothesis-generating analysis of approximately 25 % of patients with low/normal levels of base line 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 [81]. Additional analyses of the ANYARA phase II/III study data are ongoing with future development strategies aiming at a pivotal phase II/III study with ANYARA in combination with 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 [82]. Another study showed that 5T4 is expressed on tumor-initiating cells (TICs) in (NSCLC) xenografts, and this correlated with worse clinical outcome for the

patients [16]. Consistent with other mechanistic studies [30, 31], 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 [83]. 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 consistent with the targeting of TICs within the tumors.

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. The overall therapeutic value is enhanced by the targeting of the most aggressive and tumorigenic populations within tumors (TICs), and its testing in a clinical setting is now underway.

23.6.2 Direct 5T4 Antibody Effects

5T4 expression has recently been shown to correlate with the risk of relapse in pre-B-acute lymphoblastic leukemia (ALL) patients [36]. 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. Further using B-ALL cell lines (Sup5T4 and Sup derived from Sup-B15 pre-B-ALL), 5T4 expression was shown to correlate with a more

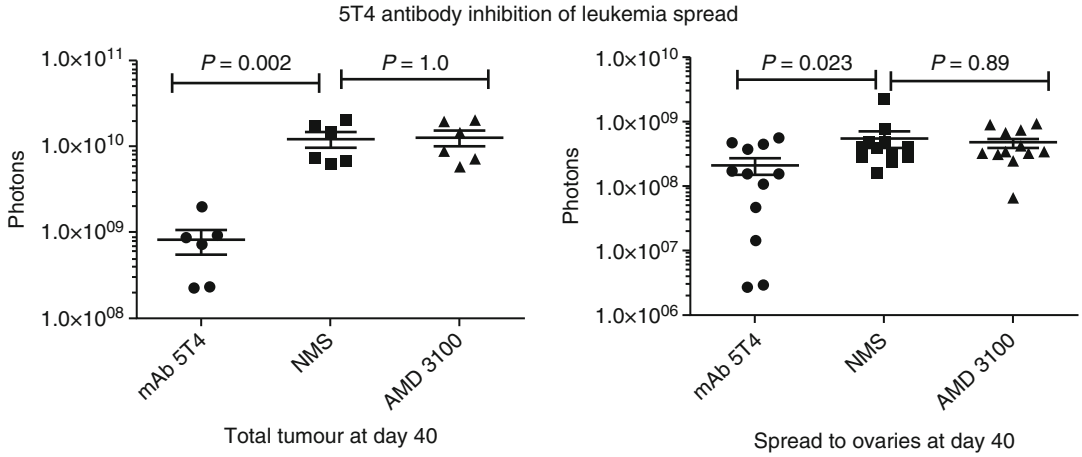

Fig. 23.4 5T4 antibody inhibition of leukemia spread. 100 μg mAb 5T4 but not normal mouse serum (NMS) (both given day 1 and then every other day for 10 days) or AMD3100 (plerixafor at 1.25 mg/kg, given daily for 10 days) blocks spread of intravenous Sup5T4 leukemia (5 × 10e6). Significant reduction in total tumor load and for spread to the ovaries at day 40 for mAb5T4 compared to either NMS- or AMD3100-treated animals (Mann-Whitney)

immature ALL phenotype, CXCR4/CXCL12 chemotaxis, increased invasion, and adhesion *in vitro*. Interestingly, 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. 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) [84]. 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. We have also shown, as for mouse embryonic cells, that some mAbs to 5T4 can block CXCR4/CXCL12 chemotaxis *in vitro*. More importantly *in vivo* antibody treatment is able to prevent the spread of 5T4-positive Sup-B15 B-ALL cells in the xenograft model [36] (Fig. 23.4). It is possible that the observed influence of spread might in part derive from inhibition of 5T4 glycoprotein function in regulating chemokine or Wnt signaling pathways. In the context of B-ALL, the use of 5T4 as a relapse risk prognostic, potential therapeutic target, and insight into its mechanistic involvement of tumor spread and relapse is 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 [63, 64]. The precise specificity and differentiation status of the TILs is largely unknown, but when successful presumably favors an antitumor effector rather than regulatory T-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 [85]. 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 is in progress with several TAA targets including CD19 (leukemia/lymphoma), PSMA (prostate), and CEA (colorectal and breast cancer) [85–87].

A high-affinity scFv specific for h5T4 [88] 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 [89]. 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 was also tested in an animal model [90]. 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 [35] have been used to construct CARs with modified murine T cells, and they were able to kill m5T4-expressing tumor cells *in vitro* [91]. The next step will compare m5T4-specific natural T cells (generated in the 5T4KO mouse; [60]) and gene-modified T cells, in therapy of an autologous m5T4B16 tumor in WT and 5T4KO mice. Overall 5T4 CAR T cells are a powerful means to bypass a number of mechanisms which allow tumors to escape T-cell killing [53] and can be readily scaled up for clinical use.

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 rationale 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 influences the immune system since it may be controlling otherwise "unseen" cancer or be required for optimal disease resolution.

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24 Emerging Biomarkers During Clinical Development of Anti-CTLA4 Antibody Therapy

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24.1 Introduction

It has now been more than 100 years since Williams Coley's proof-of-principle experiments, where patients with cancers (mostly bone and soft tissue sarcomas) were successfully treated with a vaccine composed of bacterial products able to elicit immune responses that resulted in tumor shrinkage and eradication. These treatments ultimately fell completely out of favor by the 1960s. Immunotherapy as a field continued to be investigated intensively, but no benefit was shown for any specific approach. All of this changed when the US Food and Drug Administration (FDA) approved both sipuleucel-T, a patient-derived dendritic cell-based vaccine, for castrate-resistant prostate cancer and ipilimumab, a monoclonal antibody (mAb) against cytotoxic T-lymphocyte antigen 4 (CTLA4), for advance melanoma in 2011.

CTLA4 is an inhibitory molecule that is expressed on activated T cells and regulatory T cells (T_{reg}) and is essential for the maintenance of immunologic homeostasis [1, 2]. Therapeutic strategies that block CTLA4 have been shown to increase immunologic responses and augment antitumor immunity. Two antibodies that block CTLA4, ipilimumab and tremelimumab, have been evaluated in several phase III clinical trials. These studies have shown improved overall survival for patients with metastatic melanoma treated with ipilimumab in the first- or second-line setting [3, 4]. Despite these

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improved clinical outcomes, only a subset of patients benefit from ipilimumab treatment. Meanwhile, patients may experience mechanism-based toxicity from the upregulation of immune responses [5].

While evaluations of immunotherapy have largely focused on melanoma (because of durable responses noted with immune-based treatments, such as high-dose interleukin (IL)-2) [6], there is now growing evidence that this approach is also applicable to other malignancies, including prostate, lung, and pancreatic cancer; hepatocellular carcinoma; and hematologic malignancies [5, 7–11]. There is a critical need for biomarker development to elucidate pharmacodynamic changes, understand the potential mechanisms of action, and identify novel correlates associated with clinical benefit and/or toxicity. In addition, anti-CTLA4 antibodies are the first in a group of immunomodulatory molecules currently undergoing clinical evaluation, e.g., other antibodies target programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1). Many ongoing strategies seek to combine several of these antibodies together or to study ipilimumab in combination with radiation or chemotherapy [12, 13].

Biomarkers are identified by immune monitoring assays, which currently permit the monitoring of immune cell activation and effector functions, immunosuppression, antigen-specific immunity, and analysis of these populations in the tumor microenvironment. The ultimate goal is to identify a robust biomarker predictive of a clinical outcome of interest. Continued and rational development of effective cancer immunotherapy will also heavily depend on deeper understanding and close monitoring of immune responses. In this chapter, the authors would focus on immune assays used in the monitoring of cancer patients treated with ipilimumab (and, in select cases, with tremelimumab); unless otherwise specified, these trials involve melanoma patients. We then provide our perspective on the future of monitoring of cancer immunotherapy and propose a stepwise algorithm for testing in the context of increasingly complex combination and multimodality treatments.

24.2 Absolute Lymphocyte Count

Ipilimumab augments antitumor immune responses via activation and increase in the proliferation of T cells [14]. Several studies strongly suggest that the absolute lymphocyte count (ALC) may be a pharmacodynamic (PD) marker for ipilimumab in melanoma and other solid tumors. Analysis of a randomized phase II study of three different doses of ipilimumab – from 0.3 to 10 mg/kg every 3 weeks – showed that the rate of increase of ALC following ipilimumab treatment increased in a dose-dependent manner [15]. A review of 533 melanoma patients treated on four phase II studies with various doses of ipilimumab went further to suggest that a higher rate of change of ALC was associated with clinical benefit from ipilimumab [16].

In this chapter, the authors have summarized their single-institution experience at Memorial Sloan-Kettering Cancer Center (MSKCC) of ipilimumab 10 mg/kg every 3 weeks in melanoma patients. It was observed that patients with an ALC $\geq$1,000/μL after 2 doses of ipilimumab had higher rates of clinical benefit (defined as complete or partial responses [CR or PR] or stable disease [SD]) at week 24 and longer median OS than patients with ALC <1,000/μL [17]. A similar association in patients receiving ipilimumab 3 mg/kg was noted, but not in patients who were treated with a tyrosine kinase inhibitor against the *B-raf* oncogene [18].

The idea that an ALC threshold may be predictive of benefit from ipilimumab was reinforced by another single-institution study that showed that while baseline ALC did not correlate with OS, an ALC of $\geq$800/μL after two doses correlated with superior OS ($p = 0.010$) [19]. An ALC ratio (after two doses compared to baseline) >1 also correlated with improved OS ($p = 0.028$). The association between ALC and benefit from ipilimumab has also been seen in prostate cancer. An increase of 25 % or more over baseline ALC was associated with superior OS in prostate cancer patients treated with ipilimumab and a granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting cancer vaccine [20].

24.3 Analyses of Different Cell Populations in Peripheral Blood

As the ALC is a crude biomarker, there has been strong interest in characterizing changes in specific T cell subsets in the peripheral blood using flow cytometry assays. Evaluating specific T cell subsets may provide for a more robust biomarker and may also offer important insights into the mechanistic effects of anti-CTLA4 therapy. We evaluated 35 melanoma patients who received ipilimumab at MSKCC and showed that those patients who experienced clinical benefit from ipilimumab had a greater absolute increase in the number of $CD8^+$ T cells compared to patients who did not derive benefit ($p=0.0294$). The absolute increase in $CD4^+$ T cells did not differ significantly between these two groups of patients ($p=0.2237$) [21].

24.3.1 T Cell Activation Markers

Cell surface markers expressed on T cells have also been examined to further characterize T cell responses during ipilimumab therapy. Increased levels of human leukocyte antigen (HLA)-DR and CD45RO, which are markers of T cell activation, on $CD4^+$ and $CD8^+$ T cells after ipilimumab treatment have been reported in several studies [13, 22–24]. Ipilimumab increased activated $HLA\text{-}DR^+CD4^+$ and $HLA\text{-}DR^+CD8^+$ T cells with a concomitant decrease in ($CCR7^+CD45RA^+$) naïve T cells. There was an increase in $CD4^+$ and $CD8^+$ central memory ($CCR7^+CD45RA^-$) and effector memory ($CCR7^-CD45RA^-$) after ipilimumab treatment [25]. However, there was no correlation between the degree of elevation of HLA-DR or CD45RO or memory T cell markers and clinical response to ipilimumab. A study of 12 patients treated with tremelimumab suggested similar effects, although there was some correlation with clinical benefit in this small cohort [26].

Inducible co-stimulator (ICOS) is expressed on the cell surface after T cell activation and plays a role in T cell expansion and survival. At MSKCC, 14 patients treated with ipilimumab 10 mg/kg were analyzed retrospectively. A sustained increase over 12 weeks of $CD4^+ICOS^{hi}$ T cells correlated with improved survival [27]. These results are consistent with those from another study, in which ipilimumab therapy in 75 patients led to an increase in $ICOS^{hi}$ and proliferating ($Ki67^+$) $CD4^+$ and $CD8^+$ T cells [28]. However, this study did not correlate these changes with clinical benefit. Finally, a third study demonstrated an increase in the frequency of $CD4^+ICOS^+$ but not $CD8^+ICOS^+$ T cells from baseline to after one and four treatments, respectively [25].

Ipilimumab was administered preoperatively in a trial of six patients with bladder cancer. The frequency of $CD4^+ICOS^+$ T cells increased in both the peripheral blood and bladder tumor tissue after ipilimumab treatment [29]. A study in which breast cancer patients received tremelimumab also reported an increase in $CD4^+ICOS^+$ T cells [30]. Taken together, these studies suggest that CTLA4 blockade may affect ICOS expression on $CD4^+$ and $CD8^+$ T cells differently and in a dose-dependent manner. An increase in the frequency of $CD4^+ICOS^+$ T cells may be a biomarker to indicate biologic activity in the setting of anti-CTLA4 therapy. These results are from several small retrospective analyses and warrant further prospective studies in a larger cohort of patients and correlation with clinical outcomes.

24.3.2 Regulatory T Cells

Regulatory T cells (T_{regs}) can suppress immune responses to both self- and nonself-antigen and are crucial in the maintenance of immunologic tolerance [31]. In cancer, T_{regs} are thought to have a deleterious effect by suppressing antitumor immune responses [32]. The study of T_{regs} has exponentially increased since the identification of phenotypic markers, specifically $CD4^+CD25^+$ cells [33] and the transcription factor FoxP3, which is generally accepted as the best available marker for T_{regs} [34]. Patients with cancer often have an increased number of T_{regs} in the peripheral blood or even within the tumor microenvironment [35–37].

T_{regs} constitutively express FoxP3 as well as CTLA4 on their cell surface and intracellularly. Therefore, anti-CTLA4 therapy may enhance antitumor immunity in part by depleting T_{regs}. A phase I study that treated 11 patients with various malignancies showed that T_{regs} – defined as $CD4^+CD25^+CD62L^+$ cells – declined at early time points (72 h after infusion) but rebounded to a level at or above baseline valued at the time of next dose [12].

In contrast, several other studies showed that ipilimumab therapy did not deplete $FOXP3^+$ T_{regs} *in vivo* but in fact induced their proliferation and expansion. In a study of melanoma patients receiving ipilimumab, a significant increase in relative FoxP3 expression was detected in post-therapy compared with pre-therapy samples in the $CD4^+CD25^+$ population [23]. Similarly, a phase I study of escalating doses of ipilimumab and GM-CSF in prostate cancer patients showed that $CD4^+FOXP3^+$ T cells were expanded at lower doses of ipilimumab, whereas activated effector $CD4^+$ cells were expanded only at higher ipilimumab doses [38].

Taken together, these two studies suggest that ipilimumab may function not so much through depletion of T_{regs} but augmentation/expansion of effector T cells. It may also be that CD25 and FoxP3 may not be the best markers for human T_{regs} in the setting of cancer immunotherapy. Several new markers such as a leucine-rich repeat-containing molecule (LRRC32 or GARP), Helios, CD39, and CD73 have recently been defined as markers of the T_{reg} phenotype [39–41]. Whether these markers define a cellular population that is suppressed by ipilimumab remains to be seen.

24.3.3 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs represent a phenotypically heterogeneous cell population that includes immature and mature myeloid cells, activated granulocytes, macrophages, as well as cells expressing markers of immature DCs. MDSCs can also function as antigen-presenting cells. Human MDSCs have been found in patients with head and neck squamous cell carcinoma, non-small cell lung cancer, and metastatic adenocarcinomas of the pancreas, colon, and breast [42, 43]. Human MDSCs have an immature phenotype, including lineage negative (Lin^-), $CD14^-$, $HLA\text{-}DR^-$, $CD15^+$, $CD34^+$, $CD11b^+$, $CD33^+$, and $CD13^+$ cells [44, 45]. Phenotypically, MDSCs exert an immunosuppressive effect, mainly through the production of suppressive molecules, such as ARG1 or cytokines such as transforming growth factor-β and IL-10.

In melanoma patients, the proposed phenotype is $CD14^+/HLA\text{-}DR^{low/-}$, based upon this cell population's ability to suppress lymphocyte function. $CD14^+/HLA\text{-}DR^{low/-}$ cells have been shown to increase in patients with melanoma, and the quantity of these cells has been shown to correlate with melanoma disease activity [46]. In a pilot study of 26 melanoma patients receiving ipilimumab, a lower baseline MDSC frequency was associated with improved OS (HR 1.07, $p = 0.002$), even when adjusting for pre-treatment ALC and LDH levels [47]. Efforts are ongoing to evaluate this finding in a larger cohort of patients and to determine whether this finding is specific for ipilimumab treatment *vs*. other anti-melanoma therapies.

24.4 Antigen-Specific Immunological Monitoring

Some cancers are immunogenic because they express a variety of tumor-associated antigens (TAAs). These include differentiation antigens that are tumor and tissue specific (e.g., Melan-A and gp100 in melanoma); cancer-testis (CT) antigens, which are expressed in a variety of human malignancies but not in normal adult tissue except for the testis and placenta (e.g., NY-ESO-1 and MAGE antigens); and products of gene translocation or mutation (e.g., Bcr/Abl in chronic myelogenous leukemia or mutated *p53* in multiple cancers). Metastatic melanoma has been shown to be an immunogenic malignancy, associated with spontaneous immunity to these tumor-specific antigens [48–50]. Cancer vaccines in melanoma are also able to induce a strong immune response [51, 52].

CTLA4 blockade may directly potentiate TAA-specific $CD4^+$ and $CD8^+$ T cell responses. In turn, activated $CD4^+$ cells may provide help to B cells, leading to enhancement of antibody production that can further increase T cell responses. Therefore, the analysis of antibody and T cell responses against TAAs may provide for an additional level of specificity in studying the immune effects of anti-CTLA4 therapies.

24.4.1 Antigen-Specific Antibody Response

Various studies have confirmed the generation of TAA-specific antibody responses following ipilimumab therapy. An early case report of a melanoma patient previously treated with an autologous GM-CSF-expressing tumor vaccine who then received ipilimumab showed an increase in antibodies against MHC class I chain-related protein A, which is overexpressed in many human cancers [53]. Induction of antibody response to NY-ESO-1 was also anecdotally reported in patients with ovarian cancer treated with ipilimumab [54].

Our group has observed increases in antibody titers against NY-ESO-1 in melanoma patients who received ipilimumab. In a recent serologic analysis of melanoma patients treated with ipilimumab, 22 of 144 patients (15.3 %) produced antibodies against NY-ESO-1. These 22 who had detectable NY-ESO-1 antibody titers were more likely to experience clinical benefit than those with no detectable NY-ESO-1 antibody titers (12/22; 55 % *vs*. 36/118; 31 %, respectively, $p = 0.0481$) [55]. In contrast, investigators at the National Cancer Institute – responding to our findings – did not find any difference in the 6 of 46 patients who had an increase in NY-ESO-1 antibody titers following ipilimumab therapy [56]. However, their study utilized different response criteria and different doses of ipilimumab, and it is ultimately difficult to make any specific comment based on the small sample size.

A similar lack of correlation between antibody responses and clinical benefit was noted in serologic analysis of blood samples from 197 melanoma patients treated on two phase II trials of ipilimumab. NY-ESO-1 antibody titers increased by at least fivefold after four treatments in 17–18.8 % of patients. Increased antibody titers were also detected for the TAAs: Melan-A, MAGE-A4, SSX2 and p53. However, there was no significant association between humoral response to tumor antigens and clinical benefit in this study either [25].

NY-ESO-1 antibody responses have also been detected in prostate cancer patients treated with ipilimumab. In a phase I clinical trial that combined increasing doses of ipilimumab with GM-CSF in prostate cancer patients, 5 of 24 patients had detectable antibody responses to NY-ESO-1 either before (three patients) or following treatment (two patients). One of the two patients who developed a NY-ESO-1 antibody response experienced a clinical response [57]. These investigators subsequently used a high-density protein microarray to assay for IgG responses to multiple antigens. They found that patients with clinical response develop antibody responses to a higher number of antigens than nonresponders. Interestingly, the majority of antibody responses were patient specific, and there was little overlap in antigens between responders and nonresponders [58].

In summary, most of the data suggest that CTLA4 blockade does result in an increase in antibody responses against TAAs and other intracellular antigens. Some – but not all – studies suggest a correlation between the development of antigen-specific antibody responses and clinical benefit. Whether such antibody responses can serve as a biomarker, as well as whether they are protective and contribute to the antitumor effect, possibly by integrating with corresponding antigen-specific $CD4^+$ and $CD8^+$ T cell responses, or are simply surrogates for the overall immune activation, remain to be determined.

24.4.2 Antigen-Specific T Cell Response

The evaluation of TAA-specific T cell responses has also been an intense focus of immune monitoring for anti-CTLA4 therapies. Intracellular cytokine staining (ICS) is widely used to analyze

antigen-specific T cell responses [59]. Flow cytometry has been expanded beyond a single parameter to now routinely allow for the detection of multiple functions. Polychrome (multiparametric) flow cytometry allows for simultaneous characterization of various T cell effector functions, e.g., by combining multimer staining with staining for intracellular cytokines or for CD107, a molecule expressed on cytologically active cytotoxic T lymphocytes [60–62]. Such assays have been found to have very low false-positive rates, very low limit of detection, and high sensitivity, reproducibility, and linearity [63], making them suitable for qualitative and quantitative analysis of cellular immune responses in clinical trials. Polyfunctional T cell subsets – T cell subsets that generate multiple cytokines or chemokines – are markers of robust immune activity.

In a retrospective analysis of polyfunctionality in ipilimumab-treated melanoma patients, it was shown that five of six patients experiencing clinical benefit demonstrated $CD4^+$, $CD8^+$, and/or antibody responses to NY-ESO-1 antigen. These T cells were polyfunctional, producing IFN-γ, macrophage inflammatory protein (MIP)-1β, and/or tumor necrosis factor (TNF)-α following NY-ESO-1 peptide stimulation. Patients without clinical benefit did not demonstrate evidence of polyfunctionality [64]. In our follow-up study of 144 ipilimumab-treated melanoma patients, NY-ESO-1 seropositive patients who also developed NY-ESO-1-specific $CD8^+$ T cell responses experienced more frequent clinical benefit (10 of 13; 77 %) than those with undetectable $CD8^+$ T cell responses (1 of 7; 14 %; $p=0.02$) as well as improved survival (hazard ratio 0.2, $p=0.01$) [55]. These data suggest that an integrated T cell and antibody response is required for benefit from ipilimumab and that an antibody response alone is largely necessary but not sufficient.

In addition to NY-ESO-1 responses, a case report described a high frequency of Melan-A-specific $CD8^+$ T cells in regressing tumor tissue and in the peripheral blood of a patient with melanoma who experienced CR to ipilimumab [65]. The predictive value of T cell responses against NY-ESO-1 and Melan-A was elevated in a study of 84 melanoma patients who received ipilimumab. Patients who had an increased frequency of NY-ESO-1- and Melan-A-specific T cells had improved survival compared to patients with no or a lower frequency of these antigen-specific T cells [66]. TAA-specific T cell responses have also been noted in prostate cancer. In the study previously described above, combination therapy with ipilimumab and GM-CSF led to the induction of NY-ESO-1-specific $CD8^+$ T cell response in one of the patients who experienced clinical response [57].

Taken together, these studies suggest that an integrated immune response involving the generation of antibodies and $CD4^+$ and $CD8^+$ T cells to specific tumor antigens may be important predictive markers of clinical response to ipilimumab therapy. Given the large number of antigenic targets in human cancers, it is important to identify and evaluate potential antigens that are expressed in a significant proportion of tumors and for which research reagents are available.

24.5 Analyses of Specific T Cell Populations in the Tumor Microenvironment

The presence of tumor-infiltrating lymphocytes (TILs) is a favorable prognostic factor in a number of cancers [67–71]. TILs are presumed to home to the tumor site, where they exert direct antitumor effects and help to control or eradicate tumors. As such, characterization of TILs in ipilimumab-treated patients has also been undertaken. Understandably, such efforts are complicated by the need for single or repeated biopsies of tumor tissue.

Early investigations of ipilimumab biomarkers revealed that the degree of tumor cell destruction was inversely related to the number of intratumoral $FoxP3^+$ Tregs. The overall extent of tumor necrosis was directly proportional to the ratio of infiltrating $CD8^+$ T cells to $FoxP3^+$ cells, with a higher ratio favoring increased tumor destruction [54].

In a prospective phase II biomarker study in advanced melanoma patients treated with

ipilimumab, baseline TIL scores were not clearly associated with clinical activity [72]. However, patients whose tumors had an increase in TILs after one ipilimumab treatment were significantly more likely to derive clinical benefit than patients without an increase or with decrease (odds ratio 13.27, $p=0.005$). Additional analysis of the TILs revealed a significant association between clinical activity and high baseline expressions of FoxP3 ($p=0.014$) and indoleamine 2,3-dioxygenase (IDO; $p=0.012$). This observation is unexpected since increased expression of IDO has been associated with an immunosuppressive tumor microenvironment or a response to chronic inflammation; similarly, FoxP3 is considered a marker for T_{regs}.

In melanoma patients treated with tremelimumab, a highly significant increase in $CD8^+$ T cells at the tumor site was detected following tremelimumab treatment. However, there was no difference between the absolute number, location, or cell density of TILs between clinical responders and patient without clinical response. The expression levels of T cell activation markers (CD45RO, HLA-DR), Ki67 cell proliferation marker, and the $FoxP3^+$ suppressor cell in tumor were similar before and after tremelimumab treatment [73].

Gene expression arrays have also been used to analyze tumor tissue. One study evaluated tumors obtained from 45 melanoma patients before and after one ipilimumab treatment [74]. Ipilimumab induced two major changes in tumors from patients who experienced clinical benefit: increased expression of genes involved in immune response and decrease expression of melanoma-specific antigens and cell proliferation genes. Patients with high baseline expression of immune-related genes were also more likely to have a favorable clinical outcome in terms of best overall response as assessed by using modified World Health Organization criteria. Most of these genes were related to either the innate or adaptive arms of the immune system, suggesting that a preexisting immune-active tumor microenvironment might favor clinical response to ipilimumab.

24.6 Future Perspectives

Our increasing understanding of the molecular mechanisms of immune recognition as well as increasingly sophisticated research tools is providing opportunities not previously available to monitor responses to ipilimumab and other anti-CTLA4 therapies. Such intensive immune monitoring will help us to fully elucidate the mechanism of action of this class of drugs and to rationally design new trials that evaluate them in combination with conventional chemotherapy and radiation treatments, as well as other experimental immunotherapies [75–79].

Chemotherapy and radiation have been shown to normalize tumor vasculature, decrease interstitial pressure, and upregulate MHC molecules (which helps to facilitate antigen presentation), TAAs, or FAS (CD95, TNF receptor superfamily member 6) on tumor cells. The death and lysis of tumor cells by conventional chemotherapy and/or radiation may also lead to the release of intracellular TAAs, which can be recognized by an activated immune system. All of these mechanisms may facilitate the immune-mediated recognition and destruction of tumor cells, providing a direct rationale to evaluate them in some combination with ipilimumab [80].

The other opportunity is to combine CTLA4 blockade with other emerging immunotherapies. T cell dysfunction or exhaustion in tumor-bearing hosts is one mechanism by which immunosuppression hinders productive antitumor immunity. Transmembrane proteins such as PD-1, PD-L1, a ligand of PD-1 implicated in tumor evasion of the immune system, T cell immunoglobulin mucin 3 (Tim-3), and lymphocyte-activation gene-3 (LAG-3) have been identified as markers of exhausted/dysfunctional T cells in chronic disease states in humans [81–85]. Experimental blockade of these molecules have been shown to partially restore T cell function and lead to tumor rejection [84, 86, 87, 88]. Simultaneous Tim-3 blockade acted in synergy with PD-1 blockade to enhance cytokine production and proliferation of $CD8^+$ T cells in ex vivo experiments of human cells [84]. Dual blockade of LAG-3 and PD-1

during T cell priming with NY-ESO-1 antigen efficiently augmented proliferation and cytokine production by NY-ESO-1-specific $CD8^+$ T cells derived from ovarian cancer patients [89].

Several antibodies are currently undergoing clinical trial evaluation. These include antibodies against PD-1 (nivolumab and MK-3475) and against PD-L1 (BMS-936559, RG7446, and MED14736) [75]. Two clinical studies of anti-PD-1 and anti-PD-L1 antibodies have already shown promise in various cancers [5, 90]. By monitoring changes in these inhibitory molecules following CTLA4 blockade, we can learn more about their role in facilitating or hindering responses to ipilimumab; these results may help to rationally design trials that combine different immune checkpoint inhibitors in various strategies, e.g., sequentially or concurrently.

24.7 Concluding Remarks

After more than a century of unrealized promise, immunotherapy has finally come of age. The approval of ipilimumab is a watershed moment that serves to confirm the validity of this approach, but also heralds a new era of rationally designed immune-based therapy that has the potential to be combined with traditional chemotherapy and/or radiation treatments and other emerging immunotherapies. In this regard, immune monitoring – the intensive use of correlative assays such as ELISA, multiparameter flow cytometry, and gene expression profiling of peripheral blood and tumor sites – should play a pivotal role in the development of new cancer immunotherapy and combination strategies.

A stepwise approach to immune monitoring is recommended as described in Fig. 24.1, starting with the absolute lymphocyte count, then proceeding to analyze different T cell subsets in the peripheral blood, including $CD4^+$, $CD8^+$, and $Ki67^+$ T cells; markers of activation such as ICOS on these populations; markers of exhaustion or co-inhibitory molecules such as PD-1, LAG-3, and Tim-3; markers of T_{reg} such as CD25, FOXP3, Helio, GARP, CD39, and CD73; and MDSC ($CD14^+HLA^-DR^{low}$ populations). Antigen-specific antibody responses in peripheral blood could be analyzed by ELISA and protein array. Where possible and available (based either on common expression of a particular TAA or actual immunohistochemical or RT-PCR characterization of tumor tissue), antigen-specific T-cell immune responses as well as evaluation of all of these cellular subsets in the tumor microenvironment should be undertaken.

Current immunological assays vary largely in their ability to consistently correlate immune response to clinical outcome. However, three concurrent phenomena will hopefully move immune monitoring forward: the advent of new immune monitoring techniques, an exponential rise in the number of patients treated with anti-CTLA4 therapies on clinical trials and in standard clinical practice, and an increasing awareness of the imperative to conscientiously and consistently bank patient tumor and blood specimens for these assays. Ultimately, it is hoped that the convergence of all these factors will enable immune monitoring strategies to identify robust and validated biomarkers that are prognostic and/or predictive and which can guide further clinical trial development.

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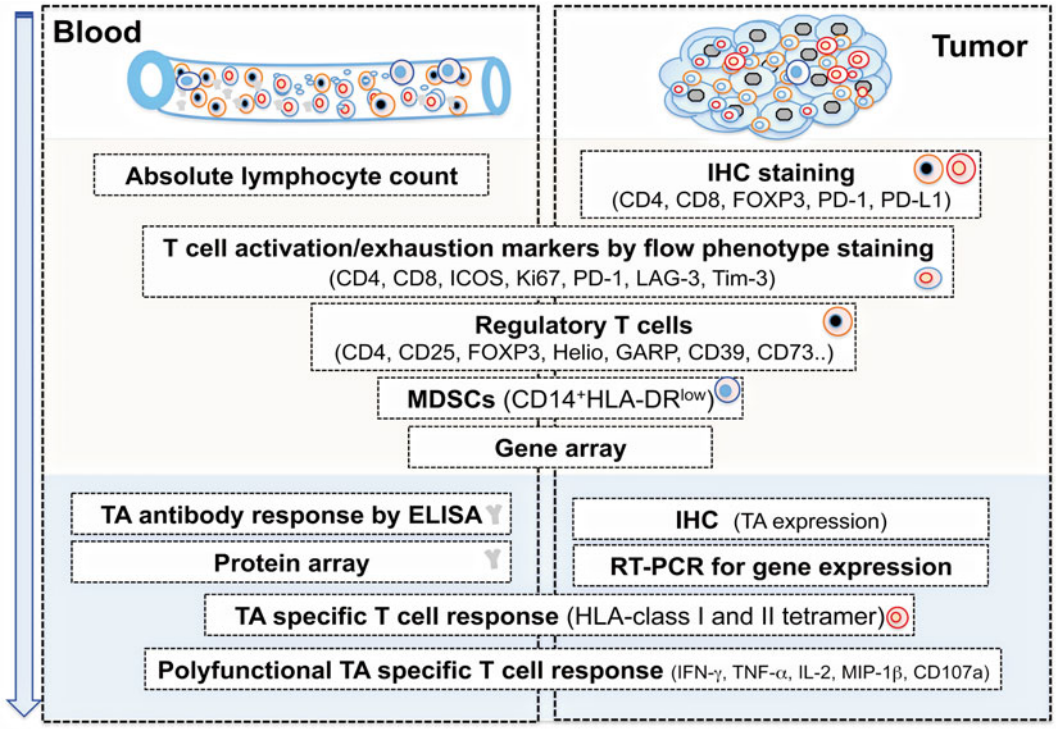

Fig. 24.1 This is a proposed schema for intensive immune monitoring. Flow cytometry can be performed on peripheral blood to analyze multiple parameters for T cell activation/exhaustion markers, regulatory T cells, and MDSCs. If tumor tissue is available, immunohistochemical characterization of the cellular populations present as well as gene expression array or RT-PCR can be performed. For tumor types such as melanoma where the expression of differentiation antigens or cancer-testis antigens occurs at a high frequency, tumor antigen (TA)-specific antibody responses in peripheral blood could be analyzed by ELISA and protein array. TA-specific T cell immune responses can also be characterized in both peripheral blood and the tumor. Immunohistochemistry and/or RT-PCR can also confirm protein or RNA expression of the TA

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New Advances in Radioimmunotherapy for the Treatment of Cancers

25

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25.1 Introduction

Radioimmunotherapy (RIT) is a molecular targeted therapy whereby irradiation from radionuclides is delivered to tumor targets by means of monoclonal antibodies (mAbs) directed to tumor antigens [1]. Over the last 20 years, RIT has significantly progressed with the development of recombinant and humanized mAbs, stable chelates for radiolabeling and pretargeting techniques with the potential to increase the therapeutic index of radiolabeled antibodies [2]. Today, RIT represents a clinical approach that deserves further studies to advance its application in earlier phase of the diseases and in combination with other therapeutic modalities. This chapter aims to discuss the most important aspects of the application of radioimmunotherapy in the treatment of cancer.

25.2 Principles of Radioimmunotherapy

The cytotoxic mechanisms of RIT involve both radiobiological and immunological processes [3]. RIT delivers a heterogeneous low-dose-rate irradiation to the targeted tumor. Although a

dose-effect relationship has not yet been clearly demonstrated, it is likely to be present even if such a relationship may be masked, in the treatment of B cell lymphoma, by the antitumor effects of cold mAbs generally injected prior to the radiolabeled antibody. Indeed, mAbs, particularly rituximab, may exert cytotoxic effects through apoptosis, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). When mAbs are labeled with radionuclides, the combination of immunological and radiobiological cytotoxicities, including bystander and abscopal effects, results in higher antitumor efficacy [3, 4].

RIT efficacy has been clearly demonstrated in hemopathies, in particular B cell lymphoma, but should be confirmed in solid tumors, more resistant to radiations and less accessible to large molecules such as antibodies [1]. RIT used as consolidation therapy, in minimal or small-size disease, has shown promising clinical efficacy in lymphoma and in solid tumors such as colon carcinoma [5–7]. In this minimal residual disease (MRD) clinical setting, biodistribution and tumor dosimetry are more favorable, tumor cells are less hypoxic and more radiosensitive [8], and immunotherapy is more efficient [9].

The choice of appropriate antibodies and radionuclide is critical [10]. The path length of penetration of the radioactive emission should match the size of the targeted tumor. In clinical practice, only iodine-131 and yttrium-90 beta emitters are used. Yttrium-90, with its long-range beta emission, is better suited for bulky disease. However, promising results have been observed using ^{90}Y-RIT in the consolidation setting in patients in partial response (PR) or complete response (CR) after induction therapy [7]. Radionuclides such as ^{131}I or ^{177}Lu with shorter-range energy emissions should be more favorable in the setting of MRD. Moreover, ^{177}Lu presents better physical properties than ^{131}I, improving the safety of RIT. Alpha particle and Auger electron emitters offer the theoretical possibility to kill isolated tumor cells and microscopic clusters of tumor cells, opening the perspective of killing the last tumor cell, which is the ultimate challenge in cancer therapy [1, 3, 10–12]. Preliminary preclinical and clinical results confirm the feasibility of this approach.

Today, two products targeting CD20 have been approved: ^{131}I-tositumomab (Bexxar®, GlaxoSmithKline) and ^{90}Y-ibritumomab tiuxetan (Zevalin®, Spectrum Pharmaceuticals, Henderson, NV, USA). ^{131}I-tositumomab is available in the United States and ^{90}Y-ibritumomab is approved in Europe, the United States, Asia, and Africa. RIT can be integrated in clinical practice using non-ablative activities for treatment of patients with relapsed or refractory follicular lymphoma (FL) or as consolidation after induction chemotherapy in frontline treatment in FL patients. Different RIT protocols are assessed in clinical trials in FL, other hemopathies, and solid tumors. Myeloablative treatment in aggressive hemopathies, RIT as consolidation after induction therapy to target MRD in hemopathies or solid tumors such as prostate carcinoma (PCa), RIT in first-line treatment, fractionated RIT to decrease hematological toxicity and increase cumulated injected activity, and pretargeted RIT to improve tumor-to-normal-tissue ratios and increase injected activity are the mainstay of research in RIT. Moreover, personalized dosimetry protocols, especially using quantitative positron emission tomography (PET) imaging, are proposed to better predict dose-effect relationships and optimize injected activities.

25.3 Radionuclides and Radiolabeling Techniques for Therapy

Killing targeted cells and preserving healthy tissues or cells is achievable by targeted radionuclide therapy (TRT) as far as proper radiopharmaceuticals (radionuclide and pharmaceutical carrier) are used. Radionuclide properties such as emission type, mean energy of emitted particles, and physical half-life matter as much as pharmaceutical carrier properties such as affinity, specificity, and stability. Moreover, some kinetic aspects are of major interest.

25.3.1 Radionuclides

As opposed to external beam radiotherapy, which uses penetrating radiations such as photons, TRT uses non-penetrating radiations: Auger electrons

and β or α particles. These particles deliver their energy within small distances, an ideal situation to preserve non-targeted tissues. For electrons in the 10 keV–10 MeV energy range traveling through soft tissues, linear energy transfer (LET) values are in the range of 0.2–2 keV/µm, whereas Auger electrons with energies ranging from 0.1 to 1 keV have LET values from 5 to 25 keV/µm. Typical LET values for 5–10 MeV α particles are 100 keV/µm. As such, Auger electrons are more suitable for inner cell irradiation, at a close distance from DNA [13]. Alpha particles also perform well at that scale but are also appropriate for small cell clusters [14]. When particles are emitted from the cell surface or in the surrounding environment, as is usually the case in TRT or in cancer treatment with radiolabeled microparticles [15, 16], electrons and ß particles come into play. To thoroughly irradiate macroscopic tumors, radionuclides such as ^{131}I, ^{177}Lu, or ^{67}Cu are theoretically best suited for the treatment of small tumor lesions and ^{90}Y or ^{188}Re for larger ones [17].

The half-life of radionuclide must also be considered. As, most often, radiopharmaceuticals are administered by systemic infusion, radioactive decay occurs along the course to the target, leading to nonspecific irradiation of healthy tissues. This appeals for use of small carriers that quickly reach the target cells, as proposed in peptide therapy [18]. On the other hand, for RIT, mAbs may take a couple of days for maximal uptake in target sites. Therefore, it is relevant to adjust the radionuclide physical half-life to the carrier biological half-life. Improved targeting approaches, referred to as pretargeting techniques [19], have been developed to circumvent this particular issue and to achieve higher tumor-to-normal-tissue activity uptake ratios in shorter times than conventional RIT, thus allowing the injection of higher activities with similar or even less damages to normal tissues.

Taking all these criteria into account, very few radionuclides (cf. Table 25.1) remain for TRT. Beta emitters such as ^{131}I or ^{90}Y have been used for a long time. ^{177}Lu and ^{188}Re are emerging and ^{67}Cu is expected to be very promising. All of them, except ^{67}Cu, are produced in nuclear reactors. When specific activity is of concern, indirect production routes can be used (e.g., ^{177}Lu can be produced by neutron capture from ^{176}Lu or by decay of ^{177}Yb produced by neutron capture from ^{176}Yb). ^{188}W used as a precursor of ^{188}Re needs very high neutron fluxes. Only ^{67}Cu is produced in accelerators and is available part of the year from BNL (USA) [20]. To secure the production of this isotope, other suppliers are needed, and Arronax is expected to develop the production of this isotope [21].

For alpha emitters, ^{213}Bi is available through a generator made of ^{225}Ac. Its short half-life makes it tricky to use; nonetheless, many studies are ongoing worldwide [22]. Despite its complex chemistry, ^{211}At may be a better candidate for alpha therapy due to its longer half-life and its production in accelerators. Other alpha emitters are available but they are linked to a cascade of alpha decays that may be a problem for specific targeting (^{225}Ac and ^{226}Th) or have a chemistry not favorable for labeling (^{223}Ra).

Regarding the targeting aspect, the specificity of the radiopharmaceutical has to be the highest possible to limit the delivery of radionuclides to healthy tissues, and affinity controls the uptake of the radiopharmaceutical in target lesions: higher affinity means higher uptake, although affinities in the nanomolar range are considered sufficient. For longer half-life radionuclides, the rate of efflux from the tumor is also very important and is not entirely related to affinity. For instance, when internalization of the radiopharmaceutical by target cells occurs, residualizing radionuclides, such as metals, affords protracted radioactivity retention in tumor sites, whereas direct radiolabeling with radioiodine results in fast excretion of radioactivity, thus reducing target cell exposure.

25.3.2 Labeling Techniques

Halogens are usually provided under the halogenide form. The easiest way to attach a halogen to a vector is to perform an electrophilic substitution on a tyrosine residue [23]. Unfortunately, even if this commonly used technology is validated

Table 25.1 Radionuclides for antibody-targeted imaging and therapy

Radionuclide	$T_{1/2}$ (h)[a]	Emissions[b]	E_{max} (keV)[c]	Range max in soft tissue (mm)[c]	Usual labeling method
Technetium-99 m	6.0	γ	140		Direct labeling or N2S2 or N3S complexes
Indium-111	67	γ	171 and 245		Polyamino-carboxylic acids: DTPA, DOTA
Iodine-123	13.3	γ	159		Direct labeling (tyrosine)
Fluorine-18	1.83	β^+	633	3.1	
Gallium-68	1.13	β^+	1,899	9.8	Polyamino-carboxylic acids: DOTA, NOTA
Copper-64	12.7	β^+	653	3.2	Many different chelating agents
		β^-	579	2.8	
Zirconium-89	78	β^+	902	4.6	Desferroxamine
Iodine-124	100	β^+	1,535 and 2,138	7.9 and 10.9	Direct labeling (tyrosine)
Scandium-44	3.97	β^+	1,473	7.6	
Iodine-131	193	β^-	610	2.9	Direct labeling (tyrosine)
		γ	362	11	
Yttrium-90	64	β^-	2,250	11	Polyamino-carboxylic acids: DOTA
Rhenium-188	17	β^-	2,120	10	Direct labeling or N2S2 or N3S complexes (chemistry analogous to that of technetium)
		γ	155		
Lutetium-177	162	β^-	498	2.0	Polyamino-carboxylic acids: DOTA
		γ	208		
Copper-67	62	β^-	392–577	1.8	Many different chelating agents
		γ	184		
Bismuth-213	0.76	α	8,400	0.1	Polyamino-carboxylic acids: CHX-DTPA, DOTA
		γ	440		
Astatine-211	7.2	α	5,870 and 7,450	0.055–0.080	Stannylated synthons: SAB, SAPS
		X	77–92		

[a]The half-life of the radionuclide must be matched with the half-life of its vector, or more precisely, it should allow for clearance of unbound activity to obtain high target to non-targeted tissue contrast ratio for imaging, and it should be matched with the vector residence time in the tumor to deliver the maximum irradiation dose

[b]Intermediate energy photons may be detected by gamma cameras. Positron annihilation photon pairs may be detected by PET cameras. Only radionuclides emitting massive particles (alpha, beta, Auger electron) deliver their ionizing energy locally enough for therapy. In that case, concomitant emission of gamma or X-rays may be used for imaging to check targeting and calculate irradiation doses absorbed by tumors and normal tissues

[c]The higher the positron energy, the longer the path between radionuclide decay and positron annihilation, resulting in poorer image resolution. For therapy, radionuclides emitting particles with very short range (alpha, Auger electron) are likely to be more effective against very-small-size tumors

with iodine-labeled non-internalizing antibodies or peptides, it does not provide satisfactory results when internalization occurs or with ^{211}At-labeled antibodies [24]. In both cases, halogen liberation leads to nonspecific irradiation of normal organs such as the thyroid for iodine [25] or the stomach for astatine [26] and reduces specific irradiation of the tumor. Several radiolabeling approaches using prosthetic groups have been proposed to solve this problem (Fig. 25.1).

Radiometals, such as ^{90}Y, ^{188}Re, ^{67}Cu, ^{177}Lu, ^{212}Bi, and other actinides, are generally provided no carrier added in chloride form. However, contaminations with metal traces resulting from the production mode decrease the specific activity of radiopharmaceuticals, which are usually between

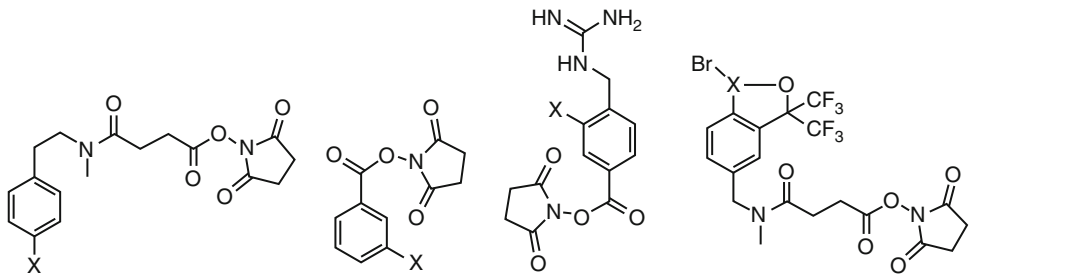

Fig. 25.1 Prosthetic groups for radiohalogen labeling

40 and 400 MBq/nmol depending on the radionuclide. Several highly specific chelating agents have been developed in order to improve specific activity [27].

Transmetallation or transchelation phenomena can occur *in vivo* when the radiopharmaceutical is in competition with metal complexing proteins, such as transferrin or ceruloplasmin [28, 29]. Thus, chelating agents with very high affinities for metals and very high kinetic stabilities have been developed (Table 25.1). The best approaches to limit these phenomena are based on a better chelation agent selection in order to improve both selectivity and stability. This choice integrates stability constant and dissociation kinetic values which have to be for the latter as low as possible.

25.4 The Treatment of B Cell Lymphoma with Anti-CD20 Antibodies

Bexxar® and Zevalin® are administered 6–8 days after a predose of cold mAbs, respectively 2×450 mg of tositumomab and 2×250 mg of rituximab, to improve biodistribution and tumor targeting. Bexxar® and Zevalin® can be integrated in clinical practice using non-ablative doses for treatment of patients with relapsed or refractory FL or as consolidation after induction chemotherapy in frontline treatment in FL patients. Hematological toxicity is the major side effect of RIT and depends on bone marrow involvement and prior treatment [30–32]. Non-hematological toxicity is generally low. Secondary myelodysplastic syndrome or acute myelogenous leukemia (AML) was reported in 1–3 % of cases [30–33]. 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 [32]. 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® in a long-term meta-analysis performed on 250 heavily pretreated patients with indolent lymphoma in 5 clinical trials, objective response (OR) rates ranged from 47 to 68 % and complete response (CR) rates from 20 to 38 % [34]. 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 a 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 high-dose treatment. This approach consists of injecting myeloablative activity of RIT or combining standard or escalated activity of RIT with high-dose chemotherapy. In a recent prospective multicenter 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 [35]. 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 [7]. 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 of stem cell transplantation in high-risk patients [36, 37]. In 2010, Zinzani et al. published the results of a phase II study assessing the efficacy and safety of Zevalin® following four cycles of R-CHOP21, in 55 high-risk elderly (age ≥60 years) patients with previously untreated diffuse large B cell lymphoma (DLBCL). Forty-eight of the 55 patients received RIT [38]. The OR rate for the entire treatment regimen was 80 %, including 73 % CR. Eight of the 16 patients (50 %) who achieved less than a CR after R-CHOP improved their remission status after RIT. With a median follow-up of 18 months, the 2-year PFS was estimated to be 85 %, with a 2-year OS of 86 %.

RIT can also be considered alone in frontline treatment. Recently, Scholz et al. evaluated, in an international multicenter phase II clinical trial, the efficacy and feasibility of Zevalin® as first-line treatment in 59 FL patients [39]. 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 hematological and reversible.

25.5 Promising Results in Hemopathies Using Other Antibodies

25.5.1 Targeting of Lymphoma with Anti-CD22 Antibodies

For lymphoma, targeting other antigens than CD20 targeted by 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 epratuzumab has good features for RIT because it is humanized, internalized by target cells, stably labeled using DOTA, and administered without a loading dose of cold antibody, at variance with Zevalin® or Bexxar® [40].

^{90}Y-epratuzumab RIT has been developed with repeated injections [41–43]. A multicenter phase I/II study was designed to assess fractionated ^{90}Y-epratuzumab in NHL relapsing patients [43]. Sixty-four patients with one to five prior therapies (median, 2), with different histologies of B cell lymphoma 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 GBq/m^2, grade 3–4 hematological toxicities were manageable with support in 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 GBq/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 GBq/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 assessing front-line treatment using fractionated RIT with ^{90}Y-epratuzumab as consolidation therapy after chemo-immunotherapy in bulky or stage III/IV aggressive B cell lymphoma. Another important perspective is the clinical evaluation of dual-targeted antibody/radioantibody therapy [40, 44, 45]. Combining an unconjugated anti-CD20 antibody therapy with a radioimmunoconjugate binding to a noncompeting antigen might improve responses by allowing optimal uptake of each agent [45, 46]. Preclinical studies showed that efficacy increased when a consolidation using anti-CD20 veltuzumab was delivered after anti-CD22 RIT [46]. The injection of cold mAb after the radioactivity dose provided higher efficacy than injection before RIT, and the amount of predose of cold mAb could be minimized [40, 45]. Thus, a reexamination of RIT in the treatment of B cell lymphoma was proposed [44], emphasizing that in RIT clinical practice, nearly 900 mg of unlabeled anti-CD20 IgG antibody is predosed to the patient before the anti-CD20 ^{90}Y or ^{131}I RIT.

25.5.2 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 at later stages of the disease in extramedullary sites [47]. The annual incidence is 4–6 cases per 100,000. The median survival of this 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 [48]. Other drugs such as inhibitors of histone deacetylase (vorinostat, panobinostat) or mAbs (elotuzumab) are under development in large prospective phase II or III studies [49].

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 [50], anti-CD38 [51], anti-CD54 [52], anti-CD74 [53], anti-CD317 [54, 55], or anti-CD319 [56] has been assessed as monotherapy or in combination with other therapeutic drugs or in preparation of autologous SCT. Because IL-6 is a major autocrine/paracrine growth factor for MM cells, immunotherapy with anti-IL-6 mAb has been performed. A transient tumor cytostasis was obtained, which did not cure the tumor [57, 58]. 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 [59]. Erba et al. have performed a RIT clinical trial using ^{131}I-L19SIP mAb specific to the EDB domain of fibronectin, reporting a stabilization of the disease in two patients at advanced stage of MM [60]. The feasibility of anti-CD138 (syndecan-1) RIT using ^{131}I-B-B4 was also recently reported, with encouraging dosimetry results [61]. Syndecan-1 belongs to the family of heparan sulfate-bearing proteoglycans. Found on epitheliums, this molecule is also present on pre-B cells and plasma cells, and it plays an important role in regulating MM [62]. Syndecan-1 is expressed in all MM tumors within the bone marrow and is present at relatively high levels on MM cell surface [62–65].

In MM, tumor cells are mostly disseminated in bone marrow either as isolated cells or as

microscopic tumor cell clusters. Beta emitters with relatively long path lengths (1 mm–1 cm) are not very suitable to target such isolated cells. By contrast, the high linear energy transfer characteristics of alpha particles enable localized irradiations while preserving surrounding tissues, and cell toxicity is achieved with only a few disintegrations at the cell surface. *In vitro* and preclinical studies demonstrated promising therapeutic efficacy of ^{213}Bi-labeled anti-mCD138 for the treatment of MM [66]. 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.

25.6 RIT of Metastatic Prostate Cancer

PCa accounts for an estimated 70,347 deaths in Europe in 2013 [67]. 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 tumor burden, decreasing prostate-specific antigen (PSA) levels, enhancing quality of life, and improving survival [68]. However, most patients evolve toward progression despite castration, with a median duration of response of 12–24 months [68]. At the stage of castration-resistant PCa (CRPC), cytotoxic chemotherapy was the only therapy [69, 70] until 2012, when the European Medicines Agency (EMA) approved the use at this stage 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 [71]. PCa is a favorable solid malignancy for which RIT may be used because it is a radiosensitive tumor with typical distribution to sites with high exposure to circulating radiolabeled 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) [72–75], but PSMA appears the most specific.

PSMA is an integral, non-secreted, type II membrane protein with abundant and nearly universal expression on prostate epithelial cells and is strongly upregulated in PCa [76–80]. Pathology studies indicate that PSMA is expressed by virtually all PCa [81]. The level of expression in non-prostate tissues is 100–1,000-fold less than in prostate tissue [76], and the site of PSMA expression in normal cells (brush border/luminal location) is not typically exposed to circulating mAb. 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 [82, 83].

A phase I trial assessing ^{111}In/^{90}Y-J591 was performed in 29 patients [84]. Dose-limiting toxicity was seen at 740 MBq/m^2, and 647.5 MBq/m^2 was determined as the maximal tolerated dose (MTD). The overall targeting sensitivity of bone and soft tissue metastasis was 81 %. Decrease of PSA was observed for two patients as objective measurable disease responses with decrease of lymph node size.

Thirty-five patients were enrolled in a ^{177}Lu-J591 phase I trial [85]. The 2,590 MBq/m^2 level was determined as MTD. Repeated dosing up to three doses of 1,110 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 2,405 MBq/m^2. The second cohort (2,590 MBq/m^2) enrolled 17 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 %. The second cohort dose (2,590 MBq/m^2) showed more 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 hematological toxicity.

These trials provide support that radiolabeled de-immunized J591 is well tolerated and non-immunogenic. Radiolabeled J591 effectively targets PCa metastases with high sensitivity and specificity, produces PSA, and declines with a dose-effect relationship.

25.7 RIT with Alpha-Emitting Radionuclides

Alpha-RIT is a therapeutic modality based on the use of an antitumor antigen mAbs coupled to an alpha emitter (radionuclide which decays by the emission of alpha particles). The rationale of this therapeutic modality is based on two prominent characteristics of the alpha particles: their short range in tissue, inferior to 100 μm, which allows for a good specificity of the treatment (once the antibody is in the vicinity of the tumor) and their high linear energy transfer (LET) between 50 and 250 keV/μm, which makes them highly cytotoxic. *In vitro* studies have demonstrated that 1–20 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$^-$ particles [86, 87]. In addition, alpha particle-induced toxicity was shown to be independent of both dose rate and oxygenation of the irradiated tissue [88].

25.7.1 Therapeutic Indication

Related to these characteristics, it is often described that alpha-RIT is particularly indicated in the treatment of MRD, hematologic cancers, and micrometastatic diseases, even though some efficacy was observed on solid tumors [22]. Despite trials using alpha-emitting radionuclides in medicine in the early twentieth century, just after the discovery of radioactivity, the first alpha-RIT clinical trial was performed in 1997. A humanized antibody specific for a human myelogenous leukemia antigen (CD33) labeled with ^{213}Bi was administered to 18 patients with AML, and results showed a reduction in circulating blasts in most patients (~80 %), whereas no extramedullary toxicity was observed [89]. Since then, seven clinical trials (Table 25.2) were initiated to treat lymphoma, melanoma [90, 91], malignant recurrent gliomas [14], and ovarian carcinoma [92]. These studies demonstrated that alpha-RIT is feasible and safe and has a significant antitumor efficacy. In addition to these alpha-RIT trials, two clinical trials using alpha-emitting radionuclides against gliomas [93] and against bone metastases in CRPC [94, 95] should be mentioned. In the last indication, the ALSYMPCA trial (phase III) using $^{223}RaCl_2$ demonstrated a significant prolongation of OS as compared with placebo and the product, Alpharadin, is now on fast track to FDA approval.

Table 25.2 Ongoing and completed clinical trials in alpha-RIT [156]

Cancer type	Radioconjugate	Phase	No. of patients	Reference
Leukemia	^{213}Bi-HuM195mAb	I	18	[89]
	^{213}Bi-HuM195mAb	I/II	31	[98]
	^{225}Ac-HuM195mAb	I	Not yet completed	[101]
Lymphoma	^{213}Bi-rituximab	I	12	[157]
Melanoma	^{213}Bi-9.2.27mAb	I (intralesional)	16	[90]
	^{213}Bi-9.2.27mAb	I (systemic)	38	[91]
Recurrent malignant brain tumors	^{211}At-81C6mAb	I	18	[14]
Ovarian carcinoma	^{211}At-MX35mAb	I	9	[92]

25.7.2 Limited Availability

These encouraging results of alpha-RIT in clinics are still limited if we consider the number of patients treated with alpha emitters up to now ($n = 142$). More than 100 alpha-emitting radionuclides are known, but once selected for appropriate characteristics, less than 10 have been evaluated [96]. Among them, the most promising ones are astatine-211, bismuth-212, bismuth-213, radium-223, actinium-225, and thorium-227. Current supplies of these radionuclides are based on nuclear weapon or nuclear fuel material reprocessing and cyclotron productions [97]. Concerning ^{213}Bi, only three centers in the world are able to produce ^{225}Ac/^{213}Bi generators, and combining all sources of production, only 100–200 patients could be treated annually. For ^{211}At, the production is also insufficient, since it is currently carried out by less than 10 cyclotrons in the world [96]. In the United States, this issue of availability was clearly identified, and recent analyses emphasized the need to develop new infrastructures for the production of alpha-emitting radionuclides.

25.7.3 Issues and Current Developments

Following the first clinical trial using ^{213}Bi-HuM195, it appeared that the limited number of ^{213}Bi atoms that could be injected was a clear issue to allow for complete remission in patients with large tumor burdens [89]. Different ways of optimization have been proposed to overcome this issue. Therapeutic associations of alpha-RIT with chemotherapy were shown to be efficient *in vitro*, and a clinical trial was initiated including alpha-RIT after partial cytoreduction with cytarabine [98]. Another solution relies on the use of ^{225}Ac, ^{227}Th, or ^{223}Ra. These radionuclides yield several daughter radionuclides with four or more alpha particle emissions in their decay scheme [99, 100]. The main issue here is to manage the distribution, metabolism, and clearance of the daughters since consecutive decays result in the loss of the chemical bond to the antibody [101]. Different solutions have been proposed. For instance, the ^{225}Ac atomic nanogenerator was described using the 225-mAb that internalizes in target cells, thus trapping the radionuclide and its daughters within the target [101].

As for beta-RIT, pretargeting strategies are currently evaluated in order to enhance the therapeutic window of RIT, i.e., to increase the tumor-to-organ ratio in terms of activity delivery [100]. The faster delivery of activity to tumors may also be an advantage when using short-lived alpha-emitting radionuclides (^{213}Bi, ^{211}At).

Finally, increased efforts are made to better understand the toxicity induced by alpha-RIT. Due to its short range, the distribution of radioimmunoconjugates at the sub-organ scale is expected to be crucial for toxicity. Different dosimetry models of organs (kidneys, bone marrow) are developed to determine the dose distribution following a RIT treatment at the cell scale [102, 103].

25.8 High Efficacy of Pretargeting Approaches in Metastatic Thyroid Carcinoma

Medullary thyroid carcinoma (MTC) represents less than 10 % of all thyroid carcinoma. 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 [104–108]. Moreover, Barbet et al. demonstrated that calcitonin (Ct) serum level doubling times (DT) was an independent predictor of OS [109]. 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 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 initial surgery. Giraudet et al. confirmed the prognostic value of biomarker DT in metastatic MTC [110].

Targeted therapy using multikinase inhibitors can be applied in progressive patients and vandetanib

has been approved [111–116]. MTC cells express high amounts of CEA, and anti-CEA radiolabeled mAbs have shown promising results [117, 118]. Pretargeted RIT (pRIT) was developed to improve the tumor-to-normal-tissue ratios and to deliver increased tumor-absorbed doses to relatively radioresistant solid tumors. Pretargeted system involves a first injection of an unlabeled bispecific monoclonal antibody (BsmAb), followed by a second injection of a radiolabeled bivalent hapten peptide [1, 19, 119–121]. Using this system, the radiolabeled 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 was started in 1996 to evaluate pRIT using the murine anti-CEA x anti-indium-DTPA F6×734 BsmAb and a bivalent indium-DTPA hapten labeled with iodine-131, in 26 metastatic MTC patients [122–124]. A good tumor targeting was observed. Dose-limiting toxicity was hematological, and maximum tolerated activity was estimated at 1.8 GBq/m^2 in this population of patients with high frequency of bone marrow involvement. Some tumor responses were observed, mainly in patients with a small tumor burden and after repeated courses of pRIT. Because of a relatively high hematological 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 tumors to determine optimal BsmAb dose, hapten activity, and pretargeting interval [22]. 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 antihuman antibody) in 33 %.

In 2006, OS of the 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 Tumor Group, GTE) [125]. 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 theses encouraging results, a prospective phase II multicenter 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-14×m734 and 1.8 GBq/m^2 ^{131}I-di- indium-DTPA hapten 4–6 days later [126]. Disease control according RECIST criteria (objective response + stabilization) was observed in 32 patients (76.2 %), including a durable CR of at least 40 months in 1 patient (2.4 %) and durable stable disease (≥6 months) in 31 patients (73.8 %). Tumor uptake assessed by post-pRIT immunoscintigraphy was a significant predictor of response. As previously reported, toxicity was mainly hematological, 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.

Today, a new generation of compounds is available for pRIT. Humanized, recombinant, trivalent BsmAb (anti-CEA TF2) and bivalent histamine-succinyl-glutamine (HSG) peptides have been produced [127, 128]. 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 [127–129]. Moreover, the HSG peptide allows facile and stable labeling with different radiometals, such as ^{177}Lu or ^{90}Y, having favorable physical features that could improve pRIT efficacy [130].

25.9 Immuno-PET: The Future for Dosimetry Assessment and Patient Selection

For more than two decades, mAbs have been labeled 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. While 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 mAbs imaging (immuno-PET). Indeed, immuno-PET has several advantages over conventional immunoscintigraphy with gamma emitters. The improved spatial resolution makes the delineation of tumors 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 a 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 [131, 132] and reconstruction algorithm [133]. 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 [134–136]. Table 25.1 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 [137–141], whereas ^{89}Zr [142, 143] and ^{124}I [144–146] are well suited to the labeling of large molecules such as intact mAbs. Copper-64 with an intermediate half-life of 12.7 h can be used for labeling of a large number of molecules with different sizes. Within the scope of a "theranostic" approach, pairs of β^+-/β^--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 [134–136].

25.9.1 Immuno-PET and Development of New Drugs

PET could provide information about tumor 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 [135, 136]. For example, it has been demonstrated recently that immuno-PET could be useful for visualizing CD138-expressing tumors 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 [147].

25.9.2 Patient Selection for Therapy

Until now, only invasive methods as biopsy with immunohistochemistry analysis could identify patients with lesions which had the highest chance of success with antibody-based therapy. Immuno-PET can offer a noninvasive 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 labeled with ^{68}Ga or ^{89}Zr could identify, noninvasively, those lesions that are likely to respond to therapy [138, 148]. It is also a powerful innovation for improving knowledge about efficacy and *in vivo* behavior of mAbs. Based on immuno-PET, the treatment strategy could be tailored for individual patients before administering expensive medicines [149].

25.9.3 Determination of the Cumulated Activity Concentration for RIT

Recently, a study assessing humanized A33 mAb labeled 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 [150]. This offers a unique opportunity to determine the maximum injected activity considering the dose-limiting organs like bone marrow [135]. Similarly, the injected activity could be adapted for each patient given a desired dose to tumor when mAbs imaging is used as a prelude for RIT [151]. As an example, it has been shown that ^{90}Y-Zevalin distribution could be predicted by ^{89}Zr-Zevalin [152]. Thus, immuno-PET holds promise for allowing comparison between different dosing regimens and mAbs constructs [153].

25.9.4 Therapy Response

Immuno-PET represents a noninvasive 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 signaling [154]. 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 tumor 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 mAbs therapy. A pilot study was recently proposed in that respect [155].

25.10 Concluding Remarks

RIT appears a most promising targeted therapy in the treatment of hemopathies and solid tumors, especially at the stage of MRD. In B cell lymphoma, clinical results show that RIT has significant efficacy but moderate response duration as a monotherapy in rituximab-refractory B cell lymphoma. A higher therapeutic impact may be achieved using RIT in myeloablative treatment, as consolidation after chemo-immunotherapy, 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.

In solid tumors, 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 to RIT and optimize the injected activity. Finally, RIT may have the potential of killing the last tumor cells, now identified as chemoresistant and radioresistant tumor 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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Psychoneuroendocrinoimmunotherapy of Cancer

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26.1 Introduction

Currently on the basis of the most recent discoveries showing evidence of a great variety of cancer-related alterations of immune and neuroendocrine functions, almost all oncologists are in agreement to consider human tumors as a systemic disease rather than a simple locoregional pathology, even though tumor mass is still a locally limited disease [1]. In that case, from a philosophic point of view, the question is, what is a tumor, as well as its cause or the consequent effect on the pathological immunobiological status of this disease which is considered a systemic disease from the beginning? In other words, is it cancer dissemination which generates a systemic disease or a previous existence of a systemic psychoneuroimmune disorder, which may contribute to determine the neoplastic disease by altering the physiological immune mechanisms responsible for the natural biological resistance against cancer onset by blocking the evolution from the single transformed cell into a clinically evident tumor mass? Obviously, because of the concomitant evidence of tumor mass and cancer-related immunobiological alterations at the time of the diagnosis of the neoplastic disease, it is not possible to establish whether the altered immunobiological functions may precede and predispose to tumor development from the single transformed cancer cell or be induced by tumor dissemination itself. In contrast, in experimental conditions it has been already demonstrated that both spontaneous

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and carcinogen-induced cancer onsets are associated with important changes in neuroendocrine functions and in neurotransmitter pathways, the prevention or the pharmacological correction of them may inhibit and counteract the carcinogenic process [2]. Obviously, within the great number of cancer-related immunoendocrine anomalies, some alterations would simply be an epiphenomenon of the neoplastic disease, whereas other immunobiological disorders would play a critical role in the pathogenesis of cancer proliferation itself. Hence, the problem is to identify which immune and neuroendocrine alterations occurring during the clinical course of the neoplastic disease may be provided by a potential biological and prognostic significance. Because of the importance of the immune system in the control of cancer cell dissemination and of the neuroendocrine status in influencing the anticancer immune reaction, alterations capable of conditioning the prognosis of neoplastic disease itself within various cancer-related endocrine, neuroendocrine, and immune anomalies have to be initially identified [3]. In fact, it has been confirmed that the immune responses, including the anticancer immunity, are physiologically under a psychoneuroendocrine control, which would be responsible for both their stimulation and inhibition [3]. Remarkably, until the late twentieth century, the majority of physicians were in agreement that the prognosis of a neoplastic disease does not depend only on tumor biogenetic characteristics themselves but primarily on the efficacy of host immunobiological response itself. Unfortunately, as a consequence of the rapid development of a great number of new anticancer drugs capable of acting on fundamental biological processes responsible for cancer cell growth, such as tumor growth factor receptor status and cancer neoangiogenesis, the maximal concentration of the clinical investigations has been almost completely limited to the analysis of cancer biological parameters, such as oncogene expression and tumor growth factor receptor activation [4], by completely forgetting the importance of host immunobiological response, which cannot be separated from the analysis of tumor biological properties, since tumor mass would not exist independently from the biology of cancer patients. With the proposal of psychoneuroendocrinoimmune (PNEI) therapy of human neoplasms, a new potential anticancer therapeutic biological strategy is identified, capable of influencing and inhibiting cancer cell proliferation and dissemination by simply correcting the most important immune and neuroendocrine alterations provided by a potential pathogenetic and prognostic significance occurring during the clinical course of the neoplastic disease. Obviously, the possibility to correct the main cancer-related altered immune and neuroendocrine functions to reestablish a physiological immune anticancer immune efficacy has previously required an adequate clinical investigation of the immune and neuroendocrine status of cancer patients. Moreover, because of the existence of a correlation between tumor biogenetic properties and host biological response against cancer growth, it is essential to concomitantly investigate tumor mass characteristics and immune biological status of cancer patients themselves. As far as tumor cell characteristics are concerned, at present it is known that the activation of some tumor growth factor receptors and the development of angiogenic processes are associated with a poor prognosis in advanced cancer patients. On the other hand, to adequately establish the clinical relevance of a single immunobiological anomaly, a complete knowledge of the physiopathology of the anticancer immune response is necessary.

26.2 The Physiopathology of Anticancer Immunity

Today, it is known that immune system-induced cancer cell destruction is mainly mediated by T lymphocytes, through both antigen-dependent and antigen-independent cytotoxicities, recognized, respectively, by T cytotoxic lymphocytes ($CD8^+$ cells) and by LAK cells, which are mainly generated from NK cells under stimulation of

IL-2 released by T helper lymphocytes (CD^+ $CD25^-$ cells) [5, 6]. The anticancer immunity is also promoted by dendritic cells, because of their antigen-presenting cell function, which may act by making more immunogenic possible tumor antigens as well as by releasing IL-12, which activates T cytotoxic lymphocytes and stimulates the differentiation of T helper cells [5, 6]. On the contrary, the antitumor immune response is inhibited by the activation of the macrophage system and by the action of a subtype of T lymphocytes, the so-called T regulatory (Treg) lymphocytes ($CD4^+$ $CD25^+$), which counteract the anticancer immunity by producing immunosuppressive cytokines, such as TGF-β and IL-10, as well as through a direct cell-cell contact [7]. In fact, the generation for Treg cells is mainly dependent on the activity of some monocyte-macrophage precursors, which stimulate Treg generation through the release of immunosuppressive cytokines, such as IL-10 and TGF-β, which represent one of the main endogenous immunosuppressive agents [8]. Then, a perfect definition of the mechanisms involved in the anticancer immunity is essential to identify those altered biological parameters provided by a potential prognostic influence on the clinical course of the neoplastic disease within the great number of cancer-related biochemical anomalies. In addition, the recent discoveries on the relations between immune and psychoneuroendocrine systems have demonstrated that the immune responses, including the anticancer immunity, are regulated by the psychoneuroendocrine system, which constitutes the biochemical mediation of the psychological and spiritual life of patients [9]. In more detail, the anticancer immunity is inhibited by the brain opioid system [10] and stimulated by the functional neuroendocrine axis constituted by the brain cannabinergic system and pineal gland [4, 11, 12]. In particular, the pineal gland has been proven to produce several anticancer natural agents, the most prevalent ones consisting of the indole hormones melatonin (MLT) [4] and 5-methoxytryptamine (5-MTT) [12], which are provided by both anticancer antiproliferative and immunostimulatory functions.

26.3 The Fundamental Phases of Tumor Onset and Dissemination

Great advances in the knowledge of tumor biology may allow to synthetize the clinical history of the neoplastic disease into six main pathogenetic dynamics [1–12]: (1) preexistence of an endogenous psychoneuroimmunosuppressive status, due to an altered psychoneuroendocrine regulation of the anticancer immunity depending on psychosocial factors, mainly consisting of a progressive decline in the pineal neuroimmune function, rather than on a primary deficiency of the immune cells themselves; (2) spontaneous or carcinogen-induced malignant transformation of a single cell within some tissue, which essentially consists of loss of apoptosis and persistent activation of some growth factor receptor or protein kinase involved in cell replication; (3) alteration of connexin structure of the intercellular junctions, induced by the same activated protein kinases; (4) modification of the extracellular matrix determined by tumor cell-induced alteration of intercellular junction structure and consequent activation of tumor neoangiogenesis, induced by the same intercellular matrix modification; (5) direct production of immunosuppressive molecules by tumor mass cancer cells, namely, IL-10 and TGF-β, with a following determination of a biological irreversible endogenous immunosuppressive status, due to a damage of lymphocyte cell functions, which promotes the metastatic dissemination of cancer cells; and (6) tumor expression of Fas ligand (Fas-L) and consequent apoptosis of Fas-receptor-expressing T lymphocytes after tumor-immune cell interaction with Fas-L-expressing cancer cells [13], with a following progressive lymphocytopenia, mainly due to a decline in T helper lymphocyte cell count. Unfortunately, at present the most commonly used standard anticancer strategies are directed against only two of the six major mechanisms responsible for cancer progression, consisting of inhibition of tumor angiogenesis and inhibition of growth factor receptor activity. Then, according to the knowledgements achieved on cancer biology, at present

the reevaluation of an immunological approach in the treatment of cancer clearly appears to be fundamental to win cancer by counteracting the overall possible mechanisms of its apparently uncontrolled growth.

26.4 Main Cancer-Related Immunoneuroendocrine Alterations

The progressive immune alterations involving both cytokine secretion and lymphocyte subset differentiation may explain the lack of the generation of an effective anticancer immune reaction in advanced cancer patients. Cancer-related immune disorders would reflect the prevalence of the immune mechanisms suppressing the anticancer immunity with respect to those responsible for its activation, and they would fundamentally consist of abnormally high blood levels of the main suppressive cytokines, namely, IL-10, IL-6, and TGF-β, which are associated with a progressive decline in the concentrations of the most important antitumor cytokines, IL-2 and IL-12 [5, 6]. Due to the concomitant evidence of several neuroendocrine anomalies, cancer-related immune alterations could be at least in part a consequence of an altered neuroendocrine regulation of the immune system, particularly on those involving the adrenal and the pineal functions, which are provided by immunosuppressive and immunostimulatory action on the anticancer immunity, respectively. In fact, cancer dissemination has been proven to be characterized by an increased cortisol secretion associated with a lack of its physiological circadian rhythm [14] and by a progressive decrease in the endocrine pineal function, mainly consisting of a decline in the nocturnal production of its most investigated indole hormone, MLT [4, 11]. Deficiency in the pineal function would represent the main cancer-related endocrine deficiency. These major neuroendocrine anomalies, including the diminished pineal MLT production during the night and the abnormally enhanced cortisol secretion, have been described in the most frequent tumor histotypes, including lung, prostate, and ovarian cancers, as well as colorectal carcinoma. According to the recent results on the psychoneuroimmune status of cancer patients, tumor progression-related neuroendocrine anomalies would be the consequence of an unbalanced ratio between the brain opioid and cannabinoid systems, with hyperactivity of the opioid system and hypoactivity of the cannabinergic one, with the generation of an endogenous suppression of the anticancer immunity as an end result of the neuroendocrine disorder, due to the inhibitory activity of the opioid system on the anticancer immunity [15].

26.5 Preliminary Clinically Applied PNEI Strategies

The rationale of PNEI therapy of human neoplasms consists of the possibility to control cancer growth by correcting the main cancer-related immune and neuroendocrine anomalies provided by prognostic significance and then responsible for the lack of generation of an effective anticancer immunity generation in the oncologic patients. While the evaluation of the antitumor therapeutic activity of both chemotherapies and target therapies with biological agents generally consists of the only radiological examinations, the efficacy of immunotherapeutic and neuroimmunotherapeutic strategies of human neoplasms may be also predicted on the basis of changes in some immune parameters, such as the simple lymphocyte count. In fact, it is known since several years that lymphocytopenia constitutes one of the most negative prognostic factors in cancer patients [1], whereas the evidence of lymphocytosis would be one of the most positive biological prognostic parameters in cancer immunotherapy with cytokines, such as IL-2 [16]. Moreover, PNEI therapy of cancer is a new therapeutic strategy of cancer carried out to induce an effective anticancer immune reaction through the simple correction of cancer-related immune and endocrine alterations, in an attempt to reestablish the neuroimmune biochemistry of health status by the exogenous administration of the endogenous antitumor cytokines and neurohormones, whose

secretion is diminished in the neoplastic disease, and to inhibit the abnormal production of some other hormones and cytokines provided by an immunosuppressive activity on the anticancer immunity. In more detail, cancer-related endocrine pineal deficiency and diminished IL-2 production may be simply corrected by an exogenous administration of pharmacological doses of MLT during the dark period of the day and subcutaneous (SC) low-dose IL-2, which is less toxic and provided by similar immunostimulating effect with respect to its intravenous injection either as a bolus or continuous 24-h infusion [17]. As previously observed in experimental conditions [4], MLT may enhance IL-2-induced lymphocyte activation and enhance its clinical efficacy with respect to IL-2 alone [18], with a consequent potential therapeutic efficacy of IL-2 in most tumor histotypes, whereas the efficacy of IL-2 alone is generally limited to malignant melanoma and renal cell carcinoma (RCC) [17]. The lower *in vivo* antitumor activity of IL-2 with respect to its anticancer potency *in vitro* could depend at least in part on cancer-related deficiency in pineal hormone production. In preliminary clinical studies carried out in untreatable metastatic cancer patients with a life expectancy of less than 1 year, the neuroimmunotherapeutic strategy with SC low-dose IL-2 plus pharmacological doses of MLT during the night may allow 1-year percentage of survival greater than 30 % in tumors other than malignant melanoma and RCC [18]. Further improved results in terms of generation of an effective anticancer immune response may be achieved by a concomitant injection of IL-12 before the administration of IL-2 [19]. Moreover, concomitant injection of IL-2 has been proven to counteract IL-12-induced lymphocytopenia, and the association between IL-2 and IL-12 has been proven to induce the maximal lymphocytosis described up to now with the different possible immunotherapeutic strategies in humans. According to data available in the literature, MLT may play an antitumor activity in humans only if it is administered at pharmacological doses of at least 20 mg/day and during the dark period of the day [4]. In addition, preliminary clinical results (unpublished data) would show a dose-response ratio in the antitumor activity of MLT.

26.6 Future Perspectives

The immunoneuroendocrine association between IL-2 and MLT is the basis regimen of the overall possible neuroimmunotherapies of human neoplasms, due to its correction of the two main cancer-related immunoendocrine deficiencies, consisting of a diminished IL-2 production by T helper lymphocytes and of MLT from the pineal gland [4–6, 11]. Further therapeutic results could be achieved merely by piloting IL-2 activity in an antitumor way, as well as by replacing the pineal deficiency not only of MLT but also of at least another fundamental pineal antitumor hormone, the 5-MTT [12]. At present, there are no clinical data on the endogenous secretion of pineal hormones other than MLT. Nevertheless, due to the evidence of pineal histological alterations in patients succumbed to cancer, it is probable that cancer progression may be associated with a more generalized endocrine deficiency of the pineal gland, than involving the secretion of 5-MTT, which in contrast to MLT is mainly released during the light period of the day and may exert an antitumor antiproliferative effect superior to that of MLT itself [12]. The importance of piloting the biological activity of IL-2 in an antitumor way is only justified by the fact that IL-2 may also induce potential immunosuppressive effect on the anticancer immunity, in particular the stimulation of Treg cell generation [7] and of the macrophage system, which may inhibit anticancer immunity through the production of several immunosuppressive agents, such as IL-6 [17]. Macrophage-mediated chronic inflammatory and immunosuppressive response may result from the administration of MLT itself [4, 11, 18] or concomitant with some neuroactive immunomodulating substances, such as the cannabinoid agonists [4, 9, 11]. Finally, cancer-related hyperactivation of the brain opioid system, which mediates an immunosuppressive psychobiological status, may be removed by the administration of the mu-opioid receptor antagonist naltrexone, which has also

been proven to enhance the biological activity of IL-2 probably by counteracting Treg cell generation. However, at present there is no agreement in the literature about the optimal schedule of naltrexone therapy in cancer patients, since both low and high doses have been suggested. In addition, Treg cell generation could also be counteracted by at least two other immunological strategies, consisting of the concomitant administration of IL-17, which may inhibit Treg cell activation, or that of specific monoclonal antibodies against Treg cells. Finally, it should also be taken into consideration that antitumor efficacy of IL-2 is also influenced by the psychospiritual status of cancer patients [9]. In more detail, the immunotherapeutic efficacy of IL-2 has been proven to be reduced by the presence of anxiety, self-punishment behavior, and repression of sexual pleasure and spiritual sensitivity, whereas it is amplified by the presence of pleasure feeling and spiritual consciousness. Then, the future psychoneuroimmunotherapies of cancer would require a concomitant psychospiritual therapeutic approach, carried out for the education of cancer patients to the rediscovery of life pleasure as a spiritual experience, by removing the opposition between sexual pleasure and spirituality [9].

26.7 Concluding Remarks

In conclusion, the aim of PNEI approach in the treatment of cancer is the replacement of the health psychoneuroimmune status, which may exert a natural biological resistance against cancer onset and development. At present, the maximal antitumor immune response in terms of lymphocytosis in humans may be achieved by the concomitant administration of low-dose IL-2 and IL-12 under a psychoneuroendocrine modulation exerted by pineal indoles, cannabinoid agonists, and mu-opioid antagonists. Moreover, since in humans the immune responses, including the anticancer reaction, can be separated from neither their physiological psychoneuromodulation nor the psychospiritual status of patients, the future PNEI therapy of cancer will mandate a concomitant psychological and spiritual approach, in an attempt to contribute in generating faith in patients to win their cancer by removing the overall unconscious self-punishments.

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Ethical Considerations in Cancer Immunotherapy

27

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Contents

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27.1 Introduction

The concept of immunological therapy of 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 also documented observation of spontaneous regression of malignant masses has 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.

Further, an extensive body of laboratory-based research supports the potential role of immune cells and their products in positively or negatively influencing the rate of cancer growth and spread [1]. And more recently, prospective clinical trials have documented the clinical utility of several immunologically based treatment strategies to produce objectively measurable effects on existing malignant mass lesions and to improve disease-specific survival.

Finally, it can be anticipated that the clinical utility of immunotherapy demonstrated to date

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represents the mere 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 often impressive 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 unique approach to cancer management. This chapter will briefly highlight a number of these issues and concerns.

27.2 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 27.1).

27.3 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., 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 >six 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.

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 [3].

As a result, until a relatively large number of human subjects have been treated with a particular immunological approach, the overall toxicity profile will remain uncertain and will mandate careful monitoring and regular updates to an ethical oversight committee responsible for insuring subject safety.

Table 27.1 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

27.4 Evaluation of Efficacy in the Clinical Trial and Non-research Settings

Extensive preclinical evaluation has provided strong support for the conclusion that immunological mechanisms 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 provocative phase III study of sipuleucel-T immunotherapy in the management of metastatic prostate cancer [4]. The study revealed the strategy to improve overall survival, but there was surprisingly no statistically significant effect on progression-free survival, a most 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. Unfortunately, the absence of a definitive answer to this question makes decision making about treatment with this drug for an individual patient quite 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. Several recent examples of exciting novel approaches employing immune mechanisms suggest the relevance of documenting the presence of a particular target in increasing the benefits of the strategy [5, 6].

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?

27.5 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 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 immune-regulatory 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 a 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 has been provided with the greatest opportunity (clinical care) will present the oncology community with a unique challenge.

27.6 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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