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Cancer Immunology

A Translational Medicine Context

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

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 volume I, interactions between cancerous cells and various components of the innate and adaptive immune system are fully described. Notably, the principal focus is very much on clinical aspects, the aim being to educate clinicians on the clinical implications of the most recent findings and novel developments in the field. To meet this purpose, this volume consists of 26 chapters. After an overview on cancer immunology in Chap. 1, the role of innate immunity in cancers is explained in Chaps. 2 and 3, followed by the adaptive immunity, including B cells, T cells, T regulatory and Th17 cells, cytokines, and chemokine receptors in Chaps. 4, 5, 6, 7, and 8, respectively. CD95/CD95L signaling pathway, MHC class I molecules, and plasmacytoid dendritic cells are separately described in Chaps. 9, 10, and 11, respectively. Chapter 12 focuses on cancer immunoediting, while Chaps. 13 and 14 explain apoptosis and autophagy in cancers. Subsequently, Chap. 15 presents the prognostic value of innate and adaptive immunity in cancers. Epigenetics and immunogenetics are explicated in Chaps. 16 and 17, respectively. In addition, immunodeficiencies (Chap. 18), immunosenescence (Chap. 19), nutrition (Chap. 20), allergies (Chap. 21), and transmissible cancers (Chap. 22) are individually described in the following chapters. Chapter 23 enlightens systems biology in cancer immunology, while immunological diagnostic tests, including flow cytometry for cancers, are mentioned in both Chaps. 24 and 25. Finally, by allocating the final chapter to immunohistochemistry of different cancers, volume I comes to its end.

The *Cancer Immunology* Series is the result of valuable contributions of 266 scientists from 91 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 the Springer publication for providing me the opportunity to publish the book.

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

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

Introduction on Cancer Immunology and Immunotherapy

1

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1.1 Introduction

Cancer is a life-threatening disease, which can involve all human organs and tissues. It is the second leading cause of death and is responsible for 25 % of all deaths in the United States. In 2012, more than 1.6 million new cases (848,170 men and 790,740 women) of invasive cancers were diagnosed in the United States alone [1]. The major cancers in adults include lung, breast, prostate, and colorectal cancer. In addition, 60,824 adolescents and young adults aged 15–29 years old were diagnosed with invasive cancers between 1975 and 2000 [2]. Among all invasive cancers, lymphoma was the most common cancer (20 %), followed by invasive skin cancer (15 %), male genital system cancer (11 %), and endocrine system cancer (11 %) [2]. Although cancer incidence has increased among people younger than 45 years old during 1975–2000, overall cancer incidence has decreased in

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men by 0.6 % per year during 2004–2008. Remarkably, the rate remained stable among females due to the high rate of breast cancer [3].

Many cancer predisposing factors have been recognized; it has been found that cancer incidence is significantly associated with age from 10 to 60 years. Additionally, male gender is at higher risk of developing cancer compared to females [2]. Race is another important factor for cancer development; before 40 years of age, non-Hispanic whites and, after 40 years of age, African-Americans/blacks have the highest incidence [4]. Other risks factors include life style choices such as tobacco use, obesity, and lack of exercise and environmental factors such as exposure to excessive sun, radiation during childhood, human papilloma virus (HPV), human immunodeficiency virus, and Epstein-Barr virus (EBV) infection [4].

Cancer can be a life-threatening health problem, especially when the tumor has metastasized to other organs. It is estimated that 577,190 patients (including 301,820 men and 275,370 women) died from cancer in the United States in 2012. Four cancers – lung and bronchus, prostate, and colorectal in men and lung and bronchus, breast, and colorectal in women – are responsible for approximately 50 % of cancer-related deaths. Fortunately, the overall cancer-related mortality has been decreasing in recent years. The death rate decreased by 1.8 % per year among men and 1.6 % per year among women. The highest mortality reduction has been found among African-Americans (2.4 % per year), followed by Hispanics (2.3 % per year); however, American Indians/Alaska natives were an exception, and the rate remained unchanged in this population [1].

Cancer survival significantly impacts patients' quality of life. Five-year mortality rates depend on several factors; survival is worse among males over 30 years of age, and the survival gets worse for patients over 45 years old in both males and females. Non-Hispanic whites have the best survival rate and African-Americans have the worst survival with survival differences as great as 20 % at 5 years after cancer diagnosis [5]. Furthermore, the type of cancer is another risk factor for patient survival. Total mortality rates vary from 6 % in thyroid cancer to 97 % in pancreatic cancer [6].

1.2 Cancer Immunity

Cancer immunology has been studied for a long time; however, the molecular and cellular basis of tumor immunity is not completely understood. Advances in understanding the basis of immunosurveillance and progress in the treatment of infectious disease have had a major impact on the development of tumor immunotherapy. The modern era of tumor immunology began in the 1950s when the role of T cell responses in tissue allograft rejection was initially identified. Since then, it has been confirmed that tumors occur in association with impaired function of T cells, indicating the importance of the immune system in the development and progression of cancer [7]. The identification of tumor-associated antigens, knowledge of effector T cell responses, and the role of regulatory and suppressor T cell populations are now shaping the use of the immune system to treat cancer.

In addition to an improved understanding of the immune system, significant advances in understanding the molecular basis of neoplasia have occurred. Precise control of cellular activity and metabolism is crucial for proper physiologic function. Notably, cell division is an important process that requires precise regulation. The main difference between tumor cells and normal cells is lack of growth control during the cell division process. This uncontrolled cell division can originate from various factors, such as chemical agents, viral infections, and mutations that lead to escape of cells from the checkpoints which properly control cell division. According to the type of tumor and proliferation rate, cancers can be benign or malignant [8]. It has been found that some tumors are caused by oncogenic viruses that induce malignant transformation. These oncogenic viruses can be both RNA and DNA viruses. Also, viral infection may lead to leukopenia and immunodeficiency, increasing the risk of malignancy. Therefore, prophylactic immunization against

oncogenic viruses (such as EBV, HPV, and HBV) might be a logical strategy for prevention of malignancy [9]. Indeed, a vaccine against the human papilloma virus has shown significant impact on preventing cervical intraepithelial neoplasia and may prevent development of cervical carcinoma.

1.3 Cancer and Immune System Impairment

It has been reported that impaired immune response can induce tumor growth and prevent effective antitumor suppression, possibly through a process of "sneaking through" which allows improved growth of small tumors rather than large tumors [10]. Tumors may also produce immunosuppressive factors, such as interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and alpha-fetoprotein, which suppress innate immune responses against cancer. This has led to investigations using neutralizing antibodies against these immunosuppressive factors [7]. In contrast, tumor-specific cytotoxic T lymphocytes (CTLs) can be genetically altered to become resistant to the TGF-β inhibitory effect by transgene expression of a mutant dominant-negative TGF-ß type II receptor (DNR). In addition, specific T cells genetically manipulated to produce IL-12 can overcome the inhibitory effects of IL-10. On the other hand, tumors may express FasL and stimulate apoptosis of tumor-infiltrating effector T cells. Small interfering RNA (siRNA) can be used to knock down the Fas receptor in tumor-specific CTL, leading to a significant decrease in their susceptibility to Fas-/FasL-mediated apoptosis [11].

The interaction between the immune system and established cancers is complex, because in addition to increasing carcinogenesis by various carcinogens among compromised subjects, cancer cells themselves can lead to severe immunosuppression. It has been reported that patients involved with primary immunodeficiency syndromes have higher risk of cancer development. In a report by Kersey et al., subjects that had an inherited abnormal lymphoid system were susceptible to malignant transformation and impairment of tumor immunosurveillance [12, 13]. In addition, tumors produce soluble factors which downregulate the interleukin-2 receptor-α (IL-2Rα), leading to suppression of T cell function. Furthermore, established tumors may result in severe protein expenditures in hosts, contributing to impairment of immune system function [14].

1.4 Immune System Reaction to Cancer

A critical question is whether cancer cells are sufficiently different from their normal cellular counterparts, and can thus be recognized by the immune system. The immune system also produces a group of complementary markers with protective effects against cancer and other immunologic or inflammatory stresses. These markers include proteins released by T cells and are generally classified as "cytokines." Cytokines include interleukins, interferons, tumor-necrosis factors (TNF), and lymphocyte-derived growth factors. The production of tumor-specific antibodies and/or activation of tumor antigen-specific T cells target tumor-associated antigens typically found on the cell membrane. Studies have suggested that vaccination in the presence of complements can lead to tumor lysis. While incompletely defined, several soluble and cellular mediators of tumor rejection have been described, including complement factors, active macrophages, T cells, and NK cells. While T cells require antigen specificity, the soluble and cellular mechanisms of the innate immune response can recognize the malignant phenotype in the absence of antigen specificity [15].

Since most tumor-associated antigens are self-proteins, the immune response is largely weak and patients may develop immune tolerance to tumor-associated antigens. Furthermore, the cells of the immune system may not adequately penetrate to the internal tumor microenvironment, resulting in slower immune-mediated tumor elimination. However, it is possible that the immune system may be more effective in controlling tumor growth rate rather than tumor regression [10]. Recently, it has been found that

nutrition also plays a crucial role in protection against human cancer, and normal levels of zinc are required for protection against the detrimental effects of various immunosuppressive cytokines [16].

1.5 Genetic and Environmental Carcinogenesis

It has been found that genetic factors are as important as environmental carcinogens. Trials have tested carcinogenesis of retrovirus infection between different breeds of animals. A unique carcinogen resulted in disparate outcomes among different breeds, indicating the importance of genetic background in the progression of cancer. Environmental factors may also suppress immune responses and dysregulate immunosurveillance mechanisms [17].

1.5.1 Cancer Cells Escape from Host Immunosurveillance

Antigens that distinguish tumor cells from normal cells depend on the histologic origin of the tumor. Tumor-associated antigens may be viral in origin, represent mutated self-antigens, be cancer-testis antigens which are expressed only by tumor cells and normal testes, or be normal differentiation antigens. Thus, tumor cells may express similar antigens to normal cells, allowing tumor cells to escape immune system attack through induction of innate and/or peripheral tolerance. A corollary to this is that immunotherapy or stimulation of immune responses to some tumor-associated antigens may lead to damage of normal tissues and organs, as exemplified by the development of autoimmunity induced by anti-CTLA-4 or anti-PD1 monoclonal antibody (mAb) treatment [18].

A number of complex mechanisms have been suggested for the escape of cancer cells from host immunosurveillance. Tumors alter their characteristics by decreased expression of immunogenic tumor-associated antigens, MHC class I molecules, beta2-microglobulin, and costimulatory molecules, which mediate the activation of T cells. Another strategy resulting in failure of tumor immunosurveillance could be the expression of very low levels of antigens, unable to stimulate an immune response. Under some circumstances, such as failure of the immune response to induce a rapid response, cancer cells may proliferate rapidly. Further strategies for escape of tumor cells from immunosurveillance are based on inhibitory tumor-mediated signaling by CTLs, as occurs through changes in cell death receptor signaling. Other strategies which allow tumor cells to evade the immune system are the secretion immunosuppressive molecules dampening tumor-reactive effector T cells and the induction of regulatory and/or suppressor cells [19].

To date, most direct evidence on tumor immunosurveillance originates from experimental studies in animal models. These models have supported the potential for antitumor immunity via vaccination, as, for example, by administration of inactivated cancer cells, or through removal of a primary tumor. In addition, antitumor immunity can be adoptively transferred through administration of tumor-reactive T lymphocytes. The complexities of immunotherapy are evident as nearly all immune system components can influence tumor growth and progression. Although there is evidence for antitumor immunity in humans and several new agents have gained regulatory approval for cancer therapy, further investigation is warranted to increase the impact of tumor immunotherapy for more cancer patients [20].

1.5.2 Cancer Immunodiagnosis

Nowadays, new immunomolecular diagnostic approaches have been suggested for tumor detection. Monoclonal antibodies marked with radioisotopes have been used for *in vivo* diagnosis of small tumor foci. In addition, monoclonal antibodies have been used for *in vitro* recognition of the cell of origin for tumors with poor differentiation. Immunodiagnostics have also been used to determine the extent of metastatic disease, especially metastasis to the bone marrow [21].

1.6 Cancer Treatment

Systemic cancer treatment is based on four general therapeutic approaches: (1) chemotherapy, which contains a wide group of cytotoxic drugs that interfere with cell division and DNA synthesis; (2) hormonal therapy, which contains drugs that interfere with growth signaling via tumor cell hormone receptors; (3) targeted therapy, which involves a novel group of antibodies and small-molecule kinase suppressors that principally target proteins crucial in cancer cell growth signaling pathways; and (4) immunotherapy, which targets the induction or expansion of antitumor immune responses [22].

1.6.1 Cancer Immunotherapy

Tumor immunotherapy is a novel therapeutic approach for cancer treatment, with increasing clinical benefits. Tumor immunotherapy is based on strategies which improve the cancer-related immune response through either promoting components of the immune system that mediate an effective immune response or via suppressing components that inhibit the immune response. Two current approaches commonly used for immunotherapy are allogeneic bone marrow transplantation and mAbs targeting cancer cells or T cell checkpoints [23]. Recently, various other approaches have been tested for cancer immunotherapy, and some are undergoing further clinical evaluation.

Initially, anticancer vaccines were considered for prevention and treatment of various tumors [23]. It is estimated that more than 15 % of human cancers are caused by viral infection [24]. Vaccine-based immunotherapy may, thus, be most useful for virus-induced cancers. Consistent with this hypothesis, a 50 % complete remission (CR) of HPV-associated vulvar intraepithelial neoplasia grade III (VINIII) has been reported [25]. An attenuated, oncolytic herpes simplex type 1, which is genetically engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF), has been developed for cancer therapy. This oncolytic immunotherapeutic agent has been injected to the tumor mass and has had beneficial effects in the treatment of melanoma and head and neck squamous cell carcinoma [26]. Although vaccine-based therapy has not been effective in some types of cancer, there are studies that have shown an overall survival benefit compared to placebo therapy [27].

Another immune-targeted approach is mAbs which block T cell checkpoints functioning to suppress T cell responses. Cytotoxic T lymphocyte-associated antigen 4 (CTLA4) is a member of a large family of molecules regulating T cell immune responses. CTLA4 is expressed on $CD4^+$ and $CD8^+$ T cells, as well as on $FOXP3^+$ regulatory T cells [28]. Administration of mAbs targeting human CTLA4 leads to the rejection of established tumors in a small cohort of patients with metastatic melanoma and demonstrated improved overall survival in patients with metastatic melanoma, resulting in US FDA approval for the treatment of metastatic melanoma [29].

Monoclonal antibodies which block other T cell checkpoints, such as the programmed cell death protein 1 (PDCD1/PD1), programmed cell death ligand 1 (PDL1/CD274), CD276 (B7H3) antigen, V-set domain-containing T cell function inhibitor 1 (B7x), and B and T lymphocyte attenuator, have also entered clinical trials. In addition, early phase studies have demonstrated significant therapeutic activity in several types of cancer, including melanoma, renal cell carcinoma, non-small cell lung carcinoma, and ovarian cancer [30]. It has been reported that PDL1 expression by tumor cells is associated with poor clinical outcome and may be associated with clinical response to anti-PD1 and anti-PDL1 therapy. Also, ligation of PDL1 leads to inactivation of tumor-infiltrating cells [31]. On the other hand, regulatory T cells have an immunosuppressive role in the tumor microenvironment. Studies of anti-PD1 and anti-PDL1 are in progress. Moreover, the combination of these agents with anti-CTLA4 and other immunotherapy strategies has yielded promising results.

The combination of antitumor vaccines with agents targeting the IL-12 receptor resulted in conflicting results. This may be due to the upregulation of IL-12 receptor by both activated

T effector cells and regulatory T cells [32]. Thus, new approaches focused on more specific targeting of regulatory T cells which reduce their suppressive effects on the immune system are necessary. Adoptive T cell therapy has been described as an effective therapeutic approach for cancer immunotherapy in early phase clinical trials. In this method, a large number of tumor-specific T cells derived from peripheral blood, or preferably from the tumor microenvironment (with or without genetic manipulation to express a high-affinity antigen-specific T cell receptor (TCR)), are adoptively transferred to patients with established tumors [33]. Recently, CD19 which is expressed by mature B cells and a majority of non-Hodgkin lymphoma (NHL) cells has been used as another novel promising therapeutic target [34]. Chemotherapy-mediated cell death leads to immune responses in a drug-induced biochemical cell death cascade-dependent manner, suggesting beneficial effects of chemotherapy and immunotherapy in combination [35]. It seems that future goals of tumor immunotherapy are headed towards chemoimmunotherapy. Potential candidates for this combination approach include antitumor vaccines, Toll-like receptor (TLR) signaling pathway agonists/antagonists, cytokines, and mAbs targeting T cell checkpoints, such as CTLA4, PD1, or PDL1/2 [36]. Also, it seems that radiation and radiofrequency ablation are future candidates for combination therapy with immunotherapy [37]. Although immunotherapy and its combination with other therapeutic approaches such as radioimmunotherapy may be beneficial for tumor treatment, there are several limitations that need to be addressed; defining the optimal target patient, optimal biological dose, and schedule, the need for better trial designs incorporating appropriate clinical endpoints, and the identification and validation of predictive biomarkers are just a few to point to [23].

1.6.2 Cancer Cell "Switch"

Cancer cells can switch on genes mostly related to the earlier embryonic stages of development. During rapid proliferation of cancer cells, precise orchestrated enzyme formation needed for suitable metabolism of its different components might get unbalanced, and products which are not observed in normal dividing cells are produced [38]. Recently, it has been reported that these biochemical "switches" lead to uncontrolled multiplication of cancer cells. One switch has been found for a type of leukemia. It has been suggested that targeting tumor switches can make treatment of cancers very simple [20]. Nonetheless, it is unclear how this may be used to optimize tumor immunotherapy.

Since cancer immunology is a highly complex process, further research is needed to more completely understand how the immune system recognizes and eradicates cancer. In this book, we will describe a variety of novel mechanisms currently under investigation for mediating aspects of tumor immunology with a particular focus on promising therapeutic approaches, producing a complete comprehensive up-to-date textbook.

1.7 Concluding Remarks

Cancer is a life-threatening health problem which is related to several genetic and environmental risk factors that manipulate immune system function. Cancers themselves produce immunosuppressor factors to impair cells division check points, leading to uncontrolled proliferation of cancer cells. Importantly, tumor cells have learned how to escape from immune system attack via presenting of similar antigens to normal cells and expression of very low levels of antigens. Therefore, diagnosis of tumors and their progression is not easy. Recently, immunodiagnostic methods are shown to be helpful in the diagnosis of cancers and determining the extent of metastasis. On the other hand, classic treatment of cancers led to unsatisfactory results, and intelligent immunological approaches, such as regulatory T-cell targeting, adoptive T-cell administration, and combination of immunotherapy and chemotherapy are addressed. Results of antitumor vaccines,

Toll-like receptor (TLR) signaling pathway agonists/antagonists, cytokines, and mAbs targeting T-cell checkpoints, such as CTLA4, PD1, or PDL1/2 are promising. However, due to the highly complexity of the cancer immunology, still a lot of gaps exist in this field that indicate the necessity of further researches for complete understanding of cancers' immunological behaviors and emerging of more novel immunotherapeutic strategies.

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2 Inflammatory and Innate Immune Cells in Cancer Microenvironment and Progression

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2.1 Introduction

Inflammation is a consistent feature of the tumor microenvironment and has been considered the seventh hallmark of cancer [1–6]. As suggested by current estimates, 25 % of cancers are associated with chronic inflammation sustained by infections (e.g., hepatitis) or inflammatory conditions of diverse origin (e.g., prostatitis) [6]. In addition, even tumors not directly connected to inflammation are characterized by the presence of cells and mediators of the inflammatory response [7].

Apart from malignant cells, host cells infiltrate tumors, including leukocytes, fibroblasts, and endothelial cells. Leukocytes, and in particular myeloid cells, are the most consistent cellular component of solid tumors. Tumor-associated myeloid cells (TAMC) mainly support tumor growth and progression, thereby contrasting the T-cell infiltrate, which mainly has antitumoral activity. TAMC all arise from hematopoietic stem cells (HSC) within the bone marrow (Fig. 2.1) and further differentiate into macrophage/granulocyte progenitors. The tumor infiltrate comprising the myeloid populations skews tumor-mediated immunosuppression, tissue remodeling, tumor progression and metastasis [8, 9]. TAMC demonstrated high plasticity, resulting in two extreme polarized macrophage (M1 and M2) and neutrophil (N1 and N2) phenotypes [10, 11]. Cross talk between the different cellular components was demonstrated, resulting in tuning of the adaptive

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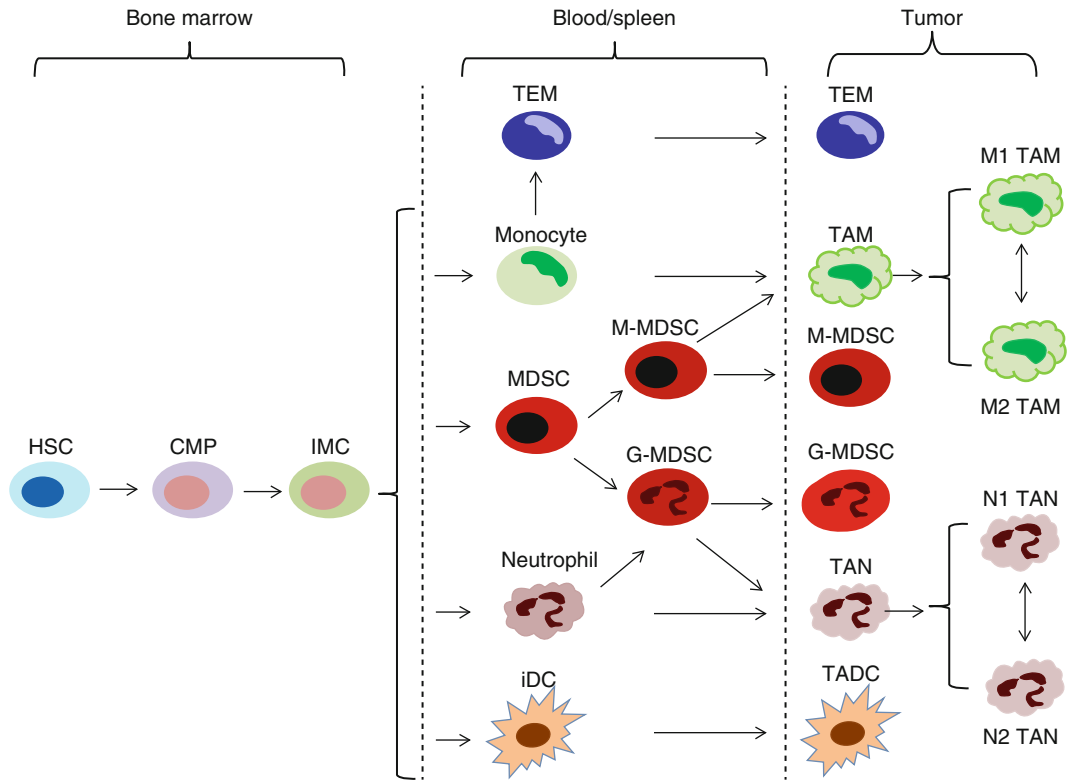

Fig. 2.1 Differentiation pathways of tumor-associated myeloid cells. Myeloid cells originate from hematopoietic stem cells (*HSC*) in the bone marrow. Here the networks that give rise to the various myeloid cell lineages in diverse compartments (bone marrow, blood/spleen, and tumor) and their precursors are illustrated. In the tumor tissue, macrophages and neutrophils display a gradient of differently polarized phenotypes whose extreme are M1–M2 for TAM and N1–N2 for neutrophils. *CMP* common myeloid progenitors, *IMC* immature myeloid cells, *TEM* Tie2-expressing monocytes, *MDSC* myeloid-derived suppressor cells, *M-MDSC* myeloid MDSC, *G-MDSC* granulocytic MDSC, *TAM* tumor-associated macrophages, *TAN* tumor-associated neutrophils, *iDC* immature dendritic cells, *TADC* tumor-associated dendritic cells

immune response, promotion of angiogenesis, and tissue remodeling [8].

Results obtained so far clearly indicate that TAMC are major players in the connection between inflammation and cancer. Ongoing efforts, which led to a better understanding of their biological properties, indicated that myeloid cell-infiltrating growing tumor could have a prognostic value, thus representing an attractive target for novel biological therapies of tumors.

In this chapter, we will mainly focus on myeloid cells infiltrating tumors and mention soluble mediators involved in their recruitment or released by TAMC, which affect tumor progression and dissemination (cytokines, chemokines, and proteases). Furthermore, new therapeutic approaches based on targeting of tumor-infiltrating myeloid cells and/or soluble mediators will be discussed.

2.2 Heterogeneity of Myeloid Cells in the Tumor Microenvironment

2.2.1 Myeloid Subsets in the Tumor Microenvironment

Solid tumors are characterized by the presence of a leukocyte infiltrate including lymphocytes and myeloid cells from early stages. Growing evidence indicated that the leukocyte infiltrate

has a prognostic value. For instance, it has been described that infiltrating T lymphocytes are associated with a favorable prognosis in colorectal cancer, melanoma, ovarian cancer, and breast cancer [12, 13]. In contrast, myeloid cells are most frequently associated with a poor prognosis [14]. TAMC (Fig. 2.1) comprise five distinct myeloid populations, namely, tumor-associated macrophages (TAM), monocytes expressing the angiopoietin-2 (Ang-2) receptor Tie2 (known as Tie2-expressing monocytes or TEM), myeloid-derived suppressor cells (MDSC), tumor-associated neutrophils (TAN), and tumor-associated dendritic cells (TADC).

Tumor-associated macrophages belong to the early infiltrating leukocyte populations within tumors, thus preceding lymphocytes, and are usually the most abundant immune population in the tumor microenvironment [6, 15]. They derive from blood monocytes actively recruited from the circulation into tumor tissues. Early studies demonstrated that appropriately stimulated macrophages are able to kill tumor cells *in vitro*; however, TAM, conditioned by the tumor microenvironment, loose the cytotoxic capability and rather exert several pro-tumoral functions, mediating cancer-related inflammation, angiogenesis, immunosuppression, tissue remodeling, and metastasis [16, 17, 6].

The heterogeneous behavior of TAM is a hallmark of myeloid cells and is oversimplified in a polarization concept with two extreme M1 and M2 phenotypes [18–20] with distinct and somehow opposite functions. M1 macrophages are classically activated by bacterial products and Th1 cytokines (e.g., LPS/interferon-γ). They are potent producers of inflammatory and immunostimulating cytokines, trigger adaptive responses, secrete reactive oxygen species (ROS) and nitrogen intermediates, and have cytotoxic effect towards transformed cells. On the other hand, M2 macrophages or alternatively activated macrophages differentiate in response to Th2 cytokines (e.g., interleukin (IL)-4, IL-13) [21]. In contrast to their M1 counterpart, M2 macrophages produce growth factors, leading to tissue repair and angiogenesis activation, have high scavenging activity, and inhibit adaptive immune responses [22, 14, 23, 11, 24]. Thus, macrophages are a very heterogeneous cell population, able to display different functions depending on the context. Macrophages can be either immunostimulatory at the beginning of the inflammatory response or immunosuppressive which dampen inflammation [25, 18, 14, 26, 27].

A similar dichotomy with polarization towards two extreme phenotypes (N1 and N2) has been also described for neutrophils [28]. Besides exerting antibacterial functions, neutrophils can infiltrate tumors playing a major role as key mediators in malignant transformation, tumor progression, and regulation of antitumor activity [29]. Tumor-associated neutrophils (TAN) have received interest only recently, mainly due to their short life span and the observation that tumor microenvironment can sustain and prolong the survival of polymorphonuclear leukocytes (PMN) [30, 31].

A particular small subset of TAMC is represented by Tie2-expressing monocytes (TEM): they express several monocyte/macrophage markers, along with the angiopoetin-2 receptor, Tie2, and are endowed with proangiogenic properties [32–35]. Tie2-expressing monocytes can be distinguished from the majority of TAM by their surface marker profile ($Tie2^+$, $CD11b^+$) and their preferential localization to areas of angiogenesis [33], while they are largely missing in nonneoplastic area adjacent to tumors [35]. Indeed, Tie2 is constitutively expressed at low levels by a substantial fraction (20 %) of circulating monocytes and is overexpressed upon monocyte homing into growing tumors or regenerating tissues [33, 36]. Following Ang-2 stimulation, $Tie2^+$ monocytes acquire an M2-like phenotype, with increased expression of IL-10, CCL17, arginase 1 (Arg-1), and scavenger and mannose receptors and low expression of proinflammatory molecules such as IL-12 and TNF-α [37, 38].

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells, having the ability to suppress T-cell functions [39, 40]. They are derived from myeloid progenitors in bone marrows which do not differentiate into mature granulocytes, macrophages, or dendritic cells. MDSC have been isolated from

blood, spleen, and bone marrow of tumor-bearing mice and infiltrate the tumor tissue, where local tumor-associated factors promote their activation [41]. In tumor-bearing mice, two main subsets of MDSC were identified: monocytic MDSC (M-MDSC), characterized by $CD11b^+$, $Ly6G^-$, and $Ly6C^{high}$, and granulocytic MDSC (G-MDSC), characterized by $CD11b^+$, $Ly6G^{high}$, and $Ly6C^-$ [42]. M-MDSC were shown to govern the ability of differentiating into monocytes (macrophages) and (DC), whereas G-MDSC do not possess this potential [43]. These subsets are functionally different: M-MDSC-mediated immunosuppression is based on upregulation of inducible nitric oxide synthase (iNOS), expression of *Arg-1*, and production of suppressive cytokines, whereas G-MDSC-mediated immunosuppression is characterized by antigen-specific responses (including ROS release requiring prolonged MDSC and T-cell contacts) [44]. Tumor-associated MDSC generally exhibit an M2-like phenotype, while M1 and M2 phenotypes could coexist in some mouse tumor models [45, 46].

Human MDSC are still poorly defined [47], even if they have been isolated from blood of patients with glioblastoma, colon cancer, breast cancer, lung cancer, or kidney cancer [48–52]. Recent studies have proposed that human MDSC have a characteristic $CD34^+$, $CD33^+$, $CD11b^+$, and $HLA\text{-}DR^-$ profile [42]. Similarly to the murine counterpart, human MDSC are divided into two main subsets: monocytic MDSC (M-MDSC), characterized by the expression of CD14, and granulocytic MDSC (G-MDSC), identified by positivity for CD15.

A small number of dendritic (DC) are found in most human and murine neoplasms. Similarly to macrophages and neutrophils, plasticity is a main feature of these cells. DC are differentially localized in tumors; for example, in breast cancer immature $langerin^+$ DC are interspersed within the tumor mass, whereas more mature $CD83^+$, $DC\text{-}LAMP^+$ DC are confined to the peritumoral area [53]. In contrast to TAM, tumor-associated dendritic cells (TADC) were found in the invasive front of papillary thyroid carcinoma [54]. Growing evidences demonstrate that the majority of TADC found within the tumor microenvironment have an immature phenotype (iDC) [55–57]. The immature stage of TADC is responsible for the tolerogenic response of adaptive immunity against tumors and strongly contributes to tumor immune evasion [58].

2.2.2 Recruitment of Myeloid Cells in Tumors

TAMC derive from monocytes and granulocytes, extravasated from the circulation and infiltrating the tumor mass. Recruitment of blood cells into tumors is mediated by chemoattractants released by tumor and stromal cells. CC chemokine 2 (CCL2), originally known as monocyte chemotactic protein 1 (MCP1), was the first relevant tumor-derived chemotactic factor described [59, 60]. Several other chemokines attracting myeloid cells have been identified, including CCL5, CCL7, CCL8, and CXC chemokine 1 (CXCL1) and CXCL12 [61–63]. Furthermore, urokinase plasminogen activator (uPA); growth factors such as colony-stimulating factor (CSF)-1, transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF, also known as FGF-2), and vascular endothelial growth factor (VEGF); and antimicrobial peptides (e.g., human beta-defensin-3) were shown to be involved in myeloid recruitment into neoplastic tissues [64, 9, 65–67].

The prototypic chemoattractant for neutrophils, CXCL8, is mainly responsible for the recruitment of TAN; other related chemokines of the CXC subfamily are also involved, including CXCL1, CXCL2, and CXCL6 [68, 69]. Moreover, tumor-derived TGF-β can promote neutrophil migration [70].

CC chemokine receptor 2 (CCR2), CCL2 receptor, CXCL12, CXCL5, and stem cell factor (SCF, also known as KIT ligand) play a pivotal role in the recruitment of MDSC into tumors [71–73]; in addition, Bv8, also known as prokineticine 2 (PROK2), might be essential for MDSC recruitment [74, 75]. Finally, the proinflammatory proteins S-100A9 and S-100A8, produced by MDSC, are implicated in an autocrine loop promoting accumulation of suppressor cells into tumors [76, 77].

TEM do not express CCR2 and are therefore recruited towards tumors by different mechanisms

[35, 78, 79]. Other CC chemokines, such as CCL3, CCL5, and CCL8, are produced by tumor cells and could play a role in TEM recruitment [80]. *Ang-2*, overexpressed by tumor cells and inflamed tissues, has been shown to exert a chemotactic effect on Tie2-expressing blood monocytes *in vitro*, suggesting that the Ang-2/Tie2 axis might be involved in recruiting TEM into tumors [81, 32, 35, 34, 82]. In addition, recent data suggest the involvement of the CXCL12-CXCR4 homing axis for TEM infiltration [82].

In recent years, it has been shown that tumor-derived factors such as VEGF, CXCL12, CXCL8, β-defensins, and hepatocyte growth factor (HGF) are secreted into the bloodstream and are believed to attract iDC into the tumor bed [83–86]. Moreover, CCL20, CCL7, as well as the receptors CCR5 and CCR6 were demonstrated to be important for TADC recruitment towards the tumor [87].

Proliferation can also contribute to sustaining TAMC levels in solid tumors. A paracrine loop has been evidenced for TAM, with production of colony-stimulating factor 1 (CSF-1) by murine fibrosarcoma cells acting on TAM-expressing CSF-1 receptor (CSF-1R) [88]. A finding confirmed more recently by Condeelis and Pollard [89] showed the effect of epidermal growth factor (EGF) produced by TAM and tumor-derived CSF-1 on recruitment and survival of macrophages during tumor growth. Indeed, macrophage proliferation has been demonstrated to occur during type II inflammation [90].

2.2.3 Tumor-Derived Factors Affecting Myeloid Differentiation and Polarized Functions

Upon arrival in the tumor, monocytes differentiate to macrophages primarily in response to CSF-1 produced by tumor cells. Although coexistence of diverse TAM subpopulations with distinct functions depending on tumor stage and geographical localization within the same tumor has been proposed, they mostly have an M2-like phenotype [91]. Many different studies demonstrated that M2 (pro-tumoral) TAM polarization is driven by cytokines and other signals released in the tumor microenvironment [92]. Among these IL-10, IL-6, CCL2, CSF-1, and prostaglandin E2 (PGE2) were reported to promote M2-like polarization [93, 94]. TGF-β is overexpressed by tumor cells and plays a crucial role in promoting an immunosuppressive phenotype, in addition to driving N2 polarization of TAN [31].

Many tumor-derived factors were implicated in MDSC expansion such as GM-CSF, M-CSF, IL-6, IL-1β, VEGF, and PGE2 [44, 95]. In addition, Bronte and coworkers recently found that cytokine-mediated induction of MDSC was completely dependent on the transcription factor CCAT/enhancer-binding protein b (C/EBPb), shown to function as a master regulator in this process [96]. Further it was proposed that a combination of at least two signals is necessary for MDSC functionality and expansion, for example, GM-CSF, inhibiting maturation of myeloid cells, and a proinflammatory molecule such as interferon-γ (INF-γ) [41].

Soluble factors released by tumor cells (i.e., IL-10, VEGF, TGF-β, etc.) contribute to keep DC in an immature pro-tumorigenic phenotype. Furthermore, in preclinical studies of breast cancer, it was shown that tumor-derived factors altered DC maturation by secretion of thymic stromal lymphopoietin (TSLP), which in turn induces the expression and secretion of the OX40 ligand, a molecule that contributes to sustain the M2-like phenotype of TAM.

2.3 Pro-tumoral Functions of Tumor-Associated Myeloid Cells

Myeloid cells exposed to the tumor microenvironment most frequently promote tumor progression. They can secrete soluble factors which support proliferation and invasion of tumor cells, activate angiogenesis, and promote resistance to therapies (Fig. 2.2). High TAM or TAN infiltration generally correlates with poor patient outcome [97, 6, 16, 11, 98–101], but few exceptions to this finding are also reported. For instance, in colorectal cancer (CRC) contrasting results reported that TAM

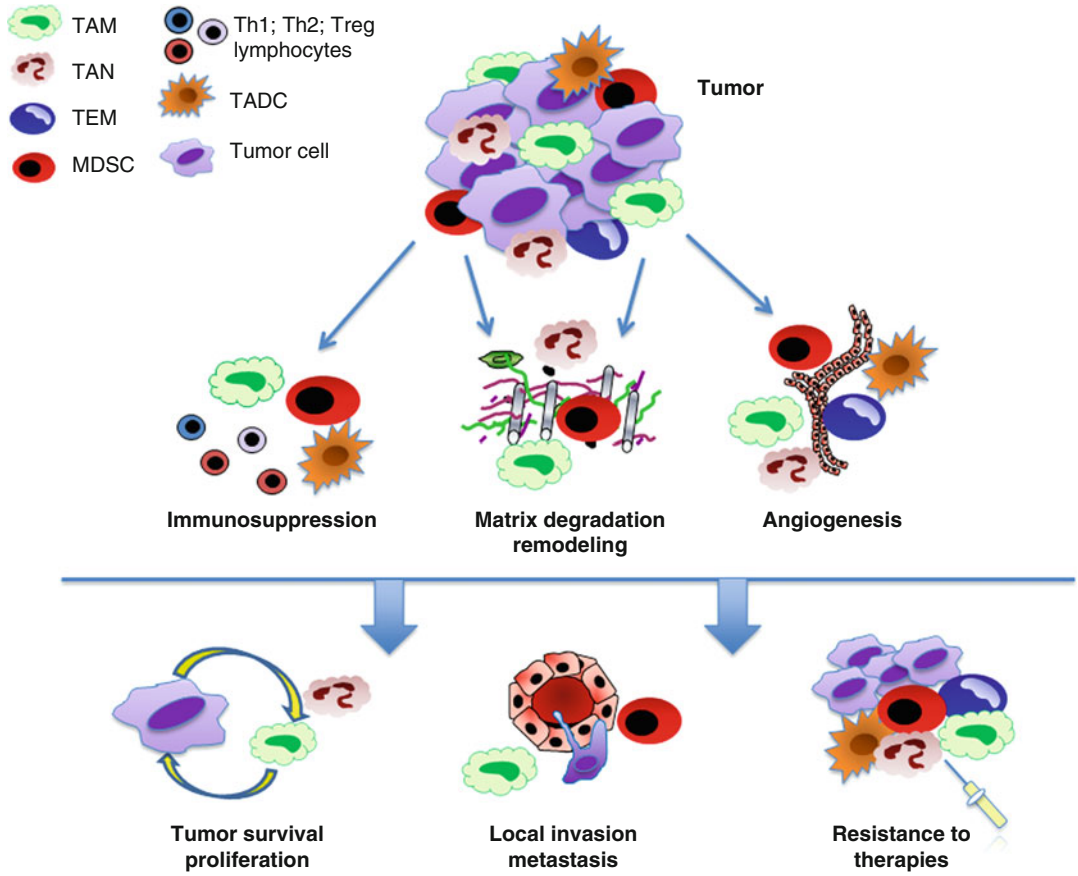

Fig. 2.2 Pro-tumoral functions of tumor-associated myeloid cells. TAMC exposed to the tumor microenvironment exert several pro-tumoral functions, including promotion of angiogenesis, matrix degradation, and suppression of adaptive immunity. These effects are mediated through the release of soluble factors (i.e., cytokines, growth and proangiogenic factors, proteolytic enzymes, etc.) and result in higher tumor survival and proliferation, local invasion and dissemination, resistance to therapies

density is associated with positive or negative patient outcome [102, 103–105]. On the same line, TAN infiltrate is associated with a favorable prognosis in patients with gastric carcinomas [106], but also with more aggressive pancreatic tumors [107]. Macrophage subsets might have distinct roles, as observed in lung adenocarcinoma were the number of $CD204^+$ TAM showed a strong association with poor patient outcome, while the $CD68^+$ TAM population did not [108]. The concept that not only the number and the presence of specific cell subsets but also the localization of infiltrating cells might have specific functions and predictive values is increasingly emerging. Accordingly, peritumoral TAM density with high expression of co-stimulatory molecules (CD80 and CD86) was associated with better patient survival in CRC, whereas the same cell population within the tumors did not have any predictive value [109, 110]. Thus, TAMC exert complex roles on growing tumors affecting different aspects of tumor progression, i.e. tumor cell proliferation and survival, angiogenesis, tumor dissemination, and resistance to therapies.

2.3.1 Tumor Proliferation and Survival

TAM were shown to have the ability to promote tumor growth directly through the production of trophic and activating factors for stromal and

cancer cells (EGF, bFGF, VEGF, platelet-derived growth factor β [PDGF], TGF-β) [111, 112, 6, 113] in response to stimuli from the tumor microenvironment. For example, IL-13 and IL-4 produced by $CD4^+$ T-cell-infiltrating tumors, such as breast cancer, led to the production and secretion of EGF by TAM [114]. Moreover, production of proinflammatory cytokines, including TNF-α and IL-6, by TAM and other cells of the tumor microenvironment (e.g., epithelial cells), sustains tumor growth and inhibits apoptosis [115–119].

Several lines of evidence suggest that TAN are required for the rapid growth of tumor cells and their depletion inhibits tumor development [120, 28]. Proteins stored within neutrophil granules (e.g., elastase) may have a role in tumor initiation [121]. In addition, neutrophil-derived ROS have been associated with DNA damage [122]. TAN were shown to be able to produce soluble factors (cytokines and chemokines, HGF, oncostatin M), driving processes like angiogenesis, wound healing, and hematopoiesis and thus exerting a role in tumor promotion and growth [123–125, 121, 101]. For instance, HGF released by neutrophils enhances the invasiveness of human cholangiocellular and hepatocellular carcinoma cells *in vitro*, and HGF levels in bronchoalveolar lavage fluids were found to correlate with neutrophil number in patients with bronchoalveolar carcinomas, which further correlates with poor patient prognosis [101].

2.3.2 Angiogenesis

To sustain the increased metabolic demand of growing tumors, the development of a tumor vasculature is required. VEGF is the primary, but not the only, angiogenic factor released by tumor cells and is involved in the "angiogenic switch" that can occur at various stages of tumor progression, depending on the tumor type and the microenvironment. Other factors are involved, including PDGF-β, bFGF, angiopoietins, and CXCL12 (SDF-1) [126]. Tumor-associated myeloid cells were shown to contribute to tumor angiogenesis by production of growth factors, cytokines, and proteases [80] such as VEGFA, Bv8, and metalloproteases (MMP) [10, 127, 65, 128].

The prototypic myeloid cell with angiogenic properties is the Tie2 monocyte [32, 35]. TEM can be found in close proximity to nascent blood vessels within solid tumors. In addition, TEM depletion completely prevented neovascularization in preclinical models (spontaneous pancreatic adenocarcinoma, human glioma grown orthotopically in the mouse) [33]. Interestingly, TEM ablation did not affect the number of infiltrating TAM or TAN, suggesting that TEM are an entity on their own and not just precursors of TAM [35]. How TEM stimulate angiogenesis has not been clarified yet, but preliminary indications in murine tumor models point to the fact that perivascular TEM secrete bFGF. It is believed that release of such factors in close proximity to vessels could directly stimulate angiogenesis or MMP9 secretion, which in turn would release growth factors entrapped within the extracellular matrix (ECM).

TAM have also a profound influence on the regulation of tumor angiogenesis [129]. It was demonstrated in several preclinical studies that TAM positively correlated with microvascular density (MVD) [130–133]. Lin and coworkers were the first to describe the direct role of TAM in driving the "angiogenic switch" in a spontaneous mammary carcinoma mouse model [134]. Likewise, depletion of monocytes by clodronate treatment in a preclinical model with Lewis lung carcinoma led to lower TAM infiltration and angiogenesis, further underlining the importance and the involvement of macrophages in tumor angiogenesis [135].

TAM express various molecules modulating angiogenesis, such as VEGF, bFGF, TNF-α, IL-1β, CXCL8, cyclooxygenase 2 (COX2, also known as PTGS2), plasminogen activator, uPA, PDGF-β, MMP7, MMP9, and MMP12 [136]. Hypoxia exerts a crucial role in the upregulation of gene transcription in TAM, promoting VEGF expression [137–141]. Other recent studies showed a direct involvement of TAM in tumor angiogenesis and neovascularization via transdifferentiation into endothelial cells when stimulated by angiogenic factors [142, 143].

More recent studies have shown that MDSC can contribute to tumor angiogenesis. In a preclinical model for colon cancer, MDSC positively

correlated with tumor growth rate and blood vessel density [144]. Moreover, tumor angiogenesis was significantly lowered by blocking Bv8 with a neutralizing antibody, a treatment that significantly reduced the number of MDSC [74]. Metalloproteases, particularly MMP9, MMP2, MMP13, and MMP14, produced by MDSC, were shown to enhance VEGF bioavailability by mobilization from the ECM [144, 145]. Increased recruitment of MDSC has also been demonstrated in the presence of hypoxia, possibly stimulating tumor angiogenesis [126, 74]. Parallel to TEM, MDSC were also observed to be localized in the vicinity of blood vessels. Under certain conditions, some MDSC acquire endothelial cell shape, start to express endothelial markers including CD31 and VEGFR2, and are eventually incorporated into the tumor endothelium [144].

TAN were shown to rapidly release VEGF from internal storage compartments, leading to endothelial proliferation and tubule formation [146, 147]. In addition, TNF-α and GM-CSF secreted by tumor cells were shown to trigger the release of proangiogenic chemokines by TAN. The number of TAN in myxofibrosarcoma positively correlated with tumor MVD [148]. Furthermore, in a xenograft mouse model of human melanoma where cancer cells were engineered to constitutively produce CXCL6, it was found that the number of TAN as well as angiogenesis was markedly increased [149]. Studies in the RIP1-TAG2 mouse model for pancreatic carcinogenesis revealed formation of dysplastic, neutrophil-bearing, angiogenic islets upon malignant transformation. In the abovementioned model, neutrophil depletion of the islets led to dramatically lowered angiogenesis [150].

In recent years, it has become more and more apparent that iDC make a profound contribution to tumor angiogenesis [85]. TNF-α and CXCL8 produced by iDC from ovarian cancer ascites triggered the release of various growth factors from EC [85, 151]. Moreover, iDC were shown to release osteopontin which promotes monocyte secretion of the proangiogenic IL-1β [152]. Finally, it was recently observed that iDC produced high levels of VEGF and CXCL8 under hypoxic conditions, which, in turn, might inhibit DC maturation and further promote angiogenesis via this autocrine loop [153, 151].

2.3.3 Cancer Cell Dissemination

The major cause of death in cancer results from therapy-resistant metastases. Stephen Paget's conclusion in the late nineteenth century that the metastatic process depends on cross talk between selected cancer cells (the "seeds") and a specific organ microenvironment ("the soil") is still valid and is experimentally confirmed [154, 155]. Tumor metastasis is a complex multistep process, during which malignant cells spread from the primary tumor site to secondary distant organs. The different steps of cancer cell dissemination can be subdivided into local invasion, entry into the bloodstream (intravasation), survival in the bloodstream, extravasation, and colonization [156]. Mesenchymal, endothelial, and immune cells are required to form an appropriate microenvironment for tumor progression [157]. Immune cells, particularly macrophages, neutrophils, T lymphocytes, and natural killer (NK) cells, are major sources of proteases that degrade the host tissue, allowing cancer cells to disseminate.

The set of proteolytic enzymes found in tumor microenvironment comprises matrix metalloproteases, serine proteases, and cysteine proteases (i.e., cathepsin) [158–162]. Matrix proteases exert essential functions in physiological conditions as active regulators of postnatal tissue development and remodeling. In addition, they are important for tissue repair in response to injury and regulate cancer progression modulating the tumor microenvironment, particularly the leukocyte infiltrate [163]. MMP were shown to activate TGF-β, which is an important regulator of T-cell and TAN functions [164]. Proteases also produce specific cleavage fragments of target chemokines with independent biological activity, ranging from anergic products (CXCL7, CXCL4, CXCL1), antagonists (CCL7), or more potent chemoattractants (CXCL8), thereby modulating the leukocyte composition within a tumor [165–167].

Besides their influence on the tumor infiltrate, proteases were shown to promote cancer cell

invasion and intravasation. The cleavage of cell-adhesion molecules like E-cadherin induces the disruption of cell-cell junctions leading to loosening of cell-cell contacts which, together with ECM protein turnover, facilitated cancer cell migration and invasion into the surrounding tissue and vasculature. Tight regulation of the single proteases within the tumor microenvironment allows the control of tumor cell invasion [168].

After invasion to the surrounding tissues, cancer cells enter the blood circulatory system directly or indirectly via the lymphatic system. Since the majority of circulating tumor cells (CTC) are eliminated by NK cells [169], only about 0.01 % of CTC survive in the bloodstream [157]. Platelets play a key role in hematogenous metastasis and contribute to the survival of CTC in the bloodstream by both thrombin-dependent and thrombin-independent mechanisms [170]. After a passage into the bloodstream, CTC adhere to vessel walls for extravasation when they are in the vicinity of secondary metastatic organs. Circulating tumor cells take advantage of the capability of neutrophils and platelets to produce and secrete adhesion molecules, such as integrins and selectins which all aid the nearby CTC to adhere and ultimately extravasate [170, 171].

The arrest of cancer cells to specific organs seems to be primarily "mechanical" [172]. However, chemokines and chemokine receptors are also involved in organ-specific colonization, which finally drive cells along tissue-specific chemokine gradients. Furthermore, a non-chemokine pathway also exists, in which immune cells support organ-specific cancer cell dissemination. One example is represented by the two inflammatory mediators S100-A8 and S100-A9, which were shown to promote metastasis through serum amyloid A 3 (SAA-3) [173].

The subsequent growth of arrested tumor cells will depend on the molecular interactions between cancer cells and the microenvironment of the new organ. Although cancer cells are sometimes said to "home" to specific organs (e.g., breast tumors metastasizing to bone), it is more likely that this organ specificity is due to efficient organ-specific growth rather than preferential "homing" of cells to a particular organ.

It has been suggested that tumor cells can influence the microenvironment of secondary organs promoting the formation of a pre-metastatic niche [174, 175]. Tumor-derived factors and HSC are crucial components of the pre-metastatic niche. VEGF derived from tumor cells promote recruitment to the secondary organs of VEGFR1-expressing HSC that induce fibronectin and MMP9 expression by resident fibroblasts, creating favorable conditions for settlement of future metastases [176]. Other soluble factors released by tumor cells can promote the formation of pre-metastatic niche. In a murine model of breast cancer, tumor cells were found to induce production of CCL17 and CCL22 in the lung; both attracting $CCR4^+$ tumor and immune cells which establish a microenvironment for metastases settlement at secondary organs [177]. Moreover, it was demonstrated that the prototypic hypoxia-induced protein lysyl oxidase (LOX), often found in tumors, leads to cross-linking of collagen IV in basement membranes, in addition to recruitment of $CD11b^+$ myeloid cells which adhere to the abovementioned collagen meshwork. The captured $CD11b^+$ myeloid cells were shown to secrete MMP2, which facilitated invasion and recruitment of metastasizing tumor cells [178].

TAMC, TAM and MDSC in particular, are important players of tumor progression and metastatic colonization through the cross talk with tumor cells. For instance, macrophages play a crucial role in conferring an invasive phenotype to epidermal keratinocytes from Snail transgenic mice [179]. TAM contribute to cancer cell dissemination by releasing enzymes involved in degradation of the ECM (i.e., MMP and cathepsin) [168, 161, 180, 76], or motility factors. Recently we found that tumor-derived soluble factors, particularly CSF-1, activate a transcription program in macrophages resulting in upregulation of a series of genes, especially *migration-stimulating factor (MSF)*. MSF is a truncated isoform of human fibronectin 1, physiologically expressed during fetal life and upregulated in M2-like macrophages [181, 182]. MSF exerts a chemotactic effect on tumor cells, indicating that macrophage products released in the

tumor microenvironment can support the pro-invasive phenotype of tumor cells [181]. An example of the cross talk between TAM and tumor cells involved in metastatic colonization is shown in breast cancer, where EGF secreted by TAM increases migration and invasion of neighboring breast cancer cells which express high levels of EGF receptor (EGFR). On the other hand, cancer cells secrete high levels of CSF-1, a main chemoattractant for TAM which expresses the cognate receptor CSF-R1. Therapies aiming at inhibiting this cross talk by blocking CSF-R1 and/or EGFR were shown to be successful [183, 184]. Macrophages and their reciprocal cross talk with tumor cells are mandatory for tumor cell migration, regardless of the factor inducing cell invasion (i.e., SDF-1).

A myeloid cell population involved in tumor progression, including invasion, is represented by MDSC. A direct role for MDSC in tumor metastasis has not been demonstrated; however, a connection was suggested by the study on mice deficient for the TGF-β receptor type 2 (TGF-β-R2), in which MDSC were concentrated on the invasive margin. In addition, it is possible to reduce lung metastases by antagonizing CXCR2 and CXCR4, two receptors involved in homing of MDSC [145]. As previously mentioned, PGE2 and the proinflammatory molecule S100A9 have been identified as main effectors of MDSC accumulation and function. Accordingly, S100A9 deficient mice rejected implantation of colorectal cancer, while administration of wild-type MDSC reverted the phenotype and colorectal cancer cells could successfully engraft [76]. In addition, TGF-β was demonstrated to be instrumental in MDSC homing, mediated via CXCL12-CXCR4 and CXCL5-CXCR2 axis in a preclinical mammary cancer model [145].

2.3.4 Suppression of Adaptive Immunity

Besides the effect on tumor growth and dissemination, TAMC have also the potential to suppress the adaptive immune response, leading to cancer immune evasion [185].

M2-like polarized tumor-infiltrating macrophages are characterized by an immunosuppressive phenotype, with production of high levels of the immunosuppressive cytokines IL-10 and TGF-β and reduced expressions of IL-12 [19, 186, 92, 187, 188]. In addition, they have reduced tumoricidal activity and are poor in antigen presentation [189]. Furthermore, TAM secret chemokines, such as CCL17 or CCL22, that preferentially attract Th1, Th2, and T regulatory (Treg) lymphocytes with defective cytotoxic functions, or such as CCL18, that recruit naïve T cells which become anergic in contact with M2 macrophages and iDC [8, 190–192].

MDSC play a prominent role in the inhibition of tumor-specific immune responses. MDSC localized within the tumor microenvironment has an M2-like phenotype and mediate immunosuppression through multiple pathways, that is, production of Arg-1 [193], iNOS [194, 195], ROI, and suppressive cytokines including IL-10 and TGF-β [196], or via the activation and recruitment of Treg [196, 197]. MDSC inhibit homing to lymph nodes of $CD4^+$ and $CD8^+$ T cells and suppress their activation [198, 199]. It was found that cysteine uptake by MDSC limited its availability for uptake by T cells, which in turn disables their activation and renders them nonfunctional. Furthermore, it was shown that posttranslational T-cell receptor modifications mediated via generation of peroxynitrite species led to anergy of effector $CD8^+$ T cells [196]. MDSC can also impair innate immunity through cross talk with macrophages which led to decreased production of IL-12 by macrophages and increased production of IL-10 by MDSC, thus driving a polarization towards an M2-like phenotype [200].

In addition to the above described mechanisms in TAM and MDSC, TADC were found to be involved in suppression of adaptive immunity. One mechanism leading to the induction of tumor-specific T-cell tolerance was via upregulation of inhibitory molecules such as B7-H1 [201] or by inducing the expression of Arg-1 [202]. Moreover, it was shown that the induction of oxygen-dependent pathways led to the downregulation of CD3 epsilon and T-cell apoptosis [203]. Furthermore, Muller and coworkers demonstrated that upregulation of indoleamine 2,3-dioxygenase (IDO) in TADC contributed to immunosuppression [204].

2.4 Selected Aspects of Therapeutic Targeting of TAMC

The above summarized data describing the pro-tumoral role of the myeloid infiltrate of tumors make clear that TAMC are reasonable targets for novel therapeutic approaches. As illustrated above, TAMC can directly promote tumor cell growth releasing growth factors and proangiogenic molecules, in addition to suppression of tumor-specific immune responses. Strategies explored in the last years are focused on the stoppage of the mechanisms leading to suppression of lymphocyte activity and, on the other side, on the reduction of recruitment of myeloid cells and repolarization of M2-like pro-tumoral cells to proinflammatory M1 macrophages. There is a wide range of preclinical and clinical research aimed at eliminating or reprogramming TAMC [39]: here we only mention some examples of the results obtained so far in this growing field of anticancer research.

Many studies have shown that targeting TAM might be a successful strategy to limit tumor growth and metastasization and to achieve better therapeutic responses [32, 44, 59, 82, 189, 205, 206, 207]. One example is represented by bisphosphonates [208] traditionally used in the clinic to treat osteoporosis, which were shown to be very effective in depleting TAM and inhibiting angiogenesis as well as metastatic spread in preclinical animal models for breast cancer [209, 210]. Furthermore, Germano and coworkers recently showed that specific targeting of macrophages with the marine antitumor agent trabectedin was very successful in four different preclinical tumor animal models [211].

An alternative strategy is to target circulating monocytes known as precursors of TAM. Two candidate molecules are the M-CSF receptor (solely expressed by monocyte-macrophages) and the chemokine CCL2, involved in monocyte recruitment within tumors. Since preclinical studies on prostate and colon cancer [212–215] identified $CCR2^{+}Ly6C^{+}$ cells as targets involved in cancer progression and metastasis, CCL2 antibodies are currently investigated for therapeutic applications in human cancer treatment. Another approach to affect TAM specifically is to try to reeducate them to become tumoricidal or, in terms of polarization, to try to repolarize them towards an M1 phenotype. Several successful trials using CpG-oligodeoxynucleotide (TLR9 agonists) were performed in combination with anti-IL-10 receptor or anti-CD40 antibodies, which reverted pro-tumoral M2-like TAM to M1 macrophages displaying antitumor activity [216–218]. Rolny et al. recently demonstrated that skewing of M2 TAM towards M1 leads to effective antitumoral activity of host histidine-rich glycoprotein (HRG), which in consequence leads to inhibition of angiogenesis and promoted antitumor immune responses [219]. Gazzaniga and coworkers reported promising results using the molecule legumain, which targets M2 polarized TAM specifically, and was able to induce a robust $CD8^{+}$ T-cell answer leading to reduced tumor growth and inhibition of tumor angiogenesis [220]. Furthermore, it was shown that zoledronic acid was able to revert M2 towards M1 TAM and inhibit breast carcinogenesis by targeting the mevalonate pathway [221]. Moreover, it was demonstrated that direct reeducation of TAM using the prototypical M1 polarizing cytokine INF-γ [222] is successful in promoting antitumor activity in minimal residual disease [8]. In line with the abovementioned results are the findings that inhibition of M2 polarization led to restoration of M1 proinflammatory phenotype and inhibition of tumor growth in several preclinical animal models [92, 223, 224].

To counteract the pro-tumoral activities of MDSC, two general strategies can be envisaged; the first consists of transforming these immature cells into mature cells devoid of suppressive activity, and the second is focused on blocking MDSC suppressive functions. Depletion of MDSC producing high levels of TGF-β (in an IL-13-dependent manner) led to the restoration of T-cell-mediated immunosurveillance in a preclinical mouse model for fibrosarcoma [225]. Several studies have shown that metabolites of all-trans-retinoic acid are able to differentiate MDSC into DC and macrophages, reducing MDSC accumulation [226, 227]. This effect was demonstrated to be beneficial for patients suffering from metastasizing renal cancer, since in

these patient less circulating MDSC were detected in the bloodstream [228]. Furthermore, one of the beneficial effects of the anticancer drug gemcitabine is its potential to eliminate MDSC without affecting T, B, NK cells, or macrophages [229].

The second possibility to counteract MDSC function is to block their inhibitory function, for example, by using COX2 inhibitors, phosphodiesterase (PDE5), and nonsteroidal anti-inflammatory drugs releasing NO [44]. Blocking of IL-1β inhibits cancer progression and metastasis [230] and decreases MDSC accumulation and suppressive activity [42]. Moreover, the proangiogenic chemokine Bv8 was shown to be important for mobilization and homing of MDSC to tumor sites and therefore qualifies as an interesting therapeutic target [74].

Complete neutrophil depletion in already immunocompromised patients is not desirable; therefore, the strategy of choice concerning TAN might be to disturb their tumor homing ability, in other words to interfere with their ability to migrate. To this purpose, preclinical experiments using anti-CXCR2 antibodies were performed and were shown to be successful [231]. Furthermore, considering the well-documented key role of TGF-β in skewing TAN towards a N2 phenotype, this cytokine keeps promising potential for treatment [70, 31].

Some studies indicate that blocking IL-10 together with the administration of CpG oligonucleotides are able to unblock the functionally paralyzed TADCs and to reactivate antitumor responses [232]. Another strategy enhancing immunotherapy might be targeting of soluble factors like VEGF, IL-10, TGF-β, gangliosides, and others, which are all tumor secreted factors leading to abnormal differentiation of DC, often leaving them in an immature state [233]. Other and more recent strategies make use of siRNA nano-complexes which lead to reprogramming of TADC from an immunosuppressive to an activated anticancer phenotype [234]. Furthermore, it was shown that in situ stimulated CD40 and toll-like receptor 3 (TLR3) TADC were successfully transformed from immunosuppressive to immunostimulatory cells [235]. More recently it was demonstrated that delivery of regulatory miRNA, particularly miRNA 155 in a nanoparticle formulation, leads to reprogramming of immunosuppressive TADC to highly active antitumoral TADC which provoked regression of established ovarian tumors [236].

In light of the recent results, tumor therapy with drugs targeting the inflammatory tumor microenvironment in combination with treatment aimed at defeating TAM, TAN, and other myeloid cells holds promise for the future.

2.5 Concluding Remarks

In recent years, it has become clear that inflammation has an essential role in tumor promotion [1–6]. The inflammatory tumor microenvironment, mainly consisting of soluble factors and host cells, has a predominant role in all aspects of the disease (progression, angiogenesis, immune surveillance). In particular, a heterogeneous group of myeloid cells is the most consistent host cell component of solid tumor [8, 9]. TAM, TEM, MDSC, TAN, and TADC display distinct specialized functions, as well as overlapping activities (e.g. angiogenesis). Tumor and stromal cells release different chemoattractants involved in the recruitment of myeloid cells from the blood into the growing tumor. Cytokines and other soluble factors released in the tumor microenvironment can contribute to induce a protumoral phenotype, promoting M2 polarization of TAM [92], N2 polarization of TAN [31], MDSC expansion [41], or preventing maturation of DCs. Thus the different TAMC populations potentially represent a target for new therapeutic approaches aimed at breaking the protumoral networks established by cancer-associated myeloid cells.

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Role of Innate Immunity in Cancers and Antitumor Response

3

Masahisa Jinushi and Muhammad Baghdadi

Contents

3.1 Introduction

Cellular components of the innate immune system serve as a "first line of defense" against tumorigenic cells. Recognition of transformed cells by pattern-recognition receptor (PRRs) on the innate immune cells activates specialized inflammatory signaling cascades, including transcription factor nuclear factor-kappa B (NF-κB) and interferon regulatory transcription factor (IRF), which lead to the release of various cytokines and chemokines attracting and activating effector lymphocytes at the tumor site. In addition, effector cells kill transformed cells through the activation of perforin or death receptor-mediated pathways, as well as secretion of cytokines necessary for the initiation of immune responses against transformed cells [1, 2]. However, some tumor cells escape from the innate immune machinery, which leads to the dysfunction of innate immune compartment, signaling pathways, and effector functions. This manipulation of innate immune systems by tumor microenvironments includes

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impairment of antigen processing and presentation by antigen-presenting cells (APCs) [3], inhibition of innate immune signaling pathways [4, 5], and anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) [6, 7]. Moreover, tumors manipulate innate immune systems to create protumorigenic environments, which lead to further tumor progression and metastasis. Therefore, it is critical to clarify the molecular mechanisms through which the interaction between tumors and innate immune systems is modified during different phases of tumorigenesis.

In this chapter, we describe the general functions of innate immunity in cancer and antitumor host response. In addition, an overview is provided on the mechanism through which coordinated actions of innate immune signals and their downstream effectors have an impact on the immunosurveillance and immune subversion within the tumor microenvironment.

3.2 Role of Innate Immune Cells in Cancer and Antitumor Immunity

3.2.1 Natural Killer (NK) Cells

NK cells are important effector cells for protection against viruses and some tumors, since NK cell-depleted mice were more susceptible to 3-methylcholanthrene (MCA)-induced tumors [8]. Chemokines such as CXCL12 and CXCL3L1 are key factors for NK migration to tumor sites [9], where they play an important role in the tumor immunosurveillance [10]. NK cells recognize and eliminate transformed cells by releasing perforin or death signal-associated receptors such as FAS and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) [11–13]. NK cells secrete interferon gamma (IFN-γ) which helps to activate T-cell-mediated immunity and suppress tumor angiogenesis [14, 15]. Moreover, various innate immune networks such as cytokines and PRR recognition systems play an important role in stimulating effector functions of NK cells as discussed later.

NK cells have the ability to distinguish transformed cells from normal cells by recognizing a variety of cell surface receptors, including killer activation receptors (KARs), killer inhibitory receptors (KIRs), natural killer group two member D (NKG2D), DNAX-accessory molecule (DNAM), etc., which will be discussed later in this chapter. For example, KIRs on NK cells has a high affinity to the specific alleles in HLA class I molecules, transducing an inhibitory signal to the NK cells and preventing it from eliminating non-transformed cells. However, deletion of a single allele in HLA class I and/or induction of activating receptors such as NKG2D ligands, which frequently occurs on transformed cells, triggers effector functions of NK cells against tumor cells [10, 16]. Recent studies have focused on "licensing" NK cells to become functionally competent through the interaction with self-MHC molecules. Ly49C is an inhibitory receptor expressed on a subset of NK cells, which interact with self-MHC molecules on target cells, and plays an unexpected role in enabling immature NK cells to develop into functioning, mature cells. On the other hand, Ly49C-negative NK cells are considered as "non-licensed" and remain at an immature stage [17]. These evolutionary processes of NK cell development and activation may help explain why donor NK cells administrated to leukemia patients during bone marrow transplantation do not always show antitumor effects [18]. The NK cell-mediated cytotoxic activities mediate the release of granule contents (perforin and granzyme) onto the surface of the tumor cell [19].

The interaction between NK cells and dendritic cells (DCs) is crucial for the amplification of innate responses and the induction of potent adaptive immunity. Immature DCs are susceptible to NK cell-mediated cytolysis [20], while mature DCs are activated by NK cells through cytokines (TNF-α and IFN-γ) and receptor (NKp30 and NKG2D)-mediated mechanisms [21, 22]. On the other hand, activated DCs trigger effector activities of NK cells, such as IFN-γ production, proliferation, and cytotoxic activities [23]. In addition, treatment with TLR3 agonist polyinosinic-polycytidylic acid (Poly (I: C)) triggers DCs to activate antitumor activities of NK cells [24, 25]. Thus, the reciprocal interaction between NK and DC

regulates the direction and quality of antitumor immunity, which is important for the development of effective cancer immunotherapy.

3.2.2 Natural Killer T (NKT) Cells

NKT cells are innate lymphocytes which share features of both NK cells and T cells. NKT cells express particular NK cell markers such as CD161 or NKR-P1, in addition to an invariant T-cell receptor alpha chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) [26]. The invariant T-cell receptor alpha chain is specific for glycolipid antigens presented by CD1d, which is an MHC class I-related molecule expressed on antigen-presenting cells and also found in some tumor cells. NKT cells were shown to play a role in the tumor immunosurveillance, since *Jα18*$^{-/-}$ mice showed increased susceptibility to chemically induced tumors and experimentally induced metastases [27]. Moreover, the administration of α-galactosylceramide, a natural lipid isolated from marine sponges which efficiently binds to CD1d and thus activates NKT cells, induces antitumor immune responses against established murine tumors [28]. The antitumor activities of NKT cells are mediated by IFN-γ production, which also activates NK and CD8^{+} T cells. NKT cell activities are also important for granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-12-based cytokine strategies [29, 30]. Recent reports have identified subpopulations of NKT cells which secrete TH1 or TH2 cytokines and thus play different roles in the pathogenesis of many diseases. For example, CD4^{-} NKT cells serve as potent effectors for triggering tumor rejection in various murine tumor models, while CD4^{+} NKT cells contribute to the pathogenesis of allergic diseases and tumors by promoting the release of IL-4, IL-5, and IL-13 [31, 32]. Indeed, IL-13 released from NKT cells antagonizes tumor immunosurveillance by promoting TGF-β secretion from Gr-1^{+} myeloid suppressor cells [33, 34]. Thus, the identification of factors influencing the differentiation of specific NKT cell subsets during tumor development is important in order to optimize the therapeutic interventions which utilize NKT cell functions against tumors.

3.2.3 γδ-T Cells

Although γδ-T cells represent a small population among T lymphocytes, they share several features with innate immune cells. γδ-T cells show high frequencies in intraepithelial lymphocytes (IELs) in the skin and gut mucosa and possess a distinct T-cell receptor on their surface with limited diversity, which may serve as a pattern-recognition receptor [35]. Moreover, γδ-T cells lack CD4 and CD8 expressed by αβ-T cells and express a number of molecules shared with NK cells or APCs, such as Fc gamma RIII/CD16 and PRRs. γδ-T cells also recognize lipid-derived antigens and function as professional phagocytes which recognize and ingest apoptotic tumor cells and may influence antitumor immune responses [36, 37].

Mice lacking γδ-T cells showed increased incidence of chemically induced sarcoma and spindle cell carcinoma, indicating the importance of these cells in tumor immunosurveillance [38]. In addition, γδ-T cells express NKG2D receptors and interact with their ligands on transformed cells, leading to enhanced cytotoxic activities and effector cytokine production [39, 40]. The activated γδ-T cells then serve as the major early source of IFN-γ, which contribute to maturation of APCs and prime αβ-T cells, and mediate cytotoxicity against tumor cells [40, 41].

3.2.4 Macrophages

Macrophages serve as a first line of defense against tumorigenesis by directly killing tumor cells and producing various antitumor mediators [42]. On the other hand, macrophages render tumor cells with the ability to acquire invasive and metastatic activities [43]. Macrophages are differentiated from immature myeloid precursors or circulating monocytes released from the bone marrow [44]. In particular, the inflammatory monocytes expressing *Ly6C are* preferentially attracted from the circulation into the tumor site by tumor-derived chemokines, such as CCL2 (MCP1-1) and CCL5 (RANTES) and CXCL12 (SDF1) [45–47]. Immature monocytes are then differentiated into either M1 or M2 macrophages

by distinct sets of cytokines when entered into distinct tumor microenvironments [48]. M1 macrophages may induce antitumor response by producing IFN-γ and IL-12 and triggering cytotoxic activities [49, 50]. In contrast, tumor microenvironments adopt multiple strategies to tip a balance in the favor of differentiating M2-type macrophages through complex network of cytokines, chemokines, and growth factors [43, 51].

Taken together, macrophages have a dual role in modulating tumorigenesis and antitumor host responses. Thus, detailed characterization of molecular machineries which govern macrophage polarization in tumors seems necessary for a thorough understanding of pharmacological targeting of macrophages and their derivatives.

3.2.5 Dendritic Cells

DCs are professional APCs contributing to the induction of both innate and adaptive immune responses against pathogens as well as tumors. DCs express Toll-like receptors (TLRs) and co-stimulatory molecules necessary for the activation of various effectors [52]. Due to the potent immunogenicity of DC, tumor microenvironments adopt multiple tactics to subvert DC functions. In addition, tumor-infiltrating DCs can both induce tumor growth and metastasis by regulating angiogenesis, host immunity, and tumor metastasis [53–56]. Moreover, indoleamine 2, 3-dioxygenase (IDO)-producing DCs cause poor tumor immunogenicity via generating Foxp3-positive regulatory T cells [57] and interacting with other innate lymphocytes such as γδ-T cells [58] and NKT cells [59].

In summary, tumor-infiltrating DCs represent a double-edged sword which can induce an immune response against tumors or tolerize the immune system against tumors and contribute to tumor growth and metastasis. Thus, a deep understanding about DC biology at tumor microenvironment is critical to optimize anticancer therapies and improve the clinical output of DC vaccines.

3.2.6 Granulocytes

Granulocytes, the key mediators of inflammation, have a potential role in the initiation of immune response cascades against tumors [60]. Granulocytes induce tumor destruction through the release of cathepsin G, azurocidin, reactive oxygen species, and inflammatory cytokines. Moreover, granulocytes, along with macrophages and T cells, are main effectors that elicit antitumor responses by DNA vaccines in murine tumor models [61]. In addition, dense infiltration of granulocytes in tumor tissues is associated with clinical responses of GM-CSF-secreting cancer cells and Bacillus Calmette-Guérin (BCG) in patients with advanced melanoma and bladder carcinoma, respectively [62, 63]. On the other hand, granulocytes contribute to tumor angiogenesis and metastasis by promoting secretion of proteinases, ROS, and cytokines that may acts as antitumor effectors in different conditions [64]. Therefore, granulocytes have both pro- and antitumor activities depending on distinct environments.

3.3 The Role of Innate Immune Receptors on Innate Immune Cells in Cancer and Antitumor Immunity

3.3.1 Toll-Like Receptors (TLRs)

Toll-like receptors (TLRs) are innate immune receptors mainly expressed on APCs, such as macrophages and dendritic cells. They play an important role in host defense against pathogens by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs). The recognition of PAMPs and DAMPs by PRRs activates inflammatory pathways such as NF-κB and IRF-mediated signals, leading to antitumor mediators like type I interferons, as well as cell survival and proliferation [65].

Various sets of TLR ligands induce the upregulation of co-stimulatory molecules and proinflammatory cytokine production by APCs, thus breaking the tolerogenic status to various tumor antigens and inducing antigen-specific antitumor immune responses [66–68]. In addition, TLR4 on DCs could interact with high mobility group box 1 (HMGB1) and facilitate antigen cross-presentation to antitumor T lymphocytes [69]. Thus, TLRs agonists serve as effective adjuvants in harnessing potent antitumor immune response and clinical responses.

In contrast, tumor cells license TLRs on myeloid cells to acquire invasive and metastatic activities by promoting the secretion of various protumorigenic mediators, such as TNF-α and S100A8 [70, 71]. Thus, the careful optimization of suitable TLRs ligands for cancer immunotherapy is critical in order to avoid protumorigenic inflammation caused by the TLRs expressed on innate immune cells in tumor microenvironments.

3.3.2 RIG-I-Like Helicases (RLHs)

RIG-I-like helicases (RLHs) are specific families of pattern-recognition receptors bearing caspase-recruitment domain (CARD) at N-terminus and helicase domains, which are responsible for detecting intracellular double-strand RNA and inducing innate immune responses. RLHs include retinoic acid-inducible gene-I (RIG-I), myeloid differentiation antigen-5 (MDA5), and laboratory of genetics and physiology-2 (LGP2 or DHX58), which are expressed constitutively in both immune and nonimmune cells. RLHs recruit specific intracellular adaptors to initiate NF-κB- and IRF-mediated inflammatory signaling pathways that lead to the synthesis of type I interferons (IFNs) and other proinflammatory cytokines [72, 73]. The utilization of RLHs ligands as adjuvants to trigger antitumor immune responses has been validated by several studies. Its administration with retinoic acid-inducible gene-I (RIG-I) ligand triphosphate RNA triggers antitumor immune response by inducing the production of IFN-α/IFN-β and various immunogenic cytokines, as well as activating antitumor immune response cells [74, 75].

Taken together, RLHs ligands may be utilized as adjuvants with other immunotherapies in order to overcome immunosuppressive tumor microenvironments.

3.3.3 NOD-Like Receptors (NLRs)

NOD-like receptors (NLRs) are especially important for the recognition of sterile inflammation such as uric acids and silica [76, 77]. NLR-mediated innate immune systems play an important role in both antitumor immunity and tumorigenicity. For example, nucleotide-binding oligomerization domain-containing protein 1 (NOD1) has a protective role against tumors, and the knockdown of NOD1 promotes tumor growth in breast cancer model *in vivo* [78, 79]. NOD-like receptor family pyrin domain containing 3 (NLRP3) serves as a sensor for activating the inflammasome pathway which regulates pro-caspase-1 cleavage and subsequent IL-1β activation [80]. NLRP3 is a negative regulator of chemical colon carcinogenesis. In a dextran sulfate sodium (DSS) and azoxymethane-induced colon cancer model, *NLRP3*$^{-/-}$ mice showed increased colitis and colitis-associated cancer, which was correlated with attenuated levels of IL-1β and IL-18 at the tumor site [81]. However, in other models, NLRP3 may also have a role in the promotion of tumors as in inflammation-induced skin cancers through the enhancement of inflammatory environment [82], which suggest a dual role for NLLRP3 in the regulation of host immunity for pro- or antitumor responses. ATP released by dying tumor cells serves as a "find-me" signal and recruits phagocytes to facilitate the engulfment of apoptotic cells [83]. Thus, ATP serves as an agonist for NLRP3 whose activation triggers IL-1β production and cross-priming of antitumor $CD8^+$ T cells [84].

3.3.4 Phagocytosis Receptors

Phagocytes are specialized eating cells responsible for removing apoptotic cells in the body through a function of ligand-receptor interaction. Dying tumor cells attacked by immune cells or targeted by cytotoxic chemotherapeutic reagents are subject to recognition and removal by phagocytic myeloid cells [85, 86]. Molecules responsible for delivering "eat me" signals, including milk-fat globule-EGF factor 8 (MFG-E8), growth arrest-specific 6 (Gas-6), T-cell immunoglobulin-mucin domain protein-4 (TIM-4), and calreticulin (CRT), recognize the phosphatidylserine (PS) on apoptotic cells by integrin αvβ3 on phagocytes [87–90]. On the other hand, the "do not eat me" signal serves as negative regulators

for phagocytes. One example includes the interaction between CD47 and signal-regulatory protein-α (SIRP-α), which provides inhibitory signals that block phagocytosis [91] (Fig. 3.1a).

Manipulation of phagocytic systems has emerged as one of the tumor immune evasion machineries, and pharmacological targeting of these pathways provides a feasible option to augment host immune responses and eradicate tumors. For example, blocking CD47 with a monoclonal antibody triggers tumor destruction by inducing phagocytosis of malignant cells [90, 92], and the treatment with anti-MFG-E8 antibodies elicits potent antitumor responses in combination with conventional anticancer drugs [93].

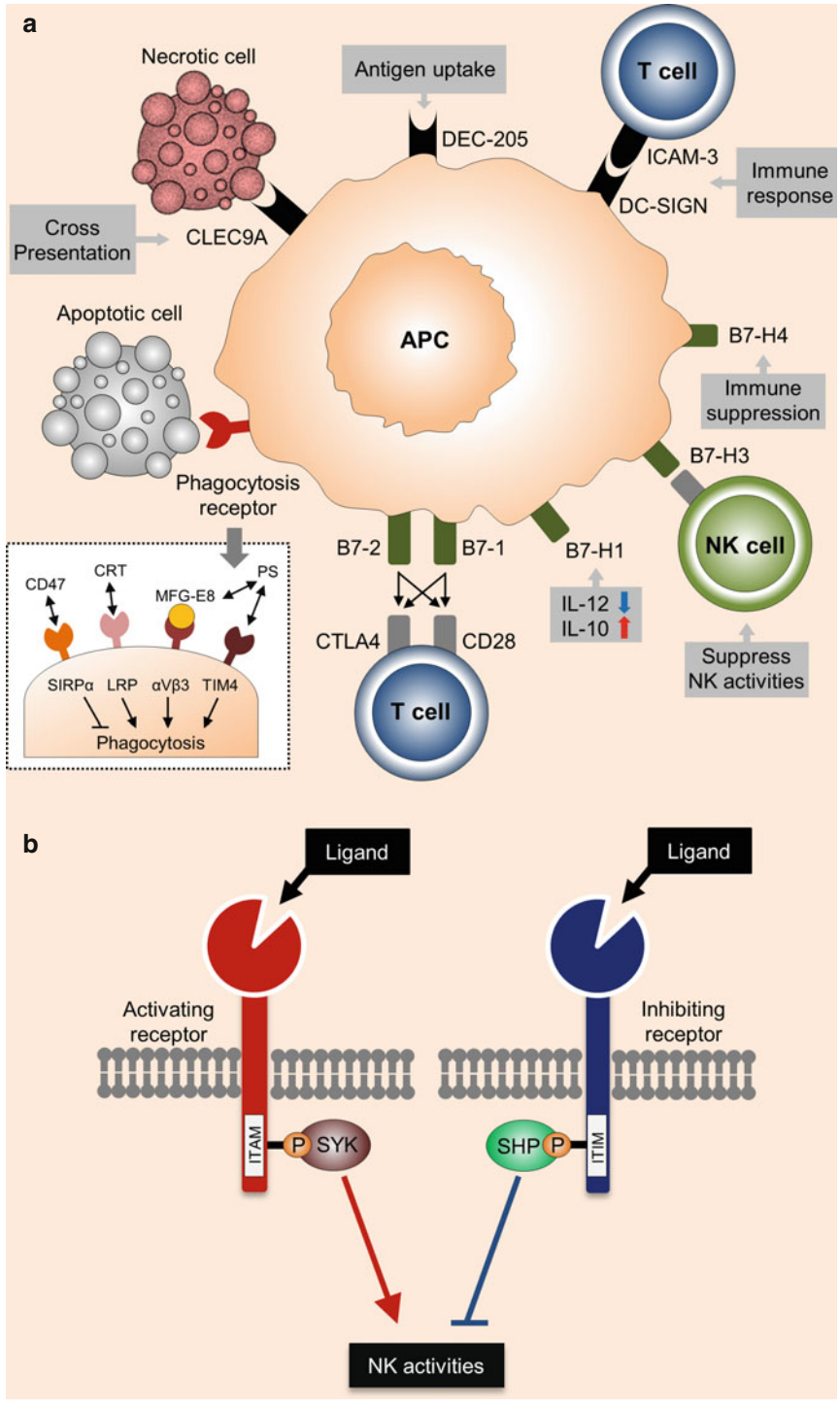
3.3.5 C-Type Lectin-Like Receptors (CLRs)

Carbohydrate-binding C-type lectin and lectin-like receptors (CLRs) are a large family of molecules expressed in innate immune cells and play an important role in the regulation of antitumor immunity. For example, the interaction between DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin) and ICAM-3 (intercellular adhesion molecule 3) facilitates the cross talk between DCs and T lymphocytes, hence influences immunogenic responses against pathogens and tumors [94]. DEC-205 is highly expressed on DCs and promotes cross-presentation of tumor antigens to cytotoxic T lymphocytes [95]. Indeed, agonistic antibody targeting DEC-205 elicits potent antitumor immunity and durable tumor regression in various murine tumor models [96]. In addition, C-type lectin domain family 9A (CLEC9A) utilizes necrotic cells for uptake, antigen presentation, and immune response, hence raising the possibility that CLEC9A-mediated recognition of immunogenic antigens may enhance antitumor immunity and clinical responses [97] (Fig. 3.1a). Therefore, CLRs serve as promising candidates for improving therapeutic responses to cancer immunotherapy. Moreover, deep understanding of the mechanism through which CLRs regulate innate immune response will lead to improvement in cancer vaccines.

3.3.6 NK Cell Receptors

NK cells possess various sets of pattern-recognition receptors which activate or suppress immune responses upon encountering

Fig. 3.1 Role of innate immune receptors in the regulation of antitumor immunity. (**a**) The functions of the innate immune system are regulated by various receptors expressed in immune cells. C-type lectin-like receptors (CLRs) regulate recognition and uptake of antigens (such as DEC-205), the interactions between immune cells (such as the interaction between DC-SIGN on APCs and ICAM-3 on T cells), and the recognition of dead cells, such as CLEC-9A which recognizes necrotic cells and enhances cross-presentation of antigens derived from necrotic cells to $CD8^+$ T cells. Members of B7 family regulate the functions of APCs, such as B7-H1 and B7-H4, which have immune suppressive effects, while other members regulate the interaction with immune cells, such as B7-H3, which interacts with NK cells and suppress its functions, and B7-1/B7-2 which regulates APCs-T-cell interactions. Phagocytosis receptors expressed on APCs interact with ligands on apoptotic cells and mediate its removal by APCs. In some cases, ligand-phagocytosis receptor interactions (such as CD47-SIRP-α) provide an inhibitory signal which blocks phagocytosis, a system utilized by tumors to evade immune machineries. (**b**) The balance between activating and inhibiting signals is critical for NK cell activities. Upon interaction with corresponsive ligands, activating and inhibitory receptors deliver a signal which is mediated by ITAM and ITIM in their cytoplasmic domain. Phosphorylated ITAM motifs in activating receptors recruit adaptor proteins which activate downstream signaling pathways, while phosphorylated ITIM motifs in inhibitor receptors recruit proteins such as SHP-1 which dephosphorylates downstream signal molecules and inhibit NK activities


a
Necrotic cell
Antigen uptake
T cell
DEC-205
ICAM-3
Immune response
DC-SIGN
Cross Presentation
CLEC9A
Apoptotic cell
APC
B7-H4
Immune suppression
B7-H3
Phagocytosis receptor
NK cell
B7-2
B7-1
B7-H1
CD47
CRT
PS
MFG-E8
IL-12
IL-10
CTLA4
CD28
SIRPα
LRP
αVβ3
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Phagocytosis
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b
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ITIM
NK activities

their target cells. The balance between activation and inhibition signals is carefully mediated by signals triggered by both activation and inhibition receptors in combination with cytokines. Signals delivered from NK receptors mainly mediate through immunoreceptor tyrosine-based activation motif (ITAM) and immunoreceptor tyrosine-based inhibition motif (ITIM). ITAM and ITIM bear conserved sequences of four amino acids repeated twice in the cytoplasmic tails of NK cell receptors. Phosphorylation of tyrosine within ITAM motifs recruits adaptor proteins such as DNAX-activating protein-12 (DAP12) and DNAX-activating protein-10 (DAP10) involved in activating downstream signaling pathways. On the other hand, phosphorylation of tyrosine within ITIM motifs recruits proteins such as SHP which dephosphorylates downstream signal molecules to inhibit NK stimulation [98] (Fig. 3.1b).

Tumor cells evolve multiple strategies to evade NK cells by modulating ligand expression, ligand shedding, and upregulation of MHC molecules, in addition to the production of immunosuppressive cytokines. Thus, it is important to understand the underlying mechanism of NK cell activation and inhibition by their receptors, which eventually regulate immunosurveillance. NKG2D is a homodimeric C-type lectin-activating receptor expressed on NK, NKT, and activated $CD8^+$ T cells [16, 99]. Ligands for NKG2D include stress-induced proteins, such as MHC class I chain-related A and B (MICA and MICB) as well as unique long 16 binding proteins (ULBPs) in human [99] and RAE1, H60, and Mult1 in mice. NKG2D ligands are upregulated in stress conditions, such as viral infection and transformation [99–102]. Several signaling pathways are involved in the induction of NKG2D ligands, including HSP70-mediated cellular stress [101] and ATM/ATR-mediated DNA damage pathways [103]. Importantly, blocking of NKG2D pathways increases the susceptibility of mice to chemically induced carcinogenesis [104], indicating the importance of NKG2D in tumor immunosurveillance. Natural cytotoxicity receptor (NCR) family consists of three activating receptors: NKp30, NKp44, and NKp46, which are able to induce a strong cytotoxic reaction by NK cells. Expression levels of NCRs are correlated with cytotoxic ability of NK cells. MHC class I molecules counteract with NCR-mediated activation signals; in addition, the loss of MHC-I molecules, frequently observed in transformed cells, activates NCRs on NK cells [105–107].

Killer cell immunoglobulin-like receptors (KIRs) are a family of cell surface molecules expressed on NK cells. KIRs have many members divided into two groups depending on the number of extracellular Ig domains (2D or 3D) or the length of their cytoplasmic tail, long *vs.* short (L or S). L-forms are shown to have inhibitory functions, while S-forms enhance cytotoxic activities of NK cells in DAP12-mediated signal pathways. KIRs regulate NK cells' killing function through the interaction with MHC class I molecules [100, 108].

The interaction between inhibitory KIRs and normal MHC-I molecules inhibits NK cell stimulation. Correspondingly, NK cell stimulation can occur due to an interaction between activating KIRs and polymorphic self-MHC class I molecules. Inhibitory KIRs were shown to be involved in the escape mechanism of acute myeloid leukemia (AML) from NK cell immune surveillance, mechanism of which includes a mismatch between donor KIRs and recipient human leukocyte antigen ligands [109]. Thus, the understanding of KIR-mediated recognition of missing self is important in the treatment of AML [110].

Ly49 family is a large group of receptors expressed in mice but not in humans [111]. Functionally Ly49 is similar to human KIRs, containing both activating and inhibitory receptors. Inhibitory Ly94 receptors possess ITIM motifs which recruit SHP-1 to trigger an inhibitory signal, while activation receptors interact with DAP12 to activate lytic machinery in NK cells [112]. Ly49H is an activating NK receptor which recognizes m157 glycoprotein encoded by

mouse cytomegalovirus (MCMV). Upon interaction with m157, Ly49H associates with DAP12 and DAP10 to stimulate NK cell-mediated cytotoxic activities against infected cells [113], suggesting a role for Ly49H in the protection against viral infection-associated tumors [114].

DNAM-1 (CD226) is an adhesion molecule expressed on the surface of NK cells, monocytes, and a subset of T cells. DNAM-1 belongs to the immunoglobulin superfamily containing 2 Ig-like domains of the V-set. DNAM-1 is reported to bind to two ligands: CD112 and CD155 [115]. CD112 and CD155 are highly expressed in some tumors like melanoma and neuroblastoma. Importantly, neuroblastoma cells that do not express CD112 and CD155 are resistant to NK cells, indicating that NK lysis of this neuroblastoma cells requires DNAM-1 interaction with its ligands on tumor cells [116].

3.3.7 B7 Family

B7 family consists of co-stimulatory and co-inhibitory receptors found on activated APC and T cells, which regulate the interaction between APCs and T cells. B7-1 and B7-2 are expressed on APCs and are involved in the stimulation of T-cell response. B7-1 and B7-2 on APCs serve as co-stimulatory molecules and play a critical role in regulating antitumor immune responses through reciprocal interaction of their receptor CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T lymphocytes [117, 118]. B7-H1 (PD-L1) expression in DCs is induced by IL-10 and VEGF at ovarian tumors [119]. B7-H1 on DCs suppresses IL-12 and promotes IL-10 secretion, creating an immunosuppressive tumor environment. Moreover, the blockade of B7-H1 enhances antitumor immunity by DC-mediated T-cell activation [119, 120]. In addition, treatment with PD-1 neutralizing antibodies has been found to decrease tumor growth and metastasis in B16 melanoma and colon cancer models [121, 122]. B7-H3 on APCs bind to an unidentified receptor on NK cells and transduce an inhibitory signal which suppress cytotoxic activities of NK cell. In addition, blocking of B7-H3 could restore the antitumor effects of NK cells [116]. Finally, B7-H4 promotes protumorigenic and immunosuppressive phenotypes of macrophages; for example, the blockade of B7-H4 normalized immunogenicity of macrophages and augmented antitumor immunity in ovarian tumor tissues [123] (Fig. 3.1a).

3.4 The Role of Effectors Produced from Innate Immune Cells in Cancer and Antitumor Immunity

3.4.1 Interferons (IFNs)

Type I IFNs are produced by many different cells in response to viral or bacterial infections. Type I IFNs (IFN-α/IFN-β) enhance proliferation and activation of innate immune cells such as DCs, macrophages, and NK cells [124]. In addition, they stimulate antigen processing and presentation to antigen-specific lymphocytes, which greatly contribute to tumor immunosurveillance [125]. The importance of type I IFNs in tumor immunosurveillance also validated enhanced susceptibility to tumorigenesis by treatment with anti-IFN-α/IFN-β neutralizing antibodies or in mice with targeted mutations of type I IFN receptor [126, 127].

Type II IFN (IFN-γ) is a cytokine involved in the activation of adaptive immune cells. IFN-γ is primarily produced by various innate immune lymphocytes such as NK, NKT, and γδ-T cells and plays a critical role in the induction of Th1 immune responses and the production of NO and ROS by macrophages, leading to enhanced cytotoxic activities against transformed cells [128]. IFN-γ has an important role in the protection against transplanted tumors or chemically induced tumors by increasing intrinsic immunogenicity of tumor cells [129, 130]. IFNGR$^{-/-}$ mice or mice deficient in IFN-γ-downstream

signaling molecule Stat-1 developed tumors more rapidly and in greater frequencies compared to wild-type mice [131, 132]. Thus, IFN-γ-mediated regulation of tumor immunogenicity has a great impact on innate immunity and tumor immunosurveillance.

3.4.2 Other Cytokines

Interleukins have an important role in regulating innate immune functions in tumor microenvironments. Several cytokines, such as IL-2, IL-12, IL-18, IL-15, and IL-21, serve as NK cell-stimulants, competent in targeting transformed cells. Mice deficient for IL-12p40 are susceptible to carcinogen-induced tumorigenesis; in addition, IL-21$^{-/-}$ mice showed reduced colitis-associated cancers [133], indicating the role of these cytokine in protecting hosts from arising tumors. With respect to the mechanisms of action, NKG2D systems are involved in the enhancement of NK cell cytotoxic activities by all cytokines suggested above, and perforin-granzyme pathways play an important role in exerting NK cell cytolysis by IL-18. Moreover, IL-21 induces NK cell effector functions by increasing sensitivities to IFN-γ, and IL-15 regulates survival, activation, and proliferation of NK cells [134]. Cytokines produced from innate immune cells serve as feasible adjuvants in activating antitumor responses in patients with advanced cancer. For example, the systemic administration of high doses of recombinant IL-2 or the adaptive transfer of IL-2-stimulated NK cell can trigger potent antitumor responses and mediate durable tumor regressions in patients with advanced melanoma and renal cell carcinoma [135]. The clinical efficacy of IL-12 has been evaluated as a monotherapy or in combination with other immunotherapies in patients with cancer; however, they did not induce durable clinical responses [136, 137].

Several cytokines antagonize immunogenic potential of tumors and innate lymphocytes. IL-10 downregulates the expression of immunogenic cytokines, such as IFN-γ, IL-2, TNF-α, and GM-CSF, and also suppresses antigen presentation by APCs. On the other hand, the carcinogen-mediated tumor incidence was increased in IL-10-knockout mice, whereas IL-10 overexpression protects mice from arising tumors [138]. Thus, IL-10 has a complex role in tumorigenesis, and the pro- and antitumor effects of IL-10 may depend on the different experimental models. TGF-β is a regulatory cytokine which has important roles in the regulation of immune responses and immune tolerance as well as carcinogenesis [139, 140]. TGF-β can inhibit the activities of NK cells through the suppression of IFN-γ production [141], as well as the downregulation of activating receptors such as NKp30 and NKG2D [142]. On the other hand, TGF-β negatively regulates recruitment and differentiation of myeloid-derived suppressor cells (MDSCs) in tumor tissues derived from mammary carcinomas, contributing to enhanced host immunity and tumor rejection [143]. Therefore, TGF-β has different roles in antitumor immunity and tumorigenicity, which are in part dependent on the phase of tumor progression and different cellular components in tumor microenvironments [144]. Vascular endothelial growth factor-A (VEGF-A) also plays a critical role in suppressing DC maturation and differentiation, therefore impacting tumor immunogenicity and host immunosurveillance [145]. Thus, various cytokines are responsible for attenuating immunogenic potentials of innate immune systems in tumors.

Several cytokines derived from innate lymphocytes contribute to smoldering inflammation and tumor progression. IL-23-IL-17 pathway operated in endogenous tumor microenvironments represents prototypical mediators which promote tumor-associate inflammation. IL-23 promotes tumor cell growth and invasion through upregulation of proteins of the matrix metalloproteinase-9 (MMP9), COX-2, and angiogenesis. In contrast, IL-23$^{-/-}$ mice showed reduced inflammation and thus attenuated tumor formation [146]. IL-17 is elevated in various tumors,

where it plays an important role in tumor growth. IL-17 can enhance tumor growth by direct effects on tumor cells and tumor-associated stromal cells by activating IL-6-Stat3 pathways [147]. Furthermore, the altered composition of commensal microbes and disruption of epithelial barrier functions facilitate differentiation of IL-17-producing T lymphocytes by IL-23 from myeloid cells in intestine, leading to increased colon tumorigenesis [148, 149].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced *in vivo* by many cells including mast cells, macrophages, T cells, fibroblasts, and endothelial cells in response to immune activation and proinflammatory cytokines. GM-CSF creates an immunosuppressive tumor microenvironment by differentiating immature myeloid-derived suppressor cells (MDSCs) into tumor tissues [150]. On the other hand, the therapeutic administration of GM-CSF has been emerged as a potent immunogenic adjuvant to stimulate antitumor immunity by enhancing APC functions [151].

Macrophage colony-stimulating factor M-CSF (also known as CSF-1) is a dimeric polypeptide growth factor which regulates the proliferation, differentiation, and survival of macrophages and their bone marrow progenitors. CSF-1 expression is elevated in different tumors and is found to be accompanied by high grade and poor prognosis [152]. Targeting of CSF-1 has been evaluated in preclinical and clinical studies [153]. The administration of anti-CSF1R-neutralizing antibody (AFS98) or a CSF-1R inhibitor (Ki20227) resulted in reduced numbers of tumor-infiltrated macrophages in an implanted osteosarcoma model and reduced vascularization, angiogenesis, and tumor growth [154, 155].

3.4.3 Chemokines

Chemokines are small cytokines secreted by many cell types in response to pathological conditions, in order to activate and attract effector cells which express appropriate chemokine receptors. Two types of chemokines have been identified: CC chemokines that are chemotactic for monocytes and CXC chemokines which attract polymorphonuclear leukocytes (PMNs). Chemokines have a central role in tumor progression through the recruitment of innate immune cells into tumor site. Most studies have focused on CCL2 and CCL5 as the major chemokines in tumor microenvironment.

CCL2 (MCP-1) is produced by tumor cells and tumor-associated stromal cells and attracts $CCR2^{+}$ inflammatory monocytes to the tumor microenvironment, which differentiate into tumor-associated macrophages and promote tumor aggressiveness, and the blockade of CCL2-CCR2 signaling by neutralizing antibodies suppresses metastasis and prolongs overall survival of tumor-bearing mice [156]. The levels of CCL2 expression and macrophage infiltration into tumors are correlated with poor prognosis and metastases in human breast cancer, suggesting significance of CCL2-mediated immune regulation in cancer patients [157].

CCL5, another important chemokine, plays an important role in the recruitment of monocytes into the tumor microenvironment [158]. CCL5 induces expression of CCL2, CCL3 (MIP-α), CCL4 (MIP-β), and CXCL8 (IL-8) by monocytes, which leads to the recruitment of myeloid cells into tumor site [159]. CCL5 also induces CCR1 expression on monocytes [160]. Hence, chemokines lead to the recruitment of monocytes, which produce more chemokines to further attract more monocytes as well as other leukocytes into the tumor site. CCL5 enhances antitumor immune responses against tumors [161], while it promotes tumorigenesis and metastases in some conditions [162, 163]. These findings suggest dual function of CCL5 in cancer and antitumor immunity.

Taken together, the dynamic interactions between tumor cells and innate immune cells governed by chemokine networks play a pivotal role in the regulation of tumor immunosurveillance and tumorigenicity (Fig. 3.2).

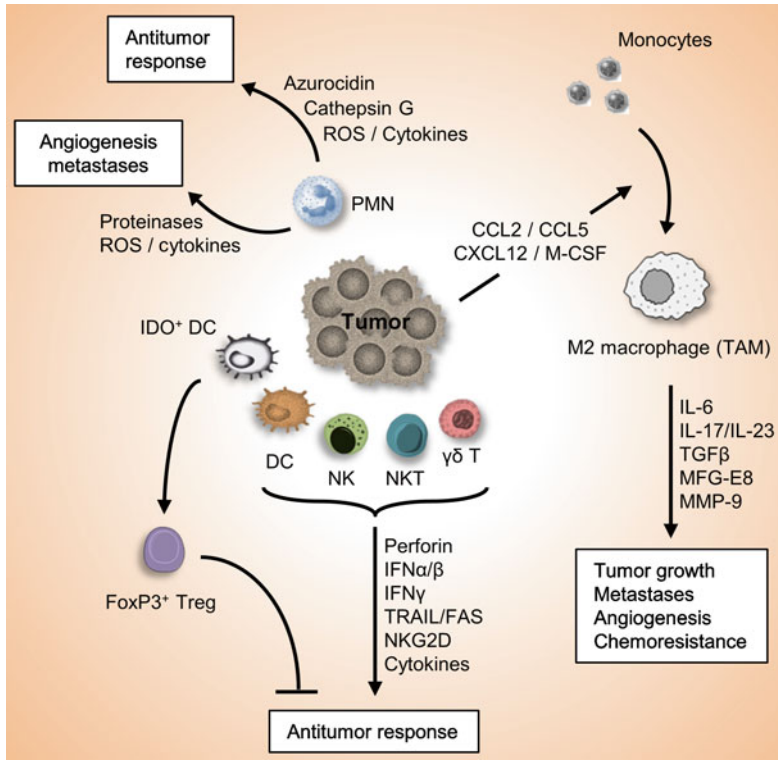

Fig. 3.2 Role of the innate immune system in cancer and antitumor immunity. Innate immune system serves as the first defense line against cancers. Innate immune cells such as DC, NK, NKT, γδ-T cells are attracted into the tumor site, where they recognize the transformed cells and release multiple factors which initiate an antitumor immune response. On the other hand, other innate immune cells may also involve in the promotion of tumor growth, angiogenesis, and metastasis. For example, IDO$^+$ DC induces differentiation of FoxP3$^+$Treg cells which suppress antitumor immunity, and molecules released by PMNs may have protumorigenic or antitumor effects. Furthermore, tumors secrete chemokines and cytokines that attract inflammatory monocytes into the tumor microenvironment and induce its differentiation into M2 macrophages, which play important roles in tumor progression, metastases, angiogenesis, and chemoresistance

3.5 Concluding Remarks

Innate immune system serves as the first line of defense against pathogens and cancers. In tumors, innate immune cells are attracted into the tumor site. Factors released from stressed cells at the tumor microenvironment, such as PAMPs and DAMPs, are recognized by another set of receptors, including TLRs, RLRs, and NLRs, which trigger distinct innate signaling pathways; these pathways lead to maturation, activation, as well as production of cytokines and chemokines from immune cells, to attract more immune cells into the tumor site and initiate an immune response against tumor cells. Thus, a deep knowledge of the role of innate immune system in tumor immunity and tumorigenesis is critical to develop new strategies for the immunotherapy of cancer.

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B Cells in Cancer Immunology: For or Against Cancer Growth?

4

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4.1 Introduction

In the 1960s, B cells were first defined in birds when researchers found that removal of the bursa in newly hatched chicks severely impaired the ability of the adult birds to produce Abs [1, 2]. A decade later, it was found that mammalian B cells are derived from bone marrow and develop into plasma cells that are the source of antibodies (Abs). Over the years, most studies on B cell function in immune response have focused on antigen presentation and antibody production. However, recent advances in B cell biology have capitalized on old findings and demonstrated that B cells can also act as effector cells or as regulatory cells [3, 4].

B cells are often overlooked in tumor immunology, likely because of the common notion that humoral and cytolytic responses work in opposition. The field of tumor immunology has focused on $CD8^+$ T cells due to their ability to directly kill tumor cells, as well as the close association between tumor-infiltrating $CD8^+$ T cells and cancer patients' survival [5]. To date, the role of B cells in tumor immunity has remained largely elusive. Results from different research groups are somewhat controversial. In this chapter, we review the roles of B cells in tumor immunology, which may either positively or negatively affect tumor growth and patient outcomes.

4.2 CD40-Activated B (CD40-B) Cells

CD40-activated B (CD40-B) cells are thought to be an excellent source of professional antigen-presenting cells (APCs) for antigen-specific tumor immunotherapy. They have demonstrated potent effects on cellular immunotherapy of cancers [6–17]. CD40-B cells induce potent expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells, including naïve $CD8^+$ T cells [6–9, 12, 16]. One reason that dendritic cells (DCs) are considered as excellent APCs in tumor immunotherapy is that they can powerfully prime naïve T cells, while resting B cells cannot. Resting B cells poorly express costimulatory molecules, resulting in immune tolerance regarding the induction of naïve T cells. Recent studies have shown that activation of mouse and human B cells using CD40L *in vitro* upregulates the expression of major histocompatibility complex (MHC) I, MHC II, and costimulatory molecules on B cells [6–9, 13, 14, 16]. These B cells present exogenous antigens by MHC class I or II molecules and stimulate antigen-specific T cells [7, 8]. CD40-B cells induce T cell proliferation, interferon-γ (IFN-γ) production, and specific cytotoxic T lymphocyte (CTL) responses [6–9, 11–15]. In mouse models, it has been shown that CD40-B cells directly present antigen to naïve $CD8^+$ T cells, in order to induce the generation of potent T effectors which are able to secrete cytokines and kill target cells [16]. Moreover, CD40-B cells express the full lymph node homing triad CD62L, CCR7/CXCR4, and leukocyte function antigen-1 (LFA1), suggesting that they could co-localize with T cells in the T cell-rich areas of secondary lymphoid organs [11, 15]. This will facilitate CD40-B cell and T cell contact for antigen presentation.

Using a metastatic mouse model, Li et al. provided direct experimental evidence that the augmented antitumor activity by anti-CD40 monoclonal antibody (mAb)-stimulated tumor-draining lymph node (TDLN) cells requires the presence of APCs, e.g., B cells as well as DCs. They found that anti-CD40 mAb augments antitumor responses of TDLN cells via ligation to CD40 on both B cells and DCs [17].

Typically, TDLN cells are composed of approximately 60 % $CD3^+$ T cells, 30 % $CD40^+$ B cells, and 5 % DCs. In a murine sarcoma model, anti-CD3-/anti-CD40-activated MCA205 TDLN T cells secreted significantly higher amount of IFN-γ in an antigen-specific manner (in response to MCA205 tumor, but not to MCA 207 tumor), in comparison with solely anti-CD3-activated TDLN T cells (Fig. 4.1a). However, when B cells were depleted from MCA205 TDLN cells, anti-CD3/anti-CD40 activation could not increase the IFN-γ anymore. This effect is very similar to DC depletion (Fig. 4.1a). *In vivo*, adoptive transfer of anti-CD3-/anti-CD40-activated MCA205 TDLN T cells mediated significantly higher MCA205 tumor regression in a pulmonary metastasis

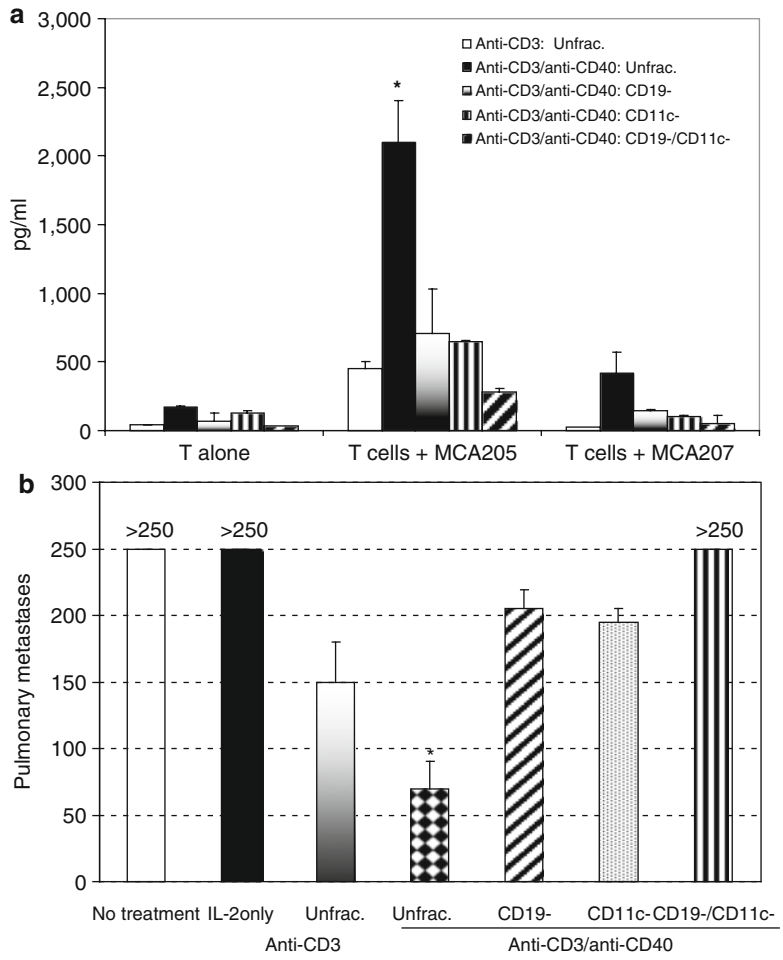

Fig. 4.1 Anti-CD40 mAb augmented antitumor responses of anti-CD3-activated TDLN cells via ligation to CD40 on both B cells and DCs. (**a**) Activated total unfractionated (Unfrac) TDLN cells were co-cultured with MCA 205 *vs*. MCA207 tumor cells to determine IFN-γ production. B cells were removed by CD19 depletion ($CD19^-$), and DCs were removed by CD11c depletion ($CD11c^-$). (**b**) Activated total TDLN (Unfrac) cells or B cell, DC-depleted TDLN cells ($CD19^-$ and/or $CD11c^-$) TDLN cells adoptively transferred into tumor-bearing mice for therapy. *$p<0.05$ compare with any other group in (**a**, **b**), respectively (Adapted by permission from the American Association of Immunologists, Inc. Copyright 2005: Li et al. [17])

setting, compared to anti-CD3-alone-activated TDLN T cells (Fig. 4.1b). However, B cell removal significantly reduced the therapeutic efficacy conferred by CD40 engagement, and so did DC removal. Together, these studies indicate that B cells, as well as DCs, are required in the generation of potent antitumor T effector cells from TDLN cells via simultaneous targeting of CD3 on T cells and CD40 on B and dendritic cells.

In a separate study, Iuchi et al. reported that host B cells were required for adoptive transferred T cells to mediate optimal antitumor immunity [18]. Tumor-bearing mice were treated with adoptive transfer of T cells accompanied with IL-2 and IL-21 administration in wild-type and B cell knockout ($B^{-/-}$) animals, respectively. They found that tumor growth inhibition was significantly diminished in the B cell-deficient mice after T cell + IL-2 + IL-21 combined therapy (Fig. 4.2).

In contrast to DCs, large numbers of B cells can be obtained from the blood of patients after ex vivo expansion (up to 1,000-fold) in the presence of CD40L [6]. For example, only about 10^6 DCs can be generated from 10 ml of blood, while 10^9–10^{10} B cells can be produced from the same volume of the blood sample. Additionally, CD40-B cells can be continuously expanded in long-term culture (>65 days) without the loss of APC functionality [6]. Therefore, CD40-B cells have the advantage over DCs in terms of isolation, generation, and long-term expansion. These characteristics

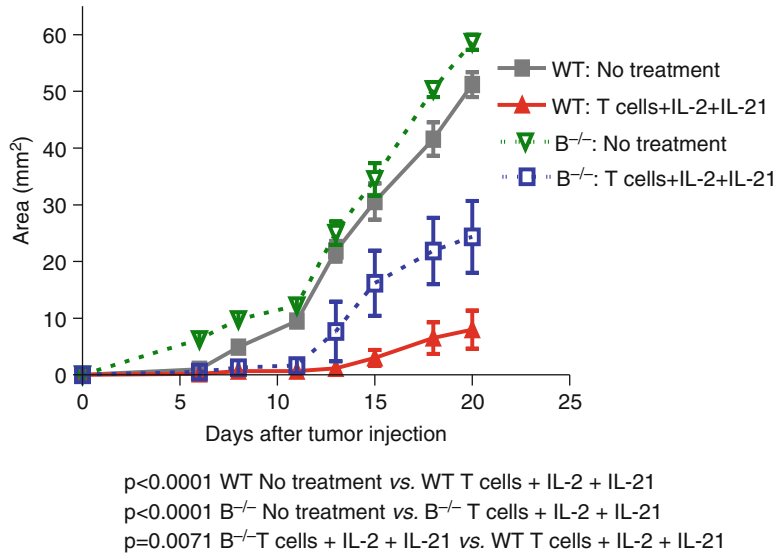

Fig. 4.2 Requirement for host B cells in T cell transfer + IL-2 and IL-21 administration-elicited antitumor immunity (Adapted by permission from the American Association for Cancer Research: Iuchi et al. [18])

make CD40-B cells a promising alternative as cell-based vaccines.

In current B cell vaccine preparations, activated B cells can be loaded with antigens by pulsing with peptides, proteins, tumor lysates, or by transfection with DNA or RNA, or transduction with viral vectors [9, 10, 19]. Coughlin et al. loaded RNA on CD40-B cells from pediatric patients. Vaccination using these B cells resulted in simultaneous targeting of multiple antigenic epitopes and induced CTLs [9]. Chung et al. reported that B cells stimulated with iNKT (CD1d-restricted invariant T cells) ligand alpha-galactosylceramide (alphaGalCer) could directly prime CTLs and generate long-lasting cytotoxic antitumor immunity *in vivo* [10]. Furthermore, Garbe et al. reported that semi-allogeneic fusions of microsatellite instability (MSI) tumor cells with B cells primed B cells to induce MSI-specific T cell responses [19].

4.3 Tumor Killer B Cells

B cells can directly kill tumor cells through antibody (Ab)-independent mechanisms [20]. Recent studies have shown that B cells express death-inducing ligands and can therefore mediate cell death under many circumstances. Evidence has emerged that B cells express Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), programmed death ligands 1 and 2 (PD-L1 and PD-L2), and granzyme B (GrB), which are potentially involved in B cell-mediated direct cytotoxicity against tumor cells [21–29].

Due to the well-known fact that B cells can produce Abs which lead to CDC and ADCC, as well as the recent findings that B cells may kill tumor cells directly through antibody-independent mechanisms, it is hypothesized that appropriately sensitized and activated B cells can function as effector cells to mediate antitumor immunity. Indeed, Li et al. [30] proved that *in vivo* sensitized and *in vitro* activated B cells could mediate tumor regression in cancer adoptive immunotherapy. *In vivo* sensitized TDLN cells were activated and expanded *in vitro* with LPS/anti-CD40, resulting in B cell proliferation and differentiation. These activated B cells were then adoptively transferred into tumor-bearing recipients for therapy. These tumor-primed and tumor-activated B cells significantly reduced lung metastases in an adoptive immunotherapy model (Fig. 4.3). Furthermore, total body irradiation (TBI) could enhance the antitumor activity of the adoptively transferred B cells. This study represents one of the early studies demonstrating that effector B cells could confer antitumor immunity after adoptive transfer into tumor-bearing mice [30].

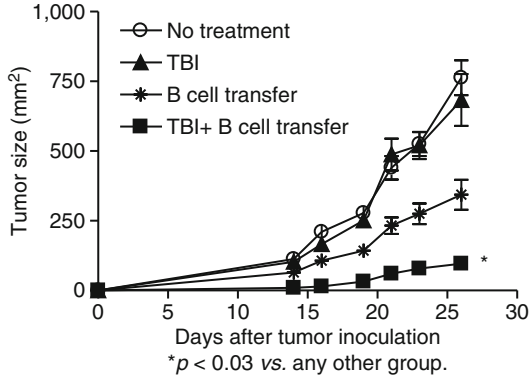

Fig. 4.3 TBI (total body irradiation) significantly augmented the therapeutic efficacy of adoptively transferred B cells in the s.c. D5 tumor model (Adapted by permission from the American Association of Immunologists, Inc. Copyright 2009: Li et al. [31])

Using a murine 4T1 pulmonary metastatic model, it was found that adoptive transfer of 4T1-primed and LPS-/anti-CD40-activated TDLN B cells significantly inhibited 4T1 pulmonary metastasis in tumor-bearing mice [31] (Fig. 4.4). The efficacy mediated by B cells was comparable to that mediated by an equal number of T cells, which served as a positive control in the experiment (Fig. 4.4a). Of note, adoptively transferred 4T1 TDLN T + B cells mediated inhibition of the spontaneous pulmonary metastasis of 4T1 in a dose-dependent manner (Fig. 4.4b).

This study also showed that activated 4T1 TDLN B cells caused tumor cell lysis directly *in vitro* in the absence of Ab and other effector cells and this direct cytotoxicity was tumor specific (Fig. 4.5). In these experiments, 4T1 mammary carcinoma murine tumor-primed TDLN B cells were activated with LPS and anti-CD40 mAb, washed thoroughly, and then co-cultured with 4T1 tumor cells or irrelevant tumor controls, Renca (renal cell carcinoma) and TSA (sarcoma). The effector B cells killed 4T1 cells directly in a dose-dependent way and were significantly more effective than their killing of the control tumors. These data support the conclusion that tumor antigen-primed and *in vitro* activated B cells are able to kill tumor cells independent of Ab or complement. However, the mechanism(s) by which the killer B cells lyse tumor cells directly in such a setting remains to be identified.

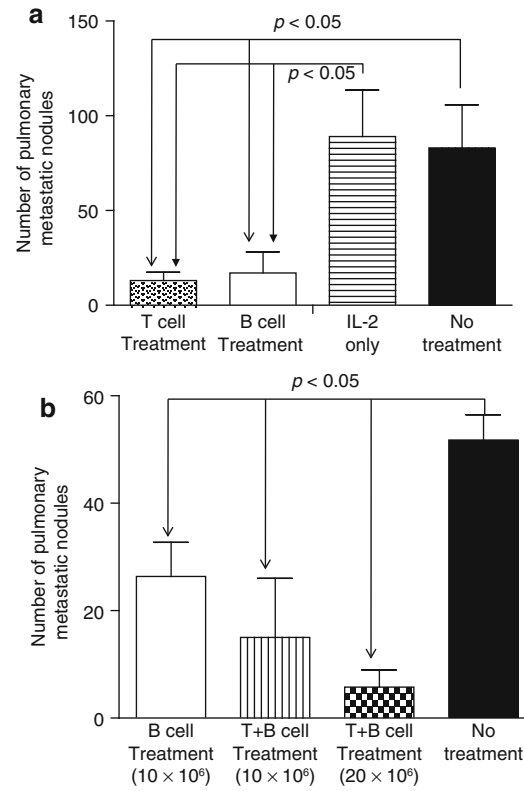

Fig. 4.4 (**a**) Adoptively transferred 4T1 TDLN B cells mediated effective inhibition of the spontaneous pulmonary metastasis of 4T1 breast cancer cells similarly to equal numbers of T cells. (**b**) adoptively transferred 4T1 TDLN T + B cells mediated inhibition of the spontaneous pulmonary metastasis of 4T1 better then B cells alone, and the efficacy was dose dependent (Adapted by permission from the American Association for Cancer Research: Li et al. [31])

In line with these findings, Kemp et al. demonstrated that CpG-A oligodeoxynucleotide (CpG-A ODN) stimulation of human PBMCs leads to high levels of functional TRAIL/Apo-2L expression on B cells, and these B cells mediate TRAIL-/Apo-2L-dependent tumor cell lysis [25].

Additional studies support the observation that B cell can function as effector cells in antitumor responses. For example, Penafuerte et al. reported that B effector cells activated with a chimeric protein consisting of IL-2 and the ectodomain of TGF-β receptor II (also known as FIST) induce potent antitumor immunity [32]. In this study, the B effector cells were characterized by the production of TNFα and IFN-γ and potent antigen presentation properties

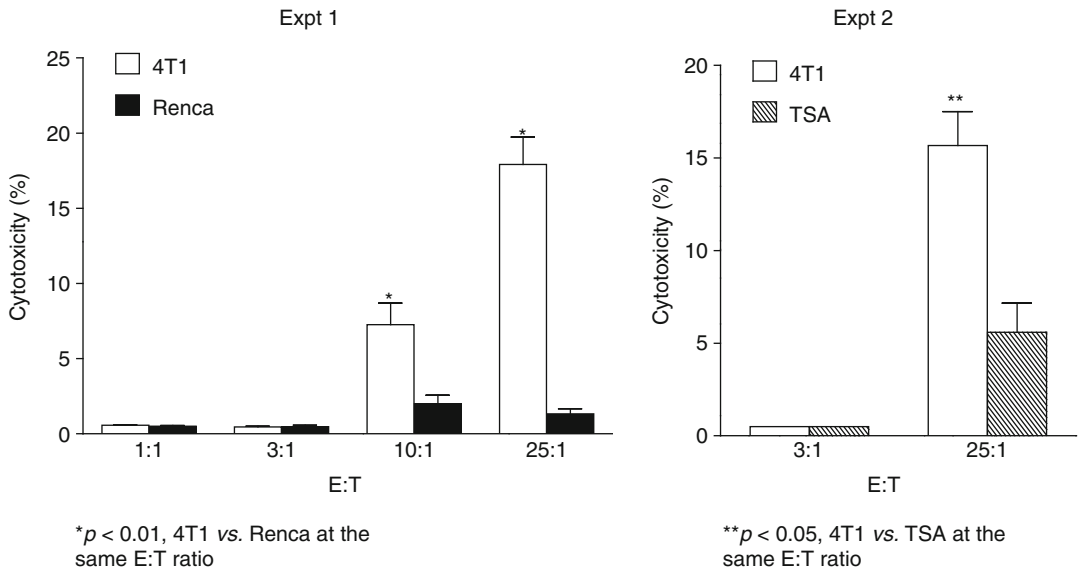

Fig. 4.5 Activated 4T1 TDLN B cells mediate direct and tumor-specific cytotoxicity of 4T1 cells (Adapted by permission from the American Association for Cancer Research: Li et al. [31])

[32]. In addition, Forte et al. found that administration of a specific CD73 inhibitor, adenosine 5′-(α, β-methylene) diphosphate (APCP), to melanoma-bearing mice induced significant tumor regression [33]. They observed that after APCP administration, the presence of B cells in the melanoma tissue was more than that observed in control mice. This was associated with the production of IgG2b within the melanoma, implying a critical role for B cells in the antitumor activity of APCP [33]. Together, these studies suggest that the mechanisms underlining B cell-mediated antitumor immunity may involve multiple cellular and molecular events, as well as direct killing of the tumor cells.

4.4 Tumor-Infiltrating B Cells (TIL-Bs) in Cancer

Tumor-infiltrating B cells (TIL-Bs) have revealed controversial roles in antitumor immunity. They have been found in breast cancer [34–36], ovarian cancer [37], lung cancer [38], colorectal cancer [39, 40], cervical cancer [41], cutaneous melanoma [42], and prostate cancer (CaP) [43]. A few studies have indicated that TIL-Bs are correlated with favorable survival of patients [36, 37, 42, 44, 45], lower relapse rate [41], or low metastasis [42]. In a study on immune infiltrates in high-grade serous ovarian cancer, it was revealed that intraepithelial $CD20^+$ TIL-Bs are associated with increased disease-specific survival [37]. Importantly, the association between the immune infiltrates and survival was dependent on histological subtype, because immune infiltrates were less prevalent in the other histological subtypes compared to the high-grade serous cases [37]. In breast cancer, TIL-Bs are present in about 24 % of tumors and comprise up to 40 % of the lymphocytic infiltrates [34]. TIL-Bs have been shown to undergo antigen-driven clonal proliferation and affinity maturation in situ [35]. Very recently, in a large patient cohort of different histological and biological subtypes, Mahmoud and colleagues provided evidence for a favorable outcome when high numbers of $CD20^+$ TIL-Bs were present [36]. Additionally, TIL-Bs may be involved in humoral immune response in situ. Using recombinant Ab cloning techniques, Hansen et al. reported an antigen-driven humoral immune response directed against β-actin exposed on apoptotic mammary carcinoma cells [46]. Yasuda and coworkers identified TIL-Bs which produce tumor-specific Abs against mutated p53 [47].

Maletzki et al. also reported that TIL-Bs from colorectal carcinoma show an activated immunophenotype ($CD23^+$, $CD80^+$) and produce IgGs that specifically bind to allogeneic target tumor cells [40].

On the other hand, TIL-Bs may produce cytokines contributing to tumor development. It has been reported that TIL-Bs in castration-resistant CaP produce lymphotoxin by an inflammation-responsive IκB kinase (IKK)-β-dependent pathway, which then in turn activates IKK-α and STAT3 in tumor cells to enhance hormone-free tumor survival [43]. In this study, B cell infiltration was detected in 100 % of human CaP samples, while B cells were undetectable in normal prostate or benign prostatic hyperplasia [43]. Castration-resistant CaP growth was delayed in mice reconstituted with bone marrow from $JH^{-/-}$ mice, which lack mature B cells [43]. It was further found that these CaP allografts exhibited IKK-α nuclear translocation, which was dependent on IKK-β in B cells. IKK-β deletion abolished lymphotoxin expression by B cells. When lymphotoxin-β was ablated in B cells, growth of castration-resistant CaP was delayed. Similarly, another study showed that tumor-infiltrating T and B cells were not associated with long-term survival of patients with non-small-cell lung cancer [38].

The roles of TIL-Bs may be complicated, since the tumor environment is dynamic and changes during tumor onset and progression. TIL-Bs need to contact other immune cells or tumor cells to be activated or regulated, so their contributions to immune responses are likely to vary in different cancers and during the course of cancer.

4.5 Resting B Cells and Regulatory B Cells in Cancer

In contrast to activated B cells, there is abundant evidence indicating that resting B cells can promote the development or progression of cancer. Resting B cells are small B cells in the G0 stage of cell cycle, prior to activation. Studies have shown that B cell-deficient mice exhibit enhanced T cell antitumor activity and significant improvement in survival rate [48–52]. It has been reported that there are increased effector T cells [48], increased T cell infiltration of tumors [52], higher Th1 cytokine and antitumor CTL response [49, 51, 52], and even reduced T regulatory cell (Treg) frequencies [53] in these B cell-deficient mice. Some studies explored the possible mechanisms involved. B cells present in the priming phase result in disabled $CD4^+$ T cell help for CTL-mediated tumor immunity [51]. B cells produce IL-10 which can repress antitumor immunity [49, 54]. Similarly, Abs were shown to promote primary tumor formation in a transgenic mouse model of inflammation-associated carcinogenesis [55]. Autoantibody responses to self-proteins triggered by cancer vaccines may influence the efficacy of vaccination [56]. Additionally, B cells have been shown to have other pro-tumorigenic roles. For example, enhanced NK cell antitumor activity has been reported in B cell-deficient mice [48, 50, 52]; however, the mechanisms are poorly understood.

We hypothesize that the effects of B cells on antitumor immunity depend on the presence of B cell subsets mainly involved under certain tumor conditions. In the past two decades, investigators have identified B cell subsets which are capable of suppressing the immune response. Suppression of an immune response was first reported in 1974 where spleen B cells were found to impair delayed-type hypersensitivity (DTH) responses in guinea pigs [57, 58]. This finding led to the conclusion that DTH responses and T cell function can be regulated by suppressor B cells. Subsequently, convincing data have demonstrated that IL-10-producing B cells, termed regulatory B cells (Bregs) by Mizoguchi et al. [59], can suppress inflammatory responses in experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and colitis [59–61]. Recently, Bregs and their potential immunomodulatory activities have been examined in several immune-related diseases. In the majority of these studies, the function of Bregs is dependent on IL-10 production, whereas the mechanisms are still undefined partly because of conflicting results regarding the phenotypic characterization

of IL-10-producing cells. For example, the following B cells have been reported as putative mouse Bregs: $CD1d^{high}$ subset of B cells in chronic colitis in TCRα-deficient mice [59], $CD21^{high}CD23^{low}$ B cells in contact hypersensitivity (CHS) mouse model [62], $CD21^{high}CD23^{high}$ T2-MZ precursor B cells in CIA model [63], $CD1d^{high}CD5^{+}$ B cells (termed B10 cells by Yanaba et al.) in CHS [64] and EAE models [65], $CD138^{+}CD19^{+}$ plasmablasts in *Salmonella typhimurium* infection [66], and T cell Ig domain and mucin domain protein (TIM)-1^{+} B cells [67]. For human, $CD19^{+}CD24^{hi}CD38^{hi}$ B cells have been found as putative Bregs [68, 69].

Triggering Toll-like receptors (TLRs) [70–72], the BCR [64], CD40 [73], or combinations thereof have been shown to promote IL-10 production by B cells. BCR-mediated Ca^{2+} flux appears to be required for IL-10 production, since B cells deficient in the calcium sensors stromal interaction molecule (STIM) 1 and STIM2 have a profound defect in IL-10 secretion and abrogated suppression abilities *in vivo* [74]. Nuclear factor of activated T cells (NFAT) 1, a transcription factor, is involved in Ca^{2+}-dependent IL-10 production [74]. Therefore, their proposed model for IL-10 production by B cells is that, after BCR stimulation, STIM and Orai-dependent Ca^{2+} increase by store-operated Ca^{2+} entry (SOCE) activates calmodulin/calcineurin and then NFAT1, leading to IL-10 expression. In addition, the TLR signaling pathway is also required for IL-10 secretion [70–72]. Given that TLR stimulation does not induce Ca^{2+} mobilization in B cells, crosstalk between Ca^{2+} and Ca^{2+}-independent TLR cascades may be involved in IL-10 production.

IL-10 is an immunomodulatory cytokine and inhibits Th1 polarization, prevents Th2 responses, and suppresses pro-inflammatory cytokine production by monocytes and macrophages [75]. So far, the potential role of Bregs in tumor immunology is not clear, but several studies suggest that Bregs can negatively regulate antitumor immunity. Using a mouse chemical carcinogenesis model, Schioppa et al. found that resistance to papilloma development in $Tnf^{-/-}$ mice was associated with a significant reduction in IL-10-producing B regulatory cells alongside an increase in IFN-γ-producing $CD8^{+}$ T cells in the spleen [54]. In this study, $Tnf^{-/-}$ mice were resistant to chemical carcinogenesis of the skin. LPS-stimulated $CD19^{+}$ B cells isolated from $Tnf^{-/-}$ mice produced less IL-10. These mice had a reduced absolute number of $IL\text{-}10^{+}CD19^{+}$ B cells in their spleens, and $Tnf^{-/-}$ mice were deficient for $CD19^{+}CD21^{high}$ B cells. The authors speculated that resistance to carcinogenesis in $Tnf^{-/-}$ mice may result from increased $CD8^{+}$ IFN-γ-producing T cells and decreased IL-10-producing B cells. In another study, Horikawa et al. reported that production of IL-10 by Breg inhibits lymphoma depletion during CD20 immunotherapy in mice [76]. They found that adoptive transfer of $CD1d^{high}CD5^{+}$ B cells (that are enriched for B10 cells) eliminates the therapeutic benefit of CD20 mAbs in mouse lymphoma model. The transferred B10 cells in this model downregulated the expression of MHC II molecules and CD86 on macrophages and reduced LPS-induced nitric oxide and TNF-α production by macrophages, indicating that B10 cells suppress the antitumor response at least partly by downregulation of macrophage activity. Our unpublished data support that Bregs play a negative role in antitumor immunity. In melanoma and breast carcinoma models, depletion of IL-10-producing B cells from TDLN cells resulted in the generation of potent effector B cells which dramatically inhibit tumor metastasis after adoptive transfer in two genetically distinct immune competent hosts, B6 and Balb/c mice, respectively.

Although little is known about the mechanisms by which Bregs undermine effective antitumor immunity, several possibilities are suggested by studies on inflammation and autoimmunity. Bregs impair Th1 immune responses. The initial finding about Th1 response regulated by Bregs was reported by Skok et al. [77]; they found that IL-10 produced by B cells is involved in the feedback regulation of Th1 development. It has been reported that Bregs suppress the Th1 cell-mediated immune reactions in a number of mouse models, including EAE, CIA, CHS, and diabetes mellitus [60, 61, 64, 65, 72, 78, 79]. Fillatreau et al. reported that B cell IL-10

deficiency correlates with enhanced type I autoreactivity; in addition, transfer of IL-10$^+$ B cells was found to result in resolution of EAE, characterized by enhanced encephalitogenic Th1 response [60]. Later, Lampropoulou et al. showed that TLR signaling in B cells suppresses inflammatory T cell responses (both Th1 and Th17) and stimulates recovery from EAE [72]. Similarly, using mouse model of CIA, Mauri et al. showed that transfer of IL-10-producing B cells inhibits T helper type 1 differentiation and prevents arthritis development [61]. Yanaba et al. also revealed that CD1d^{high}CD5$^+$ B cell transfer normalized inflammation in CHS model [64]. Using NOD mouse model of type 1 diabetes (T1D), Hussain et al. found that BCR-stimulated B cells produce IL-10 and attenuate islet inflammation by polarizing CD4$^+$ T cell response toward a Th2 phenotype [79].

Bregs induce the differentiation of Tregs. Given that μMT$^{-/-}$ B cell-deficient mice display reduced Treg frequencies in comparison with wild-type mice [53] and that these mice develop exacerbated EAE and Ag-induced arthritis (AIA) [60, 80], a role for Bregs in modulating Tregs was proposed. Several disease models have demonstrated that IL-10 produced by Bregs is important for the generation and/or maintenance of Tregs. Sun et al. reported that after oral tolerance induction, Treg cells increase much more in WT than in μMT$^{-/-}$ mice. However, adoptive transfer of B cells before treatment normalized Treg cell development in μMT$^{-/-}$ mice [81]. In this study, they found that sublingual tolerization with OVA/CTB (Ag conjugated to cholera toxin B subunit) enhances the tolerogenic activity of B cells and their production of IL-10, which was associated with the generation of Ag-specific Foxp3$^+$CD25$^+$CD4$^+$ Tregs [81]. This relationship between Bregs and Tregs is further supported by the results from mouse models of airway sensitization. These results showed that Bregs prevent and reverse allergic airway inflammation via FoxP3$^+$ T regulatory cells [82, 83]. Additionally, Bregs can induce the differentiation of T regulatory 1 (Tr1) cells [84–86]. Gray et al. reported that autoimmune inflammation could be protected by the induction of Bregs which induce T cell-derived IL-10 [84]. Blair et al. used the transitional 2 immature (T2) B cells stimulated with agonistic anti-CD40 (T2-like Bregs) to convert autologous effector T cells into Tr1 cells [86]. Sayi et al. also showed that B cells activated by *Helicobacter* TLR-2 ligands produce IL-10 and induce IL-10-producing CD4$^+$CD25$^+$ Tr1 cells depending on TCR signaling and a direct T-B cell interaction through CD40/CD40L and CD80/CD28 pathways [85].

4.6 Concluding Remarks

B cells are phenotypically and functionally heterogeneous. Characterization of B cell subpopulations is shown in Table 4.1. B cells play multiple roles in tumor immunity (Fig. 4.6). On one hand, accumulating literature indicate that B cells are significantly involved in antitumor responses. In this regard, B cells present tumor antigens to T cells to generate antitumor CTLs. Upon tumor antigen stimulation, B cells can differentiate into plasma cells to produce antibodies to target tumor cells via ADCC and/or CDC. In addition, B cells may act as killer cells to directly cause tumor cell lysis in the absence of antibodies. B cells migrate to tumor tissue and become TIL-Bs which may induce humoral immune response or act as killer cells in situ. On the other hand, regulatory B cells have been described which downregulate antitumor responses by producing immunomodulatory cytokine IL-10, suppressing Th1 immune responses, and enhancing Treg and Tr1 responses. Further characterization of B cell subsets responsible for these conflicting functions demonstrated in tumor immunity and understanding of the molecular mechanisms involved would help develop novel clinical strategies for cancer immunotherapy.

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Competing Interests

The authors have declared that no competing interest exists.

Table 4.1 Phenotypic characterization of B cell subpopulations

	Marker		Source	References
Resting B cells	Human	$CD19^+CD38^-IgD^+CD27^-$	Tonsils	[87, 88]
		$CD38^-IgM^+IgD^+CD27^-$	Blood	[88]
	Mouse	$IgM^{low}IgD^{high}HSA^{low}CD21^{int}CD23^{bright}Mel14$ $(CD62L)^{bright}CD44^{int}CD69^-$	Lymph node	[89]
		$IgM^{high}IgD^{high}CD23^{bright}$	Spleen	[90]
CD40 B cell	Human	$CD19^+CD38^+CD80^+CD86^+CD71^+$ $CD95^+CPM(carboxypeptidase\text{-}M)^+$	Tonsils	[87]
		$CD19^+CD23^+CD54^+CD58^+CD80^+$ $CD86^+MHCI^{high}MHCII^{bright}$	Blood	[6]
	Mouse	$B7.1^{high}B7.2^{high}ICAM^+MHCI^{high}$ $MHCII^{bright}$	Spleen	[90, 91]
Putative Breg	Human	$CD19^+CD24^{high}CD38^{high}$	Blood	[68, 69]
	Mouse	$B220^+CD1d^{high}CD21^{intermediate(int)}$ $CD62^{low}IgM^{int}CD23^{high}$	Lymph nodes[a]	[59]
		$B220^+CD21^{high}CD23^{low}$	Spleen in CHS model	[62]
		$B220^+CD21^{high}CD23^{high}$ $IgM^{bright}CD1d^{high}$	Spleen in CIA model	[63]
		$CD1d^{high}CD5^+$ $CD19^+$ $B220^+$	Spleen in CHS model	[64]
		$CD1d^{high}CD5^+$ $CD19^+$	Spleen in EAE model	[65]
		$CD138^+CD19^+$	Spleen of mice infected with *Salmonella*	[66]
		TIM-1(T cell Ig domain and mucin domain protein)$^+CD19^+$	Spleen	[67]
TIL-Bs	Mostly unknown. Related to cancer types and progression			
	Human	$CD19^+CD20^+$ $CD23^+CD80^+$	From colorectal carcinomas	[40]
Killer B	Unknown			

[a]From TCRα-deficient mice

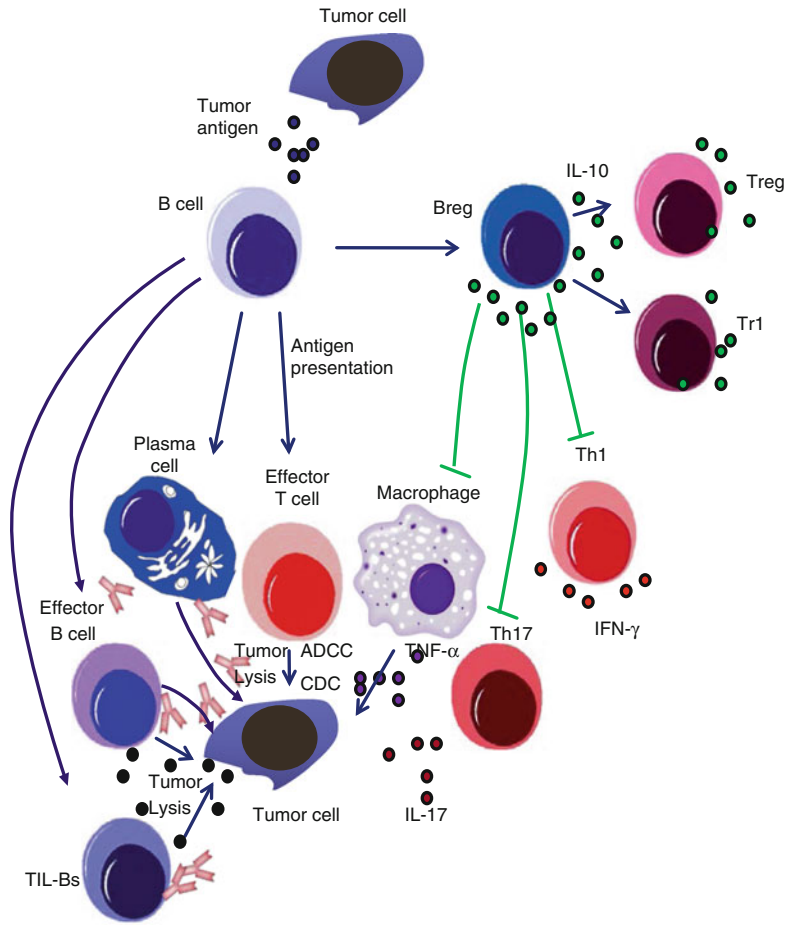

Fig. 4.6 Potential roles played by B cells in tumor immunity. *ADCC* antibody-dependent cellular cytotoxicity, *CDC* complement-dependent cytotoxicity, *TIL-Bs* tumor-infiltrating B cells

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The Role of Exhaustion in Tumor-Induced T Cell Dysfunction in Cancer

5

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5.1 Introduction

T cells are divided into two major functional types: helper and cytotoxic T cells. Helper T cells ($CD4^+$) release an array of cytokines and orchestrate diverse immune responses, which integrate both adaptive and innate effector mechanisms. Cytotoxic T cells ($CD8^+$ effector T cells) are primarily involved in the recognition and elimination of body cells compromised by intracellular pathogens or oncogenic transformation.

Thus, T cells are essential components of the immune system, which have been the major focus of immunotherapeutic strategies to boost endogenous antitumor immunity. However, despite homing to tumor sites, infiltrating T cells seldom control tumor growth, as a consequence of the tumor microenvironment, which contains a wide array of suppressive mechanisms that allow tumors to escape T cell effector functions.

Even when T cell anergy has been considered responsible for T cell hyporesponsiveness in cancer patients, cancer is also regarded as a chronic disease, similar to chronic viral infections, where T cells are continuously stimulated. Thus, with chronic stimulation, tumor-specific T cells gradually become less functional until they undergo cell death, a phenomenon known as T cell exhaustion. This chapter will focus on the latter mechanism and its participation in cancer-induced T cell dysfunction.

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5.2 T Cell Activation

T cell activation requires two signals delivered by antigen-presenting cells (APCs). The first signal involves the presentation of antigen by APCs, in the form of peptides bound to MHC class I or class II molecules, to the T cell receptor (TCR), expressed on the surface of the T cell. The second signal, or costimulatory signal, stimulates T cells in conjunction with the antigen. The molecules expressed on APCs engage their corresponding costimulatory receptors on the surface of T cells generating costimulatory signals. CD80 (B7-1) and CD86 (B7-2) are well-characterized costimulatory signal molecules, which interact with CD28 expressed on the T cell membrane [1] (Fig. 5.1). CD28 is the primary costimulatory molecule for naïve T cells; this molecule is essential for initiating T cell responses. The interaction of CD80 and CD86 with CD28, together with TCR signaling, promotes the expansion and differentiation of antigen-stimulated T cells into effector and memory cells. The interaction of CD28 with its ligands (1) enhances the production of interleukin-2 (IL-2) and other cytokines, (2) promotes energetic metabolism, (3) induces the cell cycle progression, (4) promotes T cell survival, and (5) maintains T cell responsiveness upon subsequent restimulation [1].

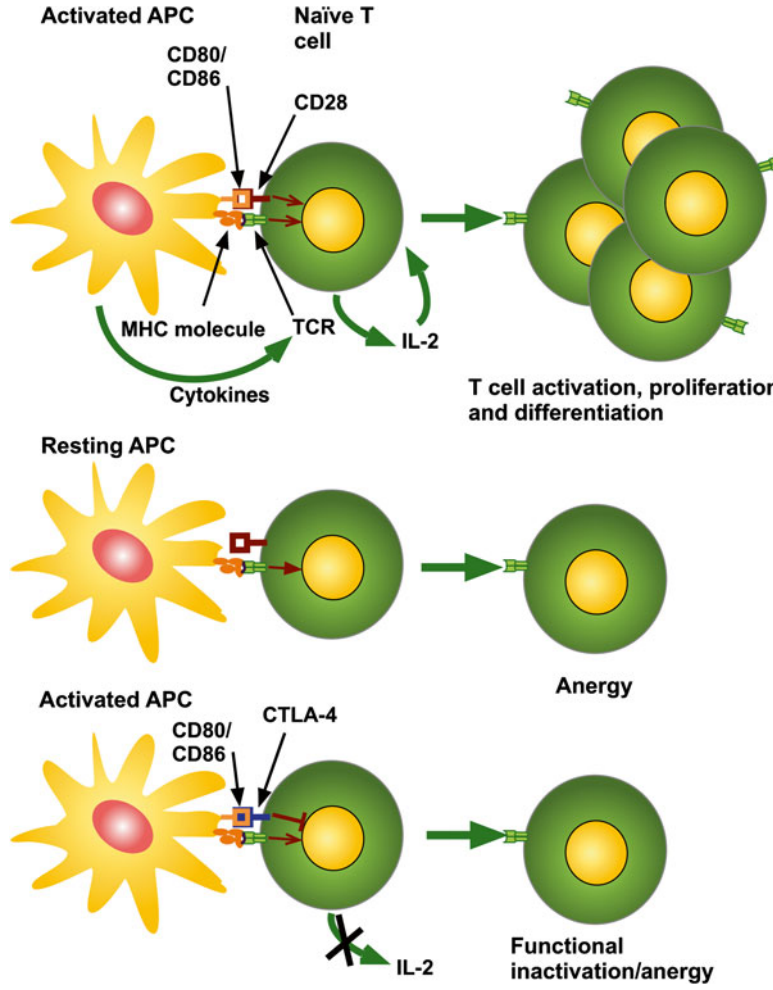

Fig. 5.1 T cell activation requires recognition of the antigen and costimulatory signals. Inflammation generated by tissue damage or infections activates antigen-presenting cells (*APCs*) and stimulates the expression of costimulatory molecules, such as CD80/CD86. Presentation of the antigen to the T cell receptor (*TCR*), in the context of major histocompatibility complex (*MHC*) molecules and CD80/CD86 that interact with CD28, stimulates the expansion and differentiation of naïve T cells (*top panel*). Resting APCs express few or no costimulatory molecules and fail to activate T cells, this leads to anergy (*middle panel*). CTLA-4 is a coregulatory molecule that binds CD80 and CD86 and is upregulated on activated T cells. CD80/CD86-CTLA-4 interactions inhibit T cell responses and mediate tolerance

Although costimulatory molecules were initially identified as stimulators of T cell responses, some costimulatory (coregulatory) receptors inhibit T cells [1]. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is a CD28 homolog that also binds CD80 and CD86. Nevertheless, CTLA-4 expression is inducible after T cell activation, and is involved in the induction and maintenance of tolerance, as its ligation inhibits IL-2 production and blocks cell cycle progression [1].

After the discovery of homologs of CD28/CTLA-4 and their ligands, many other coregulatory molecules have been identified, some of which include the inducible T cell costimulator (ICOS or CD278) with its ligand CD275 (ICOS-L, B7h, B7-RP), the inhibitory programmed death-1 (PD-1, CD279) with its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), and the B- and T-lymphocyte attenuator (BTLA, CD272) which binds the herpesvirus entry mediator (HVEM). BTLA is an additional receptor of the immunoglobulin superfamily that negatively regulates T cell activation. In addition, HVEM interacts with another negative regulator of T cells, CD160. Recent studies of the lymphocyte activation gene-3 (LAG-3, CD223) suggest that this molecule also plays an important role in the regulation of T cell responses. Moreover, the T cell immunoglobulin domain and mucin domain-3 (TIM-3), with its ligand galectin-9, are involved in terminating Th1 cell responses and establishing tolerance [2, 3].

T cells that recognize antigen in the absence of costimulation either fail to respond and undergo cell death or enter a state of unresponsiveness, a phenomenon known as anergy. Thus, costimulation is a key factor in the outcome of T cell interactions with the antigen. Significant efforts have been undertaken to characterize costimulatory molecules in order to augment antitumor responses; recent evidence has demonstrated the importance of coregulatory molecules in the inhibition of immune responses. Thus, interfering with these regulatory pathways has gained interest as a potential strategy for cancer therapy [4].

5.3 T Cell Anergy

T cell anergy induces peripheral tolerance; this mechanism protects the host from autoimmune diseases. In addition, anergy has been suggested to play an important role in the induction of T cell dysfunction in cancer patients. T cell anergy is a tolerance mechanism in which, after antigen encounter, the T cell is intrinsically and functionally inactivated [5]. The cell remains alive in this hyporesponsive state for an extended period of time. Anergic T cells neither produce nor respond to proliferative signals and are unable to exert effector functions, such as cytolysis or cytokine secretion. A characteristic of anergy is that it must be cell autonomous, which distinguishes this process from immunoregulation mediated through other regulatory cells, such as regulatory T cells (Tregs) [5, 6].

There are at least five distinct sets of circumstances that lead to T cell anergy [5, 7]: (1) TCR ligation in the absence of full costimulation; (2) exposure to partial agonists, peptides with minor sequence differences from native agonist antigenic peptides that exhibit reduced avidity for TCR ligation; (3) full signaling without IL-2 receptor-driven cell division; (4) TCR ligation in the presence of IL-10 or transforming growth factor-β (TGF-β); and (5) anergy induced through CTLA-4 or other coregulatory molecules (Fig. 5.1).

Thus, anergy is the consequence of factors that negatively regulate proximal TCR-coupled signal transduction, together with active transcriptional silencing, which is reinforced through epigenetic modifications [8]. This state of nonresponsiveness is molecularly distinct from T cell exhaustion. T cell anergy is induced upon the first encounter with the antigen and is quickly initiated, in contrast with T cell exhaustion, which is progressive. Gene expression profiles show that anergy and exhaustion are partially distinct. Genes, such as *Rnf128* (*Grail*), *Egr2*, and *Egr3*, are upregulated in anergic but not in exhausted T cells, whereas NFAT is upregulated under both conditions [9]. The detailed characterization of the differences between anergy and T cell exhaustion will have important implications for therapeutic interventions in immune-mediated diseases and chronic infections.

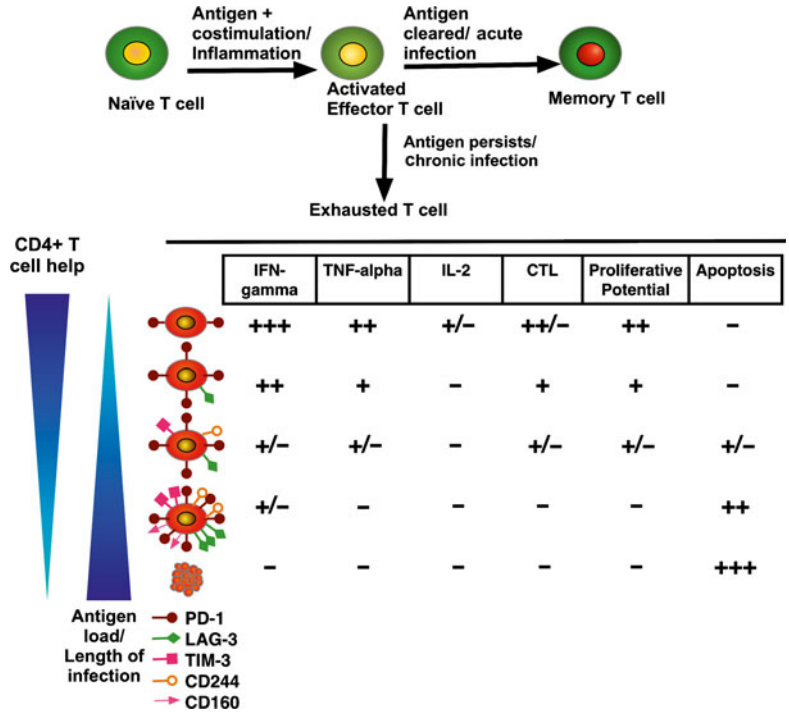

Fig. 5.2 T cell exhaustion during chronic inflammation. In an acute inflammatory process, naïve T cells are primed by an antigen, costimulatory molecules, and cytokines that promote differentiation into effector T cells. After clearance of the antigen and once inflammation is resolved, a subset of effector T cells differentiates to become memory cells. During chronic processes, such as viral infections, the antigen persists, and T cells go through several stages of dysfunction, losing effector functions (cytolysis and secretion of cytokines) and proliferative potential in a hierarchical manner. Finally, deletion of T cells by apoptosis occurs. As antigen load increases or $CD4^+$ T helper subpopulation decreases, T cells become more exhausted. Expression of coregulatory receptors is correlated with the level of exhaustion. The scale of each activity is presented from high (+++) to low (–)

5.3.1 T Cell Anergy in Cancer

Anergy has been proposed to play a role in the impairment of T cell function in human cancers. Tumor cells are poor APCs, as these cells express antigens on MHC class I molecules but do not express costimulatory molecules to provide a second signal for full T cell activation; thus, tumor-infiltrating lymphocytes (TILs) are rendered anergic [10]. In addition, immature myeloid-derived dendritic cells (mDCs), plasmocytoid dendritic cells (pDCs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) potentially induce anergy in TILs [8, 11, 12]. Several studies have shown that human tumor cells, mDCs, pDCs, MDSCs, and TAMs express high levels of coregulatory molecules, such as PD-L1, PD-L2, ICOS-L (B7-H2, CD275), and B7-H3 (CD276), indicating a poor costimulatory and a high inhibitory anergy-promoting environment. Evidence that cancer induces T cell anergy comes from studies where the transfection of CD80 in tumor cells or the blockage of the B7 family coregulatory molecules results in reduced tumor growth or tumor rejection in mice models [2, 11–14].

Analysis of the functional state of TILs has demonstrated that these cells are characterized by impairment of cytolytic activity, decreased cytokine secretion, reduced expression of IL-2Rα (CD25), and diminished activation of extracellular signal-regulated kinase (ERK) after TCR activation. Thus, T cell anergy occurs in the tumor microenvironment of some human tumors [14–16].

Nevertheless, direct evidence that T cell anergy occurs in cancer has been difficult to obtain due to the lack of surface markers to identify anergic T cells [8].

Based on mouse tumor models, the induction of antigen-specific T cell anergy has been suggested to be an early event in the progression of tumors, which occurs in the equilibrium phase of immunoediting, before immunosuppression takes place in advanced tumors (escape phase) [10, 17]. However, Klein et al. showed that highly immunogenic tumors evade immunosurveillance due to antigen overload and an insufficient number of tumor-specific T cells, resulting in the exhaustion of the immune cells [18]. Thus, from a temporal perspective, T cell anergy predominantly occurs during the early stages of tumor progression, whereas T cell exhaustion might play a crucial role in T cell dysfunction during the late stages of cancer [10].

5.4 T Cell Exhaustion

T cell exhaustion has been defined as a stage of T cell differentiation where T cells have poor effector functions, sustained coregulatory receptor expression, and a transcriptional state distinct from that of functional effector or memory T cells [19]. Originally, this phenomenon was identified in chronic viral infections in mice and later in chronic viral infections in humans, e.g., human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) [19–22]. Chronic bacterial and parasitic infections have been demonstrated to induce T cell exhaustion; also, cancer has been suggested to induce a similar phenomenon [20, 23].

During chronic infections, antigen-specific $CD8^+$ T cells initially acquire effector functions, but gradually become less functional as the infection progresses. The dysfunction of exhausted T cells is hierarchical, showing the initial loss of properties, such as cytotoxic activity, proliferative potential, and interleukin 2 (IL-2) synthesis; followed by diminished tumor necrosis factor-alpha (TNF-α) secretion and subsequent loss of interferon-gamma (IFN-γ) production during the late stages of exhaustion. Finally, during the most extreme stages of exhaustion, deletion of T cells occurs through apoptosis [19, 24] (Fig. 5.2). Like $CD8^+$ T cells, $CD4^+$ T cells also lose function during chronic infections; however, there is little information about the mechanisms of exhaustion in this T cell subpopulation [19].

Exhausted T cells possess a molecular profile that is distinct from those of memory, effector, and anergic T cells [9]. First, many membrane inhibitory receptors are upregulated, for instance, PD-1, LAG-3, and TIM-3. Second, transcription of genes encoding molecules involved in TCR signaling (such as Lck and NFATc) and cytokine receptors (IL7 and IL-15 receptors) are downregulated. Third, the pattern of genes involved in chemotaxis, migration, and adhesion is changed. Fourth, there is an altered pattern of differentiation compared with memory or effector T cells. Finally, exhausted T cells present deficiencies in translational, metabolic, and bioenergetic processes, such as the Krebs cycle [9].

5.4.1 Mechanisms for Inducing T Cell Exhaustion

Immunoregulation is critical in T cell exhaustion. Coregulatory receptors play a key role in many aspects of adaptive immunity, including self-tolerance, prevention of autoimmunity, and cancer. The mechanisms of regulation through coregulatory receptors have not been characterized in detail; nevertheless, several studies suggest that these receptors attenuate T cell responses in many ways. Accumulating evidence highlights the pivotal role of the PD-1/PD-L1 pathway in maintaining an immunosuppressive tumor microenvironment. This pathway has been proposed to be the most important coregulatory pathway involved in T cell exhaustion [25, 26].

A transmembrane receptor of the Ig superfamily, PD-1 (CD279), is upregulated in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) [25]. PD-1 interacts with its ligands PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD273), which are members of the B7 family [26]. PD-1 is rapidly upregulated on activated

T cells; then, after antigen clearance, the expression of this receptor is reduced on effector T cells. Upon subsequent antigen stimulation, effector T cells show upregulated PD-1 expression. Thus, the continuous stimulation of T cells (naïve or effector) during chronic infections induces the accumulation of PD-1$^+$ T cells [19]. High levels of PD-L1 expression on APCs (or tumor cells) might sustain PD-1 expression on T cells, impair T cell effector maturation, and allow the progression of chronic infection [27–29].

Studies in mouse tumor models demonstrated that the inhibition of PD-L1 or PD-1 using blocking monoclonal antibodies (mAbs) increases the cytolytic activity of CD8$^+$ T cells and reverses T cell dysfunction [30, 31]. Subsequently, Barber et al. showed that the inhibition of PD-1 using anti-PD-1 mAbs in chronically infected mice enhances the proliferation and effector functions of exhausted T cells [25]. Since the publication of these seminal reports, many other studies have shown that PD-1 with its ligand (PD-L1) is crucially involved in T cell exhaustion in chronic human pathogen infections and cancer [21–24, 32–34].

In addition to PD-1, many other cell surface inhibitory receptors also participate in T cell exhaustion. These coregulatory receptors regulate distinct cellular functions. For instance, PD-1 pathway affects T cell survival and proliferation, whereas LAG-3 affects cell cycle progression, but has less influence on apoptosis [19]. Several receptors belonging to the tumor necrosis receptor family are upregulated in exhausted T cells, such as Fas, TNF-R, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors; hence, these death receptors have been implicated in the induction of exhaustion, as T cells might become prone to activation-induced cell death (AICD) [19, 35, 36].

TIM-3 is an inhibitory molecule that downregulates effector Th1 responses; upregulation of this molecule has been found in HIV-specific and HCV-specific CD8$^+$ T cells in patients with progressive HIV and HCV infections, respectively. Importantly, the coexpression of TIM-3 and PD-1 has been associated with severe CD8$^+$ T cell exhaustion in terms of the proliferation as well as secretion of effector cytokines, such as IFN-γ, TNF-α, and IL-2 [19]. Interestingly, CD8$^+$ T cells expressing both coregulatory receptors also produce the suppressive cytokine IL-10 [37].

Remarkably, functional effector T cells express coregulatory receptors during activation; however, as indicated above, the prolonged and increased expression of multiple coregulatory receptors is a key feature of CD4$^+$ and CD8$^+$ T cell exhaustion. However, exhausted T cells do not necessarily coexpress all of the coregulatory molecules. The pattern as well as the level of expression of coregulatory receptors simultaneously expressed in the same CD8$^+$ T cell might considerably influence the severity of dysfunction [38].

Several factors, such as duration of the infection, level of antigen exposure, availability of CD4$^+$ T cell help, and the type of APCs that present the antigen, have been implicated in the severity of T cell exhaustion. Ligand availability for coregulatory receptors could also influence the degree of exhaustion, as well as environmental factors such as the presence of immunoregulatory cytokines [19]. In chronic viral infections, IL-10 expression is associated with T cell dysfunction [38, 39]. In addition, TGF-β has also been linked to exhaustion in chronic infections in humans [40, 41]; nevertheless, the mechanisms underlying IL-10 and TGF-β-mediated T cell exhaustion are unclear. Interestingly, both cytokines are secreted by several human tumors [42, 43].

5.4.2 Identification of Exhausted T Cells

Exhausted T cells show a poorly differentiated phenotype (CD27^{hi}CD28^{lo}CD57^{lo}CD127^{lo}CCR7$^-$ CD45RA$^+$ or CD27$^+$CD45RO$^+$) correlated with T cell dysfunction. Although PD-1 upregulation in T cells was initially considered as a hallmark of T cell exhaustion, this molecule is upregulated along with activation markers, such as CD38 or HLA-DR [44]. In healthy adults, the percentage of PD-1$^+$ cells varies from 40 to 80 % of (CCR7$^{+/-}$CD45RA$^-$) memory T cells; remarkably, these cells do not exhibit characteristics of

exhaustion [45]. Thus, PD-1 is also associated with T cell activation and differentiation.

Many cell surface coregulatory receptors are expressed in exhausted T cells. LAG-3, TIM-3, CD244 (2B4), CD160, CTLA-4, and the recently described B- and T-lymphocyte attenuator (BTLA) are coexpressed in antigen-specific $CD8^+$ T cells during chronic infection. The pattern and level of coregulatory receptors simultaneously expressed in the same $CD8^+$ T cell considerably influence the severity of dysfunction [38]. However, depending on the chronic infections or cancer, exhausted T cells might express a different pattern of coregulatory molecules.

Genomic strategies have provided a molecular profile for exhausted T cells. These genomic studies support the notion that T cell exhaustion represents a particular state of differentiation, different from that of effector or memory T cells [9, 19].

Several transcriptional pathways have been associated with T cell exhaustion. The increased expression of transcriptional repressor Blimp-1 is associated with the upregulation of many coregulatory receptors (PD-1, LAG-3, CD160, and CD244). In addition, the transcription factor NFATc1 (NFAT2) is also upregulated but shows a dysregulated function [9]. The transcription factor T-bet also plays a role in protection against T cell exhaustion, as T-bet promotes terminal differentiation after acute infection, and the increased expression of this transcription factor inhibits the expression of coregulatory receptors during chronic viral infection. T-bet expression is downregulated through persistent antigenic stimulation, resulting in T cell exhaustion [46].

5.5 T Cell Exhaustion in Cancer

Cancer and chronic viral infections have been thought to share similar mechanisms in establishing high antigen load and an immunosuppressive environment. However, there is a fundamental difference between both diseases: viral antigens are exogenous and extremely immunogenic, whereas tumor antigens are self-molecules that are weakly immunogenic. Thus, compared with tumor-specific T cells, virus-specific T cells are more frequent and easily detectable, facilitating the ease in identification, phenotypic characterization, and isolation of T cells [10]. However, in the tumor microenvironment, infiltrating T cells become dysfunctional and show reduced effector functions. Several reports suggest that PD-L1 expression on tumor cells plays an important role in tumor-induced T cell dysfunction. PD-L1 membrane expression has been observed using immunohistochemistry on many human tumors, such as melanoma, lung, larynx, colon, breast, cervix, and stomach [26]. In breast, esophageal, gastric, and renal carcinomas, the increased expression of PD-L1 on the surface of tumor cells is strongly associated with poor prognosis [26, 47]. Thus, T cell exhaustion has been proposed as a mechanism for inducing T cell dysfunction through the PDL-1/PD-1 pathway. However, as previously indicated, PD-1 expression cannot be considered as the sole marker of T cell exhaustion in chronic diseases and cancer; hence, other markers must be considered.

In human metastatic-melanoma lesions, TILs show upregulation of PD-1 expression, accompanied with reduced production of IFN-γ TNF-α, and IL-2. Both tumor-infiltrating $CD8^+$ T cells, particularly MART-1-specific, and tumor-infiltrating $CD4^+$ T cells show significantly higher levels of PD-1 expression than $CD8^+$ and $CD4^+$ T cells from peripheral blood and normal tissues from cancer patients. In addition, a large proportion of $CD8^+$ T cells from TILs were $PD\text{-}1^+CTLA\text{-}4^+$ cells compared with normal tissues and blood. Furthermore, $PD\text{-}1^+CD8^+$ cells from TILs lacked CD25 as well as IL-7RA expression, suggesting that these cells were unable to proliferate, produce effector cytokines, and differentiate into memory cells [48]. $PD\text{-}1^+$NY-ESO-1-specific $CD8^+$ T cells, from patients with advanced melanoma, upregulate TIM3 expression and are more dysfunctional than $TIM3^-PD\text{-}1^+$ and $TIM3^-PD\text{-}1^-$NY-ESO-1-specific $CD8^+$ T cells, producing less IFN-γ, TNF-α, and IL-2 [49].

Derré et al. showed that tumor antigen (Melan-A/Mart-1)-specific $CD8^+$ T cells express high levels of BTLA and are susceptible to functional inhibition through its ligand HVEM [50].

In addition, Baitsch et al. recently showed that in melanoma, tumor antigen-specific $CD8^+$ T cells with effector phenotypes simultaneously express four or more of the inhibitory receptors BTLA, TIM-3, LAG-3, KRLG-1, 2B4, CD160, PD-1, or CTLA-4 [51]. Moreover, tumor antigen-specific $CD8^+$ T cells present a large variety of genes with a similar genetic profile as that of exhausted T cells from chronic viral infections [52]. Taken together, these reports show that tumor antigen-specific $CD8^+$ T cells are exhausted in melanoma patients.

Additional evidence for T cell exhaustion in other cancers comes from studies in patients with ovarian cancer. Matsusaki et al. reported that NY-ESO-1-specific $CD8^+$ T cells, from the peripheral blood of patients with ovarian cancer, show impaired effector functions along with coexpression of the inhibitory molecules LAG-3 and PD-1. The expression of LAG-3 and PD-1 on the surface of $CD8^+$ T cells is upregulated through IL-10, IL-6, and tumor-derived APCs. In addition, LAG-3^+PD-1^+CD8^+ T cells are deficient in IFN-γ/TNF-α secretion compared with LAG-3^+PD-1^- or LAG-3^-PD-1^- subsets [53].

In hepatocellular carcinoma, PD-1^+CD8^+ T cells are higher in tumor tissues than in non-tumor tissues, presenting decreased proliferative abilities as well as effector functions, as demonstrated by reduced granule and cytokine expression compared with PD-1^-CD8^+ T cells [54]. Nevertheless, no other marker of T cell exhaustion was analyzed.

PD-L1 expression is upregulated in Hodgkin lymphoma (HL) and several T cell lymphomas, but not in B cell lymphomas. In addition, PD-1 is upregulated in TILs as well as peripheral blood T cells from HL patients and the blockade of the PD-1 pathway restores IFN-γ production in T cells [55]. Moreover, LAG-3 is expressed on TILs from patients with this malignancy [56]. Taken together, these reports suggest that TILs from patients with HL are exhausted.

In patients with chronic lymphocytic leukemia (CLL), $CD8^+$ and $CD4^+$ effector T cells show the increased expression of CD244, CD160, and PD-1, with the expansion of the PD-1^+ BlimpH1 subset. $CD8^+$ T cells from CCL patients show defects in proliferation and cytotoxicity, but with increased production of IFN-γ and TNF-α, normal production of IL-2, and increased expression of T-bet. Thus, although $CD8^+$ T cells show features of T cell exhaustion, these cells retain the ability to produce cytokines [57]. However, head and neck cancers that are positive for human papillomavirus (HPV) present a high infiltration of PD-1^+ T cells, and the numbers of PD-1^+ cells are positively associated with a favorable clinical outcome. Nevertheless, these PD-1^+ T cells express activation markers, 50 % of this population lack TIM-3 expression, and are functional after the blockade of the PD-1/PD-L1 pathway, suggesting that PD-1^+ T cells are activated rather than exhausted [58].

Interestingly, Haymaker et al. proposed that PD-$1^{high}CD8^+$ T cells in cancer patients are not exhausted [59]. This hypothesis is based on the observation that $CD8^+$ T cells from the TILs of melanoma patients recover their proliferative potential ex vivo, despite expressing high levels of PD-1. These TILs mediate antitumor responses upon adoptive transfer into patients [60, 61]. Under this hypothesis, infiltrating and peripheral blood $CD8^+$ T cells, expressing PD-1, BTLA, along with other coregulatory receptors, are not exhausted. Instead, these cells are highly activated effector-memory cells T cells that can be stimulated through immunotherapy [59]. Nevertheless, these observations have been primarily achieved in melanomas. In other cancers, the reduced proliferative and effector capacities persist, even after stimulation, and immunotherapeutic strategies have failed to induce potent antitumoral responses [53, 57, 62, 63].

Some of the phenotypic, functional, and molecular changes that occur in T cells during chronic infections are exhibited in TILs as well as peripheral blood T cells from several cancer types. The initial aim of tumor immunotherapy was to prevent anergy and tolerance towards tumor antigens. However, the efficacy of this strategy is potentially limited by T cell exhaustion [10]. Interestingly, Hailemichael et al. showed that in mice vaccinated with gp100 melanoma peptide, the persisting tumor antigen at vaccination sites induces the sequestration of

$CD8^+$ T cells, resulting in the dysfunction and death of these cells [63].

PD-1 blockage results in the recovery of T cell effector functions *in vitro* and in animal models in several tumors, thus significantly enhancing antitumor immunity [30, 31, 49, 64]. This knowledge has been translated into several clinical trials [34, 65]. Recently, Brahmer et al. showed that the antibody-mediated blockade of PD-L1 induced durable tumor regression along with prolonged disease stabilization in patients with selected advanced cancers, including non-small cell lung cancer [65]. Thus, understanding T cell exhaustion in cancer will contribute to the advancement of tumor immunotherapy.

5.5.1 A Particular Case: T Cell Exhaustion in Lung Cancer Patients

Lung cancer is the leading cause of cancer-related mortality in developed countries and the second leading cause of death in countries with emerging economies. Lung cancer is one of the most commonly diagnosed cancers worldwide, representing 13 % of all cancer cases and approximately 18 % of all cancer deaths [66]. Some reports show that the presence of TILs with memory phenotype in lung cancer is predictive of a favorable clinical outcome [67–69]. Also, it has been shown that $CD8^+$ T cell subpopulation is decreased in the pleural compartment with respect to peripheral blood from lung cancer patients, whereas the $CD4^+$ T cell subpopulation is increased [70, 71].

Both in TIL and in the pleural compartment, $CD8^+$ T cells are functionally impaired and are poorly responsive or unresponsive to several T cell-activating stimuli, even though memory cells infiltrate lung tumors. $CD8^+$ T cells present low proliferation rate, diminished production of some Th1 cytokines, and reduced cytotoxic potential [70, 72–74]. Pleural effusion $CD8^+$ T cells from lung cancer patients express cell markers associated with a memory phenotype ($CD45RA^-CD45RO^+CD27^+$Granzyme A^{low}Perforin$^-$), similar to those markers found in $CD8^+$ T cells from chronic viral infections [24], which suggests that $CD8^+$ T cells are exhausted.

Recently, pleural effusion $CD8^+$ T cells, derived from lung cancer patients, have been shown to be susceptible to AICD. This phenomenon is preferentially observed in memory as well as terminally differentiated $CD8^+$ T cells. AICD is associated with upregulation of FasL and TRAIL molecules. Interestingly, $CD4^+$ T cells from malignant pleural effusions are not prone to AICD [75]. Thus, chronic stimulation by the lung tumor mass may sensitize $CD8^+$ T cells to AICD, as it has been shown in TILs from various types of human cancers [75]. Nevertheless, evaluation of exhaustion in lung tumor-specific $CD8^+$ T cells has not been possible, since lung tumor-associated antigens are not shared among all lung cancer patients [62].

Here, it is shown PD-1 expression on $CD8^+$ and $CD4^+$ T cells from pleural effusions and peripheral blood of lung cancer patients who were admitted to the National Institute of Respiratory Diseases "Ismael Cosío Villegas." Pleural fluid was obtained by thoracocentesis used for routine diagnostic procedures. Diagnosis was established by histological examination of pleural biopsy or cytological observation of malignant cells in pleural effusion. None of the patients received any type of anticancer therapy before the study or had clinical signs of acute or chronic infection, which might interfere with the PD-1 analysis. For comparison, two groups of patients with acute (pneumonias) and chronic (tuberculosis) infections that presented pleural effusion were included. In lung cancer patients, PD-1 was expressed on average at about 40 % of pleural effusion $CD8^+$ T cells, which was significantly higher compared to percentages of $PD\text{-}1^+CD8^+$ T cells from pleural effusions secondary to acute or chronic tuberculosis infections. Also, PD-1 was expressed in more than 30 % of peripheral blood $CD8^+$ T cells from lung cancer patients; in contrast, approximately 23 % of peripheral blood $CD8^+$ T cells from healthy subjects expressed PD-1 (Fig. 5.3). With respect to $CD4^+$ T cells, the percentages of $PD\text{-}1^+$ cells were significantly higher in malignant effusions compared to tuberculosis and acute effusions

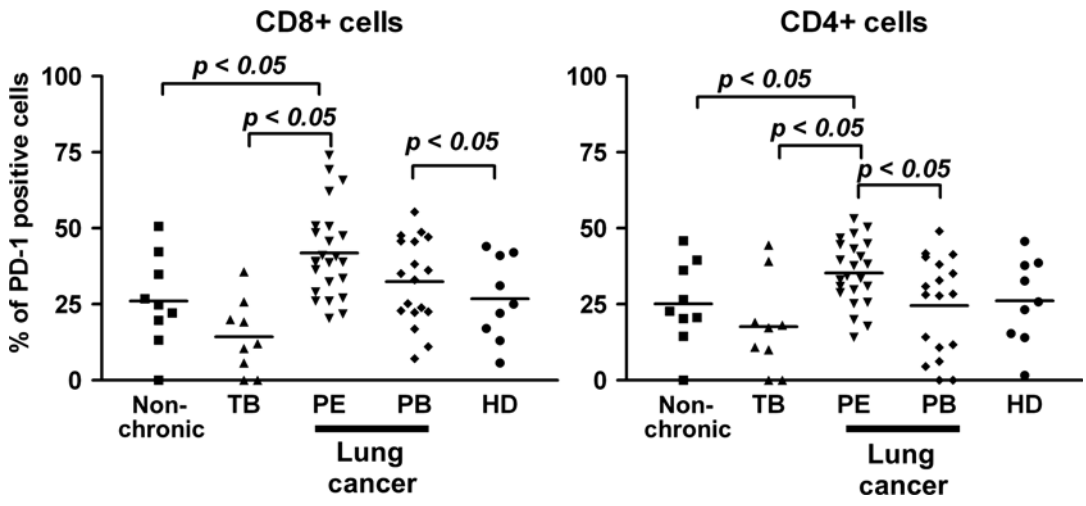

Fig. 5.3 Determination of PD-1 on CD4⁺ and CD8⁺ T cells. Pleural effusion (*PE*) and peripheral blood (*PB*) of lung cancer patients ($n=23$), patients with acute diseases (non-chronic $n=8$), patients with tuberculosis (*TB*, $n=9$), and PB from healthy donors (*HD*, $n=9$) were evaluated. For PD-1 membrane staining, peripheral blood mononuclear cells (PBMCs) or pleural effusion mononuclear cells (PEMCs) were incubated with anti-CD4 PE or anti-CD8-PECy5 monoclonal antibodies (mAbs), in addition to FITC-conjugated anti-PD-1 or isotype control mAb. Cells were washed, fixed with 1 % paraformaldehyde, and analyzed using flow cytometry. FSC *vs*. SSC dot-plot graphs were done for cellular debris and necrotic cell exclusion. From a lymphocyte gate containing 50,000 lymphocytes, CD4⁺ or CD8⁺ cells were gated from a CD4⁺ or CD8⁺ *vs*. SSC *dot-plot* graph. For the analysis of PD-1 expressions, and to rule out nonspecific antibody binding and autofluorescence, quadrants were set according to isotype control

(Fig. 5.3). Similar percentages of PD-1⁺CD4⁺ T cells were found in peripheral blood, between lung cancer patients and healthy donors. Thus, a greater percentage of CD8⁺ and CD4⁺ T cells from the pleural compartment are PD-1⁺, which is a consequence of the underlying pathology, rather than the anatomical compartment.

Recently, Zhang et al. reported that tumor-infiltrating CD8⁺ T cells derived from patients with non-small cell lung carcinoma express increased levels of PD-1 [76]. These CD8⁺ T cells are impaired in cytokine production as well as proliferative potential. Blockade of the PD-1/PD-L1 pathway by anti-PD-L1 antibody partially restores cytokine production and cell proliferation. However, PD-1 expression cannot be considered as the sole marker of T cell exhaustion; additionally, TIM-3 has been shown to mark exhausted CD8⁺ T cells [38]. In a study by Gao et al., TIM-3 was found to be highly upregulated on both CD4⁺ and CD8⁺ T cells from lung tumor tissues, but almost undetectable on T cells from peripheral blood. However, TIM-3 expression on CD8⁺ T cells was not associated with any clinical pathological parameter in lung cancer patients (e.g., tumor size, lymph node metastasis, and tumor stage) [77].

In this chapter, TIM-3 expression on CD4⁺ and CD8⁺ T cells derived from pleural effusion of lung cancer patients is shown (Fig. 5.4). Percentages of TIM-3⁺ cells were significantly higher in pleural effusion CD8⁺ T cells in comparison with CD4⁺ T cells from the same cancer patients (Figs. 5.4 and 5.5). Interestingly, pleural effusion CD8⁺ T cells from cancer patients showed higher percentages of TIM-3⁺ cells compared to those from the nonmalignant group (tuberculosis). Hence, TIM-3 is likely to be upregulated in response to tumor-derived environmental factors absent in pleural effusions from patients with tuberculosis. Nevertheless, in contrast with results reported by Gao et al., who found that in lung tumor tissues the majority of TIM-3⁺TILs are

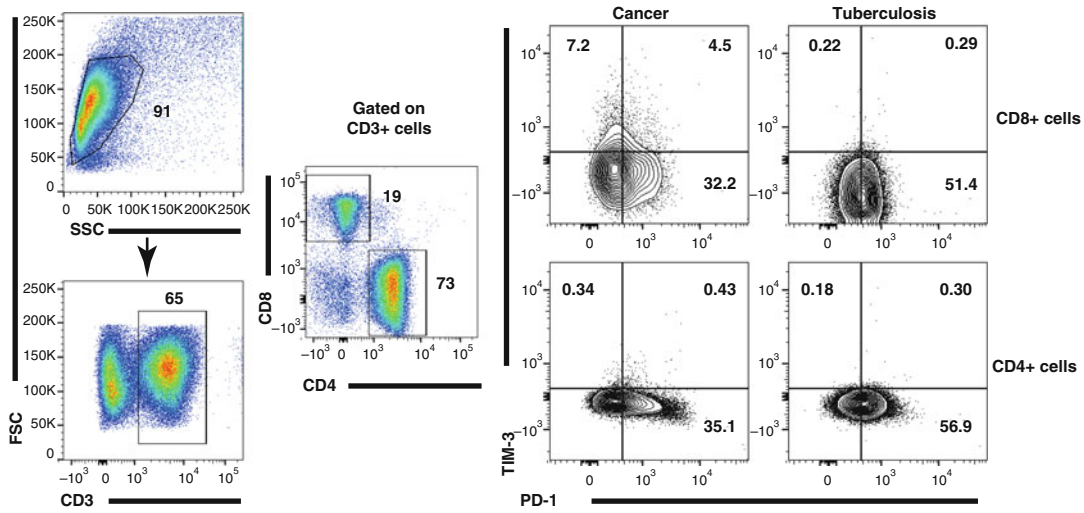

Fig. 5.4 Gating strategy for the analysis of PD-1 and TIM-3 expression on T cells. PEMCs were plotted first on SSC *vs*. FSC with selection of the lymphocyte population. Gated cells were then plotted on CD3 (PE-Texas Red) *vs*. FSC and further gated on CD4 (Alexa 700) *vs*. CD8 cells (APC-Cy7). CD4$^+$ (*lower row*) or CD8$^+$ (*upper row*) cells were plotted on PD-1 (FITC) *vs*. TIM-3 (APC) 5 % contour outlier plots; quadrants were set according to isotype controls. Immunostaining was done as indicated in Fig. 5.3. Representative data from patients with lung cancer or tuberculosis are shown

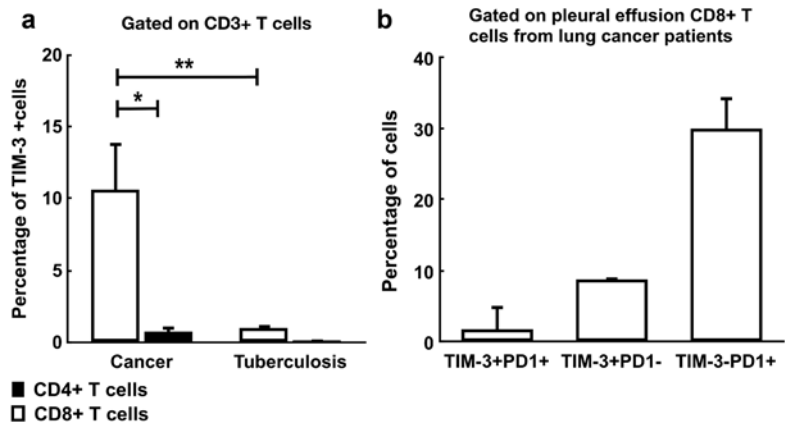

Fig. 5.5 (**a**) Percentage of TIM-3$^+$ cells on CD4$^+$ and CD8$^+$ T cells from lung cancer ($n=9$) and tuberculosis patients ($n=5$). (**b**) Percentage of cells expressing PD-1 and TIM-3 in pleural effusion CD8$^+$ T cells. Determination of TIM-3 and PD-1 was done as indicated in Fig. 5.4. * $p=0.015$, ** $p=0.023$. Bars depict mean ± standard error

PD-1$^+$ [77], in the pleural compartment, most PD-1$^+$CD8$^+$ T cells did not coexpress TIM-3 (Figs. 5.4 and 5.5). Thus, PD-1$^+$CD8$^+$ T cells might be activated in a microenvironment that does not provide the sufficient signals to fully differentiate into effector T cells. Because PD-1 was not coexpressed in TIM-3$^+$ CD8$^+$ T cells (Fig. 5.5), further studies are required to define whether this subset belongs to a population of exhausted CD8$^+$ T cells. Nevertheless, TIM-3 expression is likely responsible for the absence of CD8$^+$ T cell responses in lung cancer patients.

Interestingly, the administration of PD-1 antibody as a blocking agent against PD-1 pathway has shown durable partial tumor regression in patients with lung cancer, which was long thought to be a "non-immunogenic" tumor [65]. Thus,

reactivation of immune responses in lung cancer patients, via blocking PD-1, TIM-3, or other regulatory pathways, in combination with other therapeutic modalities, such as radiotherapy or chemotherapy, will provide major clinical benefits to lung cancer patients.

5.6 Concluding Remarks

T cell exhaustion is a stage of T cell differentiation where T cells show poor effector functions, sustained coregulatory receptor expression, as well as a transcriptional state distinct from memory, effector, and even anergic T cells. From a temporal perspective, T cell anergy possibly occurs during the early stages of tumor progression, whereas T cell exhaustion might play a crucial role in T cell dysfunction during the late stages of cancer. Several types of cancer have been shown to induce T cell exhaustion; this phenomenon is attributed to the tumor microenvironment, which has been shown to provide and maintain the required conditions for T cell exhaustion. Among other conditions, tumor mass is a source of antigens that chronically stimulate infiltrating T cells. In most cancers, tumor cells expressing PD-L1 have been associated with a negative disease outcome. Many tumors also secrete IL-10 and TGF-β, immunosuppressive cytokines that are associated with exhaustion in chronic viral infections.

The reduced functions of T cell observed *in vitro*, the correlation of the clinical prognosis of cancer patients with the expression PD-L1 in tumor cells, and the limited success of T cell-based immunotherapy provide evidence that T cell exhaustion plays an important role as a tumor evasion mechanism from the host immune system. However, caution must be taken with studies defining T cell exhaustion based only on the marker PD-1; thus, it is necessary to evaluate several cell surface and functional markers to define whether T cells are exhausted rather than activated. Baitsch et al. first showed that tumor-specific $CD8^+$ T cells from melanoma patients share similarities with chronic exhaustion observed in viral infections [52]. Nevertheless, it is not clear whether exhausted T cells share similar molecular and genetic patterns in patients with chronic infections and other types of cancer.

Understanding the mechanisms of tumor-induced T cell exhaustion will conduce to the development of vaccine-induced T cells aimed at promoting tumor rejection. Preliminary clinical findings with blockers of immune-regulatory pathways, such as the PD-1/PD-L1 pathway, suggest that this strategy is promising for enhancing antitumor immunity with the potential to produce long-lasting clinical responses.

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Regulatory T Cells and Th17 Cells in Cancer Microenvironment

6

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Contents

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6.1 Introduction

Organs and tissues in the body are highly heterogeneous in producing tissue factors that affect the development and maintenance of immune cells. In general, organs and tissues in the body maintain highly tolerogenic conditions. This is important to prevent unwanted autoimmune or inflammatory responses to harmless antigens and immune stimulants. Tumors, formed in tolerogenic tissue environments, are naturally hypoimmunogenic and utilize a number of mechanisms to actively suppress the generation of effector T cells [1, 2]. Tumors maintain tolerogenic environments to avoid antitumor immune responses. Tumors harbor high numbers of $FoxP3^+$ T cells (commonly called Tregs). Despite the tolerogenic nature of the tumor microenvironment, tumors variably produce many factors that affect T-cell differentiation and maintenance. The numbers of effector T-cell populations in tumors are relatively more variable. Certain cancers are linked to chronic inflammation [3]. Cancers formed in certain tissues, such as the intestine and in patients with chronic infection, are exposed to microbes, which can form inflammatory conditions in tumors. Cancers formed in these tissues would be influenced by inflammatory conditions. Necrotic tumor cells also induce inflammation through damage-associated molecular pattern (DAMP) receptors such as TLR2, TLR4, and the receptor for advanced glycation end products (RAGE) [4]. Inflammatory tumors

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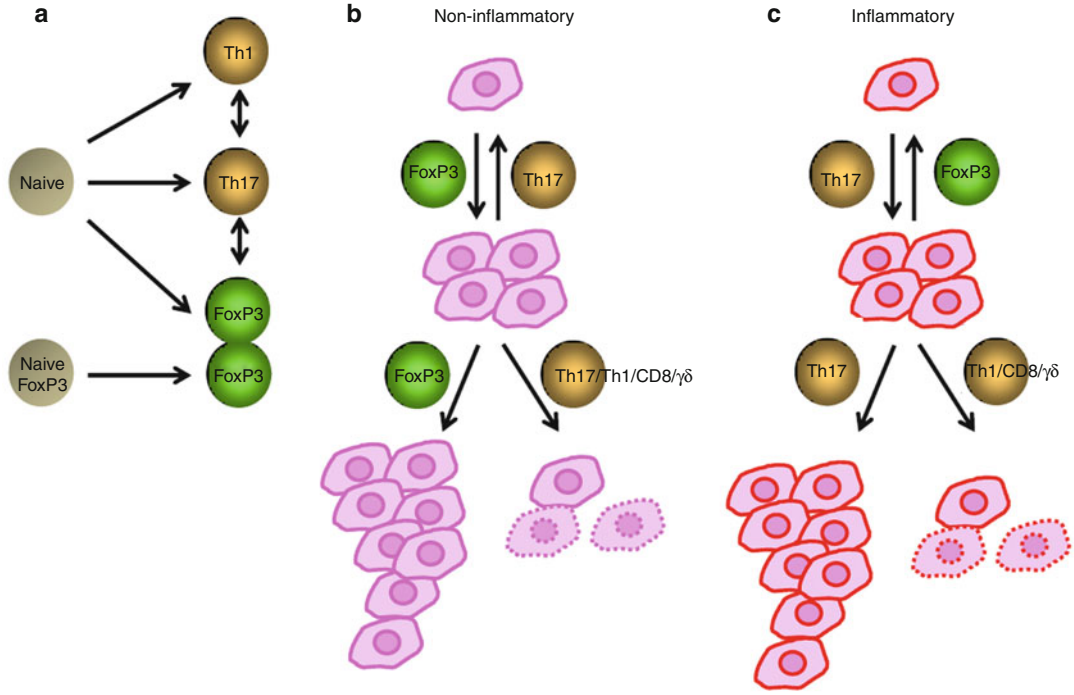

Fig. 6.1 Potential roles of FoxP3⁺ T cells and Th17 cells in tumors. (**a**) FoxP3⁺ T cells are made in the thymus as naïve-type FoxP3⁺ T cells, which migrate to lymphoid tissues. These FoxP3⁺ T cells can become the memory type after activation in secondary lymphoid tissues. Induced FoxP3⁺ T cells with memory-type FoxP3⁺ T cells and Th17 cells are made from naïve CD4⁺ T cells. FoxP3⁺ T cells suppress effector T cells and other immune cells and decrease tissue inflammation. Th17 cells produce IL-17 to induce inflammatory responses. FoxP3⁺ T cells and Th17 cells can trans-differentiate into other T-cell subsets such as Th1 and T-FH cells in appropriate cytokine and antigen priming conditions. (**b**) FoxP3⁺ T cells can promote tumor growth by suppressing antitumor immune responses at early and late stages. On the other hand, Th17 cells can induce immune responses that lead to eradication of tumor cells in a manner similar to other effector, CD8⁺, and γδ T cells. (**c**) In inflammatory conditions, FoxP3⁺ T cells and Th17 cells have the potential to play different roles. Th17 cells cause inflammation in tissues; hence, inflammatory tumors are formed and stimulated to grow. FoxP3⁺ T cells suppress the function of Th17 cells and other inflammatory T cells, leading to suppression of the tumorigenic process in inflamed tissues

harbor FoxP3⁺ T cells and effector T cells, including Th17 cells and Th1 cells [5, 6]. FoxP3⁺ T cells can suppress the function of antitumor effector T cells and other immune cells to promote tumorigenesis (Fig. 6.1). On the other hand, FoxP3⁺ T cells can suppress tissue inflammation to prevent the emergence of tumor cells following chronic tissue inflammation. Effector T cells produce inflammatory cytokines that promote tumorigenesis by increasing tissue inflammation and angiogenesis, but they can also promote antitumor immunity. An inverse correlation was observed between frequencies of FoxP3⁺ T cells and effector T cells such as Th17 cells and Th1 cells [7–9]. In certain cancers, FoxP3⁺ T cells increase, whereas Th17 cells decrease in number as cancers advance to more aggressive stages [9]. The presence of FoxP3⁺ T cells and Th17 cells in tumors and associated tissues not only reflects the nature of tumor microenvironments but also indicates the types of active T-cell-mediated immune responses in tumors. In this chapter, we will discuss tumor factors that regulate T-cell differentiation into Tregs and Th17 cells, migration of the T-cell subsets into tumors and associated lymphoid tissues, and the functions of Tregs and Th17 cells in regulating antitumor immune responses.

6.2 Diversity of Tumor Microenvironments and Tumor Tissue Factors

Tumor microenvironment is highly heterogeneous, depending on tumor types and sites of formation. Together with tumor cells, fibroblasts, myofibroblasts, endothelial cells, mast cells, and other tissue cells make up tumors. Moreover, immune cells are an important component of tumors and are mainly composed of T cells, B cells, innate lymphoid cells, and myeloid cells. Tumor-associated myeloid cells are heterogeneous as well and contain immature and mature myeloid cells.

Myeloid-derived suppressor cells (MDSC) are immature myeloid cells and highly enriched in tumors [10]. MDSC are composed of heterogeneous myeloid cells at various different stages. Compared to mature myelocytes such as dendritic cells (DCs) and macrophages, MDSC do not highly express cytokines, co-stimulatory molecules, and MHC class molecules. Therefore, they poorly support antitumor effector T-cell responses. Moreover, MDSC express various molecules that dampen immune responses. MDSC produce peroxynitrite for nitration and nitrosylation of many proteins in the tumor environment [11, 12]. A major target protein for nitration and nitrosylation is TCR, which becomes ineffective at activating T cells after the modifications [13]. They also express Arg1, inducible nitric oxide synthase (iNOS), and TGF-β1, among others [14]. Tumors also harbor many macrophages, which can be made from MDSC or myeloid progenitor cells [15]. Dendritic cells express indoleamine 2,3-dioxygenase (IDO) to regulate available tryptophan [16]. Other immune cells such as mast cells, NK cells, $CD8^+$ T cells, and B cells are frequently found in many tumor types.

The tumor environment is low in both oxygen and pH. Tumor cells rapidly divide and therefore vigorously consume oxygen supplied via blood vessels. Tumor cells mainly utilize the aerobic glycolysis pathway to generate energy [17]. This can accumulate lactic acid and protons, leading to low extracellular pH [18]. The most common pH range in tumors is 6–6.5. The low acidic tumor environment leads to immune cell anergy. For example, cytotoxicity and cytokine secretion by $CD8^+$ T cells are impaired at the low pH range [19].

Cells in the tumor microenvironment produce various cytokines and growth factors [20]. Some of these factors are drained into lymphatic vessels and form tumor-associated microenvironmental milieu in lymph nodes. If tumors have tumor-specific or tumor-associated antigens, these antigens are drained or transported into lymph nodes and presented to T cells via antigen-presenting cells (APCs). Effector and regulatory T cells can be made during this antigen priming process. The cytokine milieu is critical in determining the fate of differentiating T cells in tumor-draining lymph nodes. Again, the type and amount of cytokines and other factors produced in tumors are highly diverse among tumor types. Expression of IL-1α, IL-1β, IL-6, IL-11, and TNF-α was observed in colon carcinoma, colon adenoma, ovarian cancer, and gastric cancer [21–27]. IL-2 and IL-15 are expressed in melanoma. IL-10 and TGF-β are expressed in myeloma, colon cancer, lung cancer, and mammary carcinoma [28, 29]. Expression of IL-17, IFN-γ, and IL-4 has been observed in certain tumor types [30–32]. Expression of M-CSF, GM-CSF, and IL-3 has been observed as well [33–35]. These tumor-derived hematopoietic cytokines regulate myeloid cell-mediated inflammation and affect T-cell activity in tumors. Chemokines such as CXCL chemokines (CXCL1, 3, 6, 8, 10, and 12) and CCL chemokines (CCL1, 2, 5, 17, 25, and 28) are expressed in various tumor types [36–39]. Growth and angiogenic factors such as VEGF, EGF, and HGF are broadly expressed in a number of cancer types [40, 41]. The cell types producing these factors are not limited to tumor cells but can be from various cell types in tumors. For example, tumor-associated macrophages produce both inflammatory and immunosuppressive cytokines such as IL-1, IL-6, IL-10, and TGF-β [42].

T-cell receptor (TCR) activation signals are modified by the signals from co-stimulatory and co-inhibitory molecules, which are expressed by tumor cells and tumor-associated APC [43]. These molecules include B7-1, B7-2, programmed cell death-1 ligand (PD-L1), PD-L2, ICOS-L, B7-H2, B7-H3, B7-H4, and B7-H6. Among these,

PD-L1-PD and B7-1/2-CTLA-4 play important roles in the formation of Tregs in tumor microenvironments [44–46]. Moreover, TNF receptor family members such as OX40, GITR, 4-1BB, and CD40 are expressed in tumors and regulate antitumor immune responses [47, 48].

Inflammatory mediators are produced in tumors. Cyclooxygenase-2 (COX-2) is highly expressed in malignant tumors [49, 50]. COX-2 expression is induced in hypoxic conditions or by cytokines and growth factors [51]. COX-2 generates prostaglandin H2 from arachidonic acid, which is processed to generate major inflammatory mediators such as PGD2, PGE2, PGI2, and TXA2. These mediators regulate angiogenesis and various aspects of inflammatory responses in tumors [49].

Some tumor types are under the influence of microbe-associated molecular pattern (MAMP) receptor ligands if tumors are formed in barrier tissues such as the intestine or in patients infected with pathogens. In mucosal tissues, decreased barrier functions due to tumorigenesis or preexisting inflammation can lead to bacterial invasion and induction of inflammatory responses. Furthermore, tumors that are associated with infection by papillomavirus (uterine cervical carcinoma), hepatitis B virus (hepatocellular carcinoma), Epstein-Barr virus (Burkitt's lymphoma), human T-cell leukemia virus (adult T-cell leukemia), or herpes virus (Kaposi's sarcoma) would be influenced by viral MAMPs. MAMPs and DAMPs activate Toll-like receptors (TLRs) [52]. TLR activation can induce tissue inflammation that promotes cancer [53]. MYD88 signaling is also required for activation of dendritic cells for proper formation of effector T cells. Without proper MYD88 signaling, Th2 cells ineffective in antitumor immunity can be made [54]. TLR signaling can work together with STAT3 and Notch pathways to activate MAPK and NFkB, which promote the survival and proliferation of tumor cells [55].

Retinoic acid is an anticancer agent. Retinoic acids such as all-trans retinoic acid (ATRA) and 9-cis RA are produced from retinol (vitamin A) by retinol metabolizing enzymes such as ADH and RALDH [56]. Epithelial cells and APCs in the intestine highly express these enzymes [57]. RALDH2 expression is induced during immune responses to increase the concentration of RA available in local tissue environments. Inflamed tissues or tumors are low in expression of RA-producing RALDH but are high in expression of RA-catabolizing CYP26 [58, 59]. In sum, the tumor microenvironment is made of highly diverse factors. Some are from tumor cells, while others are from tissue cells and immune cells. These factors have profound effects on T cells in tumors and associated lymphoid tissues as discussed in detail later in this chapter.

6.3 Generation of Tregs and Th17 Cells

FoxP3$^+$ Tregs are made in the thymus as natural FoxP3$^+$ T cells. They are also induced in the periphery from naïve CD4$^+$ T cells. In addition, IL-10-producing Tregs (Tr1 cells) are made from naïve CD4$^+$ T cells. Tregs produce suppressive cytokines such as IL-10, IL-35, and TGF-β [60–62]. These Tregs play critical roles in preventing autoimmune diseases. Tregs are generally made whenever effector T cells are formed during immune responses. This is important to limit the potentially inflammatory activities of effector T cells.

Induction of effector T cells and Tregs occurs mainly in secondary lymphoid tissues. One reason for this is that naive CD4$^+$ T cells that become effector T cells and Tregs migrate mainly to secondary lymphoid tissues. However, memory/effector T cells can trans-differentiate into each other at any tissue sites upon antigen priming (Fig. 6.1a). Th1 cells are the most readily made effector T cells from naïve CD4$^+$ T cells. IL-12, a cytokine produced from DCs, promotes the generation of Th1 cells. Th2 cells are made when IL-4 is abundant. Th17 cells are generated when IL-6, TGF-β, and other inflammatory cytokines are present during T-cell priming. MAMPs and TLR activation in tissues promote the generation of Th17 cells. Th1 cells are efficient in the promotion of cell-mediated immunity through production of IFN-γ Th17 cells that are effective at inducing inflammatory conditions through producing IL-17. A number of inflammatory cytokines, neutrophil-attracting chemokines, and

inflammatory mediators are induced by IL-17 [63]. IL-2 is required for the induction of T-cell proliferation. IL-7 and IL-15 drive proliferation of T cells in an antigen-independent manner in lymphopenic conditions [64, 65]. IL-2 suppresses the formation of Th17 cells [66]. IL-4, while inducing Th2 cells, suppresses the formation of induced FoxP3$^+$ T cells and Th1 cells [67, 68]. IL-27 promotes the generation of Tr1 cells [69, 70]. Expression or activation of specific transcription factors is required for the generation of specialized effector T cells and Tregs. For example, RORγt, STAT3, and AHR are important for Th17 cells. FoxP3 and STAT5 are important for the formation of induced Tregs. c-Maf and aryl hydrocarbon receptor (AHR) are important for formation of Tr1 cells [61, 60, 71]. Beyond cytokines, many other factors can modulate the generation of Tregs and Th17 cells. This subject will not be discussed in detail, as the generation of Tregs and Th17 cells during basic immune responses is exhaustively discussed elsewhere.

6.4 Impact of Tumor-Derived Factors on Regulation of T-Cell Differentiation

Most T cells in tumors are memory T cells [72]. Both antigen-specific and nonspecific bystander T cells would be present in tumors. In general, the presence of memory T cells and CD8$^+$ T cells is linked to positive prognosis in cancer patients. This indicates that it is beneficial to have these T cells in tumors. About 30–50 % of CD4$^+$ T cells in various tumors formed in animals are FoxP3$^+$ T cells [72]. Th17 cells are also found in tumors, particularly tumors formed in mucosal tissues [73, 7, 74]. In contrast, Th17 cells are hard to find in transplanted tumors in animal models at ectopic sites [72]. Many factors of the tumor microenvironment can promote the generation of FoxP3$^+$ T cells. First, APCs in tumor environments are prone to generate FoxP3$^+$ T cells. During infection, DCs uptake antigens and undergo maturation in response to TLR activation. Activated DCs emigrate tissue sites of infection and migrate into secondary lymphoid tissues through lymphatic vessels. Only mature DCs express MHC molecules and co-stimulatory molecules such as B7-1 and B7-2 at high levels. In tumors, the signals to maturate DCs are diverse and not as apparent as those in infection. Thus, APCs maturated in tumor microenvironment do not highly express the co-stimulatory molecules [75]. Moreover, tumor-associated APCs express co-inhibitory receptor ligands such as PD-L1 and PD-L2 [76, 77]. This affects T-cell activation and differentiation. Therefore, DCs in or from tumors have low activation potentials for T cells. This condition typically generates induced FoxP3$^+$ T cells but not effector T cells. Other APCs in tumors, such as macrophages and MDSC, are also ineffective in generating effector T cells but are prone to induce Tregs [78].

As mentioned, the hypoxic condition in the tumors is another regulatory factor for T cells [79]. It is expected that draining lymph nodes or tertiary lymphoid tissues within tumors have low oxygen levels. T cells become FoxP3$^+$ T cells when they are activated in hypoxia [80]. This is in part mediated by a transcription factor called HIF-1α. The high glycolytic activity in tumors leads to accumulation of lactic acid [81–83]. This promotes the generation of FoxP3$^+$ T cells. TGF-β1 is a characteristic cytokine produced in the tumor environment [84–86]. TGF-β1 is the most efficient cytokine that induces FoxP3$^+$ T cells in the periphery. Along with TGF-β1, IL-10 acts to suppress antitumor immune responses and the promotion of Tregs [87, 88]. IL-10 is produced by various cell types, including T cells, myeloid cells, B cells, and tumor cells.

PGE2 is highly produced in the tumor environment. PGE2 induces FoxP3$^+$ T cells. This induction is mediated by EP4 and EP2 receptors [89, 90]. In this regard, inhibition of cyclooxygenase-2 (COX-2) decreased *FoxP3* expression in tumors and reduced tumor burden [91]. Interestingly, FoxP3$^+$ Tregs express COX-2 and produce PGE2 [92]. The PGE2 produced by Tregs suppresses effector T cells. In addition, prostaglandin D2 (PGD2) acts on DCs to induce FoxP3$^+$ T cells [93]. This effect is mediated through the D prostanoid receptor and cyclic AMP-dependent protein kinase A. In this regard, enforced expression of COX-2 in head and neck squamous cell carcinoma led to expansion of IL-10$^+$ FoxP3$^+$ T cells [94].

Commensal bacterial products that activate TLR2 are implicated in selectively promoting FoxP3+ T cells and Th17 cells. Segmented filamentous bacteria (SFB) promote Th17 cells in the small intestine [95]. Certain bacterial groups such as *Clostridium* and *Bacteroides fragilis* promote the generation of FoxP3+ T cells in the intestine [96, 97]. Tumors, formed in the intestine, female reproductive tract, and skin, are expected to be heavily influenced by commensal bacteria. In these tumors, bacterial MAMPs would activate APC and T cells to regulate the generation of FoxP3+ T cells and Th17 cells. Thus, depending on the bacterial group that is dominant in the tumor environment, FoxP3+ T cells and Th17 cells can be differentially generated.

As mentioned, retinoic acid is an important tumor factor. Retinoic acid affects T cells and tumor cells. Retinoic acid promotes the generation of FoxP3+ T cells but suppresses that of Th17 cells [98, 99]. Retinoic acid affects the development of DCs and generates tolerogenic DCs expressing Arg1 [100]. These DCs promote the generation of FoxP3+ T cells but suppress the formation of Th17 cells. This function seems to be mediated through RAR-α. It is also reported that retinoic acid at low concentrations (i.e., 0.5–5 nM) is required for normal function of effector T cells [101, 102]. Low concentrations of RA are found in bodily fluids in most tissues. In vitamin A deficiency, the migration and function of effector T cells are severely impaired. As mentioned, tumor cells express CYP26 and can decrease retinoic acid concentration in tumors and associated tissues [58]. This hyporetinoic acid condition would significantly affect the T-cell profile in tumors and associated lymphoid tissues. Moreover, retinoic acid can promote differentiation of tumor-associated MDSC into dendritic cells and macrophages [103].

6.5 Migration of Tregs and Th17 Cells into Tumors

Migration of T cells, including Tregs and Th17 cells, is regulated by trafficking receptors such as chemokine receptors and adhesion molecules [104, 105]. Adhesion molecules such as selectins and integrins mediate rolling and firm adhesion of leukocytes on endothelial cell vessels [106, 107]. Chemokines induce integrin activation between rolling and firm adhesion of immune cells on endothelial cells. Chemokines also induce chemotaxis for migration of immune cells within tissues. Organs and tissues express distinct and overlapping chemokines and adhesion molecules for regulation of immune cell migration [108]. Since tumors are formed within specialized organs and tissues, there are similarities in expression of trafficking signals between normal tissues and tumors formed within the tissues. Compared to normal tissues, however, tumors have altered expression of chemokines and adhesion molecules [109]. The trafficking signals and receptors required for T-cell migration into the intestine are well established. In the intestine, CCL20 and CCL25 are, respectively, expressed in the subepithelial cell dome (SED) of Peyer's patches and by small intestinal epithelial cells [110–113]. Endothelial cells in the intestine, Peyer's patches, and mesenteric lymph nodes express mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [114]. T cells migrating to the small intestine express *CCR9* and *α4β7* [115–117]. Memory T cells migrating to the Peyer's patches express CCR6 [118, 119]. Naïve T cells migrating to Peyer's patches, MLN, and PLN express CCR7, α4β7, and CD62L [120]. Memory T cells migrating to other tissues or inflamed tissues variably express CCR1-6, CCR8, CCR9, CCR10, CXCR3, CXCR5, and CXCR6 [108]. Effector T cells frequently express P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), CXCR3, CCR5, and CCR4 [105, 120].

The trafficking receptors of Tregs and Th17 cells have been determined. FoxP3+ T cells that are freshly made in the thymus express CCR7, CXCR4, and CD62L [121, 122]. FoxP3+ T cells activated or induced in the periphery express memory-type trafficking receptors that are frequently expressed by Th1 or Th2 cells. Th17 cells express most memory-type chemokine receptors [123, 124]. CCR6 is a characteristic chemokine receptor expressed by most Th17 cells. In general, FoxP3+ Tregs and Th17 cells follow the trafficking pattern of conventional naïve and memory/

effector T cells. Conventional naïve $CD4^+$ T cells expressing CCR7 and CD62L lose these receptors upon T-cell activation in the secondary lymphoid tissues and migrate into nonlymphoid or inflamed tissues. Various tissue factors influence the expression of trafficking receptors on $FoxP3^+$ T cells and Th17 cells [125, 126]. For example, retinoic acid acts on T cells undergoing activation to induce gut-homing receptors such as CCR9 and α4β7. $FoxP3^+$ T cells and Th17 cells express these gut-homing receptors and migrate to the intestine [98, 127]. In vitamin A deficiency, the number of $FoxP3^+$ T cells and Th17 cells in the gut is significantly decreased in part because most T cells do not migrate to the small intestine [128]. In addition, TGF-β1 is a major cytokine that induces the expression of CCR6 on $FoxP3^+$ T cells and Th17 cells [123]. Moreover, IL-2 is a cytokine that effectively downregulates CCR6 expression induced by TGF- β1. Thus, cytokines and tissue factors can co-regulate the expression of trafficking receptors on T cells.

Researchers have been searching for chemokines that regulate immune cell trafficking and antitumor immune responses [129–133]. Chemokines such as CCL3-5, CCL20, and CXCL10, often expressed in inflamed tissues, are also expressed in tumors [134–139]. Chemokines induce chemotaxis of immune cells and tumor cells. They can co-stimulate T cells and promote angiogenesis [140, 141]. CCR2-10 and CXCR3-5 regulate T-cell trafficking in various tumors [132]. Most of these receptors are highly expressed by $FoxP3^+$ T cells and Th17 cells in mice and humans [105, 123, 124, 121, 122, 142]. CCL17 and CCL22 are highly expressed in gastric cancer with CCR4-expressing $FoxP3^+$ T cells [131]. CCR7 is expressed by some T cells in colorectal cancers and is predictive of positive prognosis [143]. $CXCR4^+$ T cells are increased in lung adenocarcinoma [144]. Chemokines expressed in tumors also attract hematopoietic progenitors, myeloid cells, NK cells, and $CD8^+$ T cells [136, 145, 10]. An important point is that chemokine signals in cancer patients are highly diverse among different tumors. They are also affected by tissue sites and inflammatory responses in tumors. Therefore, it is difficult to find universal trafficking signals which govern T-cell trafficking in most tumors.

Our group investigated the trafficking receptors expressed by tumor-infiltrating $FoxP3^+$ T cells [72]. $FoxP3^+$ T cells account for 25–50 % of $CD4^+$ T cells infiltrating A20, CT26, 4T1, and B16 tumors. Most of these $FoxP3^+$ T cells are memory $CD44^+$ $CD62^-$ T cells, which are downregulated for CD62L and CCR7. Downregulation of CCR7 was critical for the migration of $FoxP3^+$ T cells into tumors, as $CCR7^{high}$ $FoxP3^+$ T cells were not efficient at migrating into tumors [72]. Downregulation of CCR7 and CD62L occurs in tumor-draining lymph nodes during antigen priming. Therefore, migration of T cells into secondary lymphoid tissues is required to acquire a proper trafficking receptor phenotype for migration into tumors. While downregulated for CCR7 and CD62L, tumor-infiltrating $FoxP3^+$ T cells express CCR8 and CXCR4 at high levels [72]. This trafficking receptor phenotype reflects the differentiation status of the tumor-infiltrating T cells and/or the trafficking receptor requirement for $FoxP3^+$ T-cell migration into the tumors. Induction of $FoxP3^+$ T cells from $FoxP3^-$ T cells in tumors was assessed, and the results indicate that this induction is inefficient [72]. Thus, the tumor-infiltrating $FoxP3^+$ T cell in these tumors is largely from the $FoxP3^+$ T cells made in the thymus or secondary lymphoid tissues rather than $FoxP3^+$ T cells induced directly in tumors. However, this can be quite different in other types of tumors where the tumor microenvironment is more conducive in priming T cells for differentiation into Tregs. In tumors, $FoxP3^+$ T cells appear highly stable in maintaining their FoxP3 expression. While detailed information on Th17 cell migration into tumors is not available, Th17 cells would probably utilize the same tissue- or inflammation-associated trafficking signals utilized by Th17 cells for regulation of general immune responses. Th17 cells are prevalent in the gastrointestinal (GI) tract and other mucosal tissues. High numbers of Th17 cells were observed in aggressive forms of GI cancers [73, 7, 74]. Thus, these tumors would have trafficking and cytokine signals appropriate for recruitment and maintenance of Th17 cells or their progenitors.

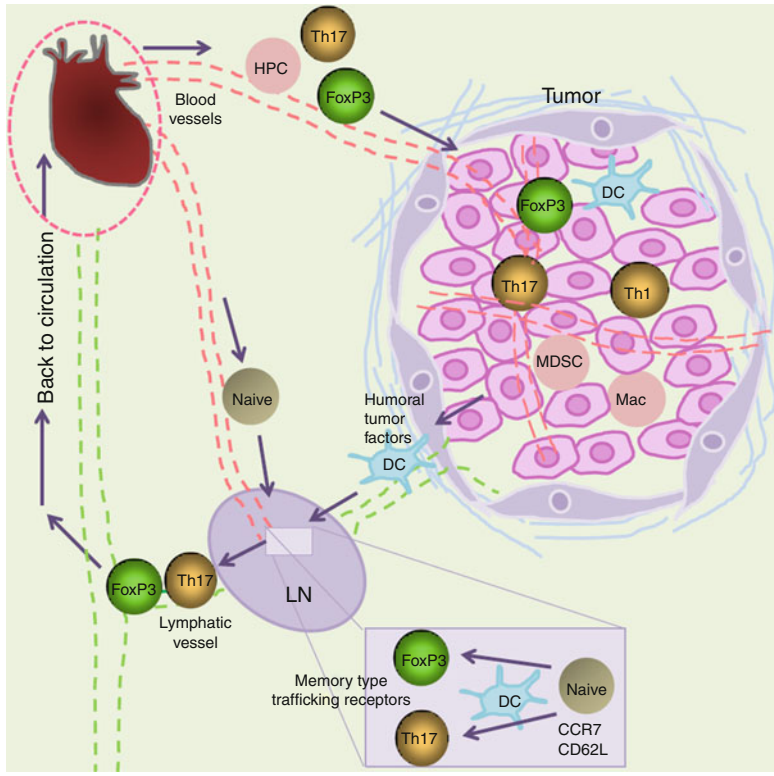

Fig. 6.2 Migration of FoxP3⁺ T cells and Th17 cells into tumors. Natural FoxP3⁺ T cells made in the thymus can migrate into lymph nodes, but cannot migrate directly into tumors unless tumors are formed in lymphoid tissues. FoxP3⁺ T cells can migrate into tumors after they are antigen primed in secondary lymphoid tissues and gain the memory/effector-type trafficking receptors. Loss of CCR7 and CD62L occurs during antigen priming and is required for migration of antigen-primed FoxP3⁺ T cells into tumors. Induced FoxP3⁺ T cells in the tumor-draining lymph nodes can migrate into tumors, as they are down-regulated for CCR7 and CD62L but upregulated for memory/effector-type trafficking receptors such as CCR4, CCR5, CCR8, CCR10, and/or CXCR4. Dendritic cells (DCs) transport and present tumor tissue antigens and play important roles in the generation of FoxP3⁺ T cells and Th17 cells in lymph nodes. Soluble tumor tissue factors are collected in tumor-draining lymph nodes, and some affect T-cell priming and differentiation. In tumors, macrophages (Mac), DCs, and MDSC suboptimally activate T cells in tumors. These APCs play potentially important roles in maintaining the phenotype of FoxP3⁺ T cells and Th17 cells in tumors. There is no such thing as tumor-specific trafficking receptors. Instead, T cells variably use conventional trafficking receptors to migrate into different tumors

Migration of FoxP3⁺ T cells and Th17 cells into tumors and draining lymph nodes is summarized in Fig. 6.2.

6.6 Impact of Tregs and Th17 Cells on Antitumor Immune Responses

The presence of T cells in tumors is a highly reliable prognostic factor for survival of cancer patients [146, 147]. There is a strong positive correlation between patient survival and frequencies of memory CD4⁺ T cells and CD8⁺ T cells in many cancer types. Tumorigenesis is increased in pan-T-cell- or γδ-T-cell-deficient animals or humans [148]. Strikingly, αβ T cells have a small negative effect on tumor numbers, but a greater positive effect on tumor size. This implies that αβ T cells are composed of heterogeneous subsets with different functions, and some of these T cells may even promote tumor growth. FoxP3⁺ T cells and other regulatory T cells are likely the T cells that suppress antitumor immune responses. FoxP3⁺ T cells can inhibit antitumor immune responses and promote tumor growth

[149]. Many FoxP3$^+$ T cells are self-reactive and effective in preventing autoimmune diseases. The same function can be used to promote tumor growth. This is because tumor cells basically express self-antigens, and FoxP3$^+$ T cells can effectively suppress immune responses to self-antigens [150]. In the same line, the frequencies of FoxP3$^+$ T cells in many tumor types are inversely correlated with patient survival rates [151, 147]. However, lack of correlation or positive correlation has been noticed as well [152, 153]. A good example is colorectal carcinoma, in which high frequencies of FoxP3$^+$ T cells are associated with a favorable prognosis [5]. It is expected that FoxP3$^+$ T cells can even prevent the formation of some tumors by suppressing tissue inflammation at early stages of tumorigenesis. Therefore, FoxP3$^+$ T cells have the potential to either promote or suppress tumorigenesis depending on tumor type, tissue site, and immune response. The potentially complex functions of Tregs in tumorigenesis are depicted in Fig. 6.1.

It has been observed that Th17 cells can promote CD8$^+$ T-cell-mediated antitumor immune responses in a mouse model [154]. Moreover, polarization of CD8$^+$ T cells into Tc17 cells increased their antitumor immunity [155]. Th17 cells may become Th1 cells or activate CD8$^+$ T cells to increase antitumor immunity. Paradoxically, Th17 cells can cause inflammation to initiate development of inflammatory tumors at early stages of tumorigenesis. In colorectal cancer, Th17 cells are linked to poor prognosis, whereas Th1 cells are positively linked to patient survival [156]. The major cytokine product of Th17 cells, IL-17, can induce tissue inflammation and the expression of certain angiogenic factors, including CXCL8, MMP-2, MMP-9, and VEGF [157]. The function of Th17 cells in cancer can be complex and appears to be determined again by cancer type, stage, and site. The potentially complex functions of Th17 cells in tumorigenesis are depicted in Fig. 6.1.

Apart from their effector functions, the frequencies of FoxP3$^+$ T cells and Th17 cells reflect the context of the tumor microenvironment. Noninflammatory tumors with low expression of IL-6 and other inflammatory cytokines would have high numbers of FoxP3$^+$ T cells, whereas inflammatory tumors with high expression of inflammatory cytokines would harbor high numbers of Th17 cells. Tumors are heterogeneous in the tumor microenvironment even within the same group of cancers, and not all tumors fit into the inflammatory *vs*. noninflammatory tumor model. While there is an inverse correlation between FoxP3$^+$ T cells and Th17 cells, both T-cell subsets can be increased or decreased depending on the balance of cytokines and other tissue factors. An example for this situation is invasive ductal breast carcinoma [157].

6.7 Concluding Remarks

As discussed throughout this chapter, FoxP3$^+$ T cells and Th17 cells play both positive and negative roles in regulating antitumor immune responses (Fig. 6.1). Despite the presence of these T cells, some tumors still develop and grow. Thus, these T cells by themselves are not sufficient to effectively mount antitumor immune responses. More detailed studies on FoxP3$^+$ T cells and Th17 cells in various tumors can provide systematic information regarding the tumor microenvironment and therapeutic interventions. It is important to develop novel strategies to boost the beneficial effects of the T-cell subsets and to suppress their tumor-promoting effects. The key is to alter tumor microenvironment to regulate these T-cell subsets. This is expected to be achieved through control of antigen-presenting cells, metabolism, cytokines, chemokines, co-stimulatory/inhibitory receptors, inflammatory mediators, and nuclear hormone receptor ligands such as retinoic acid. Regulation of multiple factors at the same time would provide more effective strategies in tipping the T-cell balance toward tumor-eradicating immune responses. A one-size-fits-all approach is not likely to be effective in changing the microenvironment and T-cell activity in all tumors. In this regard, another point is that antitumor therapy strategies should be tailor-made based on cancer type, tissue site, and tumor microenvironment. It is expected that application of wrong immunotherapy strategies to regulate the T-cell subsets could even worsen the prognosis of cancer patients. More research

into classification of cancer types based on tumor microenvironment and immunological milieu would be highly useful.

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Role of Cytokines in Tumor Immunity and Immune Tolerance to Cancer

7

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Contents

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7.1 Introduction

Strong evidence has been accumulated demonstrating that cancer cells in humans and animals are recognized in general as nonself by the immune system [1, 2]. Both innate and adaptive immune reactions to cancer have been described. Many cases of spontaneous tumor regression in patients with cancer have been reported. In addition, such spontaneous regressions normally occur following an infection. Moreover, immunosuppressed patients are at increased risk for virally induced tumors [3]. In fact, the presence of highly adaptive immune cell infiltrates within the tumor can be a positive prognostic indicator of patient survival [4]. Murine models of spontaneously arising or chemically induced tumors have been useful in demonstrating that the immune system naturally surveys for aberrant cells and has an important role in preventing tumor formation [2].

An antitumor immune response is initiated when the cells of the innate immune system become alerted to the presence of a growing tumor, at least in part owing to the local tissue damage that occurs as a result of stromal remodeling process integral to the basic physiology of solid tumor development [2, 5]. Once solid tumors reach a certain size, they begin to grow invasively and require an enhanced blood supply that arises as a consequence of the production of angiogenic proteins [6]. Invasive growth causes minor disruptions within the surrounding tissue that induces

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inflammatory cytokines and chemokines leading to recruitment of cells of the innate immune system [7]. The innate response includes several cellular factors, such as natural killer (NK) cells, natural killer T (NKT) cells, γδ T cells, macrophages, dendritic cells (DCs), and neutrophils [8]. These cells can reject tumors either by direct killing of the tumor cells or by inhibition of angiogenesis. The components of innate immunity use pattern recognition receptors and other cell surface molecules to detect tumor cells. Cancer cells express families of stress-related genes such as MHC class I-related stress-inducible surface glycoprotein A and B (MICA and MICB), which function as ligands for NKG2D receptors expressed on NK cells [9]. In addition, NK cells can be triggered for cytolytic activity by DCs depending on direct cell contact through their expression of cell surface molecules such as CD48 and CD70 which are ligands for NK cell-activating receptors 2B4 and CD27, respectively [9]. The DCs that have been recruited to the tumor site become activated either by exposure to the cytokine milieu created during the ongoing attack by the innate immune system or by interacting with NK cells. The activated DC can acquire tumor antigens directly by ingestion of tumor cell debris or potentially through indirect mechanisms involving transfer of tumor cell-derived heat shock protein/tumor antigen complexes [10]. The activated antigen-bearing DCs then migrate to the draining lymph nodes, where they trigger the activation of tumor antigen-specific $CD4^+$ Th1 cells. In addition, DCs activate $CD8^+$ cytotoxic T lymphocytes (CTL) via cross-presentation of tumor antigenic peptides on MHC class I molecules [11]. Activated tumor-specific $CD4^+$ and $CD8^+$ T cells home to the tumor site where they kill tumor cells. Mice lacking adaptive immunity (RAG-2 gene-deficient mice lacking T cells) were more susceptible to carcinogen-induced and spontaneous primary tumor formation. Thus, the development of adaptive immunity may provide the host with the capacity to completely eliminate the developing tumor. However, the development of clinically evident cancers indicates that these innate and adaptive immune responses are not always enough to prevent disease progression as cancer cells manage to escape host-tumor immunity.

Tumors use several mechanisms that facilitate immune escape and prevent tumor elimination including impairment of antigen presentation, activation of negative co-stimulatory signals, and elaboration of immunosuppressive factors [12]. In addition, tumor cells may promote the expansion and/or recruitment of regulatory immune cell populations which can contribute to the immunosuppressive network; these populations include regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and distinct subsets of immature and mature regulatory DCs [12]. All these host-derived immune cell populations can impair antitumor effector cell responses, both locally at the tumor microenvironment and systemically in the lymphoid organs. In fact, both tumor-promoting and tumor-inhibitory immune cell populations have been found in patients with various cancers. Several recent studies have found correlations between particular immune cell infiltrates in tumors and patient prognosis. Infiltration of $CD8^+$ T cells and mature DC is associated with a favorable prognosis in patients with cancer. However, an extensive macrophage infiltration correlates with poor patient prognosis in most of the cancers analyzed. Thus, the complexity of the immune cell populations infiltrating tumors with their synergistic or opposing effects may influence tumor growth differently, depending on their cytokine secretion. A number of immune-enhancing cytokines have been shown to promote or inhibit antitumor immunity in multiple experimental models and in patients with cancer. This chapter reviews the role of antitumor cytokines IL-12 and IL-27 in tumor immunity and immunotherapy while discussing the role of pro-tumor cytokines (TGF-β, IL-17, IL-23, IL-35, and IL-10) that have pathogenic significance in cancer progression.

7.2 Cytokine Regulation of the Antitumor Immune Response

Cytokines comprise a large family of intracellular communicating molecules that play important roles in immunity, inflammation, and repair, as well as general tissue homeostasis. In addition,

cytokines' functions extend to many other aspects of biology, including cancer [13]. In the tumor microenvironment, cytokines are produced by host stromal and immune cells, in response to molecules secreted by the cancer cells. In addition, cancer cells also produce cytokines in the same environment. Increased levels of circulating cytokines and their receptors have been found in patients with various types of cancer, both at diagnosis of the primary disease and in those with metastases, compared with healthy people [14, 15]. The cytokine repertoire present at the tumor site determines the type of host response directed against the tumor. Immunosuppressive cytokines secreted by tumor cells or tumor-infiltrating immune cells can impair the host antitumor response, whereas cytokines promoting the development of T-cell-mediated immunity can induce or enhance antitumor immunity. Studies of cytokine-deficient mice have revealed dual role for the immune system in suppressing and promoting tumor growth.

7.2.1 IL-12

7.2.1.1 Overview

IL-12 is a heterodimeric cytokine containing a 35 kD and a 40 kD subunit that signals through a receptor of the type I family of cytokine receptors. The principal sources of IL-12 are APCs such as DCs and macrophages. Secretion of IL-12 is generally activated via the physiological stimuli of CD40 and toll-like receptors which recognize structurally conserved molecules derived from microbes [16]. IL-12 plays a major role in the development of antitumor immune responses [17]. Numerous studies report that IL-12 promotes an effective destruction of cancer cells through the induction of the innate and adaptive arms of antitumor immunity. In addition, IL-12 has potent antiangiogenic activity. Due to these features, IL-12 has been used as a systemic cancer therapeutic agent, but the clinical development of IL-12 has been hindered by its significant toxicity and disappointing antitumor effects seen in cancer patients. However, emerging studies suggest that IL-12 in combination with other cytokines boosts antitumor immunity by contributing to the development of NK cells and CTLs without any toxic side effects.

7.2.1.2 IL-12: Linking Innate and Adaptive Antitumor Immunity

IL-12 plays an essential role in the interaction between the innate and adaptive arms of antitumor immunity [17] (Fig. 7.1). It induces IFN-γ production by NK cells and T cells. In fact, NK cells and T cells were first shown to express high-affinity receptors for IL-12 [18]. Tumor eradication after vaccinations supported by IL-12 is dependent on NK cells in several animal models [19–21]. IL-12 enhances *in vitro* lysis of both NK cell-sensitive and NK cell-resistant tumor cells. Consistent with animal studies, in patients with cancer, IL-12 enhances the cytolytic activity of NK cells and increases the expression of CD2, LFA-1, and CD56 molecules which mediate NK cell migration [22]. Moreover, IL-12 was shown to enhance the cytotoxicity mediated by NK cells from healthy donors against cancer cells derived from patients with cancer.

In addition to its effect on NK cell cytotoxicity, IL-12 enhances T-cell-mediated cytotoxicity and has an enhancing effect on $CD8^+$ T cells [23]. DCs play a crucial role in facilitating the interaction between $CD4^+$ T cells and antigen-specific $CD8^+$ T cells. Priming of CTL is enabled by the ligation of CD40 on DC and its ligand CD154 on activated $CD4^+$ T cells [24, 25]. The induction of IL-12 synthesis that occurs as a result of CD40 ligation suggests an important role for IL-12 in the molecular mechanisms responsible for CTL priming [26]. It was then shown that IL-12, in the presence of antigen, acts directly on naive $CD8^+$ T cells to promote clonal expansion and differentiation [27]. In addition, priming of $CD8^+$ T cells in the absence of IL-12 rendered them unresponsive to the same antigen [28]. Agonistic CD40 antibodies (Abs) were shown to substitute the function of $CD4^+$ T cells in murine models of T-cell-mediated immunity, resulting in rapid expansion of CTLs that cleared established lymphomas and provided long-term protection against tumor rechallenge [29, 30]. These observations provided an explanation for the impaired

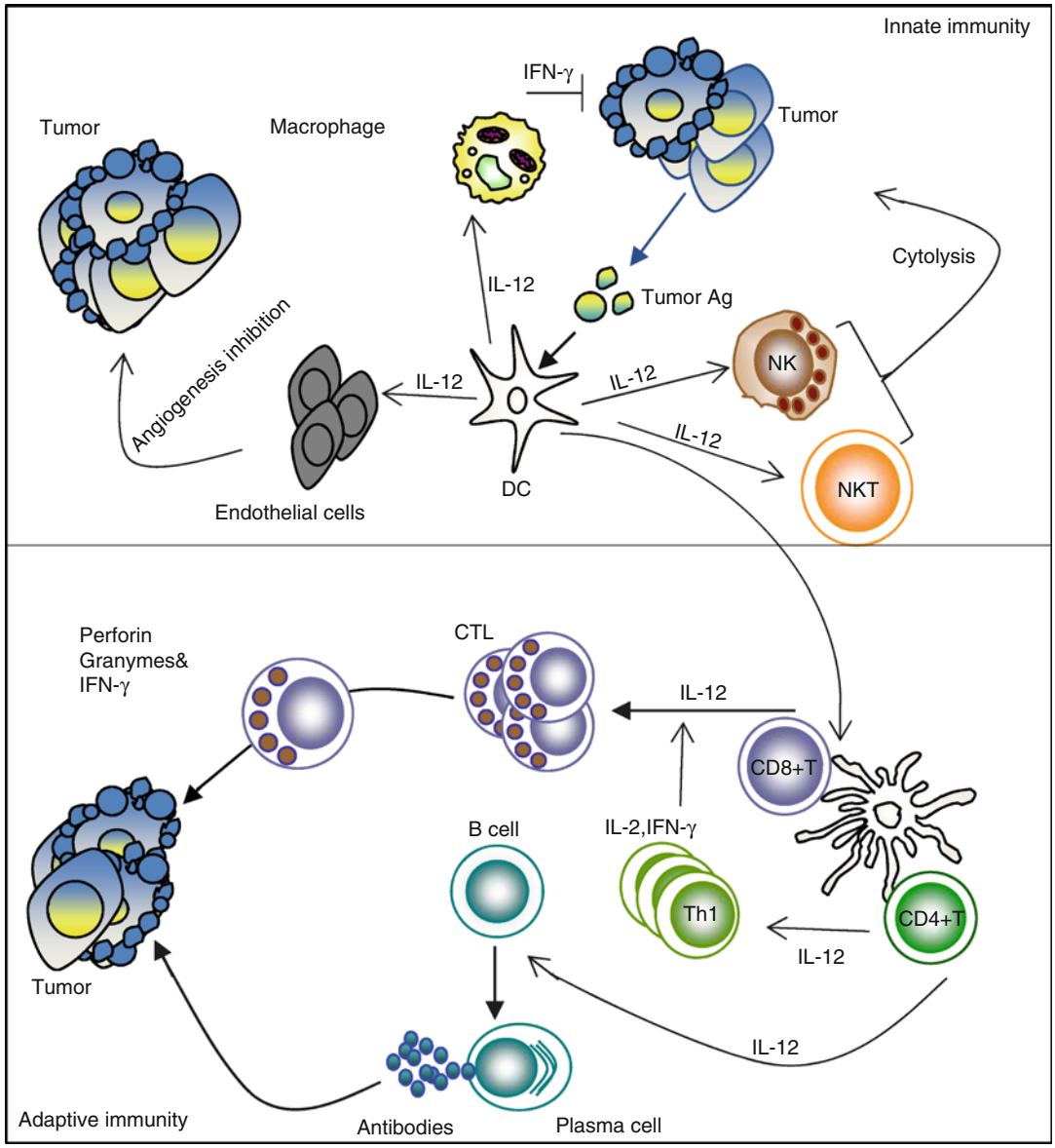

Fig. 7.1 IL-12 links innate and adaptive antitumor immunity. IL-12 utilizes several mechanisms to induce antitumor effects. IL-27 activates innate effectors, such as NK cells, NKT cells, and γδ – T cells and promotes their cytolytic activity and cytokine production. IL-12 induces IFN-γ production in macrophages that can have a cytotoxic effect on tumor cells. IL-12 induces the production of antiangiogenic molecules from endothelial cells. In addition, IL-12 has a direct toxic effect on the some tumor cells. Furthermore, IL-12 secretion by DCs can induce adaptive arms of antitumor immunity. IL-12 can augment Th1 response necessary for cellular immune response. Il-12 stimulates the differentiation and lytic capacity of CTL and promotes immune memory. Finally, IL-12 can mediate antibody-mediated tumor clearance via B-cell activation

tumor antigen-specific CTL activation in CD40-deficient mice and confirmed the key role of the CD40-IL-12 pathway in the regulation of antitumor immunity. A series of experiments, conducted by different groups, indicated that the injection of IL-12 directly into subcutaneous tumors results in CTL response against the tumor in mice [31–33].

The rejection of tumors requires $CD8^+$ T cells whose activation and maintenance depends on $CD4^+$ T cells. Upon stimulation, naïve $CD4^+$ T cells differentiate into different lineages of T helper subsets including Th1, Th2, Th17, and Tregs. These distinct $CD4^+$ T-cell subsets have varied impact on tumor growth. While Th1 cells promote $CD8^+$ T-cell-mediated immunity to tumors, the other $CD4^+$ T-cell subsets Th2 and Tregs negatively regulate $CD8^+$ T-cell function. In the presence of IL-12, naïve $CD4^+$ T cells differentiate into IFN-γ-secreting Th1 cells [34]. Th1 cytokines, IL-2, and IFN-γ, stimulate the cytolytic activity of NK cells. High production of IFN-γ by $CD8^+$ T cells and a Th2 to Th1 shift in the cytokine secretion profile of $CD4^+$ T cells were also seen in the IL-12-treated mice [35]. By altering the balance between Th1 and Th2 cytokines, IL-12 plays a critically important role in antitumor immune responses. A shift from Th1 to Th2 cytokine production has been reported in progressive cancer patients, and a vaccine inducing Th2 to Th1 shift in a murine model of tumor was shown to induce tumor rejection [36]. In addition, Th2 cytokines have been shown to accelerate tumor growth in multiple experimental models [37]. In fact, $CD4^+$ T cells can directly interact with $CD8^+$ T cells via CD40-CD154 interactions [38], which directly contrast with the early notion that $CD4^+$ and $CD8^+$ T cells are brought together on the same antigen-presenting cell for the effective delivery of IL-2 to neighboring $CD8^+$ T cells. Moreover, a full $CD8^+$ T-cell response is elicited by a temporal release of IL-2 from $CD4^+$ T cells, which is consistent with the findings that neutralization of IL-2 significantly limits $CD8^+$ T-cell growth [39]. IL-12 also plays an important role in the establishment of memory $CD8^+$ T cells [40]. A strong specific CTL response was observed in patients with advanced melanoma after administration of IL-12. The number of tumor-specific CTL increased in the circulation, and influx of specific memory $CD8^+$ T cells into metastasized lesions was documented [41]. Additionally, IL-12 was shown to stimulate humoral immunity. In a model of colon carcinoma, vaccination with IL-12-transduced tumor cells cured 40 % of tumor-bearing mice. Favorable antitumor responses were related to the synthesis of Abs against tumor antigens inducing tumor cell lysis in a complement-dependent cytotoxicity assay [42].

The ability of IL-12 to facilitate cell-mediated immune responses, including enhancement of NK cytotoxicity, generation of CTL, and DC activation, suggests its role in both the innate and adaptive immunity resistance mechanisms against tumors. Experimental studies of systemic administration of the cytokine have indicated that IL-12 exerts potent antitumor activity against a variety of metastatic tumors and can even prevent spontaneous tumor development in HER-2/neu transgenic mice. In addition, models based on intra-tumor cytokine delivery or *in vivo* transfer of cytokine-secreting tumors have indicated that IL-12 has significant dose-dependent antitumor activity against a wide spectrum of murine tumors including melanoma and breast, ovarian, and bladder tumors [17, 43, 44]. All these studies have demonstrated that IL-12 can inhibit tumor growth and improve the survival of tumor-bearing animals that are dependent on not only its ability to activate the innate and adaptive arms of antitumor immunity but also through its antiangiogenic activity.

7.2.1.3 IL-12 and Angiogenesis Inhibition

Accumulating evidence indicates that the antitumor effects of IL-12 are mediated, at least in part, through mechanisms involving angiogenesis and its direct effects on tumors. Angiogenesis is an essential process for tumor growth and metastases. In addition, it is the result of a complex balance between angiogenic and antiangiogenic factors. The balance between angiogenic and angiostatic molecules in the tumor microenvironment can determine tumor growth and survival. The antiangiogenic properties of IL-12 were first observed by Voest et al. who demonstrated that IL-12 treatment almost completely inhibited neovascularization in immunocompetent mice, severe combined immunodeficient mice, and T-cell-deficient nude mice [45]. However, suppression of angiogenesis by IL-12 was dependent on its ability to induce IFN-γ expression.

Accordingly, administration of IFN-γ reproduced the antiangiogenic effects promoted by IL-12. Moreover, it was shown that inhibition of tumor growth by IL-12 or IFN-γ required an intact signaling from IFN-γ receptors expressed in neoplastic cells. This indicated that IL-12 could inhibit tumor growth by inducing neoplastic cells to produce antiangiogenic factors. Two of the most relevant factors were identified as the IFN-γ-inducible genes, IFN-inducible protein 10 (IP-10) and monokine induced by interferon-γ (MIG) [46, 47]. Local and systemic treatment with IL-12 was associated with the expression of IFN-γ, IP-10, and MIG in the tumor; in addition, intratumor delivery of MIG into subcutaneously growing tumor in nude mice led to tumor necrosis associated with vascular damage. Administration of neutralizing Abs to IP-10 and MIG substantially reduced the antitumor effects of IL-12 [48]. IP-10 and MIG interact with their receptor CXCR3 to mediate their angiostatic activity. These results support the notion that these chemokines, both ligands of the receptor CXCR3, contribute to the antitumor effects of IL-12 through their inhibitory effect on tumor vasculature. In addition to IFN-γ stimulation, IL-12 promotes the expression of interferon regulatory factors 1 (IRF-1) and 4 (IRF-4), which are necessary for Th1 cell differentiation [49]. IRF-1 has tumor suppressor activities in cancer cells *in vitro* and decreases the tumorigenicity of cells inoculated into athymic nude mice [50, 51]. Similarly, IRF-4 suppresses c-Myc-induced leukemia in animal models and inhibits BCR/ABL-induced B-cell acute lymphoblastic leukemia [52, 53].

Emerging evidence indicates the involvement of lymphocyte-endothelial cell crosstalk at the beginning of the process of angiogenesis inhibition by IL-12. It has been shown that neutralization of NK cell function reversed IL-12 inhibition of angiogenesis in athymic nude mice. Immunohistochemistry analysis revealed that neovascularization inhibited by IL-12 displayed accumulation of NK cells and IP-10-positive cells. In addition, experimental Burkitt lymphomas treated locally with IL-12 displayed tumor tissue necrosis, vascular damage, and NK cell infiltration surrounding small vessels [54]. These results documented that NK cell cytotoxicity of endothelial cells is a potential mechanism by which IL-12 can suppress neovascularization. The antiangiogenic program activated in lymphocytes by IL-12 can also directly affect gene expression in neoplastic cells. In fact, upregulation of signal transducers and activators of transcription-1 (STAT-1) and angiopoietin 2 together with down-modulation of vascular endothelial growth factor (VEGF) has been observed in neoplastic cells exposed to soluble factors released by IL-12-stimulated lymphocytes [55]. In addition, IL-12 treatment reduced the production of metalloproteases, playing a role in matrix remodeling, a process required during neoangiogenesis [56]. Moreover, the activation of integrin αVβ3 on endothelial cells is reduced by the IL-2-induced IFN-γ, which leads to decreased endothelial cell adhesion and survival [57]. IL-12-induced secretion of IFN-γ leads to an increase in p53 activity, which subsequently results in tumor suppression due to the induction of apoptosis in cancer cells [58]. Furthermore, IL-12 dramatically decreased tumor-supportive activities of tumor-associated macrophages (TAMs), which are involved in tumor angiogenesis and metastasis. The antiangiogenic mechanisms mediated by IL-12 are complex and dependent not only on the direct effect on endothelial cells of the proinflammatory cytokine/chemokines induced by IL-12 but also on the recruitment of immune effector cells such as NK and T cells.

7.2.1.4 Regulation of IL-12 in Tumor Microenvironment

Although controlled Th1 and CTL responses can exert a significant antitumor immunity, the same responses, if exaggerated, may result in host-tissue destruction and autoimmunity. Therefore, as a part of immune homeostasis, the inflammatory responses need to be counter-regulated. Tregs play a major role in controlling unwanted immune response to self-antigens [59]. Studies have revealed a significant role for Tregs in defective immune responses to tumor antigens. Treg functions are mediated in part through secre-

tion of immunosuppressive cytokines IL-10 and TGF-β. Both TGF-β and IL-10 can inhibit DC antigen presentation, IL-12 secretion, and effector functions of both $CD4^+$ and $CD8^+$ T cells [12]. Thus, it is possible that as an immunosuppressive environment develops in the growing tumor, DCs secreting IL-12 become scarce. This might be due to an absence of DC activation signals, CD40, or inhibition of activated $CD4^+$ T cells which could themselves activate DC. Moreover, the CD40-CD40L interaction between DCs and T cells leads to the induction of not only IL-12 but also IL-10, a pro-tumor cytokine that may act in an autocrine or a paracrine manner to downregulate IL-12 secretion from DCs [60]. Indeed, reduced CD40 expression on DC or CD40-L on T cells from tumor-bearing hosts may explain the reason for reduced levels of IL-12 observed in patients with cancer [61]. In accordance with this, reduced expression of IL-12 was observed in patients with advanced cancer types including glioblastoma, renal cell carcinoma, head and neck squamous cell carcinoma, gastric cancer, melanoma, colorectal cancer, hepatocellular carcinoma, and gastric cancer [15]. Moreover, IL-12 production by stimulated peripheral blood mononuclear cells decreased significantly in patients with gastric and colorectal cancer with advanced disease. In addition to the immunosuppressive cytokines TGF-β and IL-10, other factors present in the tumor microenvironment can downregulate IL-12 production, for example, prostaglandin E2 (PGE2) produced by tumor cells or tumor-associated host cells (macrophages, endothelial cells, and stromal cells) known to inhibit IL-12 production [62].

7.2.1.5 Clinical Studies with IL-12

Based on the provocative preclinical studies, IL-12 was evaluated in patients with different malignancies. However, clinical experience with IL-12 in humans is limited. Several phase I clinical trials of IL-12 in patients with solid tumors and hematological malignancies have been reported [63]. IL-12 administration in patients with advanced colorectal cancer (CRC), melanoma, and renal cell carcinoma resulted in only one partial response (renal cell carcinoma) and one transient complete response (melanoma), among the 40 enrolled patients. However, common signs and symptoms of toxicity such as fever/chills, nausea, vomiting, fatigue, and headache were observed [64]. Administration of IL-12 resulted in stabilization of the disease in several renal cancer patients and partial regression of a metastatic lesion, but has not proceeded further in clinical development due to signs and symptoms of toxicity, including fever, vomiting, and elevation of hepatic enzymes [65]. Clinical trials of IL-12 treatment in combination with rituximab in patients with B-cell non-Hodgkin lymphoma (NHL) did not result in clinical response [66]. However, several clinical studies revealed positive results with IL-12 administration. During IL-12 treatment in patients with NHL, 21 % of the patients had a partial or complete response without major side effects [67]. Similarly, subcutaneous IL-12 treatment resulted in complete response in 56 % of the treated patients with T-cell lymphoma with minor toxicity [68]. Furthermore, clinical trials on metastatic melanoma revealed that IL-12 administration induces tumor shrinkage in patients accompanied with increased frequency of circulating antitumor CTLs [41]. The low efficacy of IL-12 in the abovementioned clinical trials may be due to an immunosuppressive microenvironment in advanced tumors. In addition, IL-12 may self-limit its own therapeutic efficacy by inducing IL-10 and other suppressive factors. For example, IFN-γ induced by IL-12 can activate immunoregulatory molecules such as programmed death ligand-1 (PD-L1) and indoleamine 2,3 dioxygenase (IDO) on a variety of cells (DC, T cells, and endothelial cells) [69]. Both PD-L1 and IDO can abrogate antitumor immunity through various mechanisms. Furthermore, other factors such as environment and diet may alter the effectiveness of IL-12-mediated anticancer immunity. Although systemic administration of IL-12 in patients is limited by its significant toxicity, emerging studies in animal models indicate that IL-12 in combination with other cytokines boosts antitumor immunity without any toxic side effects [44]. Thus, selective targeted delivery of IL-12 to tumors and/or reducing the

dose of IL-12 while combining it with other therapeutics may yield better outcome.

7.2.2 IL-27

7.2.2.1 Overview

IL-27 is a member of the IL-12 family cytokine that plays potent antitumor effects against various tumor models via different mechanisms, depending on the characteristics of each tumor [70]. Unlike IL-12, IL-27-mediated antitumor functions are independent of IFN-γ, and IL-27-treated mice do not manifest any toxic side effects. IL-27 is mainly produced by activated APCs including DCs and macrophages. DCs secrete IL-27 on exposure to physiological stimuli such as type I and type II interferons (INF) and CD40 [71–73]. In addition, IL-27 expression is induced in APCs upon stimulation by various TLR ligands such as poly(I:C), lipopolysaccharide (LPS), and CpG-DNA, which are agonists of toll-like receptors (TLR)3, TLR4, and TLR9, respectively [74–76].

7.2.2.2 IL-27 in Antitumor Immunity

IL-27 has a wide array of functions necessary for the induction of antitumor immune response. IL-27 has been shown to act on NK cells to enhance their cytotoxic activity both *in vitro* and *in vivo*; in addition, therapeutic administration of IL-27 increased NK cell susceptibility of tumors [77]. By activating NK cells, IL-27 might enhance adaptive immunity to tumors in part; the killing of tumor target by NK cells could provide DCs with increased access to tumor antigens; thus, IL-27 serves as a link between innate and adaptive antitumor immunity. In addition to NK cell activation, IL-27 acts on $CD8^+$ T cells and induces the generation of CTL through enhancing the expression of effector molecules such as granzyme B and perforin [78]. Similar to mice, IL-27 promotes IFN-γ and granzyme B production from human $CD8^+$ T cells [79]. The overexpression of IL-27 in highly immunogenic murine tumor cells facilitated CTL development with enhanced IFN-γ production [80, 81]. In line with these observations, $IL\text{-}27R^{-/-}$ mice failed to regulate tumor growth *in vivo*, reiterating the importance of IL-27 signaling in the generation of antitumor immunity [82]. Most recently, DC-derived IL-27 has been shown to induce NK and NKT cell-dependent antitumor immunity against methylcholanthrene-induced fibrosarcoma and transplanted B16 melanoma [83]. Moreover, IL-27 in combination with other cytokines such as IL-2 and IL-12 boosts antitumor immunity by contributing to the development of CTLs and NK cells [84].

In addition to the direct effect of IL-27 on $CD8^+$ T-cell activation, the influence of IL-27 on $CD4^+$ T-cell responses might provide further therapeutic opportunities. Initial studies have indicated that IL-27 leads to the differentiation of Th1 cells [85]. IL-27 synergizes with IL-12 to enhance IFN-γ production [86]. Moreover, it has been shown that IL-27 inhibits Th2 polarization of naïve $CD4^+$ T cells and suppresses Th2 cytokine production from *in vitro* polarized Th2 cells [87–89]. By altering the balance between Th1 and Th2 cytokines, IL-27 plays a critically important role in antitumor immune responses. In line with this, a recent study confirmed IL-27's capability in the reversion of the Th2 polarization of *in vivo* primed lymphocytes from pancreatic cancer patients [90]. IL-27-dependent enhancement of preexisting antigen-specific Th1 responses has also been demonstrated [36]. IL-27 may promote tumor regression through the inhibition of Tregs. IL-27 inhibits the generation of $Foxp3^+$ Tregs both *in vitro* and *in vivo*. IL-27 blocks Treg differentiation through a mechanism that is at least partially dependent on STAT-3 [91, 92]. In addition, IL-27 can limit Treg cell expansion by inhibiting IL-2, a cytokine necessary for Treg development [93]. In a murine model of neuroblastoma, IL-27 has been shown to inhibit IL-2-induced Treg expansion in the tumor, promoting antitumor immune responses [84]. IL-27 also induces tumor-specific Ab response which cooperatively elicits ADCC activity [94].

7.2.2.3 Direct Effect of IL-27 on Tumors

IL-27 possesses multiple antitumor effects mediated by mechanisms involving angiogenesis

and its direct effects on tumors. IL-27 has been shown to have antiproliferative activities which inhibit tumor growth and metastasis in murine melanoma [95]. The major antitumor role of IL-27 relies on its antiangiogenic property of surrounding endothelial cells and fibroblasts. IL-27 significantly inhibited tumor growth in SCID mice through the induction of antiangiogenic factors such as IP-10 and MIG from endothelial cells [96]. Consistent with these results, IL-27 has been shown to directly act on human umbilical cord endothelial cells and induce production of the antiangiogenic chemokines such as IP-10 and MIG [97]. IL-27 strongly inhibited tumor growth of primary multiple myeloma (MM) cells through inhibition of angiogenesis [98]. In addition, IL-27 downregulated a wide panel of proangiogenic genes, including matrix metalloproteinase-9 (*MMP-9*), *TGF-β*, and *VEGF* with a concomitant upregulation of the angiostatic chemokines IP-10 and MIG.

IL-27 may further promote tumor regression through the inhibition of a proangiogenic cytokine IL-17. IL-27 suppresses the Th17 key transcription factor RORγt and thus inhibits expression of IL-17 by T cells both in humans and mice [99, 100]. Accordingly, mice deficient in either the IL-27 EBI3 subunit or IL-27R have increased levels of IL-17 [101]. Among the Th17 suppressive molecules found in the tumor microenvironment, IL-27 is one of the most potent inhibitors of Th17 differentiation. IL-27 can be induced in tumor-infiltrating DCs by galactin-1, IFN-γ, and apoptotic tumor cells in the tumor microenvironment [71, 102, 103]. However, the proangiogenic molecules which dominate the microenvironment in advanced tumors can limit the availability of IL-27. Osteopontin (OPN), a proinflammatory cytokine, inhibits the expression of IL-27 in DCs while inducing Th17 differentiation [72]. OPN promotes tumor growth through mechanisms involving angiogenesis, tumor migration, and metastasis, suggesting that OPN may release the brake on Th17 cell responses by suppressing IL-27 in DCs. Both OPN and IL-27 are expressed in DCs and macrophages; thus, the outcome of Th17 accumulation in tumor microenvironment may depend on the fine balance with other myeloid cell populations especially tumor-infiltrating macrophages and DCs expressing OPN and IL-27.

7.2.2.4 Advantages of IL-27 Over IL-12 in Tumor Immunity

IL-27-mediated antitumor mechanisms are complex. Similar to IL-12, IL-27 utilizes effector mechanisms of innate and adaptive immunity to mediate antitumor immunity. IL-27 promotes tumor immunity through the induction of Th1 and CTL responses while inhibiting immunosuppressive Th2 and Tregs. Unlike IL-12, IL-27-mediated antiangiogenic functions are independent of IFN-γ. Thus, IL-27-treated mice are not observed with any toxic side effects [104]. The central role of IL-27 in orchestrating both the innate and adaptive arms of immunity together with multiple antiangiogenic functions explains the essential contribution of this molecule to the development of antitumor immunity against both high and poor immunogenic tumors. These observations together with the lack of toxicity observed *in vivo* in preclinical trials with IL-27 treatment highlight the enormous therapeutic potential of this approach.

7.3 Cytokines in Immune Tolerance to Cancer

Although certain cytokines produced in the tumor microenvironment can function to inhibit tumor growth, others can promote tumor growth and progression. Several cytokines were found to serve as growth and survival factors that act on premalignant cells, stimulate angiogenesis and metastasis, and maintain tumor-promoting immunosuppression and inflammation.

7.3.1 TGF-β

7.3.1.1 Overview

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine with broad tissue distribution that plays critical roles during embryonic development, normal tissue homeostasis, and cancer [105].

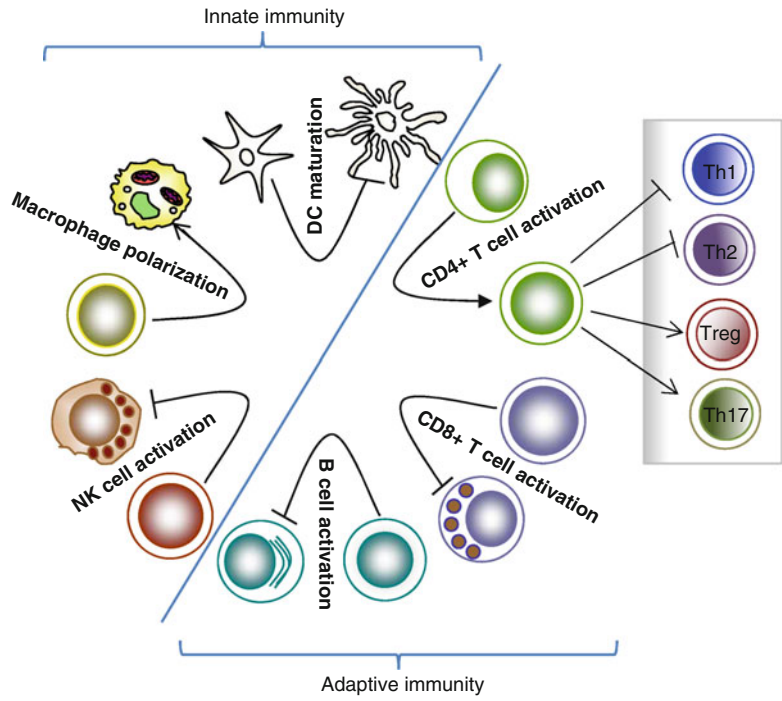

Fig. 7.2 TGF-β-mediated immunosuppression. TGF-β affects components of both innate and adaptive immune systems. TGF-β inhibits NK cell activation and its effector function. In addition, TGF-β inhibits DC maturation and antigen-presenting function while promoting polarization of M2 macrophages. TGF-β inhibits $CD8^+$ T-cell-mediated antitumor immune response. TGF-β also has a significant impact on $CD4^+$ T-cell differentiation and function. TGF-β induces Treg and Th17 differentiation while inhibiting Th1 and Th2 differentiation. Furthermore, TGF-β inhibits B-cell proliferation and antibody secretion

Elevated TGF-β serum concentrations were observed in patients with different malignancies and were associated with poor prognosis. TGF-β is released not only by a variety of cells in human and murine tumors including T cells, macrophages, and DCs but also by tumor cells themselves [106]. Almost all human cell types are responsive to TGF-β, which signals through type I and type II TGF-β receptors (TβRI and TβRII, respectively). Upon binding of TGF-β to TβRII, TβRI is recruited and activated to phosphorylate the downstream mediators, SMAD2 and SMAD3. Phosphorylated SMAD2 and SMAD3 combine with SMAD4 to enter the nucleus to modulate gene transcription [107].

The function of TGF-β in cancer is complex. TGF-β can act as a tumor suppressor or a tumor promoter depending on the stages of tumor development. Initially, it acts as a tumor suppressor since it induces apoptosis and inhibits the growth of normal and premalignant tumor cells [108]. At later stages of tumor progression, TGF-β acts as a tumor promoter. At this stage, cancer cells protect themselves and tend to acquire resistance to TGF-β growth inhibitory signals which is an important reason for the shift from being a tumor suppressor to a tumor promoter. Subsequently, cancer cells start secreting nonphysiological levels of TGF-β in an autocrine and paracrine manner which may affect the differentiation of the tumor cells and the surrounding cellular environment, respectively, leading to development of the tumor and metastasis [108]. Notably, TGF-β induces epithelial-mesenchymal transition (EMT), whereby epithelial tumor cells acquire an invasive, mesenchymal-like phenotype accompanied by changes in the expression of cell-cell adhesion molecules and secretion of metalloproteinases, leading to metastasis [109, 110]. The potent regulatory activity of TGF-β on immune cell functions represents an important mechanism of immune tolerance to tumors. The presence of TGF-β in the microenvironment of the developing tumor is predicted to disable effective immunosurveillance by multiple mechanisms, most of which converge on the impairment of tumor cell killing by innate and adaptive immune cells (Fig. 7.2).

7.3.1.2 Effect of TGF-β on Innate Immunity to Tumors

TGF-β is an important regulator of NK cell function, being a potent antagonist of IL-12-induced production of IFN-γ in NK cells [111]. In addition, TGF-β inhibits the activity of NK cells by limiting expression of the activating receptor NKG2D, NKp30, and DNAM-1 [112]. In fact, reduced expression of NKG2D is associated with elevated levels of TGF-β in patients with cancer. It has been shown that surface-bound TGF-β on MDSCs can inhibit NK cell cytolytic activity against mammary adenocarcinoma [113, 114]. Moreover, TGF-β has been shown to suppress MHC class I and MHC class II expression in a number of cell populations [115–117]. Importantly, the TGF-β-dependent decrease of MHC class I expression in tumor cells has been shown to result in reduced tumor cell lysis by NK cells [117]. Although NK cells are the major innate effectors, they require activation by DCs. TGF-β has been demonstrated to impair DC function both *in vitro* and *in vivo*. TGF-β inhibits upregulation of critical co-stimulatory molecules on the surface of DCs and reduces cytokine production and their antigen-presenting capacity [118, 119]. TGF-β can immobilize DCs, thereby interfering with their migration and the transport of antigen to draining lymph nodes for presentation to T cells. Moreover, TGF-β can also induce apoptosis of DCs [120]. In recent years, more correlative clinical data has supported the inhibitory role of TGF-β in the observed defects in cancer. Increased serum TGF-β in human colorectal cancer correlates with reduced circulating DCs [121]. Moreover, tumor-infiltrating DCs both secrete and respond to TGF-β, in either an autocrine or paracrine manner. These TGF-β-secreting DCs promote the formation of Tregs [122] that potently inhibit the function of other T cells, and that Treg production of TGF-β can inhibit NKG2D-mediated NK cell cytotoxicity, thereby enhancing tumor growth and metastasis. In addition to DCs, TGF-β can suppress or alter the activation and function of other innate immune cells such as NKT cells, macrophages, and neutrophils [106].

Macrophages, the predominant leukocyte, play a key role in tumor growth. The role of tumor-associated macrophages (TAMs) in tumors is controversial [123]. TAMs originate from recruited myeloid cells, such as blood monocytes or MDSCs by a number of chemoattractants produced by tumor cells and stromal cells. Tumor-derived chemokine CCL2 is critical for the recruitment of macrophages to the tumor site [124]. Macrophages can exert different properties when polarized with distinct inducers. Differential cytokine production is a key feature of polarized macrophages. When stimulated with IFN-γ, M1 macrophages secrete high levels of IL-12, but low levels of IL-10. In contrast, M2 macrophages express high levels of IL-10 but low levels of IL-12 [125]. Due to their different cytokine profiles, these polarized macrophages have distinct functions. For example, the IL-12 produced by M1 macrophages can promote the differentiation of Th1 cells, which can improve antigen phagocytosis and contribute to antitumor immunity. Whereas, the IL-10 expressed by M2 macrophages can promote the production of IL-4 and IL-13 by Th2 cells. Both IL-4 and IL-13 have been shown to impair antitumor T-cell responses. TGF-β promotes tumor-associated macrophage polarization to an M2 *vs*. M1 phenotype, which further promotes TGF-β production and deepens immunosuppression [126]. In most tumors, the infiltrated macrophages are considered to be of the M2 phenotype. TAMs orchestrate various aspects of cancer, such as tumor progression, angiogenesis, metastasis, and immunosuppression [127]. It has been shown that NKT cells can suppress CTL responses through mechanisms involving TGF-β. Therefore, blockade of TGF-β signaling not only enhances the frequency of antitumor CTLs but also restores the activities of the cytolytic machinery and prevents NKT cell-mediated immunosuppression [128]. Furthermore, TGF-β also inhibits effector functions of other innate immune cells such as γδ-T cells [106]. Thus a dampened innate immune response leads to poor adaptive immunity, resulting in persistence of the tumor.

7.3.1.3 Effect of TGF-β on Adaptive Immunity to Tumors

The presence of TGF-β in the tumor microenvironment can have a profound impact upon antitumor activity of T cells. It has been shown that TGF-β can suppress CTL differentiation and CTL-mediated lysis of tumor cells [129, 130]. TGF-β acts on CTLs to specifically repress the expression of different cytolytic effector molecules such as perforin, granzyme A, granzyme B, Fas ligand (FasL), and IFN-β, which are collectively responsible for CTL-mediated tumor killing [131]. Blockade of TGF-β in tumor models has been shown to reduce tumor burden by improving $CD8^+$ T-cell-mediated tumor immunity [131]. Furthermore, TGF-β could suppress IL-2 production and IL-2-induced T-cell differentiation [132]. Tumor cells transfected with TGF-β were shown to attenuate the efficacy of DC-based tumor vaccines [118]. In addition, TGF-β functionally regulates the differentiation of T helper cell subpopulations both *in vitro* and *in vivo*. TGF-β inhibits Th1 and Th2 cells, whereas it promotes Treg and Th17 cell differentiation [133]. Most recently, TGF-β has also been shown to play an important role in the development of IL-9-secreting Th9 cells [134].

Although there are many sources of TGF-β in the tumor microenvironment, it has been shown that Tregs can provide a significant source of TGF-β responsible for attenuation of tumor antigen expanded CTLs. Tregs hamper the functions of Th1, $CD8^+$ T cells, NK cells, DCs, and other key effector cells of antitumor immunity [106]. Accordingly, Treg-mediated immunosuppression has been proposed to be one of the important mechanisms involved in tumor immune evasion. An accumulation of Tregs in tumors can dampen T-cell immunity to tumors and is thus the main obstacle to successful immunotherapy [59]. The frequency of Tregs present in the peripheral blood of patients with various cancers is higher than that of normal population [135]. Notably, Tregs isolated from peripheral blood and solid tumors remain suppressive to T-cell activation *in vitro* [136]. Likewise, Tregs from tumor-bearing mice inhibited tumor rejection, indicating that Treg cells suppress tumor-specific immunity and limit antitumor resistance. In contrast, depletion of Tregs with anti-CD25 Ab in animal models enhances antitumor immunity and tumor regression, further suggesting the involvement of Tregs in tumor growth. Furthermore, when tumor-specific $CD8^+$ T cells were adoptively transferred with either Tregs or $CD4^+CD25^-$ T cells into hosts with tumor, $CD8^+$ T-cell-mediated immunity was abolished in those receiving Tregs but not $CD4^+CD25^-$ T cells [137, 138]. Collectively, these studies provide strong evidence that Tregs can attenuate the antitumor immunity by downregulating the antitumor immune responses and ultimately facilitating the development of cancer.

7.3.1.4 TGF-β, Treg, and Tumor Angiogenesis

Angiogenesis and tumor-associated immunosuppression are hallmarks of tumorigenesis. The association between angiogenesis and immunosuppression is related to hypoxia which induces both angiogenesis and immunosuppression via activation of hypoxia-induced factor 1 (HIF-1). The induction of VEGF during hypoxia is primarily mediated by HIF-1. HIF-1-induced VEGF promotes angiogenesis by inducing the recruitment of various proangiogenic bone marrow-derived cells including endothelial progenitors and myeloid cells [139]. Although hypoxia-VEGF axis has been thought to be solely involved in vascular growth and permeability, recent studies suggest its role in immunosuppression in the tumor microenvironment. Within the tumor microenvironment multiple cell types with established roles in immunosuppression have been shown to promote angiogenesis. Among the immunosuppressive cell types found in the tumor microenvironment, Tregs are considered pivotal regulators of immunosuppression. Tregs can traffic to tumors from the periphery under the influence of chemokines in the tumor microenvironment. It has been shown that tumor hypoxia leads to the recruitment of Tregs via chemokine CCL28 [140]. Forced expression of CCL28 in mouse tumor cells resulted in accelerated tumor growth and Treg accumulation associated with increased VEGF levels and angiogenesis. In addition, Tregs were shown

to express CCR4, the receptor for CCL22, and can therefore migrate to CCL22 present in the tumor microenvironment [141, 142]. Beyond recruitment of Tregs through chemokines, the tumor microenvironment promotes the continued expansion of Tregs and the generation of Tregs due to a tumor microenvironment rich in TGF-β. The recruited Tregs in turn dampen the antitumor immune response and promote angiogenesis. The accumulation of Tregs at tumors has been correlated with VEGF overexpression and increased angiogenesis in cancers, providing evidence for an association between Tregs and angiogenesis [143, 144]. Tregs can contribute to tumor angiogenesis through different mechanisms. They promote angiogenesis indirectly by suppressing Th1 cells that release angiostatic cytokine IFN-γ, as well as interferon-induced chemokines such as CXCL9 and CXCL10. Indeed, Tregs have been shown to promote tumor angiogenesis by specifically inhibiting tumor-reactive T cells. Tregs can significantly contribute to the direct promotion of tumor angiogenesis through the induction of VEGF and endothelial cell proliferation [144]. Additional therapeutic opportunities may be provided by Tregs' capability in suppressing tumor-specific immunity while promoting tumor angiogenesis by well-planned manipulations of Tregs, including depletion, blocking trafficking into tumors, and reducing their differentiation and suppressive mechanisms. It will be beneficial to tumor eradication by combining this strategy with various current therapeutic approaches. In an early phase I clinical trial in patients with metastatic breast cancer, the anti-CD25 Ab daclizumab significantly depleted Tregs and enhanced the immunogenicity of a cancer vaccine [145]. In addition, blocking Treg function using Abs targeted against glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) is under clinical evaluation in patients with cancer.

7.3.1.5 TGF-β in Clinical Trials

As a result of the wide variety of effects of TGF-β on tumorigenesis, blockade of TGF-β and its signaling pathway can be a potent approach to improve tumor immunity. There are many TGF-β signaling antagonist agents under development at both the preclinical and clinical stages. Mice with fully or partially disrupted TGF-β function have phenotypes with severe self-reactive immune responses [146, 147]. However, clinical trials of TGF-β antagonists, such as a monoclonal Ab or small molecules that interfere with TGF-β receptor signaling, in cancer patients have been tested and are ongoing. In phase I/II clinical trials, intratumoral administration of AP-12009, an antisense oligonucleotide to TGF-β, resulted in a significant increase of survival time [148]. Some clinical benefit without apparent induction of autoimmune disease was found in a clinical trial of a human monoclonal anti-TGF-β in melanoma patients [149, 150]. In addition, a vaccine that contains allogeneic tumor cells that are modified to express antisense TGF-β has been tested in a phase I/II clinical trial. Using this approach, a response rate of 30 % has been reported in non-small cell lung carcinoma (NSCLC), with no serious toxicities observed [151]. LY2157299 is a small molecule inhibitor which is selective for the kinase domain of the type 1 TGF-β receptor. LY2157299 is currently being evaluated in patients with metastatic malignancies.

7.3.2 IL-17

7.3.2.1 Overview

IL-17 is a proinflammatory cytokine produced by Th17 cells [152]. In addition to Th17 cells, IL-17 can also be produced by cells other than T helper cells, such as iNKT, $CD8^+$ T, and γδ-T cells [153–155]. Since Th17 cells produce large quantities of IL-17A, most Th17-mediated effects are attributed to this cytokine. Many factors are required for the induction and stabilization of Th17 cells. Of these, TGF-β and IL-6 are the most crucial cytokines for its differentiation. IL-6 induces production of IL-21, which subsequently favors Th17 differentiation in an autocrine manner [156, 152]. These cells require CD40-induced IL-23 to maintain their Th17 phenotype *in vivo*. The differentiation of Th17 cells into IL-17-secreting cells requires the expression of the transcription

factor ROR-γt [157]. It has been shown that Th17 cells are gradually increased in the tumor microenvironment during tumor development. In addition, Th17 cells have been found in patients with different tumors. The frequent association of raised IL-17 concentrations with negative prognosis links the increased systemic IL-17 concentrations with the later stages of cancer. Many factors released by the tumor cells and molecules secreted by tumor-infiltrating immune cells such as TGF-β, IL-6, prostaglandin E2 (PGE2), IL-21, IL-23, osteopontin, IL-1β, and TNF-α can play major roles in the induction of Th17 differentiation [158–161].

7.3.2.2 Th17 Differentiation in the Tumor Microenvironment

There are many sources for Th17 cells in the tumor microenvironment. Th17 cells found in the tumor microenvironment can either be migrated from the periphery or differentiated from naïve T cells under the influence of tumor microenvironmental factors. Th17 cells can traffic to tumors from the periphery under the influence of tumor microenvironmental chemokines such as RANTES and monocyte chemotactic protein-1 (MCP-1) [162]. In addition, high levels of chemokines CXCL12 and CCL20 have been found in tumor microenvironments, which could facilitate Th17 cell trafficking and migration into the tumor sites. Moreover, Th17 cells in the tumor microenvironment might clonally expand following stimulation by tumor-associated macrophages [163]. In addition, Th17 cells can be induced and differentiate in the tumor microenvironment [164]. It has become clear that IL-17 producing Th17 cells and Tregs share a common pathway. Although TGF-β favors differentiation of naïve T cells into Tregs, the simultaneous presence of both TGF-β and IL-6 promotes the differentiation of Th17 cells. Given the tight association of TGF-β and IL-6 with tumor incidence and progression, naïve T cells entering an established tumor are more likely to be exposed to conditions favoring Th17 differentiation. Upon stimulation with TGF-β and IL-6, $CD8^+$ T cells not only lose their cytotoxic ability but are also induced to secrete IL-17 [165]. IFN-γ expressed by Th1 or $CD8^+$ T cells inhibits angiogenesis and induces major histocompatibility complex I in tumor cells, thus favoring immune recognition and subsequent arrest of tumor growth [166]. In contrast, IL-17 favors angiogenesis and tumor growth; therefore, replacing IFN-γ with IL-17 in the tumor microenvironment may have severe consequences for immune recognition and surveillance.

7.3.2.3 Tumor-Promoting Functions of IL-17

Many functions of IL-17 in the tumor microenvironment contribute to tumor progression, besides their minor direct effect on the proliferation and survival of tumor cells [167]. The major pro-tumor role of IL-17 in cancer relies on its proangiogenic property of the surrounding endothelial cells and fibroblasts. For example, IL-17-overexpressing human cervical cancer cells and NSCLC cells show greater ability in developing tumors in immunocompromised mice compared with control cells with no IL-17 expression [168, 169]. In addition, IL-17 overexpression in fibrosarcoma cells enhances their tumorigenic growth in syngenic mice, primarily owing to the proangiogenic activity of IL-17. Moreover, the levels of Th17 cells were positively correlated with microvessel density in tumors [170]. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators [171, 172] including VEGF. In addition, they markedly promote inflammatory and tumor angiogenesis [173]. IL-17 is able to upregulate VEGF production by fibroblasts and therefore promotes fibroblast-induced new vessel formation in the inflammatory microenvironment and tumors. The IL-17-VEGF loop that modulates angiogenesis includes another angiogenic factor, TGF-β. IL-17 induces VEGF, which in turn induces TGF-β followed by VEGF-mediated angiogenesis [174]. TGF-β enhances the VEGF receptivity of endothelial cells by increasing VEGF receptor expression [175]. IL-17 also induces IL-6 and PGE2 and enhances intercellular adhesion molecule (ICAM)-1 expression in fibroblasts. All these molecules were known to have a major role in angiogenesis and tumor invasion. IL-17 appears to stimulate production of IL-8 [176]. IL-8 sig-

naling promotes angiogenic responses in endothelial cells, increases proliferation and survival of endothelial and cancer cells, and potentiates the migration of cancer cells and infiltrating neutrophils at the tumor site. Moreover, IL-17 was found to induce IL-1β and TNF-α in macrophages and cytokines which can further synergize with IL-17 to activate neutrophil-specific chemokine, thereby recruiting neutrophils to the site of inflammation [177].

One of the most important mechanisms underlying IL-17 regulation of inflammation which orchestrates the tumor microenvironment is through NF-κB signaling, the master regulator of the inflammation [178]. IL-17R signaling results in the activation of NF-κB and regulates the activities of extracellular-regulated kinase 1 (ERK1), ERK2, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases [179, 180]. While the IL-17-mediated cytokine expression is regulated primarily by NF-κB, the same cytokines can further stimulate NF-κB-mediated transcription of themselves in tumor cells and tumor-associated stromal cells, thereby creating a sustained chronic inflammatory state within the tumor microenvironment. In support of this notion, enhanced cervical cancer growth elicited by IL-17 was associated with increased expression of IL-6 and macrophage recruitment to the tumor sites [169]. Therefore, IL-17 might also function through IL-6 to promote tumor development. Chemokines can stimulate or inhibit proliferation and chemotaxis of the blood vessel endothelial cells which serve the tumor. IL-17 has been shown to selectively enhance the production of angiogenic chemokines such as CXCL1, CXCL5, CXCL6, and CXCL8 from tumor cells and epithelial cells [168, 181]. In addition, IL-17 is also known to inhibit angiostatic chemokine secretion by fibroblasts [168]. Thus, IL-17 may shift the local biologic balance between angiogenic and angiostatic chemokines toward a predominance of angiogenic chemokines in order to enhance the net angiogenic activity.

7.3.2.4 Antitumor Functions of IL-17

Although IL-17 seemed to be a potential tumor-promoting cytokine, a considerable number of reports have described tumor-inhibitory effects of IL-17. Th17-polarized cells were found to be more effective than Th1 cells in eliminating large established tumors [182]. However, the Th17-mediated tumor responses were highly dependent on IFN-γ-based mechanisms. Indeed, the effects of Th17-polarized cells were completely abrogated by the administration of IFN-γ-depleting Ab and not by IL-17- or IL-23-depleting Abs. Adoptively transferred IL-17-secreting $CD8^+$ T cells also enhanced antitumor immunity resulting in regression of B16 melanoma [183]. In addition, IL-17 has been shown to inhibit the growth of hematopoietic tumors such as mastocytoma and plasmocytoma by enhancing CTL activity [184]. Different mechanisms have been proposed for the IL-17 enhancement of tumor-specific CTLs. IL-17 has been shown to induce IL-6 from a variety of cells. Moreover, IL-17 stimulation can induce IL-12 production from macrophages [185]. Both IL-6 and IL-12 have been associated with induction of tumor-specific CTL. IL-17 promotes maturation of DC progenitors as indicated by increased expressions of co-stimulatory molecules, MHC-II antigens, and allostimulatory capacity [186]. This may lead to further improvement in T-cell priming by tumor cells producing IL-17. In addition, IL-17-transduced fibrosarcoma cells induced tumor-specific antitumor immunity by augmenting the expression of MHC class I and class II antigens [187]. These studies were focused on the effects of exogenous IL-17 in established mouse tumor cell lines. A recent demonstration shows that tumor growth in subcutaneous and lung tumor metastasis are enhanced in IL-17-deficient mice [188]. The effect is accompanied by reduced IFN-γ levels in tumor-infiltrating NK cells and T cells.

The evidence reviewed here demonstrates that IL-17-secreting Th17 cells can either stimulate or inhibit tumor growth and progression. The beneficial effects of IL-17 on upregulating host immune response may be present early in inflammation, but is eventually overcome by increasingly large tumor burden. Clearly, as discussed above, many of the inflammatory functions of IL-17 can benefit the tumor. The shift from beneficial inflammatory functions of IL-17 to a detrimental one may depend on the tumor

type and inflammatory mediators in the tumor microenvironment. The pro-tumor *vs*. antitumor effects of IL-17 are thus functions of a number of combinations of all these IL-17-induced inflammatory mediators and, perhaps, mediators which counter-regulate IL-17 production as well, operating in tandem.

7.3.3 IL-23

7.3.3.1 Overview

IL-23 is a heterodimeric protein composed of two subunits IL-23p19 and IL-12p40. IL-23 is secreted by activated DCs and macrophages. IL-23 binds the IL-23R complex, composed of IL-23R and IL-12Rβ1. Upon binding IL-23, IL-12Rβ1, and IL-23R associate, marking the beginning of the IL-23 signal-transduction cascade [189]. IL-23 plays an important role in bridging innate and adaptive responses. Therefore, IL-23 has been described as a key cytokine promoting inflammation in peripheral tissues. The activity of IL-23 in the regulation of tumor immunity is just beginning to be elucidated [190].

7.3.3.2 Pro- *vs*. Antitumor Functions of IL-23

Belonging to the IL-12 family, IL-23 performs both pro-tumor and antitumor functions. IL-23 is spontaneously produced by TAM in several mouse tumor models. Tumor-secreted PGE2 enhances the production of IL-23 and IL-1β in macrophages and DCs while downregulating IL-12 production [191–193]. While IL-12 production is decreased, IL-23 production is increased in tumors [194]. PGE2, together with IL-23, favors the expansion of human Th17 cells from PBMCs; on the other hand, PGE2 enhances IL-17 production from memory $CD4^+$ cells induced by IL-23 [161]. The involvement of IL-23 in the induction of Th17 was established when investigators showed that IL-23 promotes the production of IL-17 by activated T cells [195]. Although IL-23 is not involved in the initial differentiation of Th17 cells, it is crucial for the function, survival, and propagation of this T-cell population in the inflamed environment. In contrast to the antitumor role of IL-12, IL-23 upregulates inflammatory processes, including matrix metalloproteinase expression and angiogenesis and reduces infiltration and the function of CTLs, thus contributing to tumor growth [196]. Indeed, the mice lacking IL-23/p19 are completely resistant to carcinogen-induced tumor. The absence of tumor formation in these mice correlated with the absence of various markers including IL-17, GR-1+, and CD11b+ myeloid cells which are indicative of tumor-associated inflammation [196]. Recently, tumor-secreted lactic acid has been shown to activate the IL-23/Th17 pathway [159].

In contrast, IL-23-overexpressing tumors show reduced growth and metastasis [197–201]. The antitumor effects of IL-23 in these studies were found to be mediated through enhancement of $CD8^+$ T-cell response. In addition, intratumoral injection of IL-23-overexpressing DCs resulted in a similar phenotype [201]. Artificial overexpression of IL-23 could induce potent antitumor immunity through various mechanisms. IL-23 can mediate myeloid infiltration consisting of DCs, macrophages, and granulocytes, which instead may contribute to the inhibition of tumor growth and boost an immune reaction to these immune-sensitive tumors. In addition, overexpression of IL-23 is likely to increase the systemic IL-23 levels that could lead to the growth and survival of memory $CD8^+$ T cells.

7.3.4 IL-35

7.3.4.1 Overview

IL-35 is a recently discovered IL-12 family cytokine composed of an IL-12 p35 subunit and an IL-12 p40-related protein subunit, EBI3 [202]. IL-35 is not constitutively expressed in tissues and is produced mainly by Tregs. IL-35 induces the transformation of $CD4^+$ effector T cells into Tregs, which in turn express IL-35 but lack the expression of conventional Treg markers such as Foxp3, TGF-β, and IL-10 (Treg35 cells) [203]. The Treg35 cells generated *in vitro* can prevent the development of autoimmunity in various mouse models [204–207]. Most recently, it

has been shown that human Tregs express and require IL-35 for maximal suppressive function. Substantial upregulation of EBI3 and IL12A, but not IL10 and TGF-β, was observed in activated human Tregs compared with conventional T cells [208].

7.3.4.2 Pro-tumor Functions of IL-35

Evidence on the role of IL-35 in tumor immunity is beginning to emerge. IL-35 subunit EBI3 is expressed in Hodgkin lymphoma cells, acute myeloid leukemia cells, and lung cancer cells [209–211]. Small interfering RNA silencing of EBI3 in lung cancer cells inhibits cancer cell proliferation, whereas stable expression of EBI3 in lung cancer cells confers growth-promoting activity *in vitro* [211]. High EBI3 expression in human lung cancer cells was shown to be associated with poor prognosis [211]. Recently, IL-35-secreting Ag-specific Tregs have been observed in patients with prostate cancer [212]. Treg-derived IL-35 was shown to inhibit antitumor T-cell responses. *In vitro* generated Treg35 cells accelerate the development of B16 melanoma and prevent the generation of antitumor $CD8^+$ T-cell responses [203]. In addition, T cells that secrete IL-35 and have suppressive functions can be induced in the tumor beds of melanoma and colorectal adenocarcinoma. Blockade of IL-35 has been shown to relieve suppression mediated by Tregs [212]. Forced expression of IL-35 in tumor cells leads to significantly increased tumorigenesis in mice. IL-35 in the tumor microenvironment significantly increased the numbers of $CD11b^+Gr1^+$ myeloid cells in tumors and subsequently promoted tumor angiogenesis [213]. Furthermore, IL-35 renders tumor target cells more resistant to CTL destruction.

7.3.5 IL-10

7.3.5.1 Overview

IL-10 is an important immunoregulatory cytokine produced by many cell populations. Due to its ability in inhibiting the production of IL-2 and IFN-γ by murine and human Th1 cells, IL-10 was initially named a cytokine synthesis inhibitory factor [214]. The function of IL-10 in cancer is enigmatic. Depending on the experimental model, IL-10 displays both immunosuppressive and immunostimulating activities. On the one hand, IL-10 promotes an antitumor CTL response leading to tumor regression. On the other hand, IL-10 induces immunosuppression and assists in the escape from tumor immune surveillance, hence promoting tumor growth.

7.3.5.2 IL-10-Mediated Immunosuppression in Cancer

The cellular sources of IL-10 are Th2, Treg, Tr1, and Th17 cells; however, cytotoxic $CD8^+$ T cells can also produce IL-10, as can some subsets of DCs, macrophages, B cells, granulocytes, mast cells, keratinocytes, and epithelial cells. In addition, various cancer cells produce IL-10; among those are multiple myeloma, melanoma, human colon carcinoma, lung cancer, oral squamous cell carcinoma, renal cell carcinoma, non-Hodgkin lymphoma, and chronic lymphocytic leukemia [15, 215]. Circulating concentrations of IL-10 were raised in patients with different cancer types and were associated with adverse disease stage or with negative prognosis in those patients. It has been shown that serum levels of IL-10 increased in parallel to clinical disease progression in patients with metastatic melanoma, as well as colon cancer. Moreover, preoperative serum levels of IL-10 predicted the likelihood of colon cancer recurrence [215, 216]. IL-10 can be induced and sustained in the tumor microenvironment by a variety of cytokines. Macrophage-derived IL-6 has been shown to induce production of IL-10 by cancer cells. Similarly, IL-6 in association with TGF-β can induce IL-10 production in Th17 cells. However, TGF-β alone can induce IL-10, whereas IL-10 enhances the expression of TGF-β in a positive feedback circuit [217]. TNF-β promotes proinflammatory reactions while upregulating IL-10 in macrophages and monocytes as a negative feedback, thereby terminating the inflammatory response. In addition, IL-12 and IL-27 can also induce IL-10 production from T cells [99, 218].

IL-10 inhibits NKG2D ligand expression on tumor cells and suppresses cytotoxicity mediated

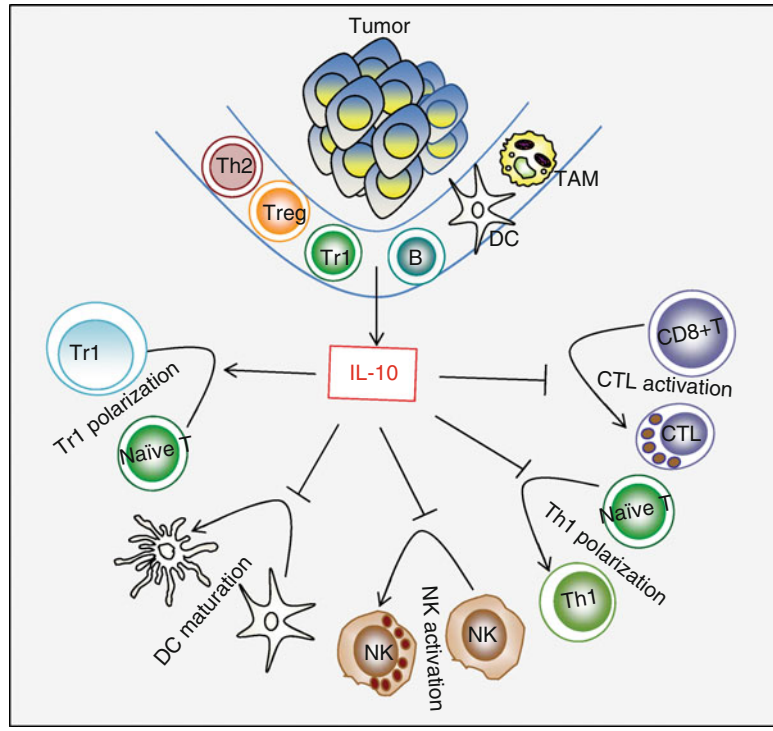

Fig. 7.3 IL-10-mediated tumor immunosuppression. IL-10 can be induced in the tumor microenvironment by many cell types including Th2 cells, Tr1 cells, Tregs, DCs, TAM, and tumor cells. IL-10 has a multitude of suppressive effects on the antitumor immune response. For example, IL-10 can inhibit the maturation of DCs and disrupt the differentiation of CTLs and Th1 cells. IL-10 can also inhibit the cytolytic activity of NK cells. In addition, IL-10 can promote tumor growth through the promotion of IL-10-producing Tr1 cells

by NK cells. Furthermore, IL-10 induces HLA-G molecules that prevent the attack by NK cells [219]. These changes allow tumor cells to survive from immunological attack by immune cells and to grow exponentially. IL-10 can act as a negative regulator in the crosstalk between innate and adaptive antitumor immunity (Fig. 7.3): For instance, T cells suppress NK and NKT cells by elaborating IL-10, which ultimately leads to impaired activation of CTL and Th1 $CD4^+$ T cells and tumor immune privilege [220]. *In vitro*, IL-10 pretreatment can convert different types of tumor cells to a CTL-resistant phenotype by decreasing the expression of HLA class I molecules on their surface [221].

IL-10 acts on DCs and macrophages and inhibits the differentiation and the antigen-presenting properties of these cells. IL-10 inhibits essential steps in immune detection such as the expression of HLA-DR and co-stimulatory molecules, CD80 and CD86, on DCs. IL-10 also prevents the production of the Th1-polarizing cytokines IL-12 and IFN-γ from DCs [222]. Administration of IL-10 before and immediately after DC cancer vaccine results in immune suppression and tumor progression, in line with a predominant inhibitory activity of IL-10 on DC-mediated antigen presentation. Moreover, IL-10-deficient DCs are shown to be more effective in inducing protective antitumor immune response in mice [60]. Exposure of DCs to tumor cell lysates resulted in increased IL-10 production and expansion of regulatory Tr1 cells. Tr1 cells have been shown to down-modulate immune responses through the production of IL-10. In addition, IL-10 has been shown to mediate the immunosuppressive activity of Tregs [223]. Therefore, DCs that encounter tumor antigens in the presence of IL-10 *in vivo* acquire tolerogenic properties and subsequently induce T-cell tolerance to tumor antigens. In addition, IL-10 significantly suppresses other inflammatory cytokines such as IL-1β, IL- 6, and TNFα expression in DCs. Moreover, inhibition of IL-10 production by T cells or malignant cells using anti-IL-10-/IL-10R-blocking Abs or anti-IL-10 antisense oligonucleotides improves antitumor immune responses in animal models.

7.3.5.3 Antitumor Functions of IL-10

Data from experimental models suggest that IL-10 may possess immunostimulating and antitumor properties. For example, overexpression of IL-10 in tumor cells leads to the loss of tumorigenicity concurrent with an increased immunogenicity accompanied by strong antitumor immune response. IL-10 has been shown to increase $CD8^+$T-cell numbers, IFN-γ secretion, and cytotoxicity in established tumors. Overexpression of IL-10 in tumor cells transplanted in mice leads to tumor rejection [222, 224]. Such observations suggest that IL-10 might maintain the number of antigen-specific CTL. Therapeutic administration of recombinant IL-10 induced antitumor immunity against fibrosarcomas in mice [225]. However, higher expressions of IL-10 correlated with tumor progression and metastasis in patients with cancer. Serum levels of IL-10 increased in parallel to clinical disease progression in patients with metastatic melanoma as well as colon cancer; in addition, preoperative serum levels of IL-10 predicted the likelihood of colon cancer recurrence. These finding may indicate that IL-10 production in the clinical setting may be detrimental. To conclude, the pleiotropic activity of IL-10 on different immune cell population and the variability of cancer models used to address the role of IL-10 in tumor immunity are likely responsible for the controversial findings reported in the literature.

7.4 Concluding Remarks

Coordinated, effective development of both innate and adaptive antitumor immune responses. While certain cytokines that are produced in the tumor microenvironment can function to inhibit tumor growth, others can promote tumor growth and progression. A more detailed understanding of tumor-cytokine and immune cell interactions in the tumor microenvironment and thereby manipulating the balance of pro- *vs.* antitumor cytokines may achieve effective cancer immunotherapy.

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Role of Chemokines and Chemokine Receptors in Cancer

8

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8.1 Introduction

Living tissues are highly organized and dynamic structures at the cellular level. Tissue renewal, remodeling, and repair, immunosurveillance, and cell-to-cell interaction and communication are examples of physiological processes relying on the fine recruitment and displacement of numerous cell types. This equilibrium is strictly dependent on the principle of "recruiting the right cell at the right place and the right moment." One major component of this principle is the chemokine and chemokine receptor system. Chemokines (CKs) for chemoattractant cytokines are small, secreted molecules historically defined on the basis of their functional chemotactic activity [1–3]. They constitute a family of over 50 members which interact with about 20 defined corresponding/cognate receptors (CKRs). This discrepancy highlights the complexity of this system as several CKs can bind to a single receptor. Conversely, one receptor

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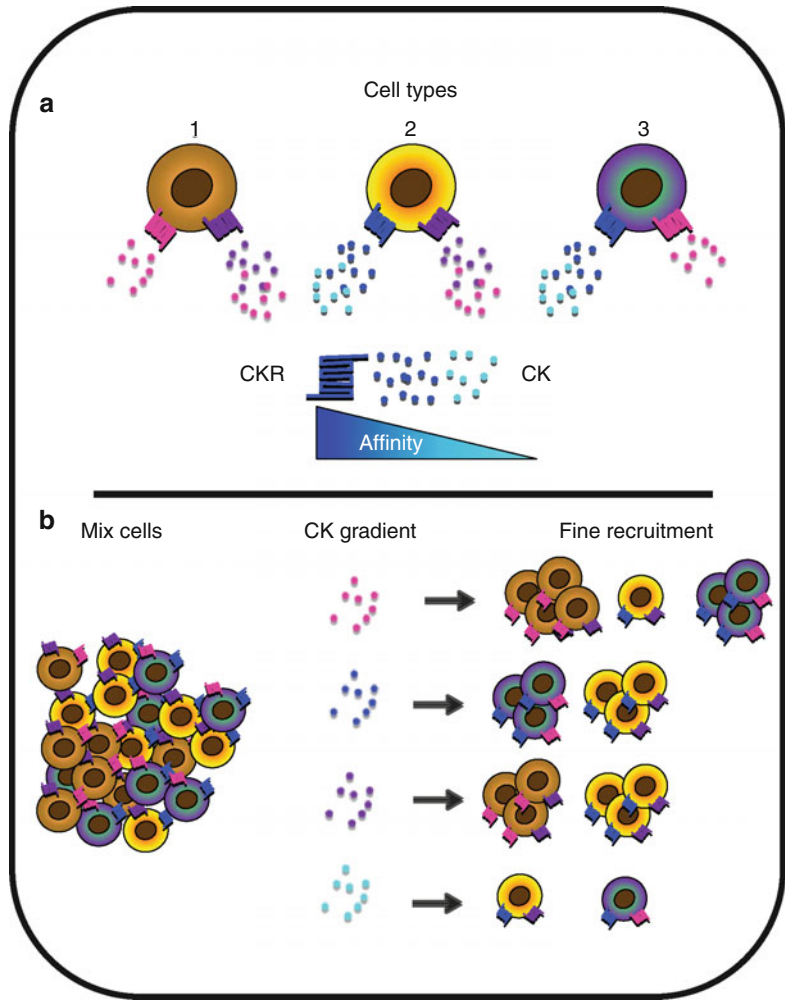

Fig. 8.1 Fine modulation of cellular recruitment by chemokines. The chemokine network is organized around several levels of complexity. (**a**) Most of the cell types (1, 2, 3) express several chemokine receptors and a same receptor is found on several cell types. Moreover, different chemokines can bind to a same receptor and most of the receptors can bind several chemokines with distinct affinity (color gradient represent differential affinity). This apparent complexity allows for the fine control of cell population recruitment. (**b**) The schematic representation illustrates the selective recruitment of cell populations according to the respective colored CK gradient. The number of cell recruited is related to the affinity of the respective CK for its receptor

can bind several different CKs. This redundancy associated with differential avidity of the CK for their CKR and the specific expression by the different cell population contributes to the fine tuning of cell migration (Fig. 8.1) and explains that a modest deregulation of the system can lead to severe pathological conditions. In addition, there is overwhelming evidence describing alternative functions of the CK/CKR couple in hematopoiesis, reproduction, angiogenesis, and immune-associated functions such as cell activation, proliferation, effector function, and survival [4, 5]. Numerous reports from the past two decades have validated the importance of the CK/CKR network with its diverse range of physiological properties and its involvement in various physiopathological disorders [6–8].

Cancer constitutes a very complex pathology in many aspects. Neoplastic cells result from the environmental, viral-induced, or inherited deregulation of genes known as "oncogenes" or "tumor suppressor genes." This primary modification often leads to uncontrolled expansion of undifferentiated cells for which the transcriptome and the proteome are highly modified in comparison with the original cell. Nevertheless, it is important to note that tumor development does not result from the simple expansion of neoplastic cell. Indeed, solid tumors (primary tumor as well as metastasis) are also constituted by a wide variety of stromal cells. Stroma is composed of non-hematopoietic cells, such as "healthy" cells of the affected tissue, fibroblasts, or endothelial cells, as well as hematopoietic cells. Hematopoietic cell

populations are mainly composed of innate immune cells, such as tumor-associated macrophages (TAMs), dendritic cells (DCs), natural killers (NK cells), neutrophils, and partners of the adaptive immune response such as T and B lymphocytes.

The relative importance of the stroma compared to tumor cells depends on the type of cancer [9], but it is now well described that several stromal cells are important predictive markers of cancer evolution (macrophages, regulatory T cells, and endothelial progenitor cells). Even though the stroma cannot be characterized properly in circulating hematological tumors, leukocytes will have an important impact on the expansion, survival, and potential homing of tumor cells to the specific tissue. This phenomenon is distinguishable from the metastatic process where the tumor cells need to cross the endothelial barrier from a primary tumor site and home to a distant tissue. The stroma contributes to the global organization and progression of the tumor known as "tumor microenvironment" through the production of growth factors, cytokines, CKs, exchange of nutrients, and tissue remodeling and repair. In contrast, immune cells are responsible for the control of tumor growth. The concept of immunosurveillance proposed by Burnett et al. [10] in the early 1970s has been widely debated. Recently, Schreiber and colleagues provided experimental evidence for the clinical emergence of cancer as a result of strong selection and modeling of tumors by the immune system in a process termed as "tumor editing" [11]. In this process, neoplastic transformation occurs, and tumor cell expansion is detected by the innate and adaptive immune systems, which either succeed in complete tumor elimination or maintain a state of equilibrium between tumor cell expansion and elimination. This phase leads to the immune selection of tumor cell variants that develop immune resistance and immunesuppressive mechanisms resulting in tumor escape and cancer progression to a clinical outcome.

Cancer is a complex process whereby undifferentiated tumor cells expand locally in specialized tissues, migrate in an active manner by leaving the primary tumor site through the endothelial barrier, establish in a distant and different specialized tissue and finally generate metastases. Inflammation generated by neoplastic transformation contributes to the recruitment of protumoral population and the production of growth factors as well as the recruitment of immune component with antitumor activity. Thus, tumorigenesis is a dynamic process involving important tissue remodeling and angiogenesis, recruitment and local migratory mechanisms, and survival and cell death for both tumor and stromal cells in which the CK/CKR network has major implication.

The CK/CKR network appears to be a promising target in cancer therapy and has already been used in standard therapeutic approaches, as well as in immunotherapy. Numerous basic and clinical interventions rely on the development of agonist or antagonist CKR in order to manipulate their critical biological function toward antitumor activity.

In this chapter, the role of the CK/CKR network in these aspects of cancer development, as well as its potential application in the improvement of cancer therapy, is described in detail.

8.2 Chemokines and Chemokine Receptors

Chemokines are small cytokines initially described for their chemotactic properties on leukocytes. During cell recruitment from the blood to inflamed tissues, CKs initiate the activation of circulating cells, promoting cell rolling, adhesion to activated endothelium, and extravasation (Fig. 8.2). In tissues, CKs determine cell directional migration, by establishing a concentration gradient (Fig. 8.3). Evidence from previous studies has shown that the control of cell mobility by CKs is implicated in developmental mechanisms and cell homeostasis, as well as in the induction and tuning of acute and chronic inflammation and control of the immune response. Numerous reviews have extensively described the CK classification, structural organization, and their associated biological properties [12, 13]. CKs are subdivided in four subfamilies based on the number and spacing between conserved cysteine

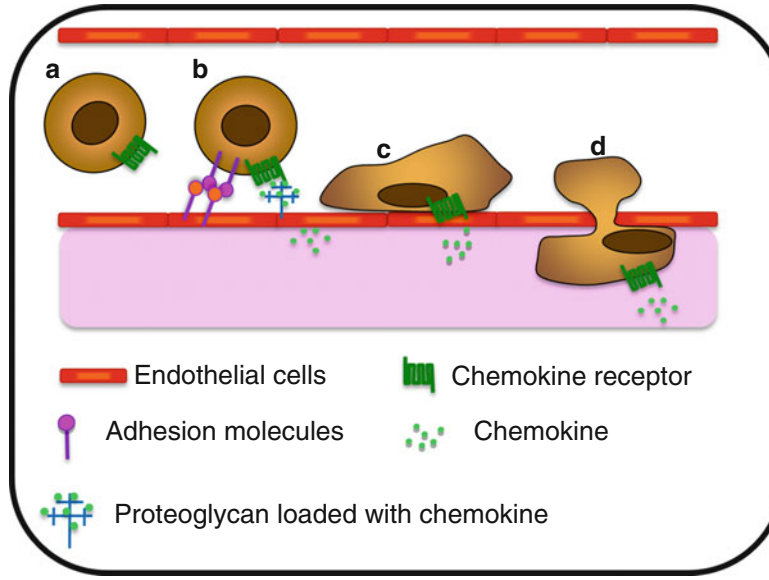

Fig. 8.2 Chemokine-associated extravasation process. (**a**) Circulating cell within the bloodstream. (**b**) Chemokine presented by proteoglycan on activated endothelial cells, induce the expression of adhesion molecules implicated in the slow rolling and the capture process. (**c**) Once stuck to the endothelium, cell exerts crawling behavior on the luminal side of the blood vessel and (**d**) extravasates and migrates through the tissue toward a chemokine gradient

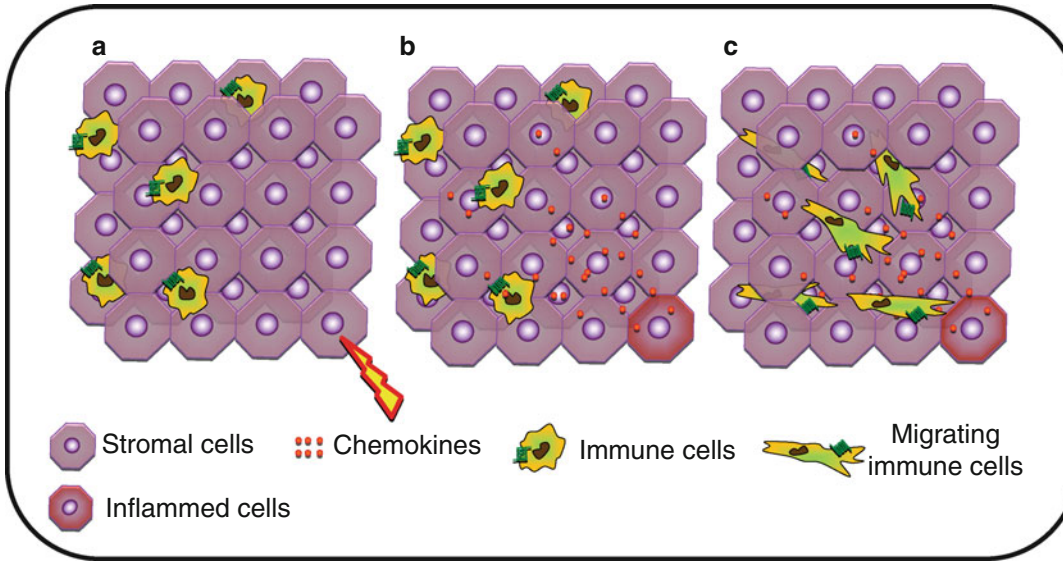

Fig. 8.3 Interstitial migration. (**a**) Upon activation, (**b**) stromal cells will produce chemokines forming a gradient within the tissue. (**c**) Tissue-infiltrated immune cells will migrate through the tissue toward the higher concentration of chemokine

in the primary amino acids sequence [14]. CKRs are seven transmembrane G-protein-coupled receptor classified according to the CK family they bind. As previously mentioned, most CKs bind to several receptors, and most of the receptors can bind several CKs with different affinities. Additionally, one cell subset can express different CKR and the same CKR is expressed by different cell subsets. This apparent redundancy is in reality a tool to tightly regulated leukocytes, stem cells, and other cell types' migrations during physiological and pathological condition.

It is now well established that CK function is not limited to cell migration. It has been clearly demonstrated that CKs directly control cell proliferation, survival and senescence, as well as cytokine secretion and phagocytic properties (Fig. 8.4). It is the balance between these

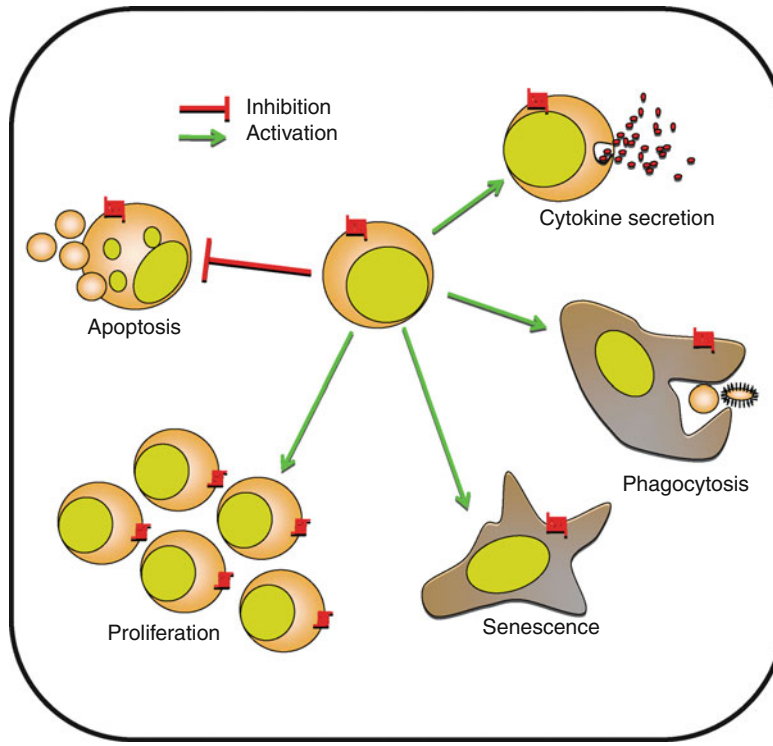

Fig. 8.4 Control of cell biology by chemokines. Besides cell migration, chemokines are implicated in multiple cellular functions including apoptosis, proliferation, and senescence. Chemokines are also directly implicated in cell activation, cytokine secretion, or phagocytosis

migratory, secretion, phagocytic, survival, and proliferation signals which explains the central roles of CK in development, tissue homeostasis, repair, inflammation, and immunity.

8.3 Control of Tumor Cell Behavior

The biological property controlled by the CK/CKR recognition system is not restricted to chemotactism. Several important processes involved in the behavior of tumor cells will be affected by these axes. In this section, the effect of CK/CKR expression on tumor cell behavior and cancer progression is discussed.

8.3.1 Chemokines and Chemokine Receptor Alterations During Neoplastic Transformation

Primary neoplastic transformation leads to strong modification of the transcriptome and proteome which is mainly shaped by immune selection of resistant tumor variants. CK and CKR are not oncogenes per se; however, modulation in the production of CK or their receptors by tumor cells is often the result of oncogenic modifications and immune selection (Fig. 8.5). The first evidence came from a human papillary thyroid cancer. The authors showed that RET (rearranged during transfection)-tyrosine kinase rearrangement promotes the secretion of numerous inflammatory cytokines, including CCL2, CCL20, and CXCL12, and increases the expression of CXCR4 [15]. Later studies have shown that Myc overexpression in pancreatic cancer has been associated with increased CK expression [16, 17]. Nevertheless, the predictive outcome of oncogenic modifications on the regulation of CK and CKR expression is difficult to assess. While RAS-RAF signaling pathway promotes CXCL8 and CXCL1 transcription in pancreatic and ovarian cancer, it inhibits CCL27 transcription in skin cancer [18–20]. Similarly, Von Hippel-Lindau tumor suppressor mutation in renal cancer [21] and TP53 mutation in cancer stem cells promote CXCR4 expression [22] while downregulating its expression in breast cancer cells [23].

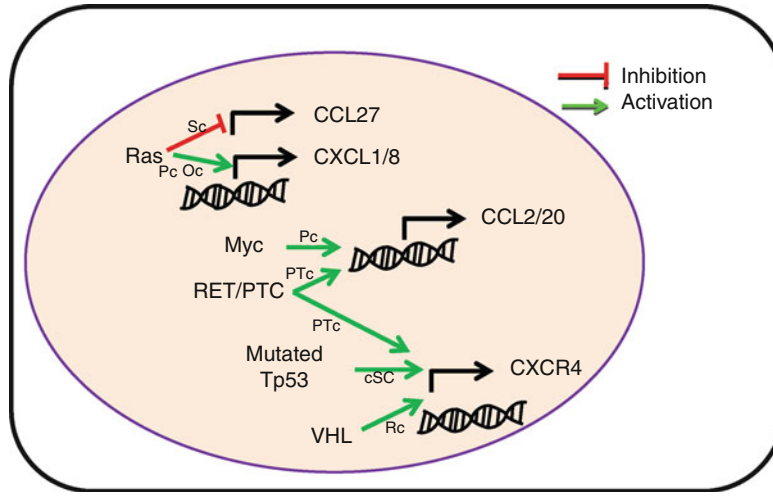

Fig. 8.5 Oncogenes induce altered chemokine and chemokine receptor expression by tumor cells. Common oncogene mutations are associated with modification of chemokine or chemokine receptor transcription, resulting in tumor promotion. *RET/PTC* rearranged RET tyrosine kinase, *VHL* Von Hippel-Lindau tumor suppressor gene, *Sc* skin cancer, *Pc* pancreatic cancer, *Oc* ovarian cancer, *HPTc* human papillary thyroid cancer, *cSC* cancer stem cell, *Rc* renal cancer

Through modification in the profile of CKR expression, tumor cells will change their sensitivity to the microenvironment and acquire new migratory and homing capabilities.

8.3.2 Metastasis/Homing

The metastasis index is undoubtedly the major factor of prognosis and determines the therapeutic attitude. Metastasis defines the process through which tumor cells leave a primary site to settle in a distant location and creates a new colony. This phenomenon is a characteristic of tumor malignancy including tumor invasion, intravasation, and homing to different sites. This has to be distinguished from the potential secondary localization of circulating tumor cells which only involves the homing mechanism.

8.3.2.1 Tumor Invasion

The first step of metastasis spreading relies on either tumor cell or stromal cell-mediated fibrosis activity and the ability of tumor cells to acquire migration and intravasation capabilities, in order to leave the primary tumor site and reach the bloodstream. Chemotaxis of tumor cells is well characterized [24]. This process requires a paracrine loop between tumor cells and stromal cells, such as macrophages shaping the microenvironment to favor metastasis [25]. Different chemical gradients may induce tumor cell chemotaxis, but the direct implication of CKs in this specific process is poorly documented. We can distinguish the indirect contribution of CK to the chemotaxis activity of cancer cells through angiogenesis, fibrogenesis, and matrix remodeling mediated by stromal cells.

CXCL12/CXCR4 is the major axis directly involved in tumor cell metastases. Overexpression of CXCR4 in rat mammary adenocarcinoma enhances the motility of tumor cells in the primary tumor [26]. This receptor is widely involved in the epithelial-to-mesenchymal transition (EMT) process, which is a major step leading to metastasis [27, 28]. Few studies have reported the implication of other CKs and CKRs such as CCL18, CCL2, or CXCR7 [29–31] through the activation of EMT-implicated signaling pathways. IL8/IL8R axis might also favor maintenance of the mesenchymal status of the tumor cell [32]. Interestingly, the integration of multiple CKR axes adds complexity to the tumor invasion process. Indeed, overexpression of CXCR4 promotes invasion. However, coexpression of CXCR7 which binds the same ligand CXCL12 impairs invasion but favors angiogenesis and primary tumor growth [26].

8.3.2.2 Homing

Once in the bloodstream, the tumor cell needs to migrate to a site that will allow its engraftment,

Table 8.1 Metastases implantation of various cancer types based on their chemokine receptor expression

		Primary tumor				
		Melanoma	Breast cancer	NSCLC	Colon	Leukemia
Metastases implantation site	Skin		CCR10/CCR7/CXCR4			
	Intestine	CCR9				
	Bone		CX3CR1			
	Lymph node		CXCR4/CCR7	CCR7	CXCR3	CCR7
	Liver		CXCR4	CX3CR1	CCR6	
	Brain	CCR4	CX3CR1	CX3CR1/CXCR4	CXCR4	CCR7
	Lung	CXCR4/CXCR2				

survival, and proliferation. In 2001, Muller et al. demonstrated for the first time that the expression of specific CKRs by tumor cells could predict the implantation of malignant cells in tissues expressing high levels of the receptor ligands [33]. Since then, several other studies have established associations between metastases, CKR expression, and implantation sites for various cancer types (Table 8.1). Consistently with their homeostatic functions, CCR7 expression by tumor cells is associated with lymph node metastases; CCR10 with skin metastasis; CX3CR1 with brain, liver, and bone metastases; CCR9 with intestine metastases; and CXCR4 with bone and liver metastases [33–36].

Overall, these observations show that CK axes generate a complex relationship between tumor cell and the environment and deserve further attention in preclinical studies as it represents an important target with clinical application.

8.3.3 Senescence, Proliferation, and Survival

Tumor expansion results in the capacity of tumor cells to proliferate infinitely without developing senescent mechanisms. Several CKs have demonstrated the ability to activate signaling pathways in favor of this goal.

Cellular senescence is generally defined as an irreversible state of G1 cell cycle arrest in which the cell is refractory to growth factor stimulation. Activation of CXCR2 by either CXCL1 or CXCL8 can result in senescence induction [37]. CXCR2 activation is thus able to act as a suppressor of malignancy in prostate and breast cancer [38, 39].

Inhibition of tumor proliferation by CXCR2 ligand is probably limited to tumor models and to early stages of tumor development. Indeed, the same CK axes display opposite effects in other tumor models. CXCR1 and CXCR2 activation by CXCL8 promotes the proliferation of gastric cancer, esophageal cancer, non-small lung cancer, and melanoma cell lines [40–43]. Other receptors of the CXC receptor family are involved in tumor cell proliferation. CXCR6 is involved in cell proliferation of pancreatic cancer cells [44], and CXCR4 is associated with tumor proliferation in numerous models, including ovarian, melanoma, glioma, renal, lung, and thyroid cancer cells [27, 45]. Few studies have investigated the implication of CCRs in the control of tumor cell proliferation. CCR6 favors colon tumor cell proliferation upon CCL20 activation [46], and CCR9 favors pancreatic cancer cell proliferations upon CCL25 activation [47].

Another role of CK in tumor cell biology is the ability to control tumor cell survival, essentially mediated through the CC receptor family. CCR10 activation promotes phosphatidylinositol-3-kinase-mediated protection from apoptosis of melanoma cells [48]. The same mechanisms are observed in squamous cell carcinoma of the head and neck after CCR7 activation [49]. CCR7 engagement by CCL21 is also implicated in the prevention of apoptosis in NLCLC, through ERK-dependant activation pathways [50].

CK direct promotion of tumor cell survival is not limited to CC chemokines; CXCL12 through CXCR4 activation promotes hepatoma, ovarian, and chronic leukemia tumor cells survival [51], and CXCR7 activation increases cell survival by reducing apoptosis [52].

Overall, these observations highlight extended functional contributions of the CK system to tumor development and reveal that they are not merely restrained to chemotaxis.

8.4 Control of Immune Cell Behaviors

As described previously, the immune system is known to shape the tumor through the "tumor editing" phenomenon. In this context, CKs are directly or indirectly implicated in the control of immune cell activation, migration to the priming site, and immune response induction. It is now clear that in most cases, the CK network is shunted by the tumor, favoring its escape from immunosurveillance and tumor progression. Nevertheless, the production of some CKs promotes the antitumor immune response and has been associated with improved patient outcome, including lower recurrence rate or increased patient survival [53].

8.4.1 Chemokines Involved in T-Cell Antitumor Immune Response

Induction of antigen (Ag)-specific antitumor immune response requires the uptake of tumor Ag by professional antigen-presenting cells (APCs) and migration from the tumor site to the corresponding draining lymph node, in order to present the processed tumor-Ag to T lymphocytes. These major immune functions can be divided into different steps for which the CKR network has important regulatory implications [54].

8.4.1.1 Migration of APCs to the Priming Site

Encounter with tumor Ag induces maturation of APCs present in the tumor environment. One feature of this maturation is the downregulation of peripheral tissue-associated CKR like CCR1, CCR5, and CCR6 and the upregulation of CCR7. Due to the constitutive expression of CCR7 ligand, CCL19, and CCL21 by peripheral lymph nodes, this switch of CKR expression by APCs promotes their migration toward the priming site. Once in the draining lymph node, APCs will locate in the preferential area to present the tumor Ag to the CCR7 expressing naive lymphocyte.

8.4.1.2 Ag Presentation to T Lymphocytes

Despite the fact that APCs display low dynamic activity, naïve lymphocytes have a high basal mobility favoring scanning of thousand APCs per hour [55, 56]. This behavior requires CCR7 expression by T lymphocytes [57]. An additional CKR-dependant mechanism favors the probability of encounter between APCs and T lymphocytes. Encounter of Ag-specific $CD4^+$ or $CD8^+$ T cells with an APC bearing their cognate Ag induces the secretion of CC-chemokines by the conjugate, namely, CCL19, CCL5, CCL3, and CCL4. These CKs will promote naïve T-cell scanning behaviors and attraction toward the conjugate [58–60], which is known to favor the establishment of memory immune response, in addition to the induction of polyclonal responses against different tumor Ags [61].

CKs are also implicated in the improvement of APC/T-cell adhesion mechanism as well as in immunological synapse stabilization, promoting T-cell priming (Fig. 8.6). CCR7 ligands secreted in the lymph node promote immunological synapse formation by T cells [62]. CXCR4 and CCR5 expressed by T cells are recruited toward the immunological synapses made with the APC. This polarization results in desensitization of T cells from external sources of CKs and improves synapse stability. A similar mechanism is observed during the interaction between tumor-infiltrated lymphocytes (TILs) and tumor cells. Indeed, the recruitment of CCR5 at the immune synapse formed between the TIL and the tumor cell results in defective responses to TIL toward a CCR5 gradient [63]. This mechanism allows for the modulation of the "GO" signals generated by

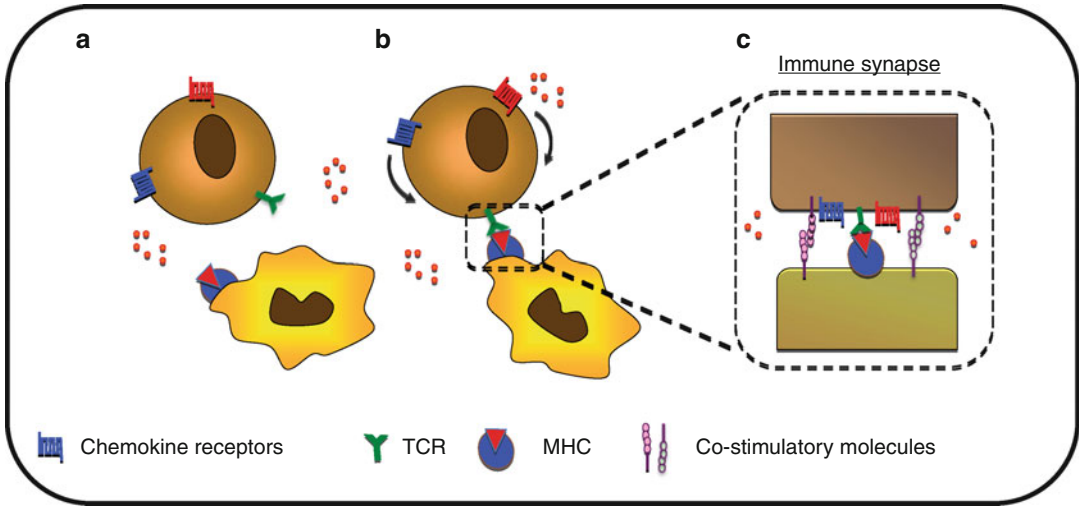

Fig. 8.6 Control of cell polarization toward immune synapse. (**a**) T-cell scan for their cognate antigen-presenting cell. (**b**) Upon recognition, T cell will polarize chemokine receptors toward the immune synapse. (**c**) This sequestration of CKR leads to reduced sensitivity to distant CK gradient and may participate in the stabilization of the immune synapse

CKs, competing with the "STOP" signals mediated by the TCR-MHC interaction [64].

8.4.1.3 Migration of Effector T Lymphocytes to Tumor

Naive T cells, after clonal expansion and differentiation into effector T cells, migrate toward the tumor site, implying that T cells downregulate the expression of the CKRs implicated in the retention at the priming site like CCR7. In addition, they upregulate various CKRs including CCR1, CCR3, CCR5, and CXCR3 allowing their movement toward the tumor site [65]. Cytotoxic T lymphocytes (CTLs) recruitment to the tumor site is consistent with this pattern of CKRs expression and is mainly mediated by CCL3, CCL5, CCL20, CXCL9, and CXCL10 [54]. Membrane-anchored CKs expression such as CXCL16 and CX3CL1 have also been shown to correlate with greater numbers of tumor-infiltrated lymphocytes and improved prognosis in colorectal cancer [66, 67]. The antitumor effect of the membrane-bound CK form *vs*. the soluble form is yet to be clearly established.

The control of TIL localization within the tumor is ill-defined. It is obvious that in most cases, TILs are mainly found at the tumor periphery; however, the underlying mechanisms remain unclear. Several clues could help us speculate on the mechanism of trapping the TILs at the tumor periphery. The recent contribution of real-time imaging showed that dense peripheral extracellular matrix might restrain TILs' access to the tumor parenchyma [68]. Whether specific niches of CKs are expressed on collagen fibers is unclear and needs further investigation. In addition, dynamic analysis showed that Ag-specific CTLs are trapped in the network of tumor-associated APCs restraining their infiltration and probably favoring immunosuppression [69, 70]. The role of CKs in this trapping is not defined, but Ag expression by APC at least induces stable engagement between the CTL and the APC. In addition, experimental evidences showed that non-tumor-Ag-specific TIL cannot infiltrate the tumor deeply without the prior tumor cells' destruction by Ag-specific CTL. These results suggest that deep infiltration of the tumor by TIL might be favored by chemotactic agents secreted upon tumor cell destruction by CTL or on extensive ECM remodeling to allow their interstitial migration [71].

Overall, considering the numerous CKs expressed by the various cell subsets of the tumor microenvironment, it is very difficult to address

specific contributions of CK/CKR couple in the interstitial migration and positioning of T lymphocytes within the tumor parenchyma. The various properties of these molecules have demonstrated that this positioning is controlled by sensitivity to the chemotactic gradient and the subsequent desensitization upon polarization toward the synapse or the downregulation of the expression of CKRs.

8.4.2 Chemokines in Innate Immune Components

Innate immune cells constitute a first barrier against tumor development. However, due to their plasticity and capacity to produce a myriad of cytokines, chronically activated innate immune cells are key modulators of cell activation and survival, as well as regulators of the ECM metabolism. Several physiological processes necessary for tumor development, such as increased cell survival, tissue remodeling, angiogenesis, and suppression of antitumor adaptive immune responses, are regulated by innate immune cell infiltrate in the tumor.

Macrophages are the main stromal cell population present in the tumor parenchyma. They can account for more than 50 % of the tumor mass. The role of TAM in tumor development is critical, as these cells, depending on their state of activation, can display antitumor properties associated with production of Th1 cytokine, high quantity of reactive oxygen species, and efficient Ag presentation or they could display protumor properties mediated by the secretion of Th2 cytokine, proangiogenic factors, growth factors that support tumor survival, and proliferation and the secretion of MMP which promote tumor invasion and metastases. Consistently, the impact of TAM on tumor development and metastases will depend on the balance between M1 antitumor macrophages and M2 protumor macrophages.

Depending on the tissue, resident macrophages are in a small proportion derived from the recruitment of circulating monocytes assuring immunosurveillance and mainly origin from self-renewal of interstitial resident macrophages derived from the yolk sac or fetal liver [72]. Within neoplastic tissues, it is suggested that TAMs are mostly recruited from the periphery. Nonetheless, knowledge of the relative proportion of native resident macrophages remains a poorly investigated field in oncology. CCL2, also called MCP-1 for monocyte chemoattractant protein-1, is probably the most frequently found CC-CK in tumors involving recruitment of circulating monocytes [73]. Interestingly, in a melanoma system where tumorigenesis is dependent on an external growth factor CCL2, there is a biphasic effect depending on its secreted quantity. High amounts are associated with a massive recruitment of TAM into the tumor with dominant antitumor activity, while lower amounts induce lower infiltration into the tumor resulting in tumor promotion through the secretion of growth factor by the macrophages [74]. These results point out the importance of the ratio between protumor and antitumor macrophages recruited into the tumor.

Other CKRs implicated in TAM recruitment are CX3CR1 and CCR1. In human glioblastoma, the level of tumor infiltration by microglial cells is dependent on CX3CR1. Patients with a functional mutation in the CX3CR1 gene associated with impaired monocyte migration have a reduced TAM infiltration into the tumor [75]. Injection of a thymoma tumor cell line (EL4) with a liver tropism to mice results in an increased infiltration of the liver by immune cells, including macrophages. In CCR1 KO mice, this recruitment during the first stage of the tumor development is massively reduced [76].

CXC chemokine receptors could also be implicated in TAM recruitment. In humans, IL-4 and IL-13, two cytokines secreted in the tumor environment, sensitize monocytes to CXCL1 and CXCL8 by upregulating their receptors (CXCR1 and CXCR2). Thus, these cytokines indirectly promote the recruitment of TAM into the tumor through CXC chemokine receptors [77].

As previously discussed, CKs not only control leukocyte recruitment into the tumor but also organize their localization within the tumor. Lack of proper vascularization at the center of the tumor induces the secretion of

several hypoxic factors like hypoxia-inducible factors (HIFs). HIFs promote the expression of CXCR4 by macrophages, favoring their recruitment toward tumor hypoxic areas [78]. On the other hand, tumor environment decreases CKR expression on monocytes. Indeed, macrophages from tumor sites express low levels of CKR [79]. Time-lapse imaging of TAMs in experimental murine model revealed that TAMs display reduced displacement but intense protrusive activity [69, 70]. Downregulation of CKR might explain this retention at the tumor site.

CKs do not only act on leukocyte attraction but are also implicated in their activation. Induction of copper/zinc-superoxide dismutase by CCL5/CCR5 activation causes tumor necrosis factor-alpha and reactive oxygen species production by macrophages [80], promoting tumor destruction. Inversely, in human monocytes, CC chemokines induce the transcription of metalloproteinase, implicated in tumor invasion and spreading. The fact that both TAM recruitment and activation are regulated by CK increases the potential interest of targeting TAM for antitumor therapies.

NK cells represent another component of the innate immune system highly involved in antitumor immune responses. NK cell recruitment to the tumor is mainly mediated through the CXCL10-CXCR3, CX3CL1/CX3CR1, and CCL3-4-5/CCR5 axes. High CX3CL1 quantity is associated with increased NK cell recruitment into the tumor in both human and mice [81, 82]. Similar phenomenon is observed with increased CCL5 and CCL3 expression by tumor cells in mouse models [83, 84]. CXCR3 is implicated in the recruitment of human NK cells to breast cancer tumor, which is mediated by CXCL10 secretion from tumor cells in response to IFN-γ produced by the NK cells themselves [85, 86]. Thus, CKs not only control NK cell recruitment but also regulate their antitumor properties. CX3CR1 activation by CX3CL1 results in improved antitumor cytotoxicity of NK cells [87, 88]. CCL3, CCL4 and CCL5 have been shown to activate NK cytotoxicity through induction of degranulation [89, 90].

8.4.3 Chemokine and Tumor-Induced Tolerance

Recruitment of tolerogenic cells such as regulatory T cells or immunosuppressive myeloid subsets is a feature or immune escape. Tumor cells secrete ligands of CKRs expressed by immature, regulatory or Th2 polarized cells. CCL22 and CCL17 produced by tumor cells recruit monocytes, as well as Th2 lymphocytes and regulatory T cells through CCR4 signaling [91]. This strategy of immune escape has been also selected in viral-induced oncogenesis process. HHV8 virus, the pathogen of Kaposi's sarcoma, encodes three viral CKs which bind to CCR3, CCR4, and CCR8 involved in the recruitment of Th2 and regulatory T cells [92].

Stromal cells produce CKs which promote the recruitment of protumoral cells. Amongst others, TAM produces CCL18 which is induced by IL10 [93]. CCL18 favors the recruitment of naïve T cells through activation of an unknown receptor. It is proposed that these naïve T cells acquired tolerogenic properties in contact with the tumor environment. $CCR6^{+}$ immature lymphoid DCs recruitment into the tumor is favored by the secretion of CCL20 from both tumor cells and TAM [94]. CCL5 recruits immature DCs as well by binding CCR1 and CCR5 [95]. Immature DCs acquire tolerogenic properties in the tumor environment and participate in the immune tolerance loops against tumor Ags [96].

Subversion of tumor immune component is a central point of tumor outcome. The above described implication of CK in cellular mechanisms should provide the basis to better understanding the clinical implication of CK network in cancer pathology. The regulation of the balance between immunogenic and tolerogenic components has deserved major attention for a long time and is the basis of immunotherapy which represents an apparent inexhaustible field of innovative anticancer strategies. Targeting the CK system in this goal is in the course of important investigation through the development of pharmaceutical compounds able to stimulate or antagonize CKR axes.

8.5 Alternative Tumor-Associated Physiological Functions of Chemokines

8.5.1 Angiogenesis

One of the features of CKs is their dual role in the angiogenic process. In the tumor environment, there is increased production of proangiogenic CK, while angiostatic CKs are downregulated. In addition to a direct angiogenic effect of CKs, this activity is indirectly potentialized by the CK-induced recruitment of leukocyte displaying angiogenic properties such as neutrophils or macrophages [97].

CK from the CXC family are probably the most described for their direct implication in tumor-associated angiogenesis. CXCLs 1, 2, 3, 5, 6, 7, and 8 display angiogenic properties. All these CKs contain a specific amino acid sequence of glutamic acid-leucine-arginine (or ELR for short) immediately before the first cysteine of the CXC motif (ELR-positive). This ELR sequence absence from the other CXC chemokines is responsible of the proangiogenic properties of most of the CXC chemokine [98].

ELR^+ chemokines mediate angiogenesis through binding to the CXCR2 receptor. ELR^+ chemokines are able to recruit endothelial precursor cells, induce cell proliferation, and promote maturation. These mechanisms could be negatively regulated by a decoy CKR expressed by endothelial cells called duffy antigen receptor for CK (DARC). Unlike most of the other CKR, DARC is not linked to G protein, and its activation does not induce calcium flux. DARC reduces angiogenesis by sequestering all the ELR^+ CKs.

One specificity within ELR^- chemokines is attributed to CXCL12 which is the only ELR^- chemokine with proangiogenic activity. CXCL12 mediates its proangiogenic effect by directly promoting the recruitment of endothelial progenitor cells [99, 100] or indirectly by promoting tumor angiogenesis through the recruitment of $CXCR4^+$ proangiogenic monocyte [78, 101] and through the secretion of vascular endothelial growth factor (VEGF) by CXCR7 activation [102].

In contrast, ELR^- chemokine secretion is often associated with attenuation of angiogenesis. ELR^- CXC chemokines are described by their angiostatic properties. ELR^- CXC chemokine secretion is induced by IFN-α and IFN-β. Through CXCR3 binding, these CKs mediate their angiostatic properties by inhibition of ELR^+ chemokine, VEGFα, and βFGF proangiogenic effects *in vitro* [103]. Interestingly, the expression of CXCR3 is dependant of the cell cycle phase, limiting the angiostatic properties of ELR^- CXC chemokines to the S/G2-M phase [104].

This important association of CKs and angiogenesis within the tumor environment sets the inhibition of ELR^+ chemokine as a robust antitumor therapy.

8.5.2 Fibrosis and Extracellular Matrix Remodeling

The association of CKs in EMT leading to fibrosis activity has been previously suggested by studies; however, there is no clear evidence that CKs play a direct role in this process.

Fibrosis and extracellular matrix remodeling are continuous processes present in the tumor parenchyma reflecting the intense dynamic and migratory activity of the neoplastic tissue. Two different types of migratory activity are defined, namely, the amoeboid and mesenchymal migration. The amoeboid migration does not require extracellular matrix (ECM) remodeling through matrix metalloproteinases (MMPs) activity due to the ability of the cell to squeeze through the ECM. The mesenchymal migration relies on previous proteolysis and degradation of the ECM to generate sufficient space for cell displacement. CK-mediated induction of MMP is mostly mediated by CC chemokines; CCL5 and CCL9 produced by mesenchymal stem cell promote tumor cell invasion in a MMP-dependant manner [105, 106]. CCL25 promotes MMP secretion in ovarian cancer cells through CCR9 binding and favors tumor cell invasion [107]. CCL21/CCR7 interaction favors MMP-9 secretion, tumor invasion, and metastases in colon cancer cells and in B-cell

chronic lymphocytic leukemia cells [108, 109]. At least, one CXC chemokine has been related to MMP activity; thus, CXCL12 is implicated in increased MMP2 activation and increased cell invasion in a pancreatic cancer cell line [110].

Studies have suggested that the extracellular matrix promotes tumor escape from the immune system by trapping antitumor leukocytes at distance from tumor cell niches [111]. However, tumor progression and metastases require degradation of this extracellular matrix surrounding the tumor. The main protagonists of these physiological activities are represented by mesenchymal stem cell (MSC)-derived cell populations. CXCL12 is implicated in the recruitment of mesenchymal stem cells (MSCs) from the bone marrow. Bone marrow-derived MSCs can account for up to 25 % of the cancer-associated fibroblasts, the main source of fibrosis within the tumor [112].

There is ongoing evidence that targeting proteolysis activity in combination with chemotaxis would provide promising results in the strategy to inhibit tumor cell invasion and metastasis.

8.6 Clinical Aspect

CKs are implicated in several aspects of tumor development. Due to these pivotal roles in tumor biology, CKs have been frequently associated with tumor evolution and clinical outcomes and have been highlighted for their potential use as prognostic or diagnostic markers. Therefore, they represent a promising target with a potential for a diverse range of therapeutic strategies.

8.6.1 Prognosis

Due to its importance across a wide range of physiological mechanisms, CK/CKR network alteration could impact tumor development. Correlative studies using genetic polymorphisms provide essential information for prognosis. Several functional polymorphisms in CKs or CKRs have been studied in order to establish correlation between functional variants and tumor risk or progression (Table 8.2).

The paragraphs below focus on the most commonly described polymorphisms, their functional relevancies, and their subsequent prognostic value in tumor risk and/or progression.

8.6.2 CC Chemokines/Chemokine Receptors

8.6.2.1 CCL2

A single-nucleotide polymorphism (SNP) in the CCL2 promoter, based on the substitution of an adenine by a guanine in position −2518 (A < −2518 < G), is associated with increased CCL2 secretion [113]. This polymorphism with an allelic frequency close to 30 % is associated with an increased susceptibility to the development of breast, gastric, and oral squamous cancer. However, it is not associated with an increased risk of developing hepatocellular and prostate cancer, glioblastoma, and melanoma. Despite this lack of association with the development of melanoma, CCL2 polymorphism is associated with increased Breslow index, suggesting its link with melanoma progression [114]. CCL2-2518G variant is also associated with increased metastases development in nasopharyngeal and breast cancer. In the former case, the deleterious effect of the polymorphism is observed only after radiotherapy [115]. Overall, the deleterious effect of the CCL2-2518G allele-associated increase of CCL2 expression is consistent with the protumoral effect of TAM in most tumors, as previously described above.

8.6.2.2 CCL5

Conflicting data arises from the study of the CCL5 G < −403 < A polymorphism on cancer risk. This mutation is thought to be responsible for the decreased secretion of CCL5 and is associated with decreased risk for leukemia and gastric cancer in women [116], as well as an increased risk for prostate and pancreatic cancer [117]. This discrepancy could reflect the balance

Table 8.2 Association between chemokines and chemokine receptor polymorphisms and tumor risk and/or progression

		CCL2 −2518 A<G	CCL5 -403 G<A	CXCL8 −251T>A	CXCL12 801G<A	CCR2 64I	CCR5 Delta 32	CX3CR1 V249I
Breast	**Risk**	–		–*	=/–*	–		
	Prog	–		–				
Hepatocellular	**Risk**	=			=/–	=/–	=	=
	Prog	=			=/–	=	=	
Gastric	**Risk**	–	+	–*/ =*				
	Prog							
Glioblastoma	**Risk**	=						=
	Prog	=						+
Prostate	**Risk**	=	–	+*	–	–	–	
	Prog							
Oro/naso pharyngeal	**Risk**			–*	–	–*		
	Prog	–		–				
Melanoma	**Risk**	=					=	=
	Prog	–					=/–	=
Pancreatic	**Risk**		–					
	Prog							
Leukemia	**Risk**		+					
	Prog				–			
Colorectal	**Risk**			+/=				–
	Prog			=	–			
Bladder	**Risk**			=		–*	–	
	Prog			=				
Lung	**Risk**			=	–*			
	Prog							
Cervix	**Risk**					–*		
	Prog					–		

Prog prognosis, + good indicator, – poor indicator, = no association, * meta-analysis

between the antitumor effects of CCL5 through recruitment of cytotoxic CTL and the protumoral effect of CCL5 through recruitment of immature DC. Nonetheless, there is no evidence supporting an association between CCL5 polymorphism and tumor progression.

8.6.2.3 CCR5

CCL5 main receptor (*CCR5*) is also subject to another relevant polymorphism. A deletion of 32 base pairs named CCR5 delta 32 results in a reading frame shift, associated with complete defect in receptor expression. The impact of the

polymorphism in tumor risk and progression is not well documented. Most studies conclude a lack of association; however, one report suggests that CCR5Δ32 could be associated with higher risks of the development of gallbladder cancer [118]. In melanoma, CCR5Δ32 is associated with reduced survival of patients with grade 4 tumor treated by immunotherapy strategies [119]. These observations might reflect the role of CCR5 in the induction of T-cell priming and memory.

8.6.2.4 CCR2

CCR2 V64I polymorphism has also been studied for its implication in tumor risk and progression. There is no known effect of the genetic variation on the CCR2/CCL2-signaling pathway, but it is associated with CCR5 instability, which could be explained by stability alteration of the CCR2/CCR5 dimer. Most of the studies conclude that there is an increased risk for people carrying the rare variant. This is the case for cervical, oral, bladder, prostate, and endometrial cancer. A recent meta-analysis with 2,661 cancer patients and 5,801 healthy controls found an overall significant association between the CCR2-V64I polymorphism and cancer risk [120]. In the subgroup analysis stratified by cancer types, there was a significant association between this polymorphism and the risk of bladder, cervical, and oral cancer.

8.6.3 CXC Chemokines

Two CXC chemokines, CXCL8 (also referred as interleukin-8) and CXCL12 (SDF-1), have been intensively investigated for their association between polymorphisms and tumor risk and development.

8.6.3.1 CXCL8

CXCL8 T<−251<A polymorphism is probably one of the most studied CK polymorphism in cancer. Its physiological effect and its impact on CXCL8 expression remain to be elucidated. There is an apparent discrepancy between studies on these effects; however, this may reflect specificity depending on the cell type or the cell activation status. The implication of CXCL8 polymorphism in cancer risk and outcome remains unclear. Unfortunately, controversies in the literature make any interpretation challenging. Several meta-analyses have been performed in order to gain some clarity, and despite some variation in the conclusion, it appears likely that the rare variant of CXCL8 promoter region is associated with increased risk of gastric and oral cancer [121–123].

8.6.3.2 CXCL12

CXCL12 is subject to a polymorphism in a 3′ untranslated region named CXCL12 3′ G801A. The rare variant is associated with increased secretion of CXCL12. Consistent with the protumoral effect of CXCL12 mentioned above, studies essentially report that CXCL12 801A variant is associated with an increased risk for several cancers (lung, breast, oral, prostate, hepatocellular, and colorectal cancer). It is also thought to favor tumor progression or metastases in lung cancer, hepatocellular carcinoma, colorectal cancer, and myeloid leukemia. The only three meta-analyses performed to date conclude that there is an increased risk for breast and lung cancer, without any significant effect on other cancer types [124–126].

8.6.4 CX3C Chemokine Receptors

The only receptor for the CX3C chemokine family is CX3CR1, which is also subject to polymorphisms associated with cancer outcome. Substitution of a valin by an isoleucine in position 249 results in increased adhesion of the couple CX3CR1/CX3CL1 and defective migration of $CX3CR1^+$ cells. The rare variant is associated with increased risk of colorectal cancer, but not hepatocellular cancer, melanoma, and glioblastoma. In this last case, the rare variant is associated with improved patient survival after tumor biopsies and decreased infiltration of the tumor by microglial cells [75]. This is consistent with the promotion of glioblastoma invasion by microglial cells [127].

8.6.5 Chemokine Circulating Expression

CK circulating levels have also been related to cancer progression. A high concentration of CCL17 is associated with the progression of Hodgkin lymphoma (HL) after treatment [128]. Interestingly,

Table 8.3 Current clinical trials evaluating the benefits of targeting chemokines or chemokine receptors cancer therapies

Inclusion criteria	Phase	Treatment
Colorectal cancer	Phase I/II	Chemokine-modulatory regimen
Stage IV adenocarcinoma of the lung	Phase I/II	GM.CD40L and CCL21
Metastatic castrate-resistant prostate cancer	Phase II	Anti-CCL2 carlumab
Solid tumors	Phase I	Human monoclonal antibody against CCL2 (CNTO 888)
Colorectal cancer patients with hepatic liver metastases	Phase I	CCR5 antagonist (Maravirok)
Previously treated peripheral T-cell lymphoma	Phase II	Anti-CCR4 monoclonal antibody KW-0761 (Mogamulizumab)
CCR4-positive adult T-cell leukemia-lymphoma	Phase II	Anti-CCR4 (KW-0761)
High-grade glioma	Phase I	CXCR4 antagonist (Plerixafor/AMD3100) and bevacizumab
Multiple myeloma previously treated with lenalidomide	Phase III	Filgrastim with or without CXCR4 antagonist (plerixafor/AMD3100)
Non-Hodgkin lymphoma	Phase III	CXCR4 antagonist (Plerixafor/AMD3100) and G-CSF
Multiple myeloma	Phase Ib	Anti-CXCR4 (BMS-936564) alone or plus lenalidomide/dexamethasone or bortezomib/dexamethasone
Multiple myeloma	Phase I/IIA	CXCR4 antagonist (BKT-140)

opposite effects are observed in melanoma, where high *CCL17* expression is associated with progression-free survival in patients with immunotherapeutic treatment [129]. Elevated concentrations of CXCL10 in the serum before treatment (monoclonal antibody therapy together with combination chemotherapy) are associated with an increased likelihood of clinical relapse and an inferior survival in patients with diffuse large B-cell lymphoma [130].Despite numerous promising results, CK and CKR genes and molecules are not currently used in clinical settings to evaluate a patients' risk of developing cancer or to predict tumor progression. This could be explained in part by the nonhomogeneous distribution of the polymorphism variants amongst ethnic communities. Additionally, in most cases, *CK* and *CKR* gene polymorphisms are not singularly powerful predictive tools. Their clinical utility is most likely to be dependent on their association with other markers.

8.6.6 Therapeutic Strategies

As discussed throughout this chapter, CKs are implicated in all steps of the tumor development, invasion, and dissemination. Several tools have been developed to target CKs or CKR as innovative strategies in cancer treatment. To date, there is no molecule targeting macrophage release; however, multiple clinical trials from phase I to phase III are recorded at clinical trial.gov website (Table 8.3). Some strategies aim to promote the production of CKs implicated in the recruitment of immune-competent cells to the tumor by injection of IFN, "celecoxib," and "rintatolimod" (NCT01545141). In another trial, patients with lung adenocarcinoma were directly injected with CKs implicated in the recruitment of antitumor effector T cells, in combination with vaccination approach (NCT01433172). Inversely, another trial aimed to inhibit the recruitment of protumoral leukocyte using an Ab against CCL2 in order to control metastatic castrate-resistant prostate cancer (MCRPC) (NCT00992186). However, this strategy failed as all the patients were removed from the study, due to progression of the tumor despite anti-CCL2 treatment.

Another approach aimed to directly target *CKR* expressed by neoplastic cells in order to control tumor or metastases development. The CCR5 antagonist, named "maravirok," originally commercialized for AIDS treatment, is under evaluation for its antitumor property in colorectal

cancer (NCT01736813). Promising results have been obtained with an anti-CCR4 Ab named "KW-0761." Injection of KW-0761 in subjects with CCR4-positive adult T-cell leukemia-lymphoma resulted in the stabilization of tumor progression in half of them. This molecule is now under evaluation in cutaneous T-cell lymphoma (NCT01728805) and in second-phase treatment for peripheral T-cell lymphoma (NCT01611142).

CXCR4 antagonists are probably the most widely used molecules in trials targeting the CK network. "plerixafor" is a FDA-approved CXCR4 antagonist for use in patients with non-Hodgkin lymphoma (NHL) and multiple myeloma. It is used as a preconditioning regimen for its ability to mobilize bone marrow resident hematopoietic stem cells and tumor stem cells toward circulation before chemotherapy. Plerixafor and other molecules targeting CXCR4 are now evaluated in several clinical trials from grades I to III phase in combination with other treatment, in various forms of leukemia and myeloma. Evaluation of CXCR4 targeting in cancer therapies is not limited to blood tumors. Plerixafor is currently being evaluated in a phase I trial in conjunction with "bevacizumab" for patients with high-grade glioma (NCT01339039).

8.7 Concluding Remarks

The advantages of targeting the CK network, through distinct strategies, have already been demonstrated as well as its limitations. A new generation of clinical trials based on a combination of approaches from standard chemotherapies to innovative immunotherapies offer new perspectives in CK network targeting strategies.

The 10 years following the discovery of the majority of CKs were characterized by extensive investigations in the involvement of these molecules in the control of cellular trafficking, specifically leukocytes. Later on, scientists demonstrated that CKs do not only control cell migration but also cell proliferation, survival, and activation state. It is now obvious that CKs act on a wider range of cell types rather than only leukocytes for which they were primarily characterized. The complex physiological processes in which CKs are involved such as tissue homeostasis, immune system maturation and surveillance, and tissue remodeling functions like angiogenesis or fibrosis are shunted in most cases toward tumor promotion. The central role of the CK network in these processes positions the CK system as an attractive target against tumor development, progression, and dissemination. Clinically, *CK* and *CKR* polymorphisms or serum levels are already associated with susceptibility or prognostic markers. Current investigations aiming at controlling tumor development by targeting the CK network are not limited to the direct effect on tumor cells. For instance, it is proposed that CKs could modulate the involvement of TAMs in tumor eradication or protection after chemotherapy suggesting that chemoattractant molecules could be used in combination with standard chemical chemotherapies to favor tumor eradication through modulation of the TAM activity. Despite numerous promising results, few molecules targeting CKRs have received FDA approval. The CXCL12 antagonism is already being used in patients with leukemia or myeloma to promote tumor cell mobilization toward the bloodstream before treatment, and the CCR5 antagonist maravirok is currently being evaluated in colorectal cancer. These low numbers of molecules targeting CKs in the market could be explained by the relatively recent discovery and characterization of the CKs. In addition, the central role of CKs in most biological functions would lead to potential numerous side effects. Given the phenomenal amount of progress made by the scientific and the medical community, it is most likely that these challenges will be overcome. Several innovative technologies allowing for more efficient and specific delivery of chemical compounds have been proposed and optimized during the last few years, such as Ab-coupled treatment and encapsulated or viral delivered constructs. Targeting the CK network using these tools will probably constitute the next step in the development of a cancer therapy with minimal side effect.

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The CD95/CD95L Signaling Pathway: A Role in Carcinogenesis

9

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9.1 Introduction

Apoptosis, or programmed cell death, plays a pivotal role in development, organ homeostasis, and immunosurveillance. The term apoptosis was coined by Kerr et al. in 1972 [1] to describe the process of cell death associated with morphological changes, including nucleus and cytoplasm condensation and protuberances from the plasma membrane producing apoptotic bodies, so-called blebs, which are rapidly phagocytosed [1, 2]. Inhibition of this cellular process is observed in different pathologies, such as cancer and autoimmunity, while amplification of the apoptotic signal was reported in neurodegenerative disorders including Alzheimer's and Parkinson's diseases [3, 4], as well as infection by human immunodeficiency virus (HIV).

The origin of the apoptotic signal has been used to distinguish two main signaling pathways. The intrinsic pathway stems from accumulation of DNA damage, deregulation of mitochondrial function, or virus infection and induces the release of proapoptotic factors from the mitochondria, whereas extrinsic signals are transmitted by the binding of apoptotic ligands to death receptors present at the cell surface. Interconnections exist between these two signaling pathways: both leading to the activation of a family of cysteine proteases specific for aspartic acid residues, called caspases [5]. The apoptotic role of mitochondria is associated with reduction in its transmembrane potential

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and the loss of its extracellular membrane integrity, leading to the release of different apoptogenic factors in the cytosol. Among them, cytochrome c associates with the caspase-9/APAF1 complex to form the apoptosome and trigger apoptosis [6].

These two signaling pathways share common features, and both require the aggregation of initiator caspases as their preliminary events. During interactions with respective ligands, members of the death receptor superfamily recruit adaptor proteins such as Fas-associating protein with a death domain (FADD) [7, 8] or tumor necrosis factor (TNF) receptor 1-associated death domain protein (TRADD) [9], resulting in the aggregation and activation of the initiator caspase-8 and caspase-10 to form the death-inducing signaling complex (DISC) [10]. In a similar manner, release of cytochrome c and ATP by mitochondria promotes the formation of the apoptosome with the cytosolic APAF-1, thereby aggregating and activating the initiator caspase-9, which in turn cleaves caspase-3 [11].

It should be kept in mind that death receptors CD95 [12], TNFR1 [13], DR4 [14], DR5 [15], and DR6 [16] have been cloned based on their ability to elicit apoptosis. Although studies have revealed the ability of Fas/CD95, DR4, and DR5 in triggering non-apoptotic signaling pathways even immediately after their cloning [17, 18], most, if not all, studies have been focused on characterizing the molecular events leading to cell death. Accordingly, several agonistic molecules were developed in order to kill cancer cells, neglecting the impact of non-apoptotic signals in pathophysiological contexts. More recent data changed this vision by evaluating the biological role of death receptor-mediated non-apoptotic signaling pathways in chronic inflammatory disorders and carcinogenesis.

In this chapter, apoptotic signaling pathways induced by death receptors are discussed. Moreover, recent evidences pointing to the non-apoptotic signals transmitted by the same receptors are brought up, which may imply their tremendous impact on tumor progression and the design of therapeutic tools.

9.2 TNF Receptor Family

Death receptors TNFR1, Fas, DR3, DR4, DR5, and DR6 belong to the tumor necrosis factor receptor (TNF-R) superfamily. These type I transmembrane proteins share common features, such as extracellular amino-terminal cysteine-rich domains (CRDs) [19, 20], which contribute to ligand specificity [21], and pre-association of the receptor at the plasma membrane [22–24] and a conserved 80 amino acid sequence located in their cytoplasmic tail called death domain (DD), which is necessary for DISC formation and initiation of the apoptotic signal [25, 26].

9.2.1 TNFR1 Signaling Pathways

TNF-α exerts its effects by binding to two receptors, TNFR1 and TNFR2 [20]. Recently, progranulin was identified as a ligand of TNFR with a higher affinity than TNF-α. Progranulin antagonizes TNF-α signaling and plays a critical role in the pathogenesis of inflammatory arthritis in mice [27]. TNFR1, a 55 kDa protein expressed in almost all cell types, presents a DD in its intracellular region; whereas TNFR2, a 75 kDa protein, is mainly detected in oligodendrocytes, astrocytes, T cells, myocytes, thymocytes, endothelial cells, and human mesenchymal stem cells [28]. Uncertainty remains on the TNFR2 signaling pathway, which has been previously reviewed [28]. The CRD1 of CD95, TNFR1, and TNFR2 is involved in homotypic interactions, leading to pre-association of the receptor as a homotrimer in the absence of ligand [23, 24, 29]. This domain has been designated as the pre-ligand binding assembly domain (PLAD) [29]. Receptors of the TNFR superfamily do not possess any enzymatic activity on their own and rely on the recruitment of adaptor proteins for signaling. Among these adaptor proteins, TRADD or FADD are instrumental in the implementation of cell death processes [7–10].

TNF is synthesized as a 26 kDa transmembrane type II protein (m-TNF) of 233 amino

acids [30] which can be cleaved by the metalloprotease TACE [31, 32] to release the 17 kDa soluble form of the cytokine (cl-TNF). In contrast to cl-TNF, which only activates TNFR1, m-TNF can bind and activate both TNFR1 and TNFR2 [33].

Activation of TNFR1 leads to the induction of cellular processes ranging from cell death (apoptosis or necroptosis) to cell proliferation, migration, and differentiation; the implementation of such different cellular responses reflects the formation of different molecular complexes after receptor activation [28]. Binding of TNF to TNFR1 causes the formation of two consecutive complexes. While the plasma membrane complex (complex I) elicits a non-apoptotic signaling pathway, a second, internalized, complex (complex II or DISC) triggers cell death [2]. In the presence of TNF, the adaptor protein TRADD interacts with TNFR1 and recruits other proteins involved in the signaling of the receptor, such as TRAF2, cIAP1, cIAP2, and RIP1, to form complex I. At the plasma membrane, this complex activates the NF-κB signaling pathway, which in turn promotes the transcription of antiapoptotic genes such as cIAP-1, cIAP-2, and c-FLIP [34]. The linear ubiquitin chain assembly complex (LUBAC) is also recruited to complex I via cIAP-generated ubiquitin chains [35]. HOIL-1, HOIP, and sharpin constitute the LUBAC complex. HOIL-1 and HOIP add a linear ubiquitin chain by catalyzing the head-to-tail ligation of ubiquitin [36] to RIP1 and NEMO (IKKγ) in complex I [37], thereby activating NF-κB.

TNF-induced caspase activation is mediated by a second, intracellular complex II, which is formed when complex I dissociates from the receptor, along with FADD and caspase-8 recruitment [2]. NF-κB activation leads to c-FLIP overexpression, preventing formation of complex II. Contrariwise, when NF-κB activation is blocked, c-FLIP, whose protein half-life is short [38], is absent, and cells experience death [2]. RIP1 is deubiquitinated by enzymes such as Cezanne [39] and CYLD [40] and the complex composed of TRADD and RIP1 moves to the cytosol to form complex II. FADD is recruited to TRADD by DD-DD interaction and binds caspase-8 [2]. Noteworthy, when caspase-8 activity is inhibited or its expression is extinguished, DISC is unable to trigger the apoptotic signaling pathway, but TNFR1 or CD95 stimulation leads to the activation of another cell death signal, namely, necroptosis [41, 42]. To prevent the induction of the necroptotic signal, caspase-8 cleaves and inactivates RIP1 and RIP3 [43]. The fine-tuned control of necroptosis by members of the apoptotic signaling pathway in the organism has been elegantly confirmed by experiments showing that the embryonic lethality of mice harboring the single KO of caspase-8 or FADD is rescued by an additional KO of the RIP3 gene [44–46].

9.2.2 TNF/TNFR: A Gold Mine for Therapeutic Tools

Many studies on TNF demonstrated its pivotal role in fueling inflammation, a multistep process that promotes autoimmunity (e.g., rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, psoriasis, and refractory asthma) and cancer. Many TNF inhibitors, such as neutralizing monoclonal antibodies (mAbs) (e.g., infliximab, adalimumab, and golimumab), have been developed to treat these chronic inflammatory disorders, demonstrating that altering ligand/receptor interactions with neutralizing mAbs is an invaluable opportunity to treat certain chronic inflammatory disorders. Other TNF-α antagonists, such as etanercept, a TNFR2-immunoglobulin Fc fusion protein, can improve the clinical course of rheumatoid arthritis [47].

While findings accumulate to decipher the molecular mechanisms involved in the induction of apoptotic and non-apoptotic signaling pathways by TNFR1 and to elucidate how the receptor can switch from one signal to the other, the mechanistic links involved in the implementation of non-apoptotic signaling pathways by CD95 remain elusive. However, recent findings have revealed its proinflammatory effects [48–54].

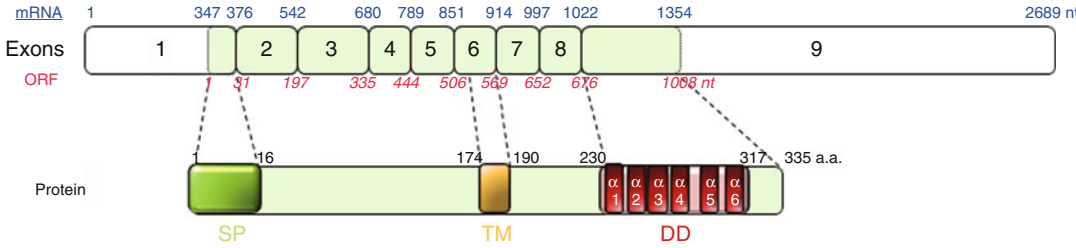

Fig. 9.1 CD95: mRNA to protein

9.3 CD95: A Death Receptor?

In 1989, identification of the mAb APO-1 by Peter Krammer et al. revealed the existence of a 52 kDa protein whose aggregation was able to transmit an apoptotic signal in cancer cells [55]. This receptor was identified in 1991 by Nagata and colleagues and called Fas (CD95 or APO-1) [12]. Its ligand, FasL, was cloned in 1993 by the same group and was found to be mainly expressed at the surface of activated T lymphocytes [56] and natural killer (NK) cells [57]; however, its expression was also detected in different tissues in which the presence of acute or chronic inflammation is undesirable including the eyes [58] and testes [59]. In addition, two mouse models, in which either the level of CD95 expression was downregulated [due to an insertion of a retrotransposon in intron 2 of the receptor gene, these mice are called *lymphoproliferation* (Lpr) [60–62]] or the CD95L affinity for CD95 was reduced [due to the germ line mutation F273L in CD95L, called *generalized lymphoproliferative disease* (*gld*), which decreases CD95L binding to CD95 [63, 64]], have provided some insight into the pivotal role played by this interaction in immunosurveillance and immune tolerance [65].

9.3.1 Structure/Function

The CD95 gene (*APT-1*) consists of nine exons, with exon 6 encoding the transmembrane domain [66] (Fig. 9.1). CD95 can be resolved under denaturing conditions between 40 and 50 kDa by SDS-PAGE. The receptor is a type I transmembrane protein harboring three CRDs. Similar to the TNF receptor [29], CD95 is pre-associated at the plasma membrane as a homotrimer, and this quaternary structure is mandatory for transmission of the apoptotic signals in the presence of CD95L [23, 24]. Homotrimerization of CD95 occurs mainly through homotypic interactions of the CD95-CRD1 [22–24]. Binding of CD95L or agonistic anti-CD95 mAbs to CD95 alters both the conformation and the extent to which the receptor is multimerized at the plasma membrane. The intracellular region of CD95 encompasses an 80 amino acid stretch designated as the DD (Fig. 9.1), which consists of six antiparallel α-helices [67]. Upon addition of CD95L, CD95 undergoes conformational modification of its DD, which induces a shift of helix 6 and fusion with helix 5, promoting both oligomerization of the receptor and recruitment of the adaptor protein FADD [68]. A consequence of the opening of the globular structure of CD95 is that the receptor becomes connected through this bridge, which increases the magnitude of its homo-aggregation. This long helix allows the stabilization of the complex by recruiting FADD. Overall, the CD95-DD:FADD-DD crystal structure provides some insights into the formation of the large CD95 clusters observed using imaging or biochemical methods in cells stimulated with CD95L. In addition, it also confirms that alteration in the CD95 conformation plays an instrumental role during signal induction [68] However, this elongated C-terminal α-helix favoring the *cis*-dimerization of CD95-DD was challenged by Driscoll et al. who did not observe the

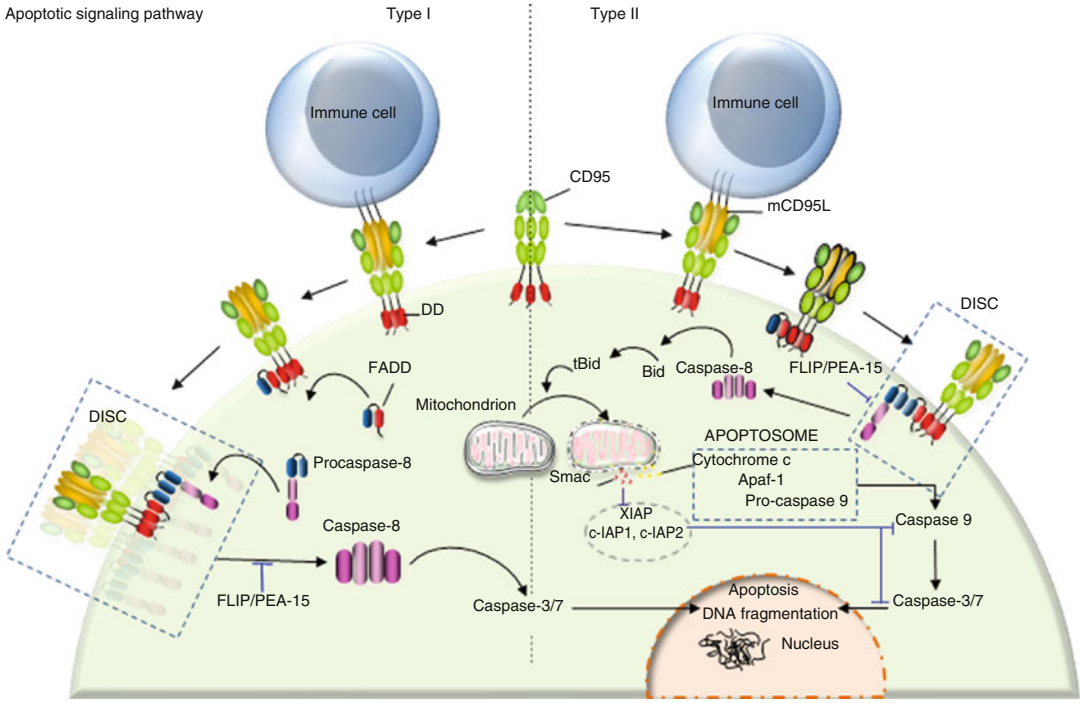

Fig. 9.2 Type I/II cells. Binding of transmembrane CD95L to CD95 leads to DISC formation. DISC consists of FADD and procaspase-8. C-FLIP and PEA-15 bind to FADD and prevent caspase-8 recruitment. At the DISC level, aggregation of procaspase-8 promotes its auto-cleavage and activation. Cleaved caspase-8 is then released in the cytosol where it promotes the cascade of caspase activation leading to apoptosis. Type I cells are characterized by an efficient DISC formation, which releases sufficient caspase-8 to directly activate caspase-3. By contrast, type II cells present a weak DISC formation, and the low amount of released caspase-8 activates the mitochondrion-dependent apoptotic pathway to amplify death signal

fusion of the last two helices at a more neutral pH (pH 6.2), compared to the acidic condition (pH 4) used in the initial study to resolve the CD95-DD:FADD-DD structure [68]. Consequently, at pH 6.2, association of CD95 with FADD predominantly consisted of a 5:5 complex, which occurred via a polymerization mechanism involving three types of asymmetric interactions but without major alteration of the DD globular structure [69, 70]. It is likely that the low pH condition used in the study performed by Scott et al. altered CD95 conformation and resulted in the formation of nonphysiological CD95:FADD oligomers [68]. Nonetheless, it cannot be excluded that a local decrease in the intracellular pH affects the initial steps of the CD95 signaling pathway *in vivo*, through promoting the opening of the CD95-DD and eventually contributing to the formation of a complex eliciting a sequence of events different from the one occurring at physiologic pH.

Once docked on CD95-DD, FADD self-associates [71] and binds procaspases-8 and procaspases-10, which are auto-processed and released in the cytosol as active caspases, which cleave many substrates leading to the execution of the apoptotic program and cell death. The complex CD95/FADD/caspase-8/caspase-10 is called DISC (Fig. 9.2) [10]. Due to the importance of DISC formation in the fate of cells, it is not surprising that numerous cellular and viral proteins were reported to hamper the formation of this structure, such as FLIP [72, 73] and PED/PEA-15 [74], which interfere with the recruitment of caspase-8/caspase-10 (Fig. 9.2).

9.3.2 Type I/II Signaling Pathways

Following the discovery of CD95 and the first steps of its signaling pathway, Peter and colleagues described that cells can be divided in two groups with regard to the kinetics through which they respond to CD95-mediated apoptotic signals, the magnitude of DISC formation and the role played by the mitochondrion in this pathway [75]. DISC formation occurs rapidly and efficiently in type I cells releasing a large amount of activated caspase-8 in the cytosol, while type II cells have difficulty forming this complex, and the amount of active caspase-8 is insufficient to directly activate the effector caspase-3 and caspase-7 [75]. Nonetheless, type II cells experience cell death upon CD95 engagement and are even more sensitive to the CD95-mediated apoptotic signal compared to type I cells [75–77]. This discrepancy can be partly explained by the fact that the low amount of activated caspase-8 in type II cells is sufficient to cleave BID, a BH3-only protein, which constitutes the molecular link between caspase-8 activation and the apoptotic activity of mitochondria. Indeed, after cleavage by caspase-8, truncated BID (tBID) translocates to mitochondria, where it triggers the release of proapoptotic factors (Fig. 9.2) [78, 79]. Although CD95 stimulation activates the mitochondrion-dependent apoptotic signal in type I and type II cells, it seems that only type II cells are addicted to this signal as they display a higher amount of the caspase-3 inhibitor XIAP compared to type I cells [80]. Among the inhibitor of apoptosis protein (IAP) family, XIAP, c-IAP1, and c-IAP2 inhibit caspase-3, caspase-7 [81, 82], and procaspase-9 [83] activity by direct binding, thereby preventing access to substrates. Furthermore, XIAP can function as an E3 ligase whose activity is involved in the ubiquitination of active caspase-3 and its subsequent degradation through the proteasome [84]. To detach XIAP from caspase-3 and restore the apoptotic signal, cells require the release of SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) by the mitochondrion [85, 86], explaining why type II cells are more addicted to this organelle compared to type I cells (Fig. 9.2).

To summarize, DISC formation and IAP amount are two cellular markers allowing a clear discrimination between type I and type II cells. Even though IAP overexpression can account for the mitochondrion dependency observed in type II cells, it remains unclear why DISC formation is hampered in type II cells and/or enhanced in their type I counterparts. Recently, high activity of the lipid kinase phosphoinositide 3-kinase (PI3K) or downregulation of its neutralizing phosphatase, phosphatase and tensin homologue on chromosome 10 (PTEN), was found in type II cells, while this signal is blocked in type I cell lines [87, 88]. The PI3K signaling pathway was reported to prevent the aggregation of CD95 [89], probably by retaining the receptor outside of lipid rafts [87, 90]. PEA-15, also known as PED, is a protein containing a death effector domain (DED) that has been shown to inhibit the CD95 and TNFR1 apoptotic signals (Fig. 9.2) [74]. Activation of PI3K and its downstream effector, serine-threonine kinase Akt, leads to phosphorylation of PEA-15 at serine 116 [87, 90]; this post-translational modification promotes its interaction with FADD, ultimately inhibiting DISC formation [91, 92].

Notably, the existence of type I and type II cells is not only an *in vitro* observation, but has been identified physiologically in human body. CD95-mediated apoptotic signal cannot be altered in thymocytes or activated T cells expressing a Bcl-2 transgene, conferring to their type I nature [93], whereas hepatocytes expressing the same transgene resist CD95-induced apoptosis and thus behave as type II cells [94, 95].

9.3.3 What Can We Learn from CD95 Mutations?

Germinal mutations in *APT-1* have been reported in patients developing a syndrome termed autoimmune lymphoproliferative syndrome type Ia (ALPS, also called Canale-Smith syndrome) [96–98]. ALPS patients show chronic lymphadenopathy and splenomegaly, expanded

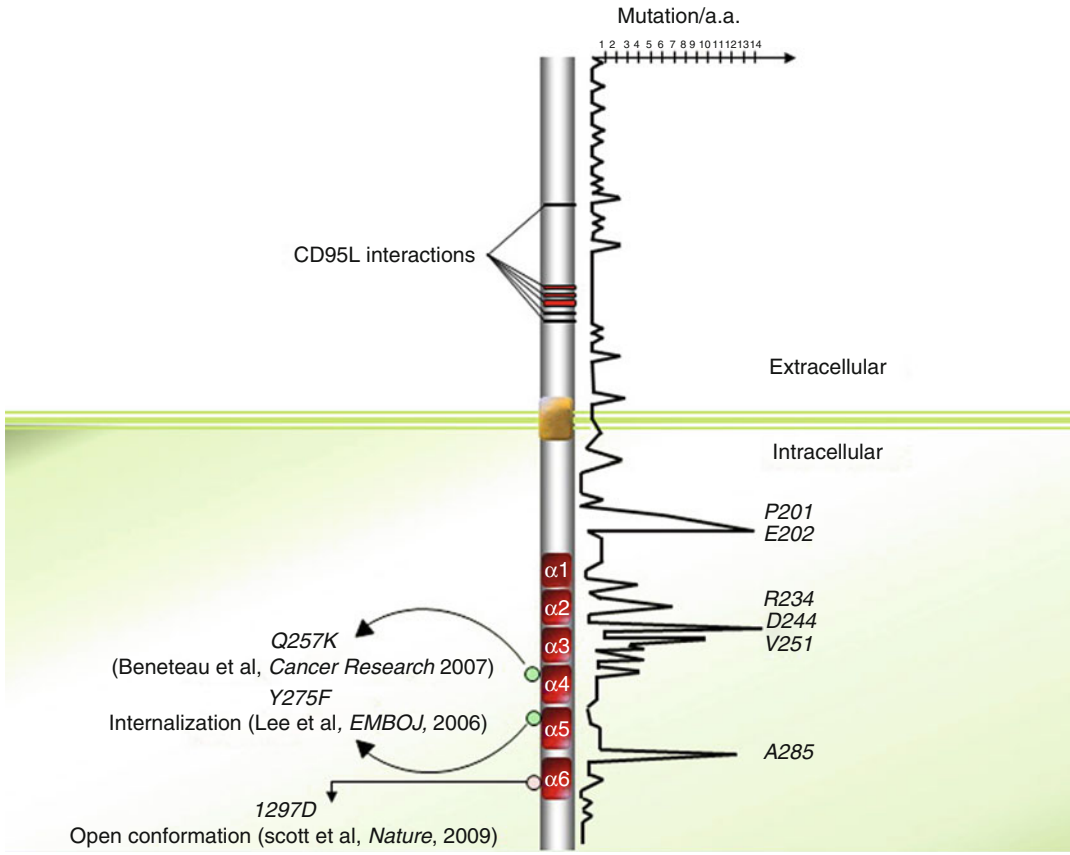

Fig. 9.3 Distribution of somatic and germinal mutations within CD95 protein sequence

populations of double-negative α/β T lymphocytes ($CD3^+CD4^-CD8^-$), and often develop autoimmunity [96, 97, 99, 100]. In agreement with the notion that CD95 behaves as a tumor suppressor, ALPS patients display an increased risk of Hodgkin and non-Hodgkin lymphoma [101]. Predominance of post-germinal center (GC) lymphomas in patients exhibiting either germ line or somatic CD95 mutations can be explained by the fact that, inside germinal centers of the secondary lymphoid follicles, the CD95 signal plays a pivotal role in the deletion of self-reactive maturating B lymphocytes [102], in addition to the fact that *APT1* belongs to a set of rare genes (i.e., PIM1, c-myc, PAX5, RhoH/TTF, and Bcl-6) subject to somatic hypermutation [103, 104], which may affect biological function. In addition to post-GC lymphomas, significant amounts of mutations in the CD95 gene were found in tumors of various histological origins (reviewed in [54]). Extensive analysis of CD95 mutations and their distribution in *APT-1* reveals that, with some exceptions, most are gathered in exons 8 and 9 encoding the CD95 intracellular region (Fig. 9.3) [105]. Remarkably, most of these mutations are heterozygous, mainly localized in CD95-DD, and lead to inhibition of the CD95-mediated apoptotic signal. Indeed, in agreement with the notion that CD95 is expressed at the plasma membrane as a pre-associated homotrimer [23, 24], formation of heterocomplexes containing wild-type and mutated CD95 prevents FADD recruitment and abrogates the ignition of the apoptotic signal in a dominant manner.

Extensive analysis and positioning of various CD95 mutations described in the literature seem to highlight mutation "hot spots" in the CD95 sequence (Fig. 9.3). Among these hot spots,

arginine 234, aspartic acid 244, and valine 251 account for a significant amount of the documented CD95 mutations. Indeed, among the 189 mutations annotated in the 335 amino acids of CD95, 30 (~16 %) are localized on these three amino acids (Fig. 9.3). Strikingly, the pivotal role played by these amino acids in stabilization or formation of intra- and inter-bridges between CD95 and FADD may explain these hot spots. For instance, both R234 and D244 contribute to the homotypic aggregation of the receptor and FADD recruitment [67]. Nevertheless, the observation of death domain hot spots is in contradiction with the study of Scott and colleagues demonstrating that the region of the CD95-DD interacting with the FADD-DD extends over a disperse surface through weak binding affinity [68].

Most ALPS type Ia patients affected by malignancies do not undergo loss of heterozygosity (LOH), which formed the hypothesize that preservation of a wild-type allele may contribute to carcinogenesis [106, 107]. In the same line, it was demonstrated that expression of a unique mutated CD95 allele blocks the induction of apoptotic signals, while it fails to prevent non-apoptotic signals such as NF-κB and MAPK [106, 107], whose induction promotes invasiveness in tumor cells [105, 108]. In addition, mutations found in the intracellular CD95-DD exhibit a higher penetrance of ALPS phenotype features in mutation-bearing relatives compared to extracellular mutations. These results suggest that unlike DD mutations, CD95 mutations localized outside the DD somehow prevent the apoptotic signal but may fail to promote non-apoptotic pathways, which may contribute to disease aggressiveness.

9.3.4 Regulation of the Initial Steps of CD95-Mediated Signaling

9.3.4.1 Lipid Rafts

In addition to CD95 downregulation or expression of the mutated allele of the receptor, the plasma membrane distribution of CD95 represents an additional pathway for tumor cells to develop resistance to CD95L-expressing immune cells. Indeed, the plasma membrane is a heterogeneous lipid bilayer comprising compacted or liquid-ordered domains, called microdomains, lipid rafts, or detergent-resistant microdomains (DRMs). These domains are described as floating in a more fluid or liquid-disordered 2-D lipid bilayer and are enriched in ceramides [109]. It has been elegantly shown that while CD95 is mostly excluded from lipid rafts in activated T lymphocytes, TCR-dependent reactivation of these cells leads to rapid distribution of the death receptor into lipid rafts [110]. This CD95 compartmentalization contributes to reducing the apoptotic threshold leading to the clonotypic elimination of activated T lymphocytes through activation of the CD95-mediated apoptotic signal [110]. Similarly, the reorganization of CD95 into DRMs can occur independent from ligand upon addition of certain chemotherapeutic drugs (e.g., rituximab [111], resveratrol [112, 113], edelfosine [87, 114, 115], aplidin [116], perifosine [115], cisplatin [117]). The molecular cascades that underlie this process remain elusive. Nevertheless, a growing body of evidence leads us to postulate that alteration of intracellular signaling pathway(s), such as the aforementioned PI3K signal [87, 90], may change biophysical properties of the plasma membrane, such as membrane fluidity, which in turn may facilitate CD95 clustering into large lipid raft-enriched platforms, favoring DISC formation and induction of the apoptotic program [118].

9.3.4.2 Posttranslational Modifications

Accumulation of CD95 mutations is not the only mechanism by which malignant cells inhibit the extrinsic signaling pathway. Posttranslational modifications in the intracellular tail of CD95, such as reversible oxidation or covalent attachment of a palmitic acid, were reported to alter the plasma membrane distribution of CD95 and thereby its subsequent signaling pathway. For instance, S-glutathionylation of mouse CD95 at cysteine 294 promotes clustering of CD95 and its distribution into lipid rafts [119]. This amino acid is conserved in the human CD95 sequence and corresponds to cysteine 304 (or C288 when

subtraction of the 16 amino acid signal peptide is taken into consideration [12, 120]). Interestingly, Janssen-Heininger and colleagues emphasize that death receptor glutathionylation occurs downstream of caspase-8 and caspase-3 activation whose catalytic activity damages the thiol transferase glutaredoxin 1 (Grx1), an enzyme implicated in the denitrosylation of proteins [119]. The consequence of Grx1 inactivation is the accumulation of glutathionylated CD95, which clusters into lipid rafts, sensitizing cells to the CD95-mediated apoptotic signal. Based on these findings, caspase-8 activation occurs prior to aggregation of CD95 and redistribution into lipid rafts, both of which are requisite to form the DISC and subsequently activate larger amounts of caspase-8. In agreement with these observations, activation of caspase-8 was reported to occur in a two-step process. First, an immediate and small amount of activated caspase-8 (<1 %) is generated when CD95L interacts with CD95 that orchestrates acid sphingomyelinase (ASM) activation, ceramide production, and CD95 clustering, which in turn promote DISC formation and the outburst of caspase-8 processing essential to mount the apoptotic signal [121].

S-Glutathionylation consists in a bond between a reactive Cys-thiol and reduced glutathione (GSH), a tripeptide consisting of glycine, cysteine, and glutamate; its attachment to the protein will alter its structure and function in a manner similar to the addition of a phosphate [122]. S-Glutathionylation is not the only posttranslational modification of CD95 on a cysteine. S-nitrosylation of cysteine 199 (corresponding to C183 after subtraction of signal peptide sequence) and 304 (C288) in colon and breast tumor cells also promotes the redistribution of CD95 into DRMs, the formation of the DISC, and the transmission of the apoptotic signal [123].

Two reports have brought into light that covalent coupling of a 16-carbon fatty acid (palmitic acid) to cysteine 199 (C183) elicits the redistribution of CD95 into DRMs, the formation of SDS-stable CD95 microaggregates resistant to denaturing and reducing treatments, and internalization of the receptor [124, 125]. Although their order remains to be fine-tuned, these molecular steps play a critical role in the implementation of apoptotic signals.

Of note, similar to S-nitrosylation, both the aforementioned S-glutathionylation at C304 (C288) and palmitoylation at C199 (C183) promote the partition of CD95 into lipid rafts and enhance the subsequent apoptotic signal. Further investigation is required to address whether these posttranslational modifications are redundant and occur simultaneously in dying cells or are elicited in a cell-specific and/or in a microenvironment-specific manner. Understanding the molecular mechanisms controlling these posttranslational modifications would be of great interest in order to identify the mechanism by which tumor cells block them, leading to their resistance to the extrinsic signaling pathway.

9.3.4.3 CD95 Internalization

Using a powerful magnetic method to isolate receptor-containing endocytic vesicles, it has been shown that CD95 promptly associates with endosomal and lysosomal markers when incubated with an agonistic anti-CD95 mAb [126]. In addition, expression of a CD95 mutant in which the DD-located tyrosine 291 (Y275) is changed to phenylalanine does not seem to alter the capacity to bind FADD but compromises CD95L-mediated CD95 internalization occurring through an AP-2/clathrin-driven endocytic pathway [126]. More strikingly, expression of the internalization-defective CD95 mutant Y291F abrogates the transmission of apoptotic signals, but fails to alter the non-apoptotic signaling pathways (i.e., NFkB and ERK), and even promotes them (Fig. 9.3). These findings provide insight into the presence of a region in the DD, interacting with AP2 and promoting a clathrin-dependent endocytic pathway in a FADD-independent manner. Regarding the role of palmitoylation in receptor internalization, the interplay between lipid alteration and the AP2/clathrin-driven internalization of CD95 remains to be elucidated.

9.3.4.4 Ca^{2+} Response

It has been recently demonstrated that CD95 engagement evokes a rapid and transient Ca^{2+} signaling, which stimulates the recruitment of

protein kinase C-β2 (PKC-β2) from the cytosol to the DISC [127]. This kinase transiently brakes DISC formation, providing a checkpoint before the irreversible commitment to cell death [128]. These findings raised the following questions: what are the Ca^{2+}-dependent molecular mechanisms transiently inhibiting DISC formation, and do tumor cells use this signal to escape the immune response and/or resist chemotherapy?

9.3.5 Programmed Necrosis Also Known as Necroptosis

In 1998, inhibition of caspase activity was shown to sensitize fibroblastic L929 cell line to TNF-mediated necrotic cell death [42]. With respect to CD95 signal, Tschopp et al. showed that FADD and RIP1 participate in the implementation of a non-apoptotic signaling pathway, which leads to a necrotic morphology without chromatin condensation and with loss of plasma membrane integrity [41]. Of note, BID cleavage was not observed in this necrotic signal. While FADD plays a crucial role in both apoptotic and necrotic pathways, RIP1 recruitment to CD95 occurs independently of this adaptor protein. Indeed, yeast two-hybrid experiments showed that RIP1 can bind directly to the CD95 DD, while this interaction is lost when a bait corresponding to mutated CD95-DD (replacement of Val 238 to Asn) is used [129]. In addition, RIP3 (RIPK3, a member of the RIP kinase family) is an indispensable factor for the induction of the necrotic signaling pathway [78–80]. A growing body of evidence supports the existence of necroptosis (programmed necrosis). In addition, identification of necrostatin, a chemical inhibitor of necroptosis [130], which specifically inhibits RIP1 kinase activity [131], has accelerated the pace of discovery in this field of cell death. Interplays exist between apoptosis and necroptosis; for instance, caspase-8, a potent inhibitor of necroptosis for both CD95 and TNFR1 [132], plays a critical role in necroptosis by its ability to process and inactivate RIP1 and RIP3 [133, 134]. At least for TNF signaling, the necrotic signal relies on the activity of CYLD, a deubiquitinating enzyme that is also cleaved and inactivated by caspase-8 [135].

Overall, these findings suggest that the apoptotic machinery controls the necrotic one. This concept has been recently established *in vivo* by double-KO experiments [44–46, 136]. The KO of FADD or caspase-8 is deleterious in mice mainly by the fact that these two apoptotic factors are beneficial in inhibiting a RIP1-/RIP3-dependent necrotic signal; thus, their loss unleashes the necroptotic program and leads to embryonic lethality. Yet, most studies on necroptosis have focused on the TNF signaling pathway, whereas the mechanism by which CD95 can elicit this cell death pathway, and how the switch in this receptor occurs between non-apoptotic, apoptotic, and necroptotic signals remains unclear. Importantly, the impact of each cell death on antigen presentation, and on the efficiency of immune response after elimination of infected or transformed cells, remains unclear.

9.3.6 CD95L, an Inflammatory/Oncogenic Cytokine?

9.3.6.1 A Ligand to Create Immune Privileges

The transmembrane CD95L (CD178/FasL) is present at the surface of activated lymphocytes [64] and NK cells [137] where it orchestrates the elimination of transformed and infected cells. In addition, CD95L is expressed on the surface of neurons [138], corneal epithelia and endothelia [58, 139], and sertoli cells [59] to prevent the infiltration of immune cells and thus to prohibit the spread of inflammation in these sensitive organs (i.e., brain, eyes, and testis, respectively), commonly called "immune-privileged" sites. The description of physiological immune privilege was followed by tumor-mediated immune privilege, since two groups reported that the ectopic expression of CD95L by malignant cells participated in the elimination of infiltrating T lymphocytes and thus could play a role in the establishment of a tumor site whose access was denied to immune cells [140, 141]. However, these observations are controversial since

ectopic expression of CD95L in allogenic transplant of β-islets [142, 143] and in tumor cell lines [144] led to a more rapid elimination of these cells than control cells, due to increased infiltration of neutrophils and macrophages endowed with antitumor activity.

9.3.6.2 At Least Two Different Ligands and Two Different Signals

Among the weapons at the disposal of immune cells, transmembrane CD95L contributes to the elimination of pre-tumor cells. Therefore, pre-tumor cells that escape the immunosurveillance will be shaped to develop resistance to CD95, a process termed immunoediting [145]. In other words, imprinting of the immune system on pre-tumor cells will select malignant cells with increased resistance towards the CD95L-induced signal. As previously mentioned, these alterations of the CD95 signal not only block the CD95-mediated apoptotic signal but also promote the transmission of non-apoptotic signals by CD95L, which may play a critical role in carcinogenesis [106–108, 146]. In agreement with this hypothesis, a complete loss of CD95 expression is rarely observed in malignant cells [147].

Accumulating evidence indicates that the apoptotic ligand CD95L behaves as a chemoattractant for neutrophils, macrophages [50, 143, 144], T lymphocytes [53], and malignant cells in which the CD95-mediated apoptotic signal is nonproductive [108, 148]. Nonetheless, the biological role of CD95L has to be clarified due to the fact that pathophysiologically the ligand is present in at least two forms with different stoichiometries. Indeed, CD95L is a transmembrane cytokine whose ectodomain can be cleaved by metalloproteases such as MMP3 [149], MMP7 [150], MMP9 [151], and ADAM-10 (A disintegrin and metalloproteinase 10) [152, 153] and released as a soluble ligand in the bloodstream. Based on the data demonstrating that a hexameric CD95L represents the minimal level of self-association required to signal apoptosis [154] and that cleavage by metalloproteases releases an homotrimeric ligand [154, 155], this soluble ligand has long been considered as an inert ligand competing with its membrane-bound counterpart for CD95 binding, thus acting as an antagonist of the death signal [155, 156]. It has been recently demonstrated that this metalloprotease-cleaved CD95L (cl-CD95L) actively participates in the aggravation of inflammation and autoimmunity in patients affected by systemic lupus erythematosus (SLE) by inducing the non-apoptotic NF-κB and PI3K [51, 53] signaling pathways (Fig. 9.4). Unlike transmembrane CD95L, induction of the PI3K signaling pathway by its metalloprotease-cleaved counterpart occurs through the formation of a complex devoid of FADD and caspase-8 which recruits the src kinase c-yes instead [53, 148]; this unconventional receptosome was designated motility-inducing signaling complex (MISC) [53, 157] (Fig. 9.4). Even though experiments by the authors did not detect any trace of caspase-8 in the MISC, this enzyme has been shown to participate in cell migration. The protease activity of caspase-8 can be abolished by its phosphorylation at tyrosine 380 by src kinase [158]. This posttranslational modification was observed in cells stimulated with EGF and in colon cancer cells exhibiting constitutive activation of src; from a molecular standpoint, this modification does not alter caspase homodimerization or recruitment in DISC [158]. Moreover, the EGFR-driven phosphorylation of caspase-8 at Y380 turns out to be a potent inducer of the PI3K signaling pathway by recruiting the PI3K adaptor p85 alpha subunit [159]. Ultimately, caspase-8 phosphorylation triggers cell migration. Nonetheless, it is noteworthy that CD95-induced migration and invasion do not appear to require an intact DD (reviewed in [160]), suggesting that either the caspase-8-dependent mode of cell migration occurs as an alternative signal for death receptors or that it only participates in non-death receptor-induced cell motility. It would be interesting to address this question in the future. To date, it can only be surmise that phosphorylation of caspase-8 at Y380 upon EGFR stimulation may prime certain cancer cells to become unresponsive to the apoptotic signal triggered by cytotoxic CD95L and meanwhile promote cell migration, an essential event in the course of cancer cell metastasis (Fig. 9.4).

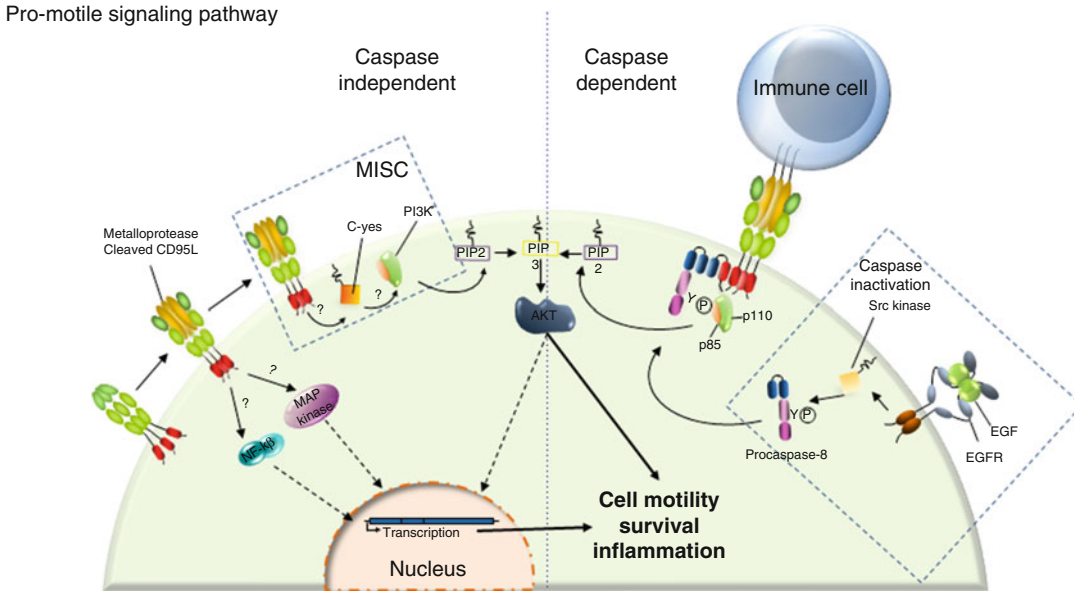

Fig. 9.4 CD95 triggers an unconventional PI3K signaling pathway. *Left panel*: In the presence of cl-CD95L, CD95 triggers MISC formation. This complex is devoid of FADD and caspase-8, but, instead, recruits the src kinase c-yes that implements the PI3K signaling pathway. CD95 engagement is also capable of NF-κB and MAPK activations through a yet unknown mechanism. *Right Panel*: It was reported that procaspase-8 can be phosphorylated by the tyrosine kinase src upon EGFR stimulation. This posttranslational modification not only blocks the catalytic activity of caspase-8 but also promotes the recruitment of the p85 subunit of PI3K. We surmise that this caspase-8 phosphorylation may favor the non-apoptotic signals induced by CD95

It is noteworthy that in a similar manner, a decrease in the plasma membrane level of CD95 or expression of a mutated CD95 allele, as observed in ALPS patients and malignant cells, inhibits the implementation of the apoptotic signal but does not affect the transmission of non-apoptotic signals, such as NF-κB, MAPK, and PI3K [106, 107, 147], suggesting that these signals may stem from a different domain than CD95-DD or rely on different thresholds to be elicited. In summary, although the CD95/CD95L interaction can eliminate malignant cells by implementation of the DISC or can promote carcinogenesis by sustaining inflammation and/or by inducing metastatic dissemination [50, 51, 53, 108, 147, 148, 161], the molecular mechanisms underlying the switch between these different signaling pathways remain enigmatic. An important question to be addressed is how the magnitude of CD95 aggregation controls the formation of "death"- *vs.* "motility"-ISCs. Addressing these questions will lead to the development of new therapeutic agents with the ability to contain the spread of inflammation or impede carcinogenesis at least in pathologies involving increased soluble CD95L such as cancers (e.g., pancreatic cancer [162], large granular lymphocytic leukemia, breast cancer [157], and NK cell lymphoma [163]) or autoimmune disorders (e.g., rheumatoid arthritis and osteoarthritis [164], graft-versus-host-disease (GVDH) [165, 166] or SLE [53, 167]). Altogether, these studies support the notion that the death function of CD95 may correspond to its "day job," while the receptor may act as "a night killer" by fueling inflammation in certain pathophysiological contexts.

Strikingly, while the soluble form of CD95L generated by MMP7 (cleavage site inside the ^{113}ELR^{115} sequence, Fig. 9.5) induces apoptosis [150], its counterpart processed between serine 126 and leucine 127 does not [51, 53, 155]. To explain this discrepancy, one may speculate that the different quaternary structures of the naturally processed CD95L underlie the

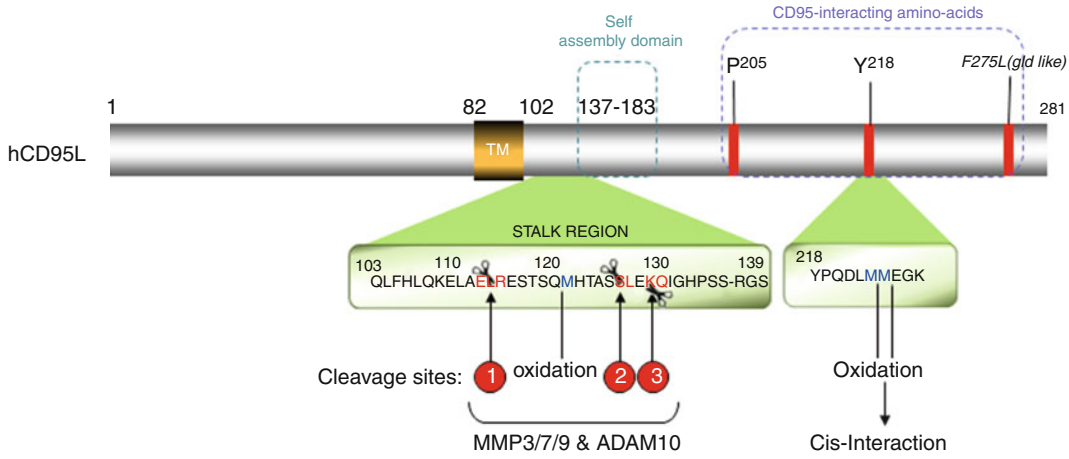

Fig. 9.5 CD95L: metalloprotease cleavage sites and domains

implementation of a "death"- *vs*. "non-death"-inducing signaling complexes and downstream signals. In agreement with this notion, soluble CD95L bathed in the bronchoalveolar lavage (BALs) of patients suffering from acute respiratory distress syndrome (ARDS) undergoes oxidation at methionines 224 and 225 (Fig. 9.5), which enhances the aggregation level of the soluble ligand followed by its cytotoxic activity [168]. The same authors observed that the stalk region of CD95L, corresponding to amino acids 103–136 and encompassing the metalloprotease cleavage sites (Fig. 9.5), participates in the multimerization of CD95L, which accounts for the damage of the lung epithelium in ARDS [168]. Of note, in ARDS BALs, additional oxidation occurs at methionine 121 (Fig. 9.5), which in turn prevents the processing of CD95L by MMP7, and explains why this cytotoxic ligand keeps its stalk region [168]. Nonetheless, preservation of this region in soluble CD95L raises the question that whether an unidentified MMP7-independent cleavage site exists in the juxtamembrane region of CD95L, near the plasma membrane, or the ligand detected in ARDS patients corresponds to the full-length CD95L embedded in exosomes [169, 170]. Indeed, this peculiar exosome-bound CD95L can be expressed by human prostate cancer cells (i.e., LNCaP), and evokes apoptosis in activated T lymphocytes [171].

Overall, these findings emphasize that it will be of great interest in the future to finely characterize the quaternary structure of the naturally processed CD95L from the sera of patients affected by cancers or chronic/acute inflammatory disorders, to better understand the molecular mechanisms implemented by this ligand and thus predict its subsequent biological functions.

9.4 Concluding Remarks

Apoptosis is a fundamental process contributing to tissue homeostasis, immune response, and development. CD95, also called Fas, is a member of the tumor necrosis factor receptor (TNF-R) superfamily. Its ligand, CD95L, was initially detected at the plasma membrane of activated T lymphocytes and natural killer (NK) cells where it contributes to the elimination of transformed and infected cells. Given its implication in immune homeostasis and immune surveillance combined with the fact that various lineages of malignant cells exhibit loss-of-function mutations, CD95 was initially classified as a tumor suppressor gene. Nonetheless, in different pathophysiological contexts, this receptor is able to transmit non-apoptotic signals and promote inflammation and carcinogenesis. Although the different non-apoptotic signaling pathways (NF-κB, MAPK, and PI3K) triggered by CD95

are known, the initial molecular events leading to these signals, the mechanisms by which the receptor switches from an apoptotic function to an inflammatory role, and, more importantly, the biological functions of these signals remain elusive.

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MHC Class I Molecules and Cancer Progression: Lessons Learned from Preclinical Mouse Models

10

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10.1 Introduction

Major histocompatibility complex (MHC) is composed of a set of molecules that play a pivotal role in the immune response against different pathogens and tumors cells. These molecules were described in mice for the first time by Gorer while performing transplantation studies with tumor cell lines injected in inbred strains of mice [1]. In the middle of the 1950s, Jean Dausset described the HLA system in humans which is equivalent to the mouse H-2 complex [2]. MHC class I (MHC-I) molecules comprise the classical (class Ia) HLA-A, HLA-B and HLA-C antigens in humans and H-2 K, H-2 D, and H-2 L in mice and the nonclassical (class Ib) -E, -F, and -G in humans and Qa and Tla antigens in mice [3]. Their structure is quite similar in human and mice, forming a trimolecular complex consisting of a 45 kDa highly polymorphic heavy chain, peptide antigen, and the nonpolymorphic 12 kDa β_2-microglobulin (β2m) light chain [4]. HLA/H-2

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class I molecules are expressed on the surface of nucleated cells [5]. It is estimated that there are up to 250,000 of each MHC-I molecule on the surface of a somatic cell [6].

MHC-I molecules bind antigens in the form of peptides, generated from endogenous proteins, present on the cell surface to $CD8^+$ T cells. In tumor cells, MHC-I molecules present tumor-associated antigens (TAAs) to T cytotoxic lymphocytes (CTLs) activating cell proliferation, cytokine production, and target cell lysis. These TAAs are generated from degraded foreign endogenous proteins by the antigen presentation machinery (APM). This process is carried out by a large number of proteins and accessories molecules [7–9]. Correct functioning of these APM components gives rise to cells with normal surface expression of the MHC-I molecules [10, 11]. Any defect in these processes will lead to non-expression of MHC-I molecules on the cell surface. These MHC-I-deficient tumor cells might be recognized by natural killer (NK) cells [12].

In this chapter, we will focus on analyzing the role of MHC-I antigens in cancer immunosurveillance in murine tumor models without obviating the great contributions done in human tumor models; the authors' laboratory is the reference to the findings described.

10.2 MHC-I Cell Surface Expression on Tumor Cells and Primary Tumor Growth

For over 30 years ago, our group of investigators is working on human and mouse preclinical tumor models in an attempt to define the mechanisms through which tumor cells evade immune system. We have found that tumor cells develop sophisticated molecular and biological mechanisms which allow them to escape immunosurveillance. Among the mechanisms studied, MHC alteration is one of the most important and frequent mechanisms, possibly playing a relevant role in the tumor-host scenario [13–15]. Any alteration affecting the surface expression of MHC-I molecules, as the expression and function of APM components, the expression of MHC-I heavy chains or β2m in tumor cells will have a profound effect on the recognition and killing of those tumor cells by T lymphocytes [16, 17]. In this context, a new phase has been proposed into the tumor evolution, called the *immunoblindness* phase, which comes after the three phases of immunoedition process [18]. During this phase, CTLs lose control over tumor cells, since losing MHC-I surface expression makes them invisible.

Our research group has a long and well-established history identifying and defining the HLA class I altered phenotypes present in human tumors. In fact, the data accumulated indicate that alterations in HLA class I expression are commonly found in most human tumors [19, 20]. Seven different altered HLA class I phenotypes have been defined in a large variety of human tumors, and the molecular mechanisms that have been found to underlie these alterations in MHC-I expression are multiple [21]. These defects can occur at any step required for MHC synthesis, assembly, transport, or expression on cell surface. Only some of these defects can be recovered by cytokines or other agents, while others remain unrecovered. Thus, MHC alterations can be classified into two main groups: reversible defects (regulatory or soft) and irreversible defects (structural or hard) [22, 23].

Many studies in human and experimental tumors have reported variations in MHC-I antigen cell surface expression [24–27]. These variations have been associated with important changes in tumor behavior and metastatic colonization [28, 29]. The crucial role of MHC-I in local tumor growth and metastasis has also been demonstrated in many different murine tumor models. The first detection of MHC-I lack in mouse tumors was described in 1976; loss of one H-2 K^k private specificity was reported in Gardener lymphoma derived from a C3H mouse [25]. Following these studies, different groups reported altered expression of MHC molecules in other tumors, i.e., the absence of some H-2^d molecules in a methylcholanthrene-induced sarcoma (MCG4) in a BALB/c mouse [30]; loss of K^k antigen (Ag) expression in a particular AKR tumor cell line designated K36.16, this tumor cell line showed resistance to killing by AKR anti-MuLV CTLs

in vitro [31]; loss of the products of the H-2 L^d locus in a BALB/c fibrosarcoma [32]; and absence of H-2 Ds Ags in SJL/J lymphomas [33].

Another field in the study of MHC-I Ags in murine tumors originates from transfection of MHC-I molecules in MHC-I-deficient murine tumors. The transfection and cell surface expression of one *H-2* ^{k} gene product in the AKR lymphoma cell line K36.16, a subline of K36 (H-2 K^k-negative) lymphoma, inhibited the syngeneic growth of this tumor [34, 35]. Studies with the methylcholanthrene (MCA)-induced T10 sarcoma demonstrated that the transfection of K^k or K^b gene into H-2 K-negative parental cells reduced tumorigenicity and abolished the formation of metastasis in syngeneic mice [36]. Similar results were obtained in other experimental models [37]. In all these studies, absence of MHC-I molecules has been interpreted as a factor which selects immunodeficient variants and represents a major escape mechanism from T cell recognition. The reconstitution of H-2 class I expression has demonstrated that even MHC-I molecules on tumor cells are responsible for regulation of NK susceptibility. Restoration of these molecules by transfection with *β2m* gene resulted in a strong decrease in susceptibility to NK lysis in S3 cell line, a negative variant for H-2 D^b and K^b of the murine thymoma EL4 [38].

The differential expression of H-2 class I K, H-2 class I D, and H-2 class I L molecules is another event present in some tumors. Studies on AKR-derived B cell lymphomas (H-2^k) have shown that D^k molecules are processed slower than K^k molecules, with a half-time of 4–5 h [39]. Other studies have shown that L^d Ags are expressed at levels three to four times lower than D^d or K^d Ags [40]. This is in line with the studies that show that in BALB/c S49 lymphoma sublines, there is a locus-specific regulation for K^d, D^d, and L^d surface molecules [41]. The differential expression of these molecules on the cell surface could be a mechanism used by the tumor cells to escape from immunosurveillance. Therefore, these studies all together could add to our knowledge about tumor biology [39]. Some examples of this locus-specific regulation have been documented in other tumor models. Green and coworkers have studied an MuLV-induced AKR tumor in which the expressed H-2 K and H-2 D Ags are differentially induced by IFN-γ [42]. In the spontaneous BALB/c line 1 murine carcinoma, it has been shown that the induction of MHC-I antigen expression by IFN-γ and DMSO differ at the molecular level. A point mutation in the D1 region of the D^d promoter diminished IFN-γ responsiveness, but did not alter induction of D^d molecule by DMSO. Thus, DMSO appears to regulate MHC-I transcription through multiple regions of the MHC-I heavy chain promoter by mechanisms distinct from IFN-γ [43]. Studies with mutant phenotypes have led to the description of factors controlling the folding, the intracellular transport, and surface expression of class I molecules [44].

Components of APM are important elements in the MHC-I cell surface expression. Alteration in the Ag presentation pathway may serve as an evasive mechanism rendering tumors unrecognizable by host immunosurveillance mechanisms. Certain murine tumor cell lines, such as the chemical-induced CMS-5, EL4, MCA102, and MCA205 cells, with deficient expression and/or function of multiple APM components, in particular the peptide transporters (TAPs) and tapasin, show reduced levels of MHC-I surface expression accompanied by low immunogenicity, hence evading T cell-mediated immune recognition *in vivo* [45]. In the B16 melanoma, MHC-I-deficient phenotype has been attributed to the downregulation or loss of the expression and function of multiple APM components [46]. In other studies, it has been shown that inoculation of C57BL/6 mice with a mixture of TAP-1-positive and TAP-1-negative tumor cell lines, generated from a transformed murine fibroblast line, produced tumors exclusively composed of TAP-1-negative cells, indicating an *in vivo* selection for TAP-deficient cells. Thus, loss of TAP function can allow tumor cells to avoid T cell immunity producing tumor cells with increased tumorigenicity [16]. In the APM-deficient mouse lung carcinoma cell line CMT.64, re-expression of TAP-1 after infection with TAP-1 adenovirus vector led to an increase of MHC-I cell surface expression and increased susceptibility to specific CTLs [47].

In addition, there are examples of tumor progression associated with increased expression of MHC Ags. For instance, one H-2 class I-deficient cell line from RBL-5 lymphoma (RMA-S), isolated after mutagenization and several cycles of selection by lysis of MHC-I-positive cells, was rejected in syngeneic C57BL/6 mice. In contrast, the H-2-positive wild-type cell line (RMA) was highly tumorigenic [48]. The transfection of this H-2 class I-deficient mutant (RMA-S) with *TAP-2* gene led to a marked increase in tumor outgrowth potential *in vivo*. This occurred despite restored antigen presentation and sensitivity to CTLs and was found to be due to escape from NK cell-mediated rejection. These data suggest that a defect in the machinery responsible for processing and loading of peptides into MHC-I molecules is sufficient to render cells sensitive to elimination by NK cells [49]. These data are in accordance with the *missing self hypothesis* [12] in which NK cells are able to distinguish class I-expressing and class I-deficient tumor cells. These cells are able to kill TAP-deficient RMA-S cells (H-2 class I negative) more efficiently compared to RMA cells (MHC-I positive). NK cells refrain from killing when target cells express self MHC-I molecules [50]. Similar results have been obtained after IFN-γ treatment in murine H-2-negative YAC-1 lymphoma cell line. In this case, re-expression of H-2 antigens abrogated NK lysis of the cells [51]. In other tumors including EL4 lymphoma [12, 48] and murine tumor cell lines expressing human papilloma virus (HPV) 16-derived E6/E7 oncoproteins TC-1 (MHC-I-positive) and MK16 (MHC-I-negative) variants, NK cells appear to be an effective tool against MHC-I-deficient cells [52, 53]. In this case, immunization with the MHC-I-negative (MK16), but not with TC-1 (MHC-I-positive), cell line inhibits the growth of MHC-I-negative tumors. NK cells are responsible for this immunity, although IFN-γ production by $CD4^+$ and $CD8^+$ T cells cannot be excluded [54]. The heterogeneity of MHC-I expression in tumor cell population and the balance of the MHC-restricted CTLs and MHC-unrestricted NK cells immune mechanisms determine the final outcome of the MHC-I expression in the primary tumor [55].

10.2.1 Studies in GR9 Tumor Model: H-2 Antigen Surface Expression and Tumorigenic Capacity

Since the generation of the GR9 tumor model in the 1980s, our knowledge about the role of MHC-I molecules in the tumor scene has increased dramatically [28, 29, 56, 57, 58]. GR9 tumor model is a subcutaneously induced methylcholanthrene (MCA) fibrosarcoma in BALB/c. The original tumor mass was directly adapted to tissue culture without any *in vivo* passage in syngeneic or allogeneic mice to avoid immunoselection [56]. Forty-three cell lines were obtained after cloning using a phase contrast microscope and limit dilution, adapted to tissue culture and criopreserved. The GR9 fibrosarcoma tumor and the GR9-derived clones have been extensively studied and characterized by our group. The H-2 class I phenotype of the different cell lines were analyzed (Fig. 10.1) [13, 56, 59]. GR9 cell line presents surface expression of the three H-2 class I molecules (K^d, D^d, and L^d), and it is composed of tumor clones with a great heterogeneity in H-2 phenotype which could be classified in four groups: highly positive clones (D8, A7, G2), middle positive clones (B10, B7, B3), low positive clones (B6, C11, C5, G10), and very low/negative clones (B9, B11) (Fig. 10.1) [13, 56, 59]. Transcriptional analysis of the H-2 class I heavy chains, β2m, and APM components genes showed a correlation between the expression of these genes and the surface expression of MHC-I molecules [59]. A coordinate transcriptional downregulation of H-2 L^d heavy chain, calreticulin, LMP-2, and TAP-1 has been found in B11, B7, and C5 clones in comparison with A7 clone. In all instances, H-2 class I K^d, D^d, and L^d molecules of all tumor cell lines could be recovered after IFN-γ treatment [59]. This data indicates that tumor cells have reversible (soft) defects underlying MHC alterations [23, 60].

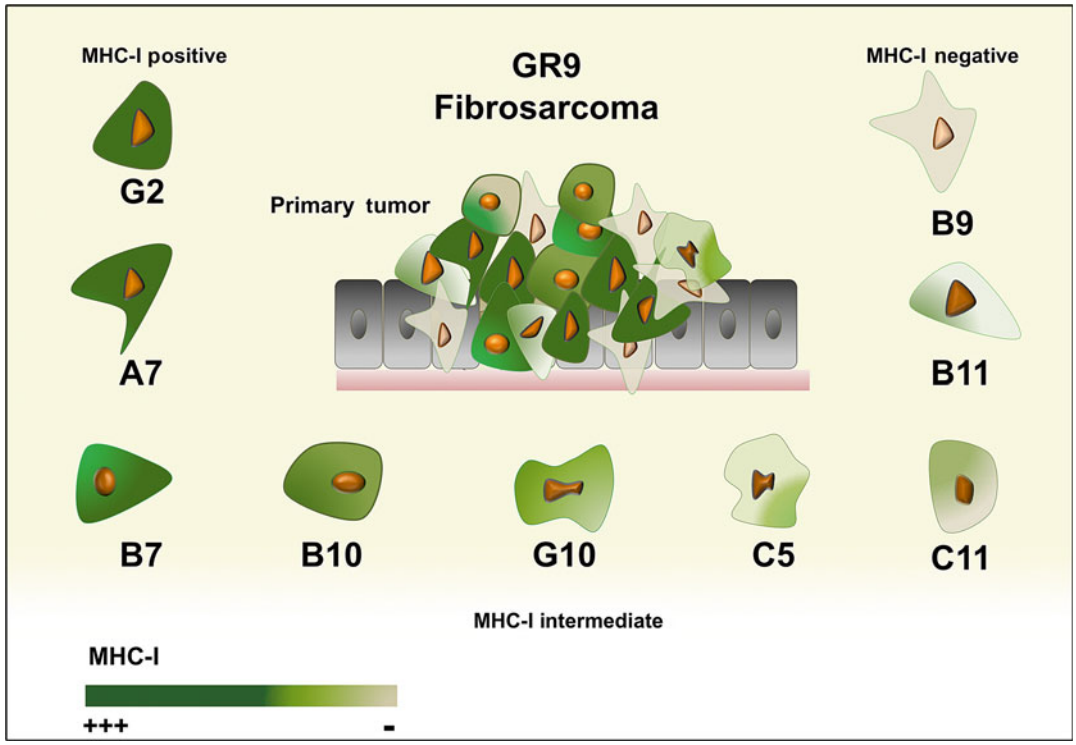

Fig. 10.1 GR9 fibrosarcoma tumor model. Cell clones are adapted to tissue culture from the primary tumor and classified according to MHC-I surface expression

More recently, we have shown that the tumor suppressor gene *Fhit* is involved in the coordinated transcriptional regulation of various APM components and/or MHC-I heavy chains [58]. Transcriptional levels of Fhit are significantly lower in tumor clones with low expression of MHC-I molecules. Results have shown that transcriptional level of *Fhit* in A7 clone is 1.4 higher than those found in B7 clones and 3.6 and 3.2 time higher than that expressed in C5 and B11 clones [59].

The intratumoral heterogeneity in H-2 class I expression presented in GR9 cell lines is not an unusual case since other MCA-induced tumors obtained in our laboratory (GRB7.1, GRB7.2, and GRIR5) presented similar levels of H-2 class I heterogeneity. These differences have a strong influence on *in vivo* tumor behavior in immunocompetent mice [13]. Local tumor growth of different clones of GR9 in syngeneic immunocompetent BALB/c mice showed an inverse correlation between the MHC-I phenotype of tumor clones and their local tumorigenic capacity [59, 61]. Comparing local tumor growth after subcutaneous injection of 6.25×10^5 cells of A7, B7, C5, and B11, we found that all cell lines grew *in vivo* locally. A7 and B7 showed similar growth rate, but different from C5 and B11. Thus, local tumors of mice injected with C5 and B11 cell clones began to grow at day 8 and were removed at days 23 and 28, respectively. In contrast, the other two clones, A7 and B7 cells, began to grow later at days 14 and 16 postinjection, respectively; the primary tumor was removed at day 39. Clones with high MHC-I expression are very immunogenic in local tumor growth experiments; in contrast, clones with decreased MHC-I expression grew rapidly *in vivo* when injected subcutaneously. The behavior is totally opposite in spontaneous metastatic capacity (see following

section). In brief, results clearly show that in this tumor model, an inverse correlation between MHC-I surface expression on tumor clones and local tumorigenic capacity exists. Moreover, these differences in local tumor growth were associated with an immune response, since the clones progressed similarly in irradiated syngeneic BALB/c mice [61].

10.3 MHC-I Expression and Metastatic Progression

Metastatic progression is a complex process during which cancer cells leave the heterogeneous primary tumor to spread to secondary sites. Thus, pathogenesis of cancer metastases involves a set of sequential events initiated when tumor cells acquire an invasive phenotype [62–64]. These invasive tumor cells detach from matrix, invade the tissue, and migrate toward blood or lymphatic vessels to finally get access to the systemic circulation. However, most tumor cells are destroyed after extravasation into circulation by the immune system or hemodynamic forces, and only a small proportion eventually extravasate and arrive at the new site [65, 66]. This last step requires complex interactions between tumor cells and distant tissue microenvironment [67, 68]. Some *in vitro* model systems have contributed to the study of individual steps of metastatic cascade [69, 70]. However, the major limitation of these models is that they do not incorporate the complex interplay between host and tumor cells; therefore, it is necessary to work with *in vivo* models. One of the most common problems about cancer research and treatment is difficulty reproducing metastatic human disease using *in vivo* models. Preclinical tumor models must mimic the fundamental steps associated with the metastatic cascade [71, 72]. Three main types of models *in vivo* have been employed to approximate the situation observed in patients with advanced metastatic disease: genetically engineered mouse models (GEMM), transplantable tumor model systems (GRAFT) or spontaneous metastasis assays, and experimental metastasis assays. At first, an oncogenic alteration is introduced (deletion or overexpression) in a specific tissue [63, 73–75]. The other alternative extensively used, GRAFTs, recapitulate all steps of secondary colonization by spontaneous visceral metastasis. In these models, tumors or tumor cell lines are transplanted into mouse, generating a primary tumor that will be excised to prolong survival of hosts, thus increasing the possibility of distant spontaneous metastases [76–79]. Experimental metastasis assay also is the other common test to investigate biological behavior of tumor cells *in vivo*. In experimental metastasis assays, tumor cells are directly injected into blood circulation to spread to organs. We considered that spontaneous metastases assay resembles all sequential steps associated with the metastatic cascade, from primary local tumor to secondary colonization. In contrast, experimental metastasis assay is a bypass in the metastatic cascade, evading the first steps: local primary tumor growth, migration, and extravasation into blood and/or lymphatic vessels. Our research group has compared the behavior of different tumor cell lines in experimental and spontaneous metastases assays, finding that it is opposite. Tumor cell lines with high spontaneous metastatic ability showed very low experimental metastatic capacity [59]. In consequence, we think that experimental metastasis assays should not be used as a model for studying metastatic advance disease.

10.3.1 MHC Class I Expression on Primary Tumor Cells May Determine Spontaneous Metastatic Capacity

During the late 1970s, heterogeneity in metastatic potential of tumor populations was demonstrated by Fidler and Kripke, using a mouse malignant melanoma [80]. Great difference between the ability of clones from B16 cell line was observed in terms of developing metastatic colonies *in vivo*. This fact suggests that a heterogeneous population composed the primary tumor where there were nonmetastatic and metastatic tumor cells. Later research on various cell lines including clones with different metastatic potentials isolated in tumor cell populations of BALB/cfC3H mammary

adenocarcinoma [81], methylcholanthrene [82], or ultraviolet-light-induced fibrosarcomas [83, 84] supported these findings. However, Haywood and McKhann were the first to suggest the possible influence of the MHC-I genes on metastatic capacities of tumor cell populations [85]. They compared metastatic capacity of five methylcholanthrene-induced sarcomas, finding that tumors more metastatic had quantitatively more H-2 surface expression. These results, as well as later evidences observed by other groups, showed that the level of MHC-I expression was implicated in the metastatic capacity of the tumor cells. Three different spontaneous tumors originated in mouse, Lewis lung carcinoma (3LL), B16 melanoma, and BW T lymphoma, have been used by Eisenbach's research group to show whether metastasis disease is influenced by MHC-associated mechanisms. They worked with different tumor cell variants of these tumors, finding that metastatic ability directly correlated with surface expression levels of the H-2 D Ags and inversely with the of H-2 K Ags [86–89]. Moreover, H-2 K-negative/D-positive clones with high metastatic ability reverted their metastatic phenotype, inducing H-2 K-restricted CTLs when transfected with the *H-2 K* gene [87, 90, 91, 92]. In brief, these results support that the metastatic phenotype is associated with H-2 D surface expression and loss of H-2 K surface expression in primary tumor cells. In this context, Kazav et al. using T10 sarcoma (H-2 b × H-2 k) [induced by methylcholanthrene in a (C57BL/6J X C3HeB/-FeJ) mouse] reported that expression of MHC-I increased the metastatic capacity of tumor cells [93, 94]. Several clones of T10 sarcoma presented differential expression of H-2^b and H-2^k haplotypes: H-2^b x H-2^k positive and only H-2^b positive. Metastatic clones characterized to express both parental haplotypes and nonmetastatic clones only showed expression of H-2^b haplotype [95]. Furthermore, metastatic potential in this tumor system was only acquired when H-2 D^k-Ags were expressed on the surface of tumor clones. Moreover, T10 clones expressing only H-2 D^k-Ags were more metastatic than clones expressing both H-2 D^b and H-2 D^k-Ags, while clones merely expressing H-2 D^b Ag were nonmetastatic [95, 96].

10.3.2 Different MHC-I Surface Expression on GR9 Tumor Clones Determines Their Spontaneous Metastatic Capacity

In our laboratory, the GR9 fibrosarcoma murine model was used to assess whether levels of MHC-I surface expression on primary tumor cells exert influence on their spontaneous metastatic capacity. Four cell clones (A7, B7, C5, and B11) with different MHC-I surface expressions were chosen for spontaneous metastasis assays (Fig. 10.1). Results showed significant differences in metastatic capacity between these clones [59]. For example, A7 clone with a strong H-2 class I surface expression was highly metastatic, generating metastases in 90 % of the hosts and resulting in 1–50 metastases per animal. Clones with intermediate or low H-2 class I expression, as B7 or C5, presented lower metastatic capacity, 50 and 20 %, respectively. In contrast, MHC-I-negative B11 clone did not present spontaneous metastatic capacity, and the B11 tumor-bearing mice remained free of metastasis at the end of the assays for more than 24 months. In brief, cell clones with high surface expression of H-2 class I molecules were also highly metastatic, but those clones with low or negative H-2 class I expression were weekly or nonmetastatic (Fig. 10.2). Our experimental evidences support the idea that levels of MHC-I surface expression of primary tumor cells directly correlated with spontaneous metastasis ability and inversely with local oncogenicity, as it was shown above [59] (Fig. 10.2). Consequently, extrapolation of oncogenic and metastatic behavior of tumor cells *in vivo* is not always possible, because they may be completely opposite.

Analysis of MHC-I cell surface expression on spontaneous metastases derived from these fibrosarcoma clones displayed that in all cases the metastases presented the same or lower MHC-I surface expression than the original clone [59]. In consequence, metastatic progression promoted a downregulation in MHC-I surface expression. Analysis of leukocyte subpopulations in tumor-bearing mice revealed a distinct behavior among

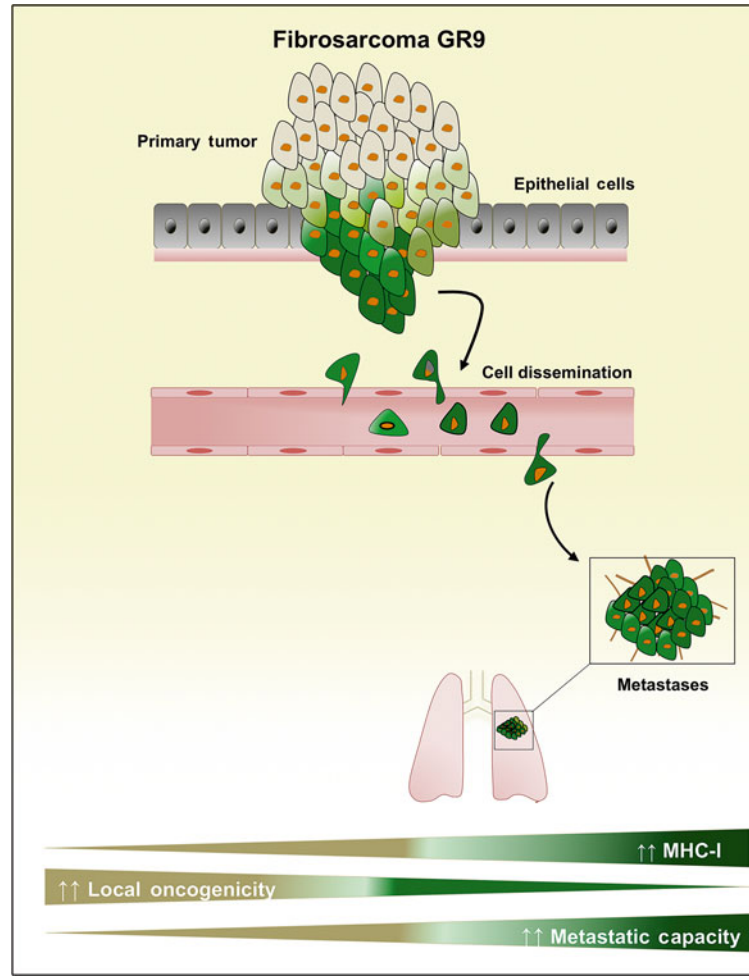

Fig. 10.2 Schematic representation of the dissemination and invasion of GR9 primary tumor cells. MHC-I-positive tumor cells from GR9 primary tumor presented a high spontaneous metastatic capacity, whereas MHC-I-negative tumor cells presented a weak spontaneous metastatic capacity

different clones. A7 and B7 produced immunosuppression characterized by decrease in T lymphocytes and increase in Treg cells [29]. In contrast, B11 tumor-bearing mice developed a strong immunostimulation characterized by an increase in T lymphocytes, dendritic, and macrophages cells (*unpublished observations*). In brief, A7 and B7 cells progressed to metastatic disease suppressing the immune response, whereas that B11 clone promoted an immune response which avoided metastatic progression. The other GR9 tumor clone studied was B9, with H-2-negative surface expression and with weak spontaneous metastatic capacity (0–1 metastasis per mouse). In contrast, this clone is highly metastatic using nu/nu BALB/c mice, ranging 5–7 per mouse [28, 97]. Moreover, metastases were H-2 class I negative in immunocompetent hosts and H-2 positive in immunodeficient hosts. Thus, we observed that H-2 phenotype of spontaneous metastases was influenced by immunological state of the hosts.

GR9 fibrosarcoma cell line, composed of different cell clones, presented intermediate levels of H-2 K^d, H-2 D^d, and H-2 L^d molecules. Analysis of spontaneous metastases assay with GR9 tumor cells revealed that GR9 cells have high spontaneous metastatic capacity; 90 % of tumor-bearing mice develop metastases, ranging

1–9 per animal. GR9 produced strong immunosuppression in tumor-bearing mice. Interestingly, 96 % of metastases derived from GR9 clone showed downregulation of MHC-I surface expression. These results suggest that MHC-I-positive clones, as A7 or B7, produced immunosuppression, favoring the growth of MHC-I low or negative clones.

Other experimental evidences from our tumor model also support the idea that in GR9 fibrosarcoma tumor, the amount of MHC-I Ags also affects NK cell cytotoxicity [98]. Since NK cells have been recognized as one of the main host immunological mechanisms against metastasis disease, this notion seems imperative [99]. In our system, tumor clones with no or low expression of MHC-I molecules were found to be sensitive to NK mediated lysis, while clones with high levels of MHC-I expression were relatively resistant [98].

10.4 Immunotherapy as a Treatment Against Cancers Expressing Different MHC-I Surface Expression

10.4.1 Immunotherapy as a Treatment Against Primary Tumors with Different Levels of MHC-I Expression

As mentioned above, MHC-I molecules present TAA to CTLs; therefore, MHC-I surface expression on tumor cells may play an important role in the outcome of immunotherapies as anticancer treatments. During treatment with vaccines containing peptides derived from TAAs, MHC-I-positive surface expression on tumor cells presenting these TAAs is crucial to make this immunotherapy effective. As a consequence, before the application of immunotherapies, MHC-I surface expression on tumor cells must be analyzed. Furthermore, two immunosuppressive mechanisms have been described recently showing evasion of tumor cells from CTLs attack, mediated by expression of noncognate MHC-I molecules or by myeloid-derived suppressor cells (MDSCs) [100, 101].

Several murine tumor models have been used to evaluate the application of different immunotherapies to recover MHC-I surface expression in MHC-I-deficient tumor cells, in order to promote an antitumor immune response. In MHC-I-negative B16 melanoma cells, intratumoral electroporation of IL-12 cDNA promoted an increase in their MHC-I surface expression, mediated by IFN-γ, leading to the eradication of established melanomas by activation of CTLs [102]. In cervical carcinoma cells, administration of synthetic oligodeoxynucleotide-bearing CpG motifs (CpG-ODNs) upregulated MHC-I surface expression causing tumor regression mediated by CTLs [103]. Other studies also have reported that CpG-ODNs immunotherapies delayed the growth or inhibited minimal residual tumor disease of both MHC-deficient and MHC-positive tumors [104, 105]. Moreover, combination of dendritic cell-based vaccines with CpG generated inhibition of tumor growth in MHC-positive and MHC-negative tumors [106]. CpG-ODN 1585 only produced regression of MHC-deficient tumors, principally activating NK cells [105]. In other assays, depletion of T(reg) cells avoided the growth of recurrent tumors after surgery of MHC-negative and MHC-positive tumors [107]. In all these assays, the action against MHC-I-deficient tumors was mediated by NK or $NK1.1^+$ cells [108]. Previous to the application of immunotherapy, MHC-I-deficient tumor cells may be treated with agents to upregulate MHC-I surface expression. Epigenetic mechanisms are frequently implicated in MHC-I downregulation of tumor cells; as a result, application of agents as 5-azacytidine (5AC) or trichostatin A could increase MHC-I surface expression [109, 110]. Treatment of 5AC with CpG-ODN or with IL-12 showed additive effect against MHC-I-deficient tumors, being the immune response mediated by $CD8^+$ T cells [111]. Other chemo-immunotherapies, based on ifosfamide derivative CBM-4A together with IL-12, also led to significant inhibition in the growth of MHC-I-deficient tumors [112].

10.4.2 Immunotherapy as a Treatment Against Metastatic Progression Derived from Primary Tumors with Different MHC-I Expression

Immunotherapy has also been used as an antimetastatic treatment against spontaneous metastasis derived from primary tumors with different MHC-I expressions. As mentioned above, studies performed by Eisenbach's et al. showed an inverse correlation between H-2 K tumor cell surface expression and spontaneous metastatic capacity [86, 89, 90, 113]. Tumor cell lines derived from H-2 K-low or H-2 K-deficient primary tumors presented high spontaneous metastatic capacity, which was reverted by transfection of tumor cells with *H-2 K* gene [86, 114, 115]. Moreover, injection of the H-2 K-transfected tumor cells that protect against metastatic disease originated from H-2 K-low or H-2 K-deficient tumors. Furthermore, therapy with IFN-γ-treated tumor cells or with H-2 K-transfected tumor cells promoted upregulation of H-2 K surface expression and protected against metastatic dissemination from parental tumor cells [113, 115]. An additional effect was reached when tumor cells were jointly transfected with IFN-γ and allogeneic *MHC class I* genes [116].

In GR9 murine tumor model, the influence of MHC-I cell surface expression on primary tumors has been investigated with respect to the success of immunotherapy as antimetastatic treatment. A7 is a fibrosarcoma clone with strong spontaneous metastatic capacity. Four treatments were used: two immunotherapies (CpG + irradiated autologous A7 cells, and PSK) [117], one chemotherapy (docetaxel), and one chemo-immunotherapy (PSK + docetaxel). A7 tumor clone was injected subcutaneously in BALB/c mice, and the primary tumor was excised when the large tumor diameter reached 10 mm. Treatment began 1 week after tumor removal, on a weekly basis during 6 weeks; 1 week after the last dose, mice were euthanized and autopsy was performed. Interestingly, all mice treated with each immunotherapy or chemo-immunotherapy appeared metastases-free (Fig. 10.3) [29]. In contrast, partial reduction in the number of metastases occurred in the mice treated with chemotherapy. In the control group, mice injected with A7 tumor cells and treated with saline solution, a high number of spontaneous metastases in all mice were observed (Fig. 10.3) [29]. In brief, the two immunotherapy protocols and the one chemo-immunotherapy protocol eradicated metastasis completely and cured the mice, whereas chemotherapy treatment reduced the number of metastases partially. When the same four treatment protocols were applied against spontaneous metastases generated from B7 fibrosarcoma clone (intermediate MHC-I expression level and with lower spontaneous metastatic capacity than A7 clone), the antimetastatic effect was not as effective (Fig. 10.3). PSK, PSK + docetaxel, and docetaxel promoted partial reduction in the number of metastases, whereas that CpG + irradiated autologous B7 cells treatment did not produce any antimetastatic effect (unpublished data). In the case of spontaneous metastases derived from GR9 fibrosarcoma, neither treatment had any antimetastatic effect. Analysis of lymphocyte subpopulations in different assays showed that growth of local tumors promotes strong immunosuppression in the three cases. However, this immunosuppression was completely reverted by immunotherapies in the case of A7-injected mice, partially reverted for B7-injected mice, and remained unchanged in GR9-injected mice [29]. All these results suggest that immunotherapies may be potential antimetastatic treatments against primary tumors with high MHC-I cell surface expression.

10.5 Concluding Remarks

In tumor cells, MHC-I molecules may present peptides derived from tumor-associated antigens, which are new proteins expressed or overexpressed in tumor cells. Presentation of these new peptides may allow recognition and destruction of tumor cells by CD8+ T lymphocytes. Loss of MHC-I expression on tumor cells is a widespread and frequent mechanism developed to escape from immunosurveillance. Alteration in MHC-I in both human and murine experimental tumors has been widely reported. Results show an

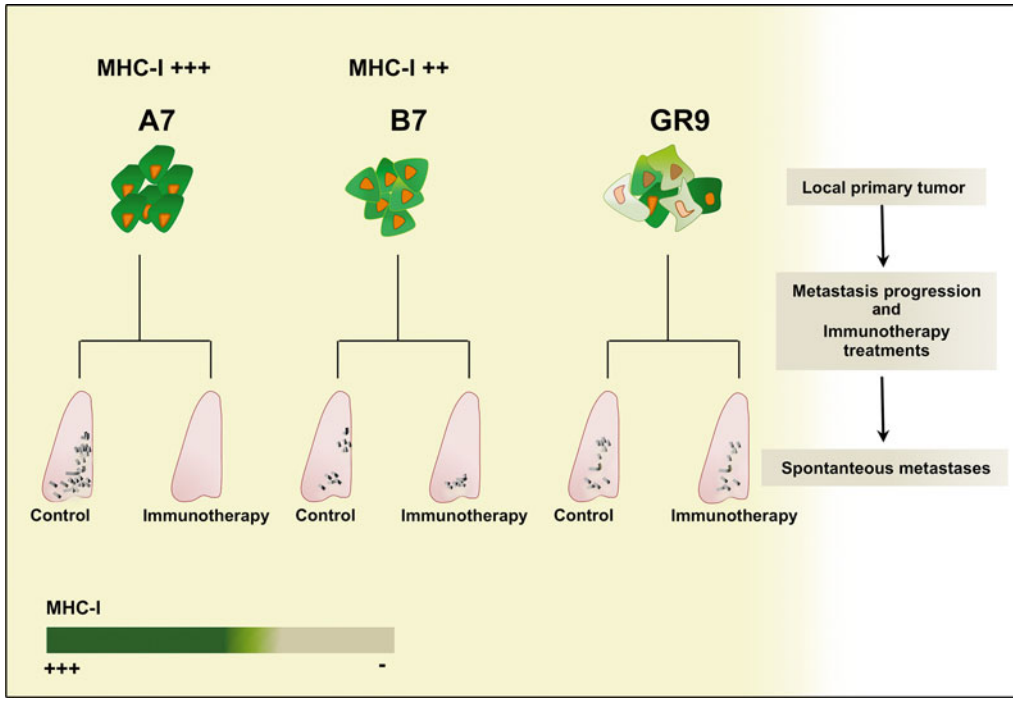

Fig. 10.3 Immunotherapy as an antimetastatic treatment against tumors with different MHC-I expression. Immunotherapy was completely effective in inhibiting spontaneous metastatic progression in A7 tumor clone (MHC-I highly positive). For B7 tumor clone (intermediate level of MHC-I expression), immunotherapy accomplished partial reduction in the number of spontaneous metastases. In the case of GR9 fibrosarcoma, immunotherapy had no antimetastatic effect

inverse correlation between MHC-I expression on tumor cells and primary tumor growth, i.e., MHC-I-negative tumors grew more rapidly compared to MHC-I-positive tumors. In contrast, a direct correlation was found between MHC-I expression on primary tumors and spontaneous metastatic capacity. Immunotherapy as an antimetastatic treatment was completely effective against MHC-I highly positive tumors and was partially effective on tumors with intermediate level of MHC-I expression.

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Role of Plasmacytoid Dendritic Cells in Cancer

11

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Contents

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11.1 Introduction

Dendritic cells (DCs) are highly specialized antigen-presenting cells (APCs) essential to generate immune responses [1], recognizing, processing, and presenting "danger signals" to the adaptive immune system. It is now clear that DCs are not a unique homogeneous cell population, but rather a pool of subsets with different origins, phenotypes, and functions [2, 3]. However, two are the most important DC subsets: myeloid-derived dendritic cells (mDCs) and plasmocytoid dendritic cells (pDCs). mDCs reside in an immature state in peripheral tissues where they behave as sentinels to actively capture and process antigens (Ags). Following exposure to proinflammatory cytokines or pathogen-derived products (pathogen-associated molecular patterns: PAMPs), they undergo a maturation process and migrate to the draining local lymph nodes via the afferent lymphatics [4]. In contrast, pDCs do not reside in peripheral tissues during homeostasis, but are encountered in the peripheral blood and lymphoid organs [1, 5]. The hallmark of pDCs is their unique capability to produce large amounts of interferon-α and interferon-β (type I IFN) in response to viruses [6]. Furthermore, pDCs can differentiate into mature DCs when stimulated by viruses [7, 8]. Thus, pDCs represent key effectors in innate immunity and the ideal cell population in connecting innate and adaptive immunity [6]. Their discovery dates back to more than 50 years ago when Lennert and Remmele [9] identified a

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previously unrecognized rare cell types with plasma cell-like morphology in the paracortical area of reactive lymph nodes. Later data revealed that these cells express both T-cell and monocyte markers and, therefore, designated plasmacytoid T cells or plasmacytoid monocytes [2, 3, 10]. In the 1980s, pathologists became increasingly aware of this enigmatic cell, and its tissue accumulation was shown to be restricted to lymphoid organs afflicted by reactive or neoplastic disorders [3, 4], as well as skin-associated lymphoid tissue [11, 12]. However, despite an increasing interest in these cells, their functional significance has still remained enigmatic.

11.2 Localization and Trafficking Patterns of Plasmacytoid Dendritic Cells (pDCs)

The development and molecular regulation of pDCs is still under investigation. FMS like tyrosine kinase 3 ligand (Flt3L) is the main growth factor that induces the differentiation of common myeloid progenitor cells into both mDCs and pDCs [13]; however, the E2–2 transcription factor is uniquely required for pDC differentiation [14]. During steady-state conditions, mouse pDCs reside in lymphoid organs and blood, as well as the liver, lung, and skin; nonetheless, their proliferation rate is very low [15]. Human pDCs reside in primary, secondary, and tertiary lymphoid organs (aggregates/follicles – lymph nodes (LNs), tonsils, spleen, thymus, bone marrow, and Peyer's patches [16], in addition to the liver and blood [17]. They can migrate from lymphoid organs toward T-cell-rich areas of secondary lymphoid tissues through high endothelial venules (HEV) and toward the marginal zone of the spleen [18]. In contrast, during pathological conditions, pDCs leave the bone marrow or the circulation and infiltrate inflamed tissues where they can "sense" danger signals, both PAMPs and endogenous danger signals (danger-associated molecular patterns: DAMPs), leading to the release of large amounts of type I IFNs [16, 18]. In this scenario they generate protective immunity as type I IFNs can activate mDCs, B, T, and NK cells [16, 18]. In particular, pDCs accumulate in inflammatory sites, e.g., lymphoid hyperplasia of the skin [11], cutaneous systemic lupus erythematosus (SLE), psoriasis vulgaris (basal epidermis and papillary dermis, but not normal skin), contact dermatitis, and allergic mucosa [19]. pDCs also infiltrate ascites associated with primary and malignant melanoma [20, 21], head and neck carcinoma [22], and ovarian carcinoma [23]. Recruitment into these sites suggests that pDCs may contribute to the ongoing inflammatory response through the release of cytokines and chemokines and lead to the activation of lymphocytes [24] or, alternatively, to the induction of tolerogenic responses [25].

An intriguing question is how do pDCs enter LNs and inflammatory sites? Chemokines are important regulators of DC trafficking *in vivo*. Similar to mDCs, blood pre-pDCs (an immediate precursor of pDCs) undergo maturation and upregulate functional CCR7 after activation with microbial stimuli or CD40 ligation, thereby acquiring responsiveness toward CCL19 and CCL21 expressed by HEVs and LN constituents [26, 27]. Furthermore, pDCs express L-selectin (CD62L), which recognizes corresponding ligands (peripheral lymph node addressin [PNAd]) on HEVs [18]. These observations may account for the localization of pDCs around HEVs and in T-cell-rich areas of LNs during pathological conditions. pDCs also express ligands for VCAM-1, an inducible molecule on endothelial cells which may enhance migration to draining LNs [25]. Pre-pDCs express several additional chemokine receptors, e.g., CCR2, CCR5, and CXCR3 [28, 29]. Nevertheless, unlike mDCs, they marginally respond to the corresponding ligands (MCP-1; RANTES, MIP-1α, and MIP-1β; Mig [CXCL9], IP-10 [CXCL10], and I-TAC [CXCL11], respectively). Instead, they migrate efficiently following the recognition of CXCR4 ligand SDF-1/CXCL12, which is expressed on dermal endothelial cells, in LN-derived HEVs, and in malignant cells [25]. Although relatively inactive on their own, CXCR3 ligands produced by Th1 cells can enhance the responsiveness of pre-pDCs to SDF-1 by 20- to 50-fold [26, 29].

During microbial infection or inflammation, the induction of CXCR3 ligands might drive the recruitment of immature pDCs to tissues responsible for SDF-1 production. In tonsils and in psoriatic skin, epithelial cells expressing SDF-1 have been associated with the expression of CXCR3 ligands [29]. However, pDCs lose their responsiveness to SDF-1 once differentiated [28]. Interestingly, pDCs express cutaneous lymphocyte-associated antigen (CLA), which binds to E-selectin on dermal endothelial cells and which may enhance their recruitment to cutaneous inflammatory lesions [30].

Adenosine has recently been identified as a potent chemotactic factor for immature pDCs via an A1 receptor-mediated mechanism [31]. Upon maturation, the receptor is downregulated, resulting in loss of migratory function. In turn, the A2a receptor is upregulated, through which adenosine reduces the production of proinflammatory cytokines [31]. Thus, adenosine, as a resultant of tissue injury from the degradation of the increased release of ATP, as well as SDF-1 and CXCR3 ligands, facilitates the recruitment of immature pDCs from blood to inflammatory sites, but subsequently limit their contribution to an inflammatory response upon maturation after an encounter with virus, bacteria, or activated T cells [31].

"Local" maturation upregulates CCR7, allowing pDCs to migrate to LNs in response to CCL19 and CCL21 and resist apoptosis [32]. At this site, pDCs could potentially present peripherally acquired Ags to T cells. Recently, IL-18 produced by mDCs in inflamed sites was shown to attract pre-pDCs and modulate their function to skew Th cells toward Th1 cells [33].

11.3 Plasmacytoid Dendritic Cells (pDCs) Phenotype

pDCs are a rare cell type representing only 0.5 % of circulating cells in healthy individuals [16]. They are round-shaped cells characterized by a prominent endoplasmic reticulum [18]. Mouse pDCs manifest most of the morphological and phenotypical features of their human counterpart [16, 18, 34]. Human pDCs are $CD4^+$, $CD45RA^+$, IL-3αR $(CD123)^+$, immunoglobulin-like transcript factor (ILT)-3^+, ILT-$1^{low/-}$, Siglec-H^+, and $CD11c^{low/-}$ cells (Table 11.1) [18]. Two additional surface markers for human pDCs are represented by BDCA-2 and BDCA-4 that correspond to the murine mPDCA-1, restricted to the peripheral blood and bone marrow-derived pDCs [18]. BDCA-2 is a C-type lectin transmembrane glycoprotein which can internalize Ags for presenting to T cells. Some data show that triggering BDCA-2 can potently inhibit *in vitro* induction of IFN-α/IFN-β expression in pDCs by viruses [35]. On the other hand, BDCA-4 does not have a substantial effect on pDC function, but can be used for the purification of pDCs by magnetic selection (Table 11.1).

In addition, recent evidence demonstrated that $CD9^+$ Siglec-H^{low} pDCs secrete IFN-α when stimulated with TLR agonists, induce CTLs, and promote protective antitumor immunity. By contrast, $CD9^{neg}$ Siglec-H^{high} pDCs secrete negligible amounts of IFN-α, induce $Foxp3^+$ $CD4^+$ T cells, and fail to promote antitumor immunity [36]. Although newly formed pDCs in the bone marrow are $CD9^+$ and are capable of producing IFN-α after aggregating in peripheral tissues, they lose CD9 expression and the ability to produce IFN-α. Therefore, recognition of the pDC surface markers is actually very important not only to distinguish pDCs from mDCs and other cell types but also to identify their function and to allow researchers to isolate them. To date, Bdca2-DTR [37] and Siglec-H-DTR models [38] are the recently developed appropriate murine models used to study the role of pDCs in the pathogenesis of various diseases. These mouse models allow the study of pDCs in pathophysiological conditions through the depletion of pDCs by diphtheria toxin (DT) using the human diphtheria toxin receptor (DTR) that is driven by the BDCA2 promoter, as the mouse receptor for DTR binds several orders of magnitude more weakly to DT. However, many studies have also been conducted by using specific depleting antibodies (Abs), such as 120G8 Ab [39], BST-2 Ab [40], and mPDCA-1 [41] *in vivo*. All these Abs bind to the same surface marker (BST-2 or CD317).

Table 11.1 Markers currently identified on pDCs

Marker	Structure/function	Ligand	Effect of activation
BDCA-2/BDCA-4	Associated with FcεRIγ to form a signaling receptor complex	ITAM	Upon ligation, they inhibit TLR activation and release of type I IFN
CD4	A glycoprotein expressed on the surface of T-helper cells, monocytes, macrophages, and dendritic cells	It recognizes the TCR-MHC class II complex and is required together with the CD3 zeta chain for the recognition of antigens	Activation of pDCs
CD 123	The IL-3 receptor (70KD) is composed of a ligand specific alpha subunit and a signal-transducing beta subunit shared by the receptors for interleukin 3 (IL3), colony-stimulating factor 2 (CSF2/GM-CSF), and interleukin 5 (IL-5)	IL-3	Amplification of inflammation
IL-T3	Characterized by its cytoplasmic ITIM domain	Fc receptor	Tolerance induction
IL-T7	Characterized by its cytoplasmic ITIM domain and is also expressed on B, T, and NK cells	IFN I	Inhibition of release of Type I IFN (negative feedback)
CD-11 c	A heterodimeric integral membrane protein composed of an alpha chain and a beta chain. It is present only on mouse, but not human, pDCs	ICAM-2 and VCAM-1	Induces cell activation; it is an adhesion receptor that is implicated in phagocytosis of latex beads and bacteria in the absence of complement. It plays an important role in the inflammatory response and can lead to the production of proinflammatory cytokines after an APC response
TLR-7	An intracellular endosomal pattern recognition receptor	Single-stranded RNA	Upregulation of CD40, CD80, CD86, and CCR7. Induction of high levels of Type I IFN. Does not induce IL-12p70 production
TLR-9	An intracellular endosomal pattern recognition receptor	Unmethylated CpG oligonucleotides from bacterial DNA	Upregulation of CD40, CD80, CD86, CD83, HLA-DR, and CCR7. Upregulation of Type I IFN, IL-6, TNFa, IL-8, and IP-10. Does not induce IL-10 secretion

Ab-depletion models seem to be less specific than DTR models, but still very efficient in pDC depletion, thus allowing the investigation of the role of pDCs during steady state and pathological conditions. The limitation of Ab-mediated pDC depletion stands on the role of some molecules, such as BST-2, which is also expressed by stromal and other immune cells after an inflammatory stimulus [40].

11.4 Activation of pDCs

Plasmacytoid dendritic cells are highly specialized at sensing nucleic acids via the intracellular pattern recognition receptors, Toll-like receptors (TLR) 7, and TLR9 [16, 34]. pDCs and mDCs have a different repertoire of TLR expression [16, 18, 34]. Human and mouse mDCs can express TLR1, TLR2, TLR4, TLR5,

TLR7, and TLR8, while pDCs selectively express high levels of TLR7/TLR8 and TLR9 [42]. TLRs are a family of receptors associated with the innate immune response [43]. In particular, TLR7 recognizes single-stranded RNA enriched with guanosine or uridine from viruses, synthetic imidazoquinolines, and guanosine analogs [43]. On the other hand, TLR9 is activated by unmethylated CpG oligodeoxynucleotide (CpG-ODN) motifs typical of viruses and bacteria [43]. Interestingly, the response of human pDCs is dependent upon the class of synthetic CpG-ODN used to stimulate them. Stimulation with CpG-A (D)/2216 ODN induces sustained high IFN-α production by pDCs, but minimal upregulation of cell surface maturation markers including CD80, CD86, and major histocompatibility complex class II (MHC-II) [44, 45] has no effect on B cells (which also express TLR9). On the other hand, stimulation with CpG-B (K)/2006, a strong B-cell activator, results in increased expression of costimulatory and Ag-presenting molecules and higher IL-8 and TNF-α secretion, but lower levels of IFN-α production by pDCs. Two distinct pathways of IFN-α/IFN-β production have been identified regarding stimulation with CpG-A *vs.* CpG-B [45]. pDCs constitutively express IRF-7 and synthesize high levels of IFN-α in response to CpG-A, which also triggers an autocrine feedback loop involving the IFN receptor-dependent pathway [42]. In contrast, IFN-α/IFN-β induction by CpG-B is independent of the IFN-α/IFN-β receptor loop [45, 46]. Recently, CpG-C, a new class of CpG ODN in which structural elements of CpG-A and CpG-B have been combined, has emerged. This sequence activates B cells and induces IFN-α production by pDCs [47]. Furthermore, non-CpG-containing ODNs have been shown to bind human TLR9 [47, 48] and to stimulate pDCs [49].

TLR7 and TLR9 are very sensitive to different stimuli; the first triggers ssRNA viruses and the latter responds to DNA viruses [50]. TLR7 and TLR9 activation recruits a cytoplasmic adaptor, myeloid differentiation primary response gene 88 (MyD88), which is able to assemble a multiprotein signal-transducing complex-inducing interferon regulatory-factor 7 (IRF-7) activation [43]. MyD88 also leads to TRAF-6-mediated NF-κB and MAP-kinases (MAPKs) activation, essential for the transcription of proinflammatory cytokines, chemokines, and costimulatory molecules [43, 51].

The exposure of pDCs to TLR7 or TLR9 ligands can lead to the production of type I IFN and proinflammatory cytokines, such as TNF-α, and chemokines, such as IL-8 (CXCL8) [1, 16, 18]. Constitutive expression of IRF7, which is different from mDCs in which induction is needed, renders pDCs high producers of type I IFN [1, 16, 18], regulating T-cell immunity, leading toward a Th1 and cytotoxic T lymphocyte polarization and activation of mDCs, NK cells, and B cells [1, 16, 18]. Remarkably, IFN-α modulates several aspects of the immune system, including pDC survival [52], mDC differentiation, modulation of Th1 and $CD8^+$ T-cell responses, cross-presentation, upregulation of MHC and costimulatory molecules, activation of NK cells, and induction of primary Ab responses [53]. However, a recent study found that type I IFN negatively controls pDC turnover in that an overproduction of type I IFNs can lead to the death of pDCs during steady-state conditions and viral infections [50]. pDC activation can also lead to the production of IL-12p70, IL-1β, and IL-6 [54]. Furthermore, recent discovery found that pDCs may mediate the release of IL-10 [26]; however, another group [55] showed that these cells do not directly produce IL-10 (Fig. 11.1).

Moreover, it was recently demonstrated that pDCs produce high amounts of granzyme B [56], which is effective only in combination with perforins mainly produced by cytotoxic T lymphocytes (CTLs). This further connects pDCs to adaptive immunity. Additionally, in the absence of an "efficient" adaptive CTL immunity, pDCs can behave as killing DCs due to the release of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and to the induction of DR5 expression, a TRAIL receptor, on the cell target [37, 56].

A diversity of C-type lectin receptors (CLRs) has been identified on DC subsets, including DC-SIGN (CD209), DEC-205 (CD205),

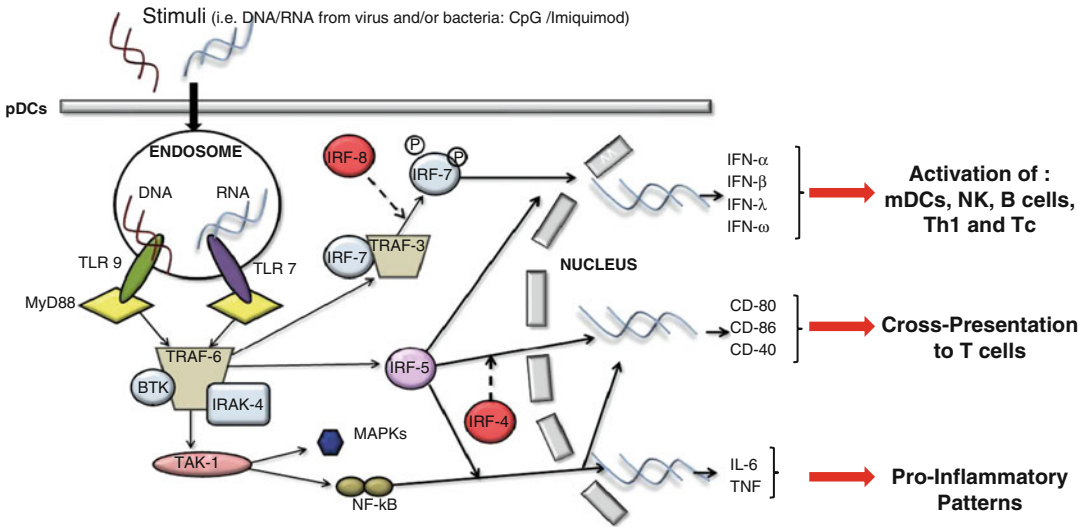

Fig. 11.1 The recognition of stimuli, such as DNA or RNA motifs from viruses and bacteria, by pDCs via TLR7 and/or TLR9, induces the activation of MyD88-dependent signalling pathways that lead to the expression of cytokines such as IL-6 and TNF-α, costimulatory molecules such as CD80, and the synthesis/release of type I IFN

langerin (CD207), mannose receptor (CD206), BDCA-2, and dectin-1. CLRs typically recognize carbohydrate-rich structures on microbes and self-antigens [35]. They have been implicated in cell adhesion and regulation of signaling events (e.g., BDCA-2), migration and homing (e.g., DC-SIGN), Ag uptake and processing for MHC-II presentation to T cells (e.g., DC-SIGN, BDCA-2, langerin, and mannose receptor), cell-cell transmission of pathogens (e.g., DC-SIGN), and tolerance to self-antigens (e.g., DEC-205). pDCs express BDCA-2 and BDCA-4, dectin-1, and possibly DEC-205 but lack DC-SIGN and langerin, found on $CD34^+$ and monocyte-derived DCs and Langerhans cells (LCs), respectively [57]. The physiologic function of CLRs on pDCs remains unknown. Anti-BDCA-2 Abs are rapidly internalized and efficiently presented to T cells, suggesting a role in Ag capture and presentation [35]. Interesting relationships between CLRs and TLRs have been documented. In mDCs, interaction of DC-SIGN with lipoarabinomannan secreted by mycobacteria inhibits lipopolysaccharide (LPS)-induced DC activation through TLR4 [58]. This mechanism may permit pathogens to evade immune responses and perpetuate tolerance to self-antigens in the face of TLR activation by microbes. On the other hand, it has been shown that dectin-1 collaborates with TLR2 in inducing proinflammatory cytokine secretion in murine macrophages and DCs [59]. Whether BDCA-2 has any connection to TLRs in pDCs remains to be elucidated. However, early reports have shown that secretion of type I IFNs by pDCs in response to influenza virus (most likely triggering TLR7/8) or to complexes of plasmid DNA and anti-DNA Abs (possibly stimulating both FcR and TLR9) is significantly inhibited by ligation of BDCA-2 with anti-BDCA-2 Ab [35]. It is worth noting that BDCA-2 is downregulated after pDCs maturation and that mature pDCs secrete less IFN-α/IFN-β in response to viral stimuli than immature pDCs do [60, 61]. BDCA-2 has an intracellular domain of 21 amino acids without known motifs implicated in signal transduction; however, ligation induces Src family protein-tyrosine kinase-dependent intracellular calcium mobilization and protein-tyrosine phosphorylation of intracellular proteins [35]. BDCA-4 (neuropilin-1) is also upregulated in blood mDCs after overnight culture and may participate in DC-lymphocyte interactions [62].

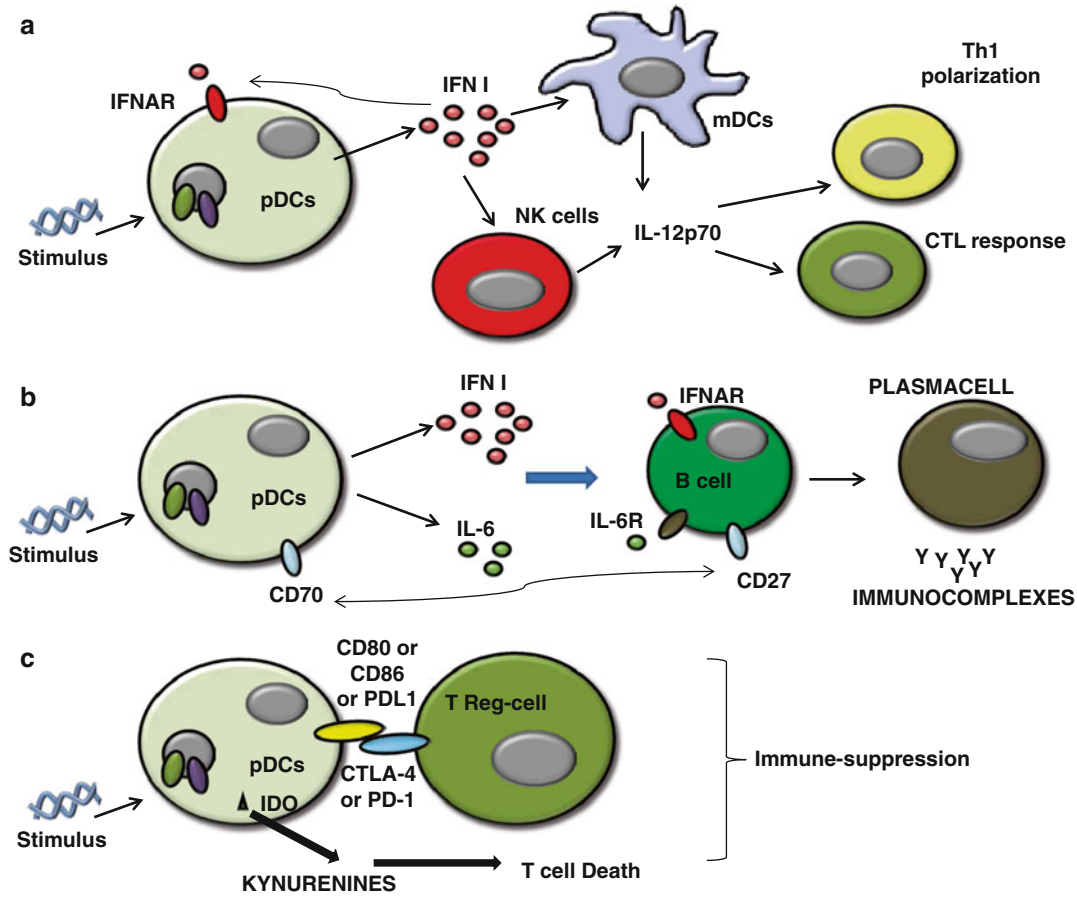

Fig. 11.2 (**a**) Activated pDCs produce high amounts of Type I IFNs which both amplify its own production in an autocrine manner via the expression of IFNAR on themselves and induce the release of other proinflammatory cytokines such as IL-12p70 from mDCs and NK cells that lead to Th1 and CTL polarization; (**b**) pDCs induce B cells to differentiate into plasma cells via the activation of IFNAR, IL-6R activation, and the interaction of CD70-CD27 on B cells; (**c**) pDCs can lead to immunosuppression via both direct interaction with Treg (CD80 or CD86^{+}CTLA-4 or PD-L1^{+}PD1) and the release of IDO-induced kynurenines metabolites which induce Th1 cell toward death

11.5 pDCs: Bridging the Gap Between Innate and Adaptive Immunity

The production of type I IFNs by pDCs represents the bridge between the innate and adaptive immune system. Type I IFN (IFN-α and IFN-β) is an important component of the innate immunity, especially during viral infections [16, 18]. In contrast to mDCs, pDCs produce high amounts of type I IFNs upon activation [16, 18], which both amplify its own production in an autocrine manner and induce the release of other proinflammatory cytokines such as IL-12p70 from mDCs and NK cells [63] (Fig. 11.2a). Activation of mDCs diverts the immune environment toward a Th1-like bias, during which IFN-γ production facilitates Th1 differentiation [16, 18, 63], long-term T-cell immunity [18, 63], and a CTL-mediated response [64], as well as proliferation and survival of T cells [63, 64]. Moreover, through the production of IL-6 and type I IFNs, pDCs induce B cells to differentiate into plasma cells which are immunoglobulin (preferentially IgG and IgM)-producing cells (Fig. 11.2b). In the process of B-cell activation, a key role is played

by the CD70 receptor expressed on pDCs, as it can induce the differentiation and the proliferation of IgG-producing B cells [65] (Fig. 11.2b).

In addition, activated pDCs can undergo other important phenotypic changes that induce them to change their phenotype toward an mDC phenotype [1]. The upregulation of MHC and T-cell costimulatory molecules enables pDCs to engage and activate naïve T cells [66–68]. There have been many controversies regarding the role of pDCs to prime T cells and cross-present Ags [68]. The expression of MHC and T-cell costimulatory molecules is not as high as in mDCs, and this is why pDCs are less efficient than mDCs at priming T cells [69]. Moreover, the repertoire of Ags that can be presented by pDC-derived MHC molecules is more restricted than those of mDCs because not all of these Ags reach the endocytic compartment into pDCs [68, 69]. However, some pDC receptors such as BDCA2, Siglec-H, and DCIR are able to bind Ags, mediate endocytosis, and process and present to T cells [68, 69].

Interestingly, activated pDCs can also promote Th2-like immune responses [63] underlining their functional plasticity. There is evidence that IFN-α stimulates the differentiation of pDCs into Th1-polarizing pDCs, whereas in the absence of IFN-α but only in the presence of proinflammatory signals, pDCs can also stimulate Th2 polarization/differentiation [70]. Moreover, some authors reported that CpG-activated pDCs exert a strong immunosuppression and induce the differentiation of allogeneic $CD4^+CD25^+$ T cells into $CD4^+CD25^+$ regulatory T cells in tumor conditions [50, 55]. Very interestingly, pDCs can directly or indirectly recruit Treg cells via PD-L1/PD-1 axis [71] (Fig. 11.2c), release of immunosuppressive cytokines, such as IL-10 [55, 71], and the membrane tolerogenic inducible costimulator ligand (ICOS-L) [72].

pDCs can also synthesize large amounts of functional indoleamine 2,3-dioxygenase (IDO), which requires autocrine release of type I IFN, upon TLR9 and CD200R ligands' stimulation [16]. IDO-derived metabolites promote T-cell death [55, 73] and suppresses T-cell immunity in normal and pathological settings. In the same manner, reduced tryptophan amounts can lead to the release of regulatory cytokines, such as IL-10 [74], associated with a tolerogenic environment.

Taken together as a whole, these data suggest that pDCs represent a key effector cell in both innate and adaptive immunity regulation [1].

11.6 pDCs and Human Diseases

A wide spectrum of human diseases including infection, autoimmunity, and cancer are associated with accumulation of pDCs in lymphoid and peripheral tissues strictly correlated to the reduction of these cells in the peripheral blood [21]. For many of these diseases, compelling evidence supports a pathogenic role of pDCs, mainly related to either the increase or reduction of proinflammatory or antiinflammatory functions of pDCs. Alternatively, pDC accumulation might exert an adjuvant immune function, as in viral infection, and in imiquimod-treated cancers, where they seem to encounter an antiviral and antitumor activity. In many other pathologies, information available is still limited, and pDC biology is largely unknown.

11.6.1 Role of pDCs in Human Infections

pDCs have been most extensively studied during HIV and chronic viral hepatitis, particularly hepatitis C virus (HCV) infections. The emerging picture suggests an important role for pDCs in these infections; however, the exact mechanism and consequences of pDC activity are controversial at present [75]. pDCs can respond to HCV and particularly to HCV-infected hepatocytes which induce pDCs to signal via an endocytosis- and IRF7-dependent mechanism, but not via the NF-κB pathway, implying a non-full functional response of pDCs that contribute to the evasion of immune responses by HCV [76]. In contrast, other studies demonstrated normal pDC functionality in chronic HCV infection [77]. The resolution of this controversy would establish pDCs either as a weak link of anti-HCV immune response or as a potentially powerful effector type that can be harnessed for immunotherapy of chronic HCV.

Similarly, pDC dichotomy is observed in HIV infection, in which some authors assume that

pDCs can be infected with the HIV and/or respond to it with robust IFN secretion [78], while others reported impaired activity of pDCs in HIV infected patients [79, 80]. Interestingly, pDCs are progressively depleted from the blood of infected patients, either through infection-induced death or due to redistribution to lymphoid organs. The key unresolved question is whether HIV-induced pDC activation is beneficial or harmful for the host. On one hand, IFN secretion by pDCs was shown to inhibit viral replication in T cells and promote pDC and cDC maturation, leading to the killing of infected T cells. In this context, it is likely that HIV may have evolved mechanisms to suppress pDC activation, e.g., through BDCA-2 ligation [81], which disables pDC functions as APCs and type I IFN-producing cells. On the other hand, the same functions of pDCs may exacerbate T-cell depletion, e.g., by disseminating HIV to uninfected $CD4^+$ T cells or by bystander T-cell killing. Most importantly, elevated IFN response by pDCs may contribute to chronic immune activation and faster T-cell depletion [82]. It is plausible that the function of pDCs in HIV infection changes from protective to pathogenic as the disease progresses. At the early stages of infection, IFN production and virus cross-presentation by pDCs may help limit virus spread and mount cytotoxic T lymphocyte responses; whereas as the virus replication escapes control, IFN secretion may drive polyclonal T-cell hyperactivation and depletion [77]. The eventual loss, redistribution, or functional impairment of pDCs at the late stages of infection would contribute to immunodeficiency. Thus, the role of pDCs in HIV and HCV infections highlights the power and the danger of pDC activation and reveals another strategy of immune system subversion by these viruses.

11.6.2 Role of pDCs in Autoimmune Diseases

Several autoimmune diseases are associated with elevated levels of type I IFNs, implying a potential role for pDCs in cytokine production [83]. To date, the strongest evidence for pDC involvement has been accumulated from the study of two diseases: psoriasis and systemic lupus erythematosus (SLE) [84]. In psoriasis, early skin lesions are highly infiltrated by activated pDCs, corresponding with decreased numbers of circulating pDCs [85]. Blocking IFN production by pDCs using anti-BDCA-2 Ab inhibited the development of skin lesions in a xenograft mouse model, providing causal proof of pDC function in the disease [85]. Gilliet's group [86] identified the activating stimulus for pDCs as complexes of self-DNA with the antimicrobial peptide LL-37. This and possibly other homologous proteins promote the aggregation of released cellular DNA and RNA into large complexes that efficiently activate pDCs [86, 87]. Although the origin of these immunostimulatory complexes and the consequences of pDC activation remain to be elucidated, the major role of pDCs in psoriasis is well established. Similarly, lupus patients show a decrease in circulating pDCs and the accumulation of activated, IFN-producing pDCs in affected tissues such as the skin [88]. The hallmark of lupus is the production of antinuclear Abs and immune complexes of such Abs with endogenous nucleic acids were shown to activate pDCs through TLR7/9 [89, 90]. These complexes may be delivered into the endosomal compartment of pDCs via Fc receptor II (FcγRII) [89, 91], and their stimulatory capacity can be augmented by the nuclear DNA-binding protein HMGB1 [92]. In addition, self-DNA forms complexes with LL-37 and other antimicrobial peptides released by neutrophils, and the resulting complexes induce IFN secretion in pDCs through TLR9 [92]. Notably, TLR-activated pDCs become resistant to glucocorticoids, which could underlie the limited efficacy of these drugs in lupus [93, 94]. The direct causal relationship between pDC-derived IFN and lupus progression/severity is hard to establish in the human system and should await for elucidation in animal models. Nevertheless, the likely connection between the formation of nucleic acid-containing immune complexes, pDC activation, and IFN secretion and the pronounced IFN signature of the disease makes a strong case for the pDC as a major player in lupus pathogenesis [77]. Overall, the aberrant conversion of self-nucleic acids into ligands for TLR7/TLR9 on pDCs (via immune complex formation, antimicrobial peptide binding, and other mechanisms to be

discovered) may represent a common pathogenesis step in psoriasis, lupus, and possibly other autoimmune diseases such as Sjögren's syndrome [95].

The activity of pDCs in viral and autoimmune diseases might teach us how and why pDCs highly populate cancerous masses playing a pivotal role for the tumor immune microenvironment.

11.6.3 Role of pDCs in Cancer

Recent studies have shown that the density and location of immune cells in primary tumors can predict patient survival [96], supporting the notion that monitoring local immune response might represent a critical step in predicting patient prognosis and likely the response to antitumor strategies [97]. pDCs have been found in a variety of neoplasms; nonetheless their function is still unknown. Solid tumors, such as head and neck, breast, ovarian, lung cancer, and skin tumors, are populated by non-active pDCs [97]. Clinical studies have suggested a direct correlation between reduced numbers of circulating pDCs and higher presence of these cells into malignant masses [1, 97]. Although the causal relationship is still under investigation, recent results from mouse models are starting to define the specific role(s) of pDCs in tumor masses. The mechanism that induces the recruitment of pDCs to the tumor site is not clear. Circulating pDCs express multiple chemotactic receptors such as CXCR4 and ChemR23 being the only biological active receptors in healthy donors [28]. CXCR4 binds CXCL12, widely expressed in tissues and which most likely represents the main axis for pDC accumulation in human tumors [25]. CXCL9, CXCL10, and CXCL11, which bind CXCR3, present on pDCs, are all IFN-inducible proteins and might be involved in pDC infiltration [98]. In addition, cytokines such as CXCL10, CXCL12, and chemokines, such as CCL2, are released by tumor and stromal tumor-associated cells, such as cancer-associated fibroblasts (CAFs), allowing pDCs to migrate from the circulation to the injured tissue [23]. Accordingly, Drobits et al. demonstrated that CCL2 produced in the inflamed skin of tumor-bearing mice facilitated pDC recruitment [56].

Once recruited, pDCs seem to be important players in cancer immunoediting as their capacity to bring together the innate and the adaptive immunity. In particular, it seems that critical role is played by type I IFNs. Endogenously produced IFN-α/IFN-β was required for the prevention of the growth of primary carcinogen-induced sarcoma [99]. In this study, host hematopoietic cells were critical targets of IFN-α/IFN-β during the development of protective antitumor responses [99]. pDCs have been widely described as professional type I IFN-producing cells; therefore, the higher presence of pDCs in the tumor mass might directly link pDCs to cancer immunoediting in that pDCs may behave as antitumor cells. However, other reports showed opposite activities of pDCs in cancer. Animal studies demonstrated that tumor-associated pDCs (TApDCs) are defective in type I IFN production but instead secrete immunosuppressive factors responsible for tumor progression [100, 101]. Similar to what described for viral infections and autoimmune diseases, the dichotomy of pDCs in cancer might underlie their phenotype and maturation state.

11.6.3.1 Antitumor Activity of pDCs

Type I IFNs are pleiotropic cytokines with a demonstrated clinical benefit to cancer patients and have recently emerged as the connection bridge between tumor cells and the immune system [102]. pDCs produce large amounts of type I IFNs upon TLR7 and TLR9 stimulation. Drobits et al. showed that the intratumoral stimulation of pDCs with imiquimod renders these cells cytotoxic and contributes to tumor regression independently from conventional adaptive immune mechanisms, but via the production of TRAIL and granzyme B secretion by pDCs via IFNAR1 signaling [56]. However, the role of TApDC-derived granzyme B in the absence of perforins not produced by pDCs still remains to be elucidated.

Another mechanism that may underlie the antitumor activity of TApDCs is their antigen-presenting activity. Although in their immature state, TApDC are still capable to internalize Ags

in vivo and to activate $CD4^+$ T cells [103]. The immature state of pDCs is reflected in that they have altered cytokine production in response to TLR-9 ligands *in vitro*, while preserving unaltered response to TLR7 ligands [104], which instead seem to have potential antitumor activity. To date, imiquimod is in phase III clinical trial against melanoma. In contrast to these results, systemic administration of CpG favored pDC-induced lung tumor progression [105], as also observed in a mouse model of breast cancer [104]. Similar to the data showed by Drobits et al., Mercier et al. proved that, although CpG did not alter TApDC activity, the intratumoral administration of a TLR7 ligand led to TApDC activation and displayed a potent curative effect in a type I IFN-dependent manner [56]. In addition, Liu et al. [106] demonstrated that the intratumoral activation of pDCs via CpG could induce NK cell-dependent tumor regression in a melanoma animal model. Remarkable is that TLR9 expression and responsiveness is impaired by tumor-derived components [107]. ILT7 on pDCs binds BST-2 expressed by tumor cells and their interaction inhibits type I IFN production by pDCs, disabling TLR9-dependent signaling pathways [108]. Moreover, tumor-derived TGF-β and TNF-α have been identified as the main *in vivo* mechanisms blocking type I IFN production by pDC in tumors through inhibition of IRF7 signaling complex, leading to a negative impact of defective pDCs in breast cancer through Treg expansion [109].

Taken altogether, these data supported the rationale to use TLR7 ligands to restore TApDC activation in both breast and skin cancer. However, it still remains to be determined how the activation of TLR7 and TLR9, which is MyD88-dependent, on pDCs, can behave differently according to the tissue specificity and on the route of administration.

11.6.3.2 Pro-tumor Activity of pDCs

Several evidence have shown the prevailing immunosuppressive activity of pDCs due to both of the impairment in type I IFN production and the release of pro-tumor factors [1]. Stimulation of lung tumor-bearing mice with systemic CpG, a TLR9 ligand, did not lead to the same results as observed by Liu et al. [106]. Activation of pDCs through CpG had the opposite effect in that pDC activation increased the recruitment of Tregs and limited the inflammatory cell influx to the lung, thereby establishing an immunosuppressive environment enabling tumor growth [1, 105, 109]. The same was observed in another mouse model of breast cancer in which *in vivo* depletion of pDCs delayed tumor growth showing that TApDC provide an immune-subversive environment, most likely through Treg activation thus favoring breast tumor progression [110]. The discrepancy in these data and the one from Liu et al. [106] could be a result of tissue-specificity and route of CpG administration which is very important in determining the tumor microenvironment, which in turn strongly influences immune cell phenotype. Moreover, in the absence of a specific stimulus, pDCs in the tumor mass have been associated with the development and maintenance of the immunosuppressive microenvironment [111]. Similar to mice, human pDCs in tumor masses are in their immature phenotype; nonetheless, a thorough study has never been conducted on the role of these cells in human tumor microenvironment. Nevertheless, it is clear that pDCs play a fundamental role in the tumor microenvironment. The specific depletion of pDCs induced lung tumor regression with a concomitant Th1 polarization that arrested tumor progression [105]. On the other hand, stimulation of TLR7, rather than TLR9, can subvert the immunosuppressive activity of TApDCs. TLR7-dependent pathway induced melanoma regression in mice [56] through the transformation of pDCs into tumor-killing cells able to produce granzyme B and TRAIL. Likewise, another group revealed that human pDCs can kill melanoma cells *in vitro* under imiquimod and IFN-α stimulation [112]. While pDCs can produce high levels of granzyme B, their role as cytotoxic immune cells remains to be determined as they lack the pore-forming perforin [112]. On the other hand, it has been proposed that under IL-3 and IL-10 exposure, pDCs release abundant granzyme B, which in turn is capable of blocking T-cell proliferation, thus suggesting a

new potential mechanism for tumor-immune evasion [112].

Several mechanisms have been postulated for the immunosuppressive nature of tumor-associated pDCs: (1) release of tolerogenic factors, (2) ILT-7 expression, (3) PD-L1 expression, (4) Siglec-H activity, and (5) induction of a Th2-like environment. Tolerogenic factors produced by tumor cells, such as PGE2 [113] and TGF-β [109], can alter type I IFN signaling pathway. Tumor-derived PGE2 and TGF-β act synergistically to block IFN-α and TNF-α secretion by pDCs [16, 109]. Opposite to IFN-α and TNF-α, IL-6 and IL-8 production are enhanced in PGE2- and TGF-β-treated pDC [114]. Both IL-6 and IL-8 promote immune-cell survival and chemotaxis but also enhance tumor cell proliferation and angiogenesis [115, 116]. Moreover, PGE2 is crucial for the secretion of other immunomodulatory factors such as SDF-1, the ligand for CXCR4, which is upregulated on both human pDCs and tumor environment [117]. Thus, pDCs can be retained in the tumor tissue via PGE2-induced sensitization for SDF-1 [29]. In further support, PGE2- and TGF-β-mediated retention of pDCs in the tumor tissue is accompanied by the suppression of the lymph node-homing receptor, CCR7 [113]. PGE2-exposed pDCs are unlikely to present Ags and to prime T cells in the regional LNs. Concomitantly, suppression of CD40 expression and the overexpression of CD80/86 on pDCs enhances and even promotes Treg activation via the negative regulatory receptor cytotoxic T-lymphocyte antigen-4 (CTLA-4) [118, 119] (Fig. 11.2c).

Another potential mechanism for pDCs favoring tumor immune escape is the release of IDO-derived metabolites [119] from both pDCs (Fig. 11.2c) and tumor cells, inducing Treg differentiation and Th1 cell apoptosis [55, 74]. Most human tumors overexpress IDO [120], explaining the elevated tryptophan catabolism in cancer patients. Interestingly, the activation of IDO in either cancerous cells or regulatory DCs can be sufficient to promote tumor immune escape [121]. Some cancer cells, such as lung cancer-derived cells, highly express ILT7L, which can bind to ILT7 that is on pDCs [122]. ILT7L is induced by IFN-γ and inhibits IFN-α production by human pDCs, indicating that the ILT7L-ILT7 interaction between cancer cells and pDCs may cause impairment of pDCs in the tumor microenvironment, possibly leading to immunosuppression and poor prognosis of cancer patients as observed in preclinical studies [119]. Moreover, under tumoral conditions pDCs can also direct mDC phenotype toward a more immature state, as already reported for human lung cancer [16, 70, 105]. However, the underlying mechanism is still not defined.

To date, pDCs can directly interact with Treg via the PD-1/PD-L1 axis [55] (Fig. 11.2c), paving the road to another mechanism of action of the newly approved monoclonal Ab, anti-PD-1 for cancer immunotherapy.

Moreover, Ag targeting to pDCs via Siglec-H inhibits Th1 cell-dependent immunity [103]. The administration of CpG increased Siglec-H expression on pDCs recruited to the lung of tumor-bearing mice, further supporting their implication in the inhibition of Th1 cell expansion [105].

pDCs activated by IL-3 and CD40 ligand (CD40L) promote the differentiation of naive $CD4^+$ and $CD8^+$ T cells into Th2 cells and anergic IL-10-producing $CD8^+$ regulatory T cells, respectively [123]. This state of anergy is mediated by IL-10, either directly (by interaction with cytotoxic T lymphocytes, CTLs) or indirectly (by inhibition of DCs) [114]. Since the tumor microenvironment is Th2-like, pDCs participate in this scenario by further augmenting immunosuppression.

Overall, these effects may allow pDCs to establish a reduced inflammatory pattern but, at the same time to favor tumor progression/establishment, as observed in asthma [124], virus infection [125], and cigarette smoke exposure [70]. To note, the aforementioned studies describe the role of pDCs which are not activated by a specific stimulus; then, it seems obvious that the activation of pDCs at the tumor site is a limiting step in tumor regression. Therefore, the dichotomy of pDCs in cancer may rely on the stimulation/activation of pDCs with specific stimuli as in the case of imiquimod.

11.7 Potential Therapies: Clinical Significance

Secreted factors by tumor cells, such as TGF-β, VEGF, and IL-10, may inhibit pDCs functions with the resulting prevailing of the suppressive immune response dictated by the same pDCs and adaptive immune cells. On the contrary, other studies reported tumor-infiltrating pDCs as functional and fully competent APCs. Production of IFN-α renders TApDCs as antitumor cells. In this context, the activation of intratumoral pDCs by means of imiquimod (TLR7 ligand) and/or CpG (TLR9 ligand) has been successfully used in the clinic to treat basal cell carcinoma and melanoma [1]. TLR signaling on pDCs can be used to induce type I IFNs and possibly protect pDCs from tumor-derived inhibitory factors (such as TGF-β and IL-10), as well as support T-cell-mediated antitumor immune response. However, this practice can only refer to the activation of TApDCs in loco, as mouse models showed that systemic administration of CpG rendered pDCs immunosuppressive, favoring lung and breast tumor progression [1, 101, 105, 109, 110].

Many therapeutic trials have been designed to potentiate CTL responses. Myeloid-derived dendritic cells-based vaccines succeeded in inducing specific T cells in patients, but without sufficient clinical efficacy [126]. A potential explanation of this failure may underlie the role of pDCs at modulating tumor immune-environment and, more specifically, mDCs activity [105]. Animal studies on several diseases, such as asthma, virus infection, and cigarette-exposed and lung cancer models, revealed that pDCs can hamper the activity of mDCs [105]. In particular, the presence of high levels of pDCs in tumor masses was associated with immature mDCs incapable of mounting an effective adaptive immune response against cancer. Specific ablation of pDCs rendered mDCs active and prone to induction of a CTL response against tumor cell proliferation [105]. Therefore, we speculate that pharmacological manipulation of pDC phenotype could result in successful antitumor therapy together with the conventional strategies. In support, our unpublished data showed that doxorubicin or oxaliplatin, drugs that are highly used in the clinical antitumor practice, had a much effective activity against lung tumor progression due to the induction of proinflammatory pDCs, activated by tumor cell death. This latter study was conducted on mouse models. Therefore, clinical correspondence could prove the potential antitumor activity of proinflammatory pDCs resulting in tumor regression. In addition, previous studies on the role of pDCs as antitumor cells only after intratumoral activation of these cells by means of imiquimod and CpG could underlie the same mechanism of action. In other words, several endogenous molecules (DAMPs) that participate to the sterile inflammation have been described as potential TLR ligands. Similarly, we could speculate that tumor cell death can induce the release of DAMPs which activate pDCs in a TLR7- or TLR9-dependent manner leading to type I IFN production by pDCs. This prevails and allows the gap between the innate and the adaptive immunity to overcome tumor-mediated immunosuppression. In this scenario, Aspord et al. demonstrated that stimulation of PBMCs from HLA-A*0201$^+$ donors by HLA-A*0201 matched allogeneic pDCs pulsed with tumor-derived peptides triggered high levels of antigen-specific and functional cytotoxic T lymphocyte responses; this resulted in melanoma regression in a humanized mouse model [127]. This semi-allogeneic pDC vaccine was more effective than conventional mDC-based vaccines, endowing a strong potential for clinical application in cancer treatment [127].

11.8 Concluding Remarks

In the last decade several studies provided evidence that pDCs actively participate in a wide spectrum of human diseases including infection, autoimmunity, and cancer. In particular, human neoplasms are populated by pDCs which presence is related to a poor prognosis. However, the role of tumor-associated pDCs (TApDCs) remains controversial. Various studies indicate that pDCs play an immunosuppressive role and facilitate tumor progression in both animal models and humans. In contrast, others found that the

presence of activated pDCs results in tumor regression in mice. Given these findings, it is clear that pDC function plays a critical role in tumor biology. Understanding pDC biology in cancer represents an important necessity and will pave the road to novel therapeutic strategies to fight malignancies.

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12 Cancer Immunoediting: Immunosurveillance, Immune Equilibrium, and Immune Escape

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Contents

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12.1 Introduction

The immune system's regulation of the cancerous process is a long-known fact. However, the role played by it in malignancies has been a matter of debate. The history of cancer immunity dates back to 1909 when Paul Ehrlich proposed the concept of immunosurveillance in cancers for the first time [1]. However, due to lack of experimental evidence, this concept fell into disrepute. In 1957 Burnet and Thomas argued that indeed, the immune system fights and eliminates certain cancers and the frequency of malignancy would have been much higher if immunity was not there [2]. In 1970s, several experiments were conducted in athymic mice to prove immunosurveillance in cancers; however, the results were not as expected, which was thought to be due to the presence of residual immunity in the animals used for these studies [3–5]. Consequently, the experiments done again on animal models with specific molecular immune defects revealed more frequent development of carcinogen-induced tumors in these immunodeficient animals [6]. However, more recently, the recognition of the dual nature of the part played by immune system in malignancies has led to the modern concept of cancer immunoediting. Since then, immunoediting in cancer has served as the foundation stone of most of the work being carried out in cancer immunity [7,8].

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12.2 Cancer Immunoediting with Its Three Es: Reflection of the Dual Role of Immunity in Cancer

The cancer immunoediting theory states that tumors are sculpted by the immune system, resulting in the selective growth of the variants which are better equipped to fight the immune system (Fig. 12.1). This selective growth advantage conferred on tumors is a consequence of a number of genetic and epigenetic events occurring within the tumors. The clue to the tumor-editing role of the immune system came from the experiments of Robert Schreiber's group on spontaneous and 3′-methylcholanthrene (MCA)-induced tumors in 129/SvEv mice (Fig. 12.2) [6]. The concept of immunoediting was introduced by Dunn et al. in 2002 to explain the antitumor as well as pro-tumor features of our immune response at different stages of cancer [3]. Since then, many studies conducted over a period of time have demonstrated the editing of tumors by host adoptive cells, leading to their complete reprogramming. A more recent

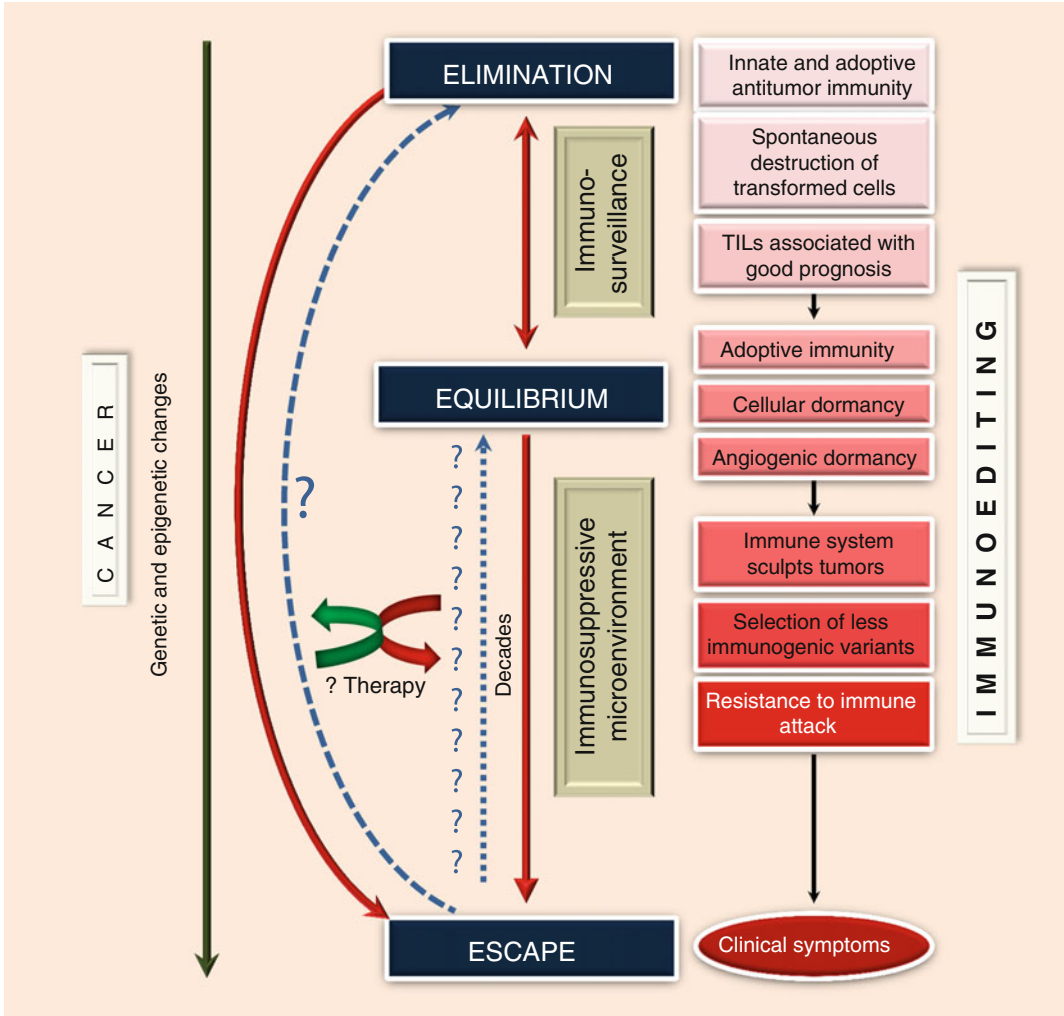

Fig. 12.1 Cancer immunoediting process with its three Es of elimination, equilibrium, and escape. Please note that although in many cases the sequence is followed, in others one or the other phase may be skipped. Although the events from equilibrium phase may proceed either toward escape or back to the elimination phase, the reversibility of the escape phase with or without therapy to other two phases is questionable

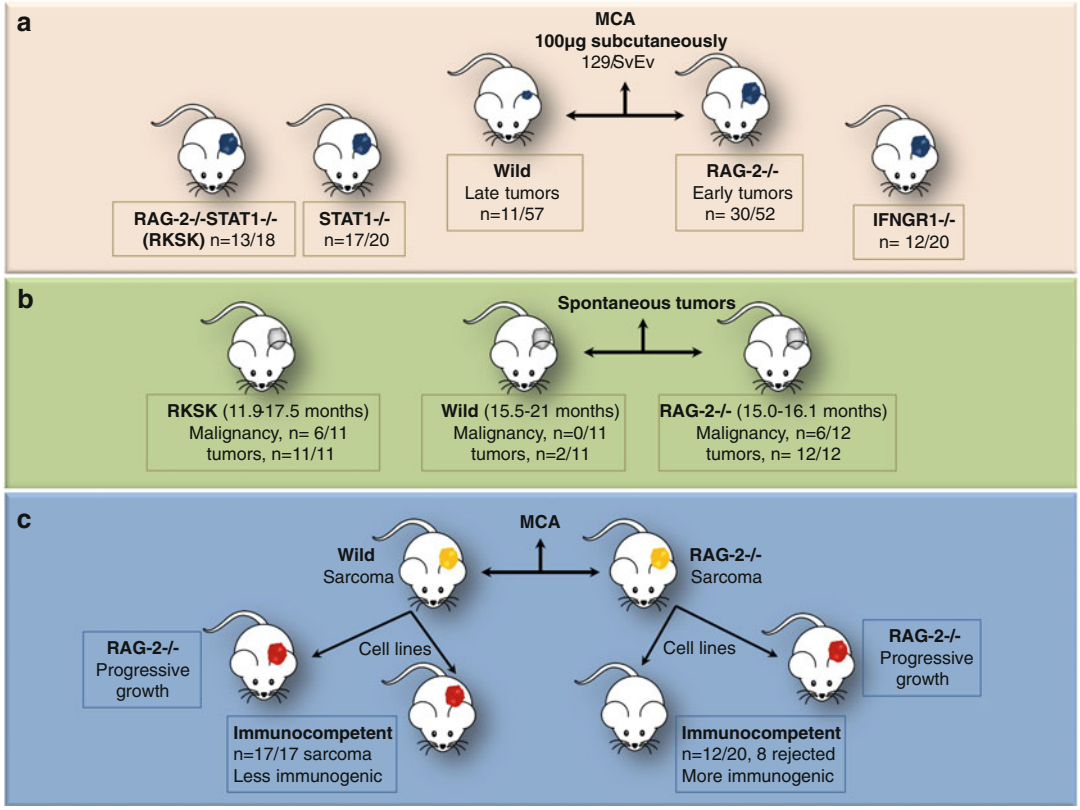

Fig. 12.2 Mice experiments by Shankaran et al. [6] demonstrating surveillance and sculpting roles of immune system. (**a**) Immunodeficient (RAG-2–/–/IFNGR1/STAT1–/– or combined RAG-2–/– STAT1–/–, RkSk) mice developed tumors earlier than wild type and with greater frequency on subcutaneous injection of MCA, thus necessitating the presence of intact T, NKT, and B cells for prevention of chemically induced tumors. (**b**) Spontaneous tumor development was also observed to be higher in RAG-2–/– and RkSk mice as compared to unmanipulated 129/SvEv wild-type mice. Moreover, the later merely developed benign tumors and no malignancy was noted. (**c**) Furthermore, cells were taken from MCA-induced tumors in wild and RAG-2–/– mice and were injected into immunocompetent and RAG-2–/– mice. Progressive tumor growth was noted in immunodeficient mice transplanted with sarcoma cells derived from wild or RAG-2–/– mice. The immunocompetent mice transplanted with sarcoma cells from wild mice also showed progressive tumor growth; however, many mice transplanted with sarcoma cells derived from RAG-2–/– mice rejected the transplanted tumor cells. This occurred due to sculpting of sarcoma by the immune system in wild mice, thus rendering it less immunogenic. Tumors from the immunodeficient mice which were not edited were more immunogenic and thus were rejected by immunocompetent mice

study has linked processes such as epithelial mesenchymal transition in tumor cells, which result in an invasive phenotype, to the immunoediting process through the involvement of cytokines such as TNF-α and TGF-β [9]. Cancer immunoediting is a broad concept which includes three "Es" of elimination, equilibrium, and escape which together sum up to all the events occurring during an immune response to cancer [3].

12.2.1 Immune Elimination: Evidences For and Against

The immune elimination phase of cancer immunoediting is sine qua non of the original immunosurveillance process. It envisages the destruction or eradication of cancer by the host immune system and is believed to occur when a cell gets transformed by overcoming its intrinsic

Table 12.1 Timeline of events depicting evolution of cancer immunity from immunosurveillance to immunoediting

Study	Hypothesis/observation/experimental evidence	Results
William B Cooley (1891) [10]	Injected cultures of heat-inactivated bacteria or bacterial culture supernatants into cancer patients	Demonstrated marked regression of tumors and prolonged survival after the treatment
Paul Ehrlich (1909) [1]	Immune system protects the host from malignancy	Gave birth to the idea of immune control of malignancies
Burnet and Thomas (1957) [2]	Immune system must be removing the carcinogenic events arising out of ongoing evolutionary genetic remodeling taking place in an individual	Formal emergence of immunosurveillance hypothesis
Several groups (1965–1973)	Induced immunodeficiency by thymectomy or heterologous antilymphocyte serum or pharmacological agents. Immunodeficient animals are more prone to develop cancers	No consensus regarding immunosurveillance
Stutman O (1975) [11]	The methylcholanthrene (MCA)-induced cancer incidence in immunodeficient nude athymic mice was not higher than the control mice	Rejection of immunosurveillance hypothesis
Kaplan et al. (1998) [12]	IFN-γ and perforin deficient animals were more prone to MCA-induced tumors as compared to controls	Resurrection of immunosurveillance in cancer
Shankaran et al. (2001) [6]	Experiments in RAG-2 null mice (lacking T, B, and NKT cells) revealed higher incidence of both MCA-induced sarcomas and spontaneous epithelial tumors in these animals	Definitive evidence of existence of cancer immunosurveillance
Dunn et al. (2002) [3]	Concept of cancer immunoediting to explain the tumor sculpting role of immune system	Coined the term immune elimination as a part of broader concept of cancer immunoediting with 3 Es of elimination, equilibrium, and escape

tumor suppressor mechanisms, before being able to establish a full-blown tumor. Although the existence of such a phenomenon has been hypothesized since long, the early experiments carried out on nude mice models which are only partially immunodeficient failed to prove it. The definitive experimental proof to its presence came from the work of Shankaran et al. in the last decade (Table 12.1, Fig. 12.2) [6]. However, despite the experimental evidence of its presence in mice, it has been difficult to demonstrate it in the clinical scenario. Still, the data obtained from various cancer registries wherein a higher cancer incidence especially of viral etiology has been observed in immunosuppressed transplant recipients suggests its existence in human subjects as well. Currently, a similar trend has been noticed in the setting of acquired immunodeficiency syndrome [13,14]. The proponents of this stage in cancer immunity state that many of the cell transformation events occurring in our body may be removed quietly by the immune system without us ever being aware about it. Spontaneous regression has been reported in some tumors including cutaneous melanoma, retinoblastoma, osteosarcoma, etc., in humans [15]. Studies have shown that both innate as well as adaptive immune response contribute to fighting off the cancer from our body.

12.2.1.1 The Key Players in Anticancer Immunity

The key players responsible for launching an effective immune response against cancer include the immune cells and soluble molecules secreted into the tumor milieu (Fig. 12.3). In case, the tumor exhibits high immunogenicity, a specific immune response occurs against it. However, if tumor immunogenicity is low, the nonspecific effector responses gain importance.

The major cell types involved in an antitumor immune response are adoptive T cells, which not only kill tumor cells directly with the help

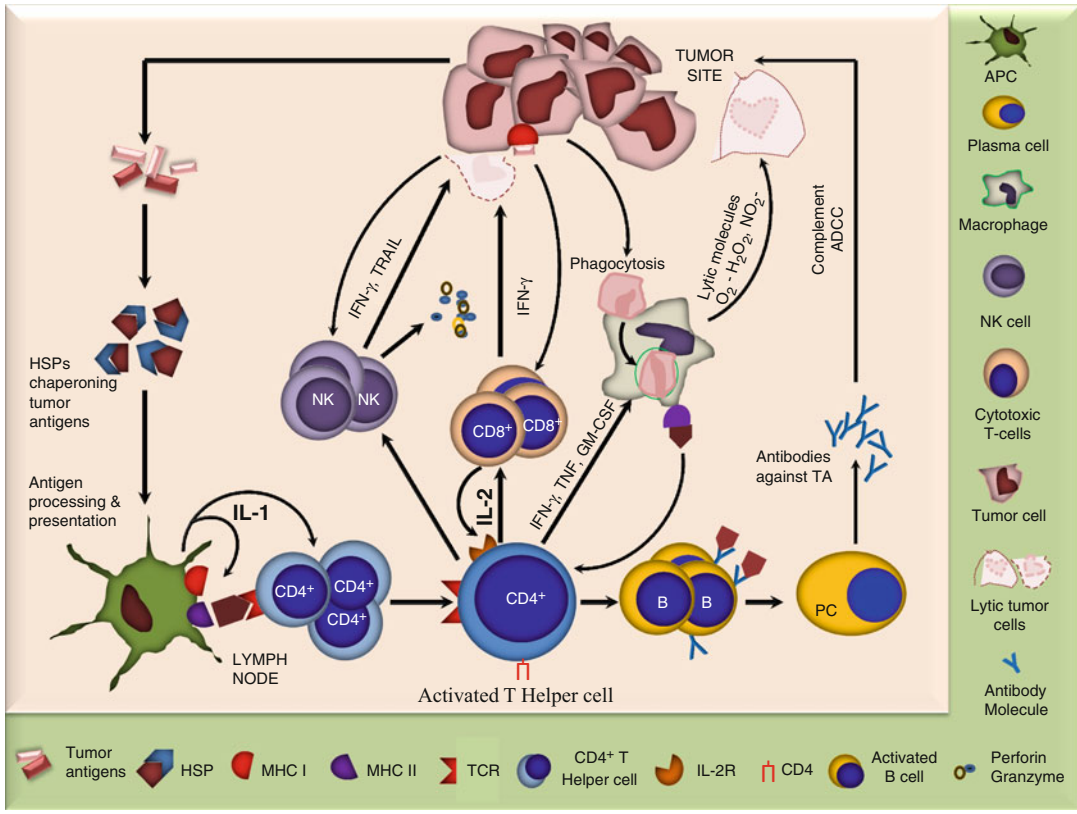

Fig. 12.3 Diagram showing key players involved in antitumor immune response. The tumor releases Ags which are chaperoned by heat-shock proteins and taken up by the APCs which process them and present to $CD4^+$ T cells. The later being the central point of immune response activate various other cells including NK cells, $CD8^+$ cells, macrophages, and B cells which act in various ways to counteract the tumors. In addition, tumors may directly activate the cytotoxic cells including $CD8^+$ and NK cells and phagocytic cells. While the former two can cause direct tumor lysis primarily via perforin and granzymes, the later may engulf tumor cells and kill them by releasing lytic molecules or may process and present tumor Ags to $CD4^+$ T cells

of TNF-α but are also essential for the activation of other components of the immune machinery. The $CD8^+$ cytotoxic lymphocytes (CTLs) are able to directly recognize tumor cells which express MHC I and can also be activated by $CD4^+$ T-helper cells. They may cause lysis of the tumor cells via perforin- and granzyme-dependent mechanisms. The $CD4^+$ T cells also secrete factors to induce proliferation of B cells and to promote their differentiation to antibody (Ab)-secreting plasma cells. The later may contribute to antitumor immunity by complement-mediated lysis or by antibody-dependent cellular cytotoxity (ADCC). The $CD4^+$ T-helper cells also activate macrophages by secreting IFN-γ, TNF, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF). The activated macrophages may phagocytize tumor cells and kill them by releasing toxic free radicals including O_2^- and NO_2^- or by becoming antigen-presenting cells (APCs) which present tumor antigens to $CD4^+$ T cells such as dendritic cells (DCs). Natural killer (NK) cells also have the potential to directly recognize and destroy tumor cells via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and IFN-γ-dependent mechanisms. Loss of MHC class I as commonly observed in tumors may be responsible for their increased susceptibility to NK-cell-mediated lysis. In addition, NK-cell activity may also be enhanced by IL-2 and IFN-γ produced by the $CD4^+$ T-helper cells. NKT and γδ T cells also recognize the danger signals released

from the tumors and become activated. The NKT cells especially the invariant or the type I NKT, which are $CD4^-$ $CD8^-$ and mainly recognize the lipid/glycolipid antigens (Ags) via CD1d molecule, have been recognized to protect against certain cancers. The protective role is however supposed to be indirectly exerted via secretion of IFN-γ and subsequent activation of NK and $CD8^+$ T cells. The γδ T cells which represent 1–5 % of peripheral blood T cells are also reported to infiltrate and cause lysis of tumors, both *in vitro* and *in vivo* [16–20].

In various clinical studies on different cancers including colon, ovary, lung carcinomas, and melanoma, the tumor-infiltrating lymphocytes (TILs) have been associated with increased time to disease recurrence, an enhanced 5-year survival, and an overall good prognosis. Also, in a study on metastatic colorectal cancer, TIL density at the invasive margin was linked to a better chemotherapeutic response. Similarly, increased infiltration by $CD3^+$ and $CD8^+$ T cells, NK cells, and γδT cells has been correlated with improved outcomes in epithelial ovarian cancers. Some of the above studies have done quantitative assessment of the TILs in tumors, thus impressed upon the need to have a scoring system for TILs in order to determine the exact tumor behavior [21,22].

12.2.2 The Equilibrium Phase: The Most Controversial and the Least Understood Phase

This phase represents an intermediate stage of immune response in cancer. During this phase, the cancer and the immune system both coexist without allowing each other to dominate. The immune system cannot eliminate the cancer during this phase; however, it does not allow it to expand or metastasize. The cancer in turn is sculpted by the immune system, thus leading to the emergence of variants resistant to the immunological attack [3].

Various studies in mice have pointed toward the occurrence of the equilibrium phase in cancer immunity. In experiments on MCA-induced tumors in mice, Koebel et al. demonstrated the presence of inert lesions in healthy mice, which grew when subjected to immunological oppression (Fig. 12.4) [23]. The study served to be an important milestone in proving the existence of the equilibrium phase in cancers. Likewise, the tumors have been observed to stay dormant for decades after remission in human cancer patients, which is believed to be due to the fact that immune system keeps them in check. The immune system is believed to synergize with chemoradiotherapy in treatment-induced remission which renders the tumors silent. However, they relapse promptly after any kind of immune insult, thereby, further proving the presence of immune dormancy. The minimal residual disease commonly observed in hematological malignancies and the emerging donor-derived malignancies in immunosuppressed transplant recipients are considered two examples of the equilibrium phase in humans. Even though the immune system prevents monoclonal gammopathy of unknown significance (MGUS) from progressing to myeloma, it fails to eliminate the MGUS cells [24,25].

Adoptive T cells, both $CD4^+$ and $CD8^+$, have been observed to play a pivotal role in cancer immune equilibrium. Immune-sufficient mice with inert tumors are shown to develop into full-fledged tumors only upon depletion of T cells/IFN-γ/IL-12. However, the depletion of innate immune cells was not found to result in the development of tumors. Moreover, tumor cells were found to be highly immunogenic during the equilibrium phase, as they are unedited by the immune system and become less immunogenic at the end of this phase [23,26,27].

In addition, the mechanisms including cellular and angiogenic dormancy also complement the immune system in maintaining cancer cells in the dormant state. In the former, the tumor cells hide themselves in specialized niches, become quiescent, and wait for the opportunity to regrow. In the later condition, expansion is not possible, due to the lack of adequate vascularization. When faced with favorable conditions, tumor cells come out of their slumber and undergo a series of genetic and epigenetic modifications which

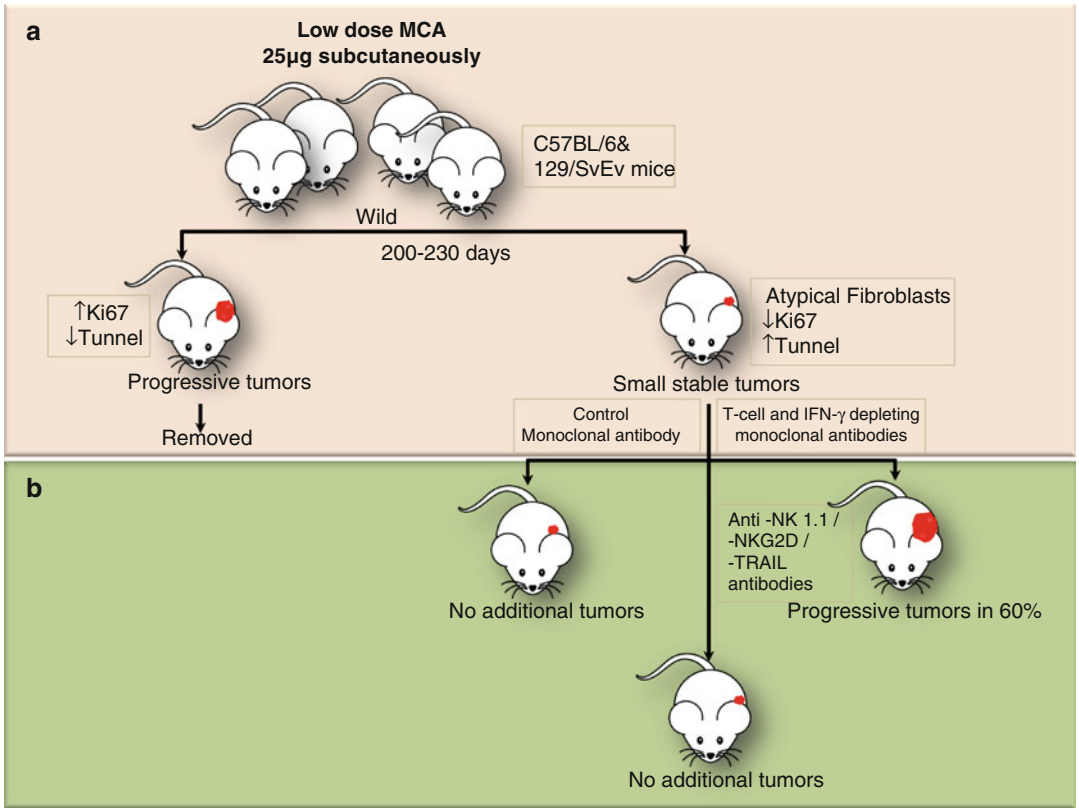

Fig. 12.4 Experiments conducted in mice by Koebel et al. demonstrating the presence of equilibrium phase in tumorigenesis. (**a**) Groups of wild-type C57BL/6 or 129/SvEv mice were injected with a single low dose of MCA. After monitoring for 200–230 days, the mice with rapidly growing sarcomas were set aside. (**b**) The remaining mice displaying small stable masses at injection site were injected with control Ab or mAbs depleting specific components of innate and adoptive immunity. The mice in former two groups did not develop any additional tumors; however, those in the last group (T cell and IFN-γ depleted) showed rapid tumor growth. This could only be explained by cancer immune equilibrium in which the tumors were not removed, but restricted by the immune process. However, on suppression of adoptive immunity progressive tumor growth was observed

increase their immune resistance, eventually leading to the next phase of cancer immunity, known as immune escape. Studies are being conducted to identify the genetic and molecular signatures of dormant tumor cells which allow them to retain their dormant status or facilitate their escape [23,26–29].

12.2.3 Immune Escape: The Best Studied Phase

The escape phase represents the final and most extensively studied phase of the immunoediting process. The unleashing of mechanisms underlying the escape phase has formed the basis for the development of various therapeutic agents with the aim to stop the progress of the neoplastic process. Due to increasing genomic instability, cancer cells acquire various characteristics enabling them to ward off the immune process or to modify it in such a way which is beneficial to tumor cells. Tumors utilize a number of strategies to evade an effective immune response (Fig. 12.5). The basis of an effective immune response against any Ag is its recognition as a nonself and its presentation to immune effector cells. Tumors escape recognition by either presenting self Ags to which the immune system is already tolerized or by modulating their antigenicity. The later

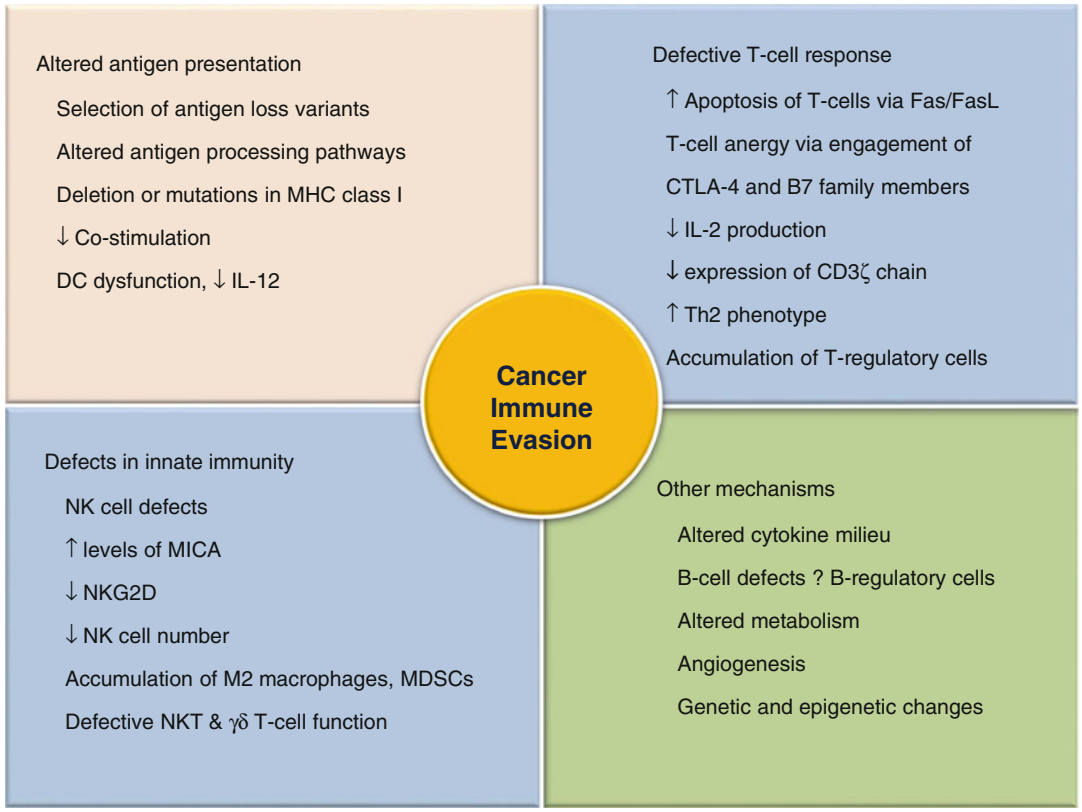

Fig. 12.5 Mechanisms of immune evasion by the cancer

involves the shedding of tumor Ags into the circulation from where they may be removed [30]. The next line of defense adopted by tumor cells is the modulation of APCs, rendering them incapable of effectively presenting cancer Ags to immune cells. The APCs like DCs are either deleted or functionally compromised in response to the factors secreted by malignant cells [31]. Tumor-induced co-inhibition of the second signal of the Ag presentation and consequent immunosuppression has now been recognized in several cancer types [32]. In addition, the tumors alter MHC molecules especially MHC class I and other components of Ag processing machinery in the APCs, so as to further incapacitate the presentation of its Ags to the immune system [33]. Besides, tumor cells plunge into an active battle against the immune process by attacking its adoptive and innate immune cells. Tumor cells subvert T cells and render them anergic through co-inhibitory molecules including cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-L1 [34]. Anergic T cells are unable to produce cytokines such as IL-2 and IFN-γ. Therefore, the autocrine and paracrine activation of $CD4^+$ cells and other immune cells including B cells, macrophages, and $CD8^+$ cells are blocked, leading to further suppression of the immune cascade [35]. Moreover, tumors also express Fas ligands on T cells, leading to lymphocyte apoptosis [36]. Not only do they suppress $CD4^+$ and $CD8^+$ cells, but also promote the suppressor T-cell phenotype such as $CD25^+Foxp3^+$ T-regulatory cells. These cells secrete IL-10, TGF-β, and VEGF which suppress the antitumor response and promote tumoral angiogenesis (Table 12.2) [37]. Besides, tumors also inhibit innate immune response by induction of quantitative and qualitative defects in NK cells, macrophages, and neutrophils. NK cells have been found to exhibit decreased cytotoxic potentiality due to the presence of tumor-secreted factors including TGF-β in the tumor

Table 12.2 Mechanisms of immunosuppression induced by T-regulatory cells and myeloid-derived suppressor cells

T-regulatory cells
Secretion of immunosuppressive molecules like IL-10, IL-35, and TGFβ
Polarization of DCs toward tolerogenic phenotypes
Direct cytolysis of effector T cells via granzyme B, TRAIL, and galectin-1
Metabolic changes like increased IDO in DCs and increased conversion of ATP to adenosine promoting immunosuppression
Stimulation of tumoral angiogenesis via VEGF secretion
Myeloid-derived suppressor cells
Inhibition of effector T-cell proliferation and function via L-arginine-dependent mechanisms
T-cell inhibition via production of ROS and TGFβ
Reduced T-cell homing via depletion of L-selectin
Promotion of Th2 and T-regulatory phenotypes via IL-10 secretion
Inhibition of DC function via IL-10
Promotion of angiogenesis via secretion of VEGF, basic fibroblast growth factor, HIF-1, etc.

microenvironment (TME) [38]. The later along with other cytokines (IL-4, IL-13, etc.) present in the tumor bed favors the accumulation of M2 macrophages, which also induce immunosuppression [39]. Recruitment of immature myeloid cells like myeloid-derived suppressor cells (MDSCs) further complements the tumor-immunodeficient environment by reducing T-cell and NK-cell activation and promoting neovascularization via factors like VEGF [40].

Other mechanisms such as anaerobic glycolysis, hypoxia, and acidity of the TME along with the existent defects in tryptophan metabolism induced by increased expression of the enzyme indoleamine 2,3-dioxygenase (IDO) further depress the antitumor immunity, thereby leading to cancer progression and metastasis [41–43].

12.3 Tumor Antigens and Cancer Immunoediting

Antigenicity of tumors has always been a matter of discussion. In the past, it was believed that since tumors are derived from self cells, the immune system is more receptive to their Ags. However, it was subsequently noticed that tumors may express Ags which are quantitatively or qualitatively different from self Ags, thus rendering them sensitive to the immune attack. Quantitative differences include significantly increased expression of Ags, which are less expressed in normal or benign conditions or reexpression of Ags only expressed at a specific stage of embryonic development (Table 12.3). Moreover, the lineage-specific Ags expressed normally in specific tissues may be expressed aberrantly in tumor cells. Qualitative differences are produced due to mutational events occurring during carcinogenesis. Over the years, several efforts have been made for the identification and mapping of the Ags expressed on tumor cells; various nomenclatures have been used to characterize them such as tumor-associated Ags and tumor-specific Ags. Antigens capable of evoking a tumor-specific immune response have also been designated as tumor rejection Ags in some textbooks, e.g., tyrosinase, MUC-1, Her-2/neu, β-catenin, caspase-8, etc. [44]. Previous studies on tumor antigens (TAs) have mainly focused on the discovery of new Ags and their classification into two subclasses, a group which can evoke a protective immune response and another group serving as potential therapeutic targets. However, the advent of cancer immunoediting theory has changed our insight on TAs, as they are now considered to be one of the prime targets of the above process. Currently, ongoing studies are attempting to differentiate between the antigenicity of the original or unedited tumors and those sculpted by the immune system [17,45,46]. Differences between the immunogenicity of tumors derived from carcinogen MCA (more immunogenic) and those arising spontaneously (less immunogenic) in mice have been described by DuPage et al. [47]. They also showed that primary sarcomas are edited by the immune system and, hence, become less immunogenic in order to escape the T-cell response. In the same line, Matsushita et al. obtained similar results in their study on tumor exomes [48]. A recent study has revealed the presence of antiinflammatory antibodies to tumor-associated Ags like NY-ESO-1, thereby suggesting the importance of humoral immune system in cancer immunoediting [49]. Novel genetic-based

Table 12.3 Examples of common categories of antigens present in tumors [44]

Antigen type	Antigen class	Antigen	Characteristics of antigens	Tumor
Tumor-associated antigens	Oncofetal antigens	CEA AFP	Expressed in fetal tissues, reexpressed in tumors	Colon cancer Germ cell tumors, HCC
	Differentiation and lineage-specific antigens	CD5	Normally in T cell but aberrantly in B cells in CLL	CLL
		Melan A, tyrosinase Gp 100 PSA	Melanocyte lineage	Melanoma Prostate carcinoma
	Cancer testes antigens	MAGE 1 NY-ESO-1	Expressed in germinal tissues and reexpressed in malignancies	Melanoma
	Heat-shock proteins	Gp 96 HSP70		Fibrosarcoma, colon cancer
	Gene amplification	Her-2/neu	Receptor tyrosine kinase	Breast cancer Ovarian cancer
	Aberrant post translational modification	MUC1	Under glycosylated mucin	Breast Pancreas
Tumor-specific antigens	Mutated oncogenes or proteins	Mutated p53 BCR-ABL β-Catenin	Point mutations Translocation 9;22 Signal transduction pathway	Many tumors CML Melanoma
		Caspase 8	Apoptosis regulation	Squamous cell carcinoma
Oncoviral proteins	HPV 16, E6 and E7 proteins		Viral transforming gene products	Carcinoma cervix

CEA carcinoembryonic antigen, *AFP* alpha fetoprotein, *Gp* glycoprotein, *PSA* prostate-specific antigen, *MAGE-1* melanoma-associated antigen 1, *NY-ESO-1* New York-ESO-1, *BCR-ABL* breakpoint cluster region-Abelson, *HPV* human papilloma virus

approaches including exome sequencing, in silico analysis, and $CD8^+$ T-cell cloning are likely to further help in understanding the alterations in tumor antigenicity occurring during different phases of cancer immunity [48].

12.4 The Tumor Microenvironment During Cancer Immunoediting

The microenvironment surrounding the tumor plays a critical role in determining cancer behavior. TME is composed of cells (tumor as well as immune), various factors secreted by them, and the stroma. The TME is a dynamic system switching from host protective to tumor friendly during different phases of the immunoediting process. During the elimination phase, the milieu of the tumor comprises of factors which promote its eradication. Collaboration of factors including IFN-γ and lymphocytes has been found to help in regulating the development of tumors. In different studies, IFN-γ - and perforin-deficient mice together with T-cell and NK-cell defects are found to exhibit a greater propensity for tumor development. Cytokines like IL-2, IL-12, and IL-7 have been found to promote antitumor immunity, suppress recruitment of suppressor cells, and inhibit tumor angiogenesis.

During the equilibrium phase, TME assumes the role of a niche, concealing relatively dormant cancer cells. The niche environment allows cancer cells to thrive without progression by maintaining a balance between the cytostasis and cytolysis.

However, molecules which precisely maintain this balance during the immune equilibrium state remain to be defined.

During the escape phase, tumor bed gets packed with factors and cells which promote immune suppression. Factors like IL-6, TGF-β, IL-8, and IL-10 help in generalized subversion of an effective anticancer immune response. Growth factors like VEGF not only promote angiogenesis but also facilitate the recruitment of T-regulatory cells and MDSCs to the tumor site. Besides, tumor cells induce downregulation of antitumor cytokines including IL-12 and IFN-γ. In addition, the abundant presence of other factors within the TME including prostaglandin E2, reactive oxygen and nitrogen species and phosphatidylserine, etc., aids cancer cells to evade the immune response. Furthermore, the stroma including cancer-associated fibroblasts, chemokines, matrix metalloproteinases, and adhesion molecules also participates in cancer's conquest over antitumor immunity.

Although the above few paragraphs have tried to provide a simplified view of the events occurring during various phases of the immunoediting process, there are several paradoxes involved. One set of factors may play an immunostimulatory and antitumor role under particular conditions, whereas they may exert an immune inhibitory and pro-tumor role under other circumstances. For example, IFN-γ which is a potent cytokine responsible for antitumor immunity is now emerging as an important player in cancer immune evasion. The pro-tumor effects of IFN-γ are believed to be related to an increase in T-regulatory cells and MDSCs and a decrease in neutrophilic infiltrate in the TME [50–53].

12.5 Clinical Relevance of the Immunoediting Process in Cancer

The introduction of immunoediting concept has added a new insight to understanding of cancer immunity. A clear understanding of the mechanisms underlying the three phases of cancer immunity is vital for designing the immunotherapeutic strategies to prevent, stop the progression, or treat cancers. In addition, it has contributed to the development of new markers for the diagnosis and prognostication of malignancies. Identification and manipulation of various molecules involved in different phases of the immune response to cancer has emerged as a promising approach for the development of novel immunotherapeutic strategies for cancer treatment and eradication. Table 12.4 provides examples of the immunotherapeutic approaches directed toward the three phases of the immunoediting process.

Deciphering the nature of the cellular infiltrate and secretory molecules produced in response to the transformation events and characterization of the mechanisms involved in the elimination of tumor cells at early stages has led to the development of novel cancer therapeutics. Moreover, quantitative as well as qualitative assessment of the immune cells present in TME may contribute to the development of algorithms demonstrating tumors' response to chemoradiotherapy. *In vivo* or *in vitro* expansion of tumor-specific effector cells is being applied as a strategy to boost up the antitumor immune response. Recognition of TAs which evoke an effective antitumor immune response has served as the basis for the development of different types of cancer vaccines. Monoclonal antibodies (mAbs) targeting diverse TAs have entered clinical trials for several cancer types. Besides, TAs such as CEA have also been used as biomarkers for early detection and for determining tumor prognosis. The concept of immunogenic chemotherapy which stimulates adaptive immunity is also gaining impetus in recent years.

The equilibrium phase has also emerged as a potential target to immunotherapists, as maintaining cancer cells in the equilibrium phase indicates prevention or delay in cancer progression and fatality. In cases treated with mAbs which exert their effect via NK cells, an adoptive T-cell response was also found to be evoked, leading to the maintenance of tumors in equilibrium phase [69]. Furthermore, development of sensitive techniques to seek out the occult tumor cells in various organs may help in their specific targeting, resulting in their complete eradication. Identification and targeting of immune or

Table 12.4 Examples of therapeutic approaches targeting different phases of cancer immunoediting

Phases of immunoediting	Approaches	Outcome
Elimination	*In vivo* or *in vitro* expansion of immune effector cells and using them for therapy	Sipuleucel T (autologous PBMCs, APCs, and recombinant fusion protein i.e. PA2024, PA, PAP fused to a GM-CSF), FDA approved for prostate cancer [54]
	DC-based approaches	
	Tumor antigen-based vaccines	
	Tumor-specific monoclonal antibodies	Trastuzumab (Her2/neu), rituximab (CD20), cetuximab (EGFR) [55–57]
	Immunostimulatory cytokines	IL-2, IL-7, IL-15 [58–60]
Equilibrium	Adoptive transfer of cancer-reactive T cells	Monitored for establishment of equilibrium phase [61]
Escape	Anti-CTLA-4	Ipilimumab approved for melanoma [62]
	Blockade of T-cell co-inhibition	mAb against B7-H1 [63]
	Depletion of T-regulatory cells	Denileukin diftitox [64]
		Lenalidomide [65]
	Inhibition of MDSCs	Sunitinib [66]
	Inhibition of IDO	1-Methyl tryptophan [67]
	Blockade of VEGF	Bevacizumab [68]

PBMCs peripheral blood mononuclear cells, *APCs* antigen-presenting cells, *DC* cendritic cell, *GM-CSF* granulocyte macrophage colony-stimulating factor, *EGFR* epidermal growth factor receptor, *CTLA-4* cytotoxic T-lymphocyte antigen-4, *mAb* monoclonal antibody, *MDSCs* myeloid-derived suppressor cells, *IDO* indoleamine-2, 3 dioxygenase, *VEGF* vascular endothelial growth factor

nonimmune events shifting the balance from equilibrium to the elimination or to the escape phase may lead to tumor removal or at least progression restriction.

As discussed in earlier sections, tumor cells apply a variety of tactics to combat with the host immune system. The assessment of factors involved in the escape mechanism served as the mainstay for the discovery of many anticancer immunotherapeutic agents. Some developed agents like ipilimumab (anti-CTLA-4) are now being used clinically along with other forms of therapy, whereas many other agents have entered different phases of clinical trials, and a large number are still in experimental stages (Table 12.4).

12.6 Concluding Remarks

In conclusion, it could be stated that enough proof is available to establish the presence of cancer immunoediting in animals as well as in humans. Understanding the sequence of events occurring during the immunoediting process and recognition of the cellular and molecular mechanisms underlying its different phases has led to a spurt in cancer immunotherapeutic approaches. Further knowledge on the genetic and epigenetic features characterizing the three Es of cancer immunoediting are warranted for the development of more precise cancer immunotherapeutic approaches in the future.

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Apoptosis and Cancer

13

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13.1 Introduction

The concept of life and death has been a topic of interest among scientists, philosophers, and theologians. It was such an intriguing subject that an Immortality Project was established in mid-2012 to find answers to human immortality. The highly funded project, headed by a well-known philosopher, employs empirical studies to address research areas such as near-death experiences, alleged out-of-body experiences, postmortem survival, and the influence of beliefs about immortality on human behavior, attitudes, and character. Scientifically, life and death are essential parts of a natural cycle of all multicellular organisms. Cell division, death, shape modification, and cell rearrangements form critical processes on which tissues are shaped and organs are made [1]. The orchestration of these processes depends on a genetic program operating on cell behavior: for example, some signaling molecules and growth factors promote cell divisions and control tissue size, whereas other proteins control the orientation of cell divisions and cell rearrangements. Control of tissue size is manifested in the process of cell competition whereby faster growing cells can out-compete slow growing

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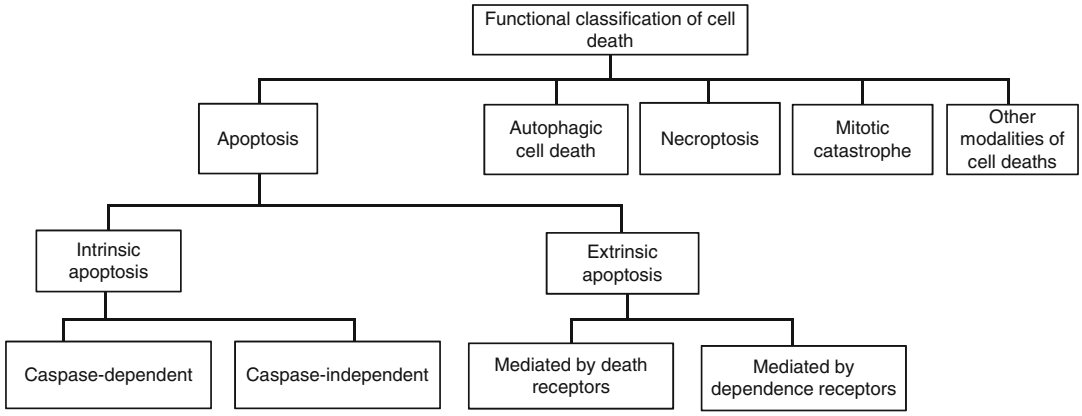

Fig. 13.1 Functional classification of cell death modalities as described by the Nomenclature Committee on Cell Death (NCCD) [19]

cells. Competition also involves apoptotic elimination of the slow growing cells and their engulfment by fast growing cells [1, 2]. Hence, cell death plays an important role in the development and homeostasis of normal tissues [3, 4]. Cells produced in excess during the development process eventually undergo cell death, thereby contribute to sculpturing of organs and tissues [5].

Historically, cell death phenomenon was first reported in 1842 by Carl Vogt [6, 7]. Subsequently, the term programmed cell death (PCD) was mentioned by Lockshin and Williams in 1965 [8]. The phenomenon describes coordinated deaths of certain larval muscles during transformation into adult moths. Kerr and co-workers later described a series of similar morphological characteristics following the death of a variety of tissue sources, which was coined as "apoptosis" [9]. About the same time, Horvitz and colleagues started a systematic search for genes controlling PCD in the nematode worms, *Caenorhabditis elegans*. The discovery of cell death defective genes, such as *ced-3*, *ced-4*, and *ced-9*, implicated that PCD is a process with strict genetic program [10]. This was quickly followed by the identification of substrates and homologous genes in mammals and realization that mutations of some of these cell death genes were contributing factors in various cancers. The 2002 Nobel Prize in Physiology or Medicine was awarded jointly to Sydney Brenner, H. Robert Horvitz, and John E. Sulston for their extensive work and discoveries on genetic regulation of organ development and PCD.

An imbalance between cell growth and cell death is implicated in a variety of human diseases including cancer, autoimmune diseases, neurodegenerative disorders, viral infections, and AIDS [11–15]. Cell death has a profound effect on cancer growth and progression [16–18]. Malfunction of the cell death machinery, as a direct consequence of mutations of the signaling molecules involved either directly or indirectly in the cell death pathways, has long been identified as an important contributing factor in cancer. Continuous efforts in deciphering the mechanisms and signaling pathways of these cell deaths have also brought forward a new paradigm of which cancer may be efficiently targeted. Novel and specific cancer therapeutics and techniques directed at members of the cell death signaling pathways have been developed, and newer generation of drugs is currently being tested in clinical trials.

Figure 13.1 illustrates the most recent cell death classifications by the Nomenclature Committee on Cell Death (NCCD). NCCD has suggested limiting the use of the term "programmed" only for those physiological instances of cell death, irrespective of the modality by which they are executed, and which occur in the context of embryonic and postembryonic development and tissue homeostasis [19]. On the other hand, the term "regulated" cell death should be used to indicate cases of cell death, be it programmed or not and whose initiation and/or execution is mediated by a dedicated molecular machinery and can be

inhibited by targeted pharmacologic and/or genetic interventions [19]. Apoptosis and its possible roles in tumorigenesis and some of the novel antitumor strategies and therapeutics will be discussed in this chapter.

13.2 Mechanisms of Apoptosis

The term "apoptosis" was introduced by Kerr and co-workers in 1972, derived from a Greek term meaning "dropping off" of leaves or petals from trees or flowers [9]. Earlier methods to define cell death rely much on morphological criteria and the use of microscopes [7]. The earliest recognized morphological changes in apoptosis involve compaction and segregation of nuclear chromatin and condensation of the cytoplasm [9, 20]. The process is followed by the convolution of the plasma membrane and cell blebbing in a florid manner, producing fragments of cells known as apoptotic bodies. These fragments are membrane-bounded and contain nuclear components [20, 21]. Apoptotic bodies are quickly taken up by nearby cells and degraded within their lysosomes, usually with no associated inflammation [9, 20].

It is important to note that despite the various types of apoptosis characterized by their biochemical features and signaling pathways, they share similar morphological features. Biochemically, apoptosis is universally characterized by the double-stranded cleavage at the linker regions between nucleosomes, resulting in the formation of multiple DNA fragments [21] and phosphatidylserine externalization [22], and is accompanied by a series of gene and protein expressions. Figure 13.2 illustrates the morphological characteristics of apoptosis and how it is compared with necroptosis and autophagic cell death.

The NCCD has formulated several rounds of recommendations to propose guidelines and unify criteria on the use of cell death terminologies [19, 23]. According to the latest NCCD publication, apoptosis is functionally classified into intrinsic or extrinsic apoptosis. Intrinsic apoptosis is either caspase-dependent

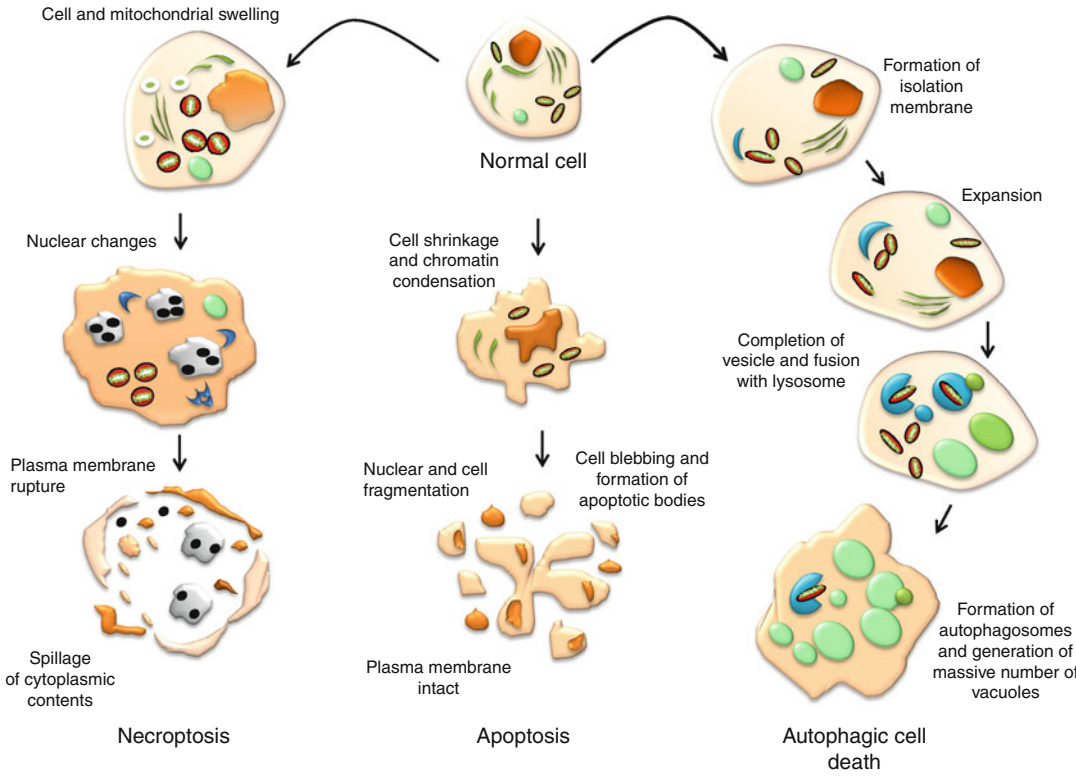

Fig. 13.2 Morphological characteristics of cells undergoing apoptosis, autophagic cell death, and necroptosis

or caspase-independent, whereas extrinsic apoptosis is categorized depending on source of trigger, as mediated either by death receptors or by dependence receptors.

13.2.1 Extrinsic Apoptosis Pathway

Extrinsic apoptosis is essentially caspase-dependent and is induced by extracellular stress signals which are mediated by specific transmembrane receptors. In the extrinsic apoptosis induced by death receptors, the signaling pathway is mediated by receptors belonging to the tumor necrosis factor (TNF) receptor superfamily and is characterized by extracellular cysteine-rich domains (CRDs) and intracellular death domain (DD). Ligands such as TNF ligand, TNF ligand superfamily member 10 (TNFSF10), FAS ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) interact with their respective death receptors [FAS/CD95, TNF-α receptor 1 (TNFR1), or TRAIL receptor (TRAIL-R1 or TRAIL-R2)], recruit Fas-associating protein with a death domain (FADD), and form the death-inducing signaling complex (DISC) [24, 25]. This complex recruits pro-caspase-8 and pro-caspase-10, leading to the activation of the executioner caspase-3, caspase-6, and caspase-7 [26, 27].

On the other hand, extrinsic apoptotic signals can be alternatively mediated by dependence receptors such as UNC-5 homolog family receptors (UNC-5A, UNC-5B, UNC-5C, and UNC-5D) and deleted in colorectal cancer (DCC) family receptors. These receptors are activated by netrins, a family of extracellular proteins that direct cell and axon migration during embryogenesis [28]. Netrins are members of the laminin superfamily and contribute to the regulation of cell-cell adhesion and tissue organization [29]. Netrin-1 has been recently identified to be an anti-apoptotic survival factor in tumorigenesis [30]. DCC and UNC-5 homologs mediate cell death in the absence of netrin-1 and the binding of the ligand to these receptors switches between a pro-apoptotic signal and the promotion of survival and motility [30]. UNC-5B (also known as UNC-5H2) complex responds to the withdrawal of netrin-1 by recruiting a signaling complex consisting of protein phosphatase 2A (PP2A) and death-associated protein kinase 1 (DAPK1) [31]. In the presence of netrin-1, the PP2A complex is repressed by the recruitment of cancerous inhibitor of PP2A (CIP2A) into the UNC-5B/DAPK1 complex, of which DAPK1 is autophosphorylated and remained inactive. Conversely, netrin-1 withdrawal is associated with a conformational change in UNC-5B, resulting in the exposure of the death domain, releasing of CIP2A, and the recruitment of PP2A to the UNC-5B-DAPK1 complex. PP2A-mediated dephosphorylation of DAPK1 results in the activation of downstream apoptotic pathway. PP2A-like activity has been linked to the formation of DISC, and is known to inhibit B-cell lymphoma 2 (Bcl-2) phosphorylation, leading to apoptotic cell death [32, 33]. In certain cell types, where the extrinsic apoptotic pathway is triggered but lower levels of DISC followed by lower levels of active caspase-8 are formed, amplification of the death signal is possible through the cleavage of Bid by caspase-8, which directly mediates Bak/Bax oligomerization, and triggers the release of cytochrome (Cyt) *c* [34, 35].

Another signaling pathway mediated by dependence receptors are the DCC and the Patched dependence receptor (Ptc). DCC encodes an approximately 200 kDa type I membrane protein, which displays homology with cell adhesion molecules in its extracellular domain, suggesting that DCC may play a role in cell-cell or cell-matrix interactions [36, 37]. DCC appears to drive apoptosis independent of both mitochondrial-dependent and death receptor/caspase-8 pathways. DCC interacts and drives the activation of caspase-3 through caspase-9 without requiring Cyt *c* or Apaf-1 [38]. Ptc, identified as a tumor suppressor, induces apoptosis, but is suppressed by its ligand, sonic hedgehog (Shh) [39, 40]. Ptc interacts with the adapter protein DRAL/FHL2 in the absence of Shh and recruits a protein complex that includes DRAL/FHL2, the CARD-containing domain protein TUCAN, and apical caspase-9. Ptc triggers caspase-9 activation and enhances cell death via a caspase-9-dependent mechanism [41, 42].

The death receptor and dependence receptor pathways converge at the activation of caspase-3, followed by cleavage and activation of downstream caspases. Caspases or cysteine aspartic acid-specific proteases are synthesized as inactive zymogens (or proenzymes) and are usually cleaved to form active enzymes or undergo autoproteolysis in a cascade manner. Initiator caspases such as caspase-8, caspase-9, and caspase-10 couple cell death stimuli to the downstream effector caspases such as caspase-3, caspase-6, and caspase-7. The major proteolysis activity that takes place during apoptosis is carried out by effector caspases. Caspase-3 appears to be the major executioner caspase during the demolition phase of apoptosis [43, 44]. Caspase-3 cleaves a number of structural proteins such as fodrin, gelsolin, rabaptin, nuclear lamin B, and vimentin [44–46]. On the other hand, caspase-6 appears to merely cleave the nuclear lamin A during apoptosis [44]. Caspase-3 also cleaves diverse regulatory proteins and enzymes, including focal adhesion kinase (FAK), protein kinase C delta, retinoblastoma protein (Rb) (a protein involved in cell survival), p21-activated kinase (PAK), U1 small nuclear ribonucleoprotein (U1snRNP), DNA fragmentation factor 45 (DFF45)/inhibitor of caspase-activated DNase (ICAD), receptor interacting protein (RIP), X-linked inhibitor of apoptosis protein (X-IAP), signal transducer and activator of transcription-1 (STAT1), and topoisomerase I [44, 45, 47]. Initially, poly (ADP-ribose) polymerase (PARP) is reported to be an exclusive substrate for caspase-7 [44], but a later study proved that it is cleaved by both caspase-3 and caspase-7 [48].

Caspase-mediated cleavage of structural proteins is essential for the apoptosis-associated morphological changes. For example, cleavage of gelsolin in multiple cell types causes cells to round up, detach from the plate, and undergo nuclear fragmentation [49]. Inactivation of rabaptin-5 causes fragmentation of endosomes during the execution phase of apoptosis [50]. Fodrin is a major component of the cortical cytoskeleton of most eukaryotic cells; it has binding sites for actin, calmodulin, and microtubules [51]. Its proteolysis contributes to structural rearrangements including blebbing during apoptosis [52, 53].

FAK is a tyrosine kinase of which its phosphorylation state and activity are linked to cell adhesion to the extracellular matrix through integrin receptors. It has a direct influence on the cytoskeleton, structures of cell adhesion sites, and membrane protrusions, leading to regulation of cell movement [54, 55]. Caspase-mediated cleavage of FAK is known to contribute to the morphological changes in apoptosis. On the other hand, PAK, a serine-threonine kinase, regulates morphological and cytoskeletal changes in a variety of cell types [56, 57]. Blocking PAK function during Fas-induced apoptosis inhibits the morphological changes, but accelerates the phosphatidylserine externalization in the membrane. Stable Jurkat cell lines that expressed a dominant-negative PAK mutant are resistant to Fas-induced formation of apoptotic bodies and cleavage of PAK [58].

PARP cleavage is believed to attenuate the cell's ability to carry out DNA repair [45, 59]. Caspase-8 is also found to cleave PARP-2, a member of the PARP family involved in DNA repair, suggesting that caspase-8 is both an initiator and effector caspase [60]. Active caspase-3 or caspase-7 proteolytically cleaves DFF45, which subsequently releases active DFF40, the inhibitor's associated endonuclease. It is responsible for the degradation of chromosomes into nucleosomal fragments, considered as the characteristic hallmark of apoptosis [61, 62]. Cleavage of both structural and regulatory proteins is essential for the apoptotic-associated chromatin condensation, DNA fragmentation, nuclear collapse, and morphological changes such as cell shrinkage and detachment, membrane blebbing, and formation of apoptotic bodies. Figure 13.3 illustrates the extrinsic apoptosis signaling pathway.

13.2.2 Intrinsic Apoptosis Pathway

Intrinsic apoptosis is known as either caspase-dependent or caspase-independent, and both signaling pathways are centrally mediated by the mitochondria. Intrinsic apoptosis can be triggered

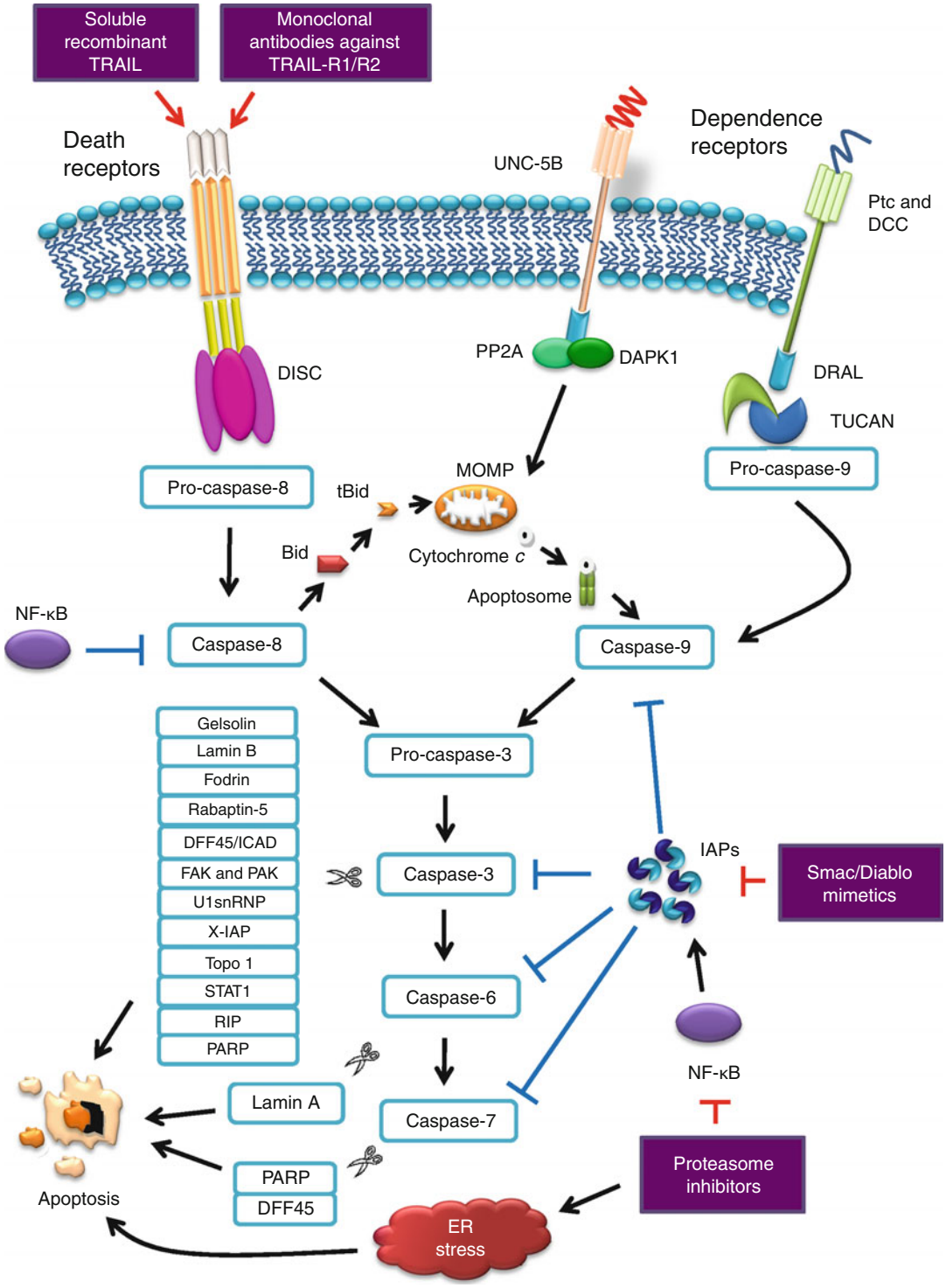

Fig. 13.3 Extrinsic apoptosis signaling pathway and antitumor therapeutic targets

by DNA damage, γ-irradiation, oxidative stress, cytosolic Ca^{2+} overload, serum deprivation, and many other intracellular stress conditions. Upon stimulation, various molecules are released into the cytoplasm including Cyt *c* [27, 63], second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (Smac/Diablo) [64, 65], apoptosis-inducing factor (AIF; promotes chromatin condensation) [66], endonuclease G (EndoG; facilitates chromatin degradation) [67, 68], and high-temperature requirement protein A2 (HtrA2/Omi) [69]. Cyt *c* binds to and activates Apaf-1 protein in the cytoplasm, inducing the formation of apoptosome which subsequently recruits the initiator pro-caspase-9, yielding activated caspase-9 and finally mediating the activation of caspase-3 and caspase-7 [35]. Loss of Cyt *c* from the mitochondria also results in the inhibition of the respiratory chain. The condition elicits and aggravates reactive oxygen species (ROS) overproduction and is thought to activate a feedforward circuit for the amplification of the apoptotic signal [70]. The function of Cyt *c* and its role in apoptosis are widely reviewed and discussed elsewhere [71–73].

Bcl-2 family of proteins plays an important role in the regulation of mitochondrial-linked apoptosis [74]. Bcl-2 subfamilies such as Bax, Bak, and Bcl-2 homolog (BH)3-only subfamily proteins (e.g., Bid) play a pro-apoptotic role, while Bcl-2 and Bcl-X_L are functionally anti-apoptotic. Activated Bax and Bak form homo-oligomer which creates pores on the mitochondrial membrane and releases toxic proteins from the mitochondria. Bcl-2 and Bcl-X_L inhibit the action by blocking the activation of Bax and Bak and preventing the release of pro-apoptotic proteins [75]. Nevertheless, the activation of Bax and Bak can be restored with the presence of pro-apoptotic BH3-only proteins. BH3-only proteins function as antagonists of specific subsets of their pro-survival relatives [76, 77]. The pore-forming activities of Bax and Bak trigger a condition known as mitochondrial outer membrane permeabilization (MOMP). MOMP can also be triggered by the opening of a multiprotein complex known as permeability transition pore complex (PTPC) [78, 79]. MOMP causes generalized and irreversible inner mitochondrial transmembrane potential ($\Delta\Psi m$) dissipation. In the inner mitochondrial membrane (IM) of a healthy cell, the frontier between the intermembrane/intercristal space and the matrix is nearly impermeable to all ions, including protons which help create the proton gradient required for oxidative phosphorylation [70]. The charge imbalance that results from the generation of an electrochemical gradient across the IM forms the basis of the $\Delta\Psi m$ [70]. A loss of the $\Delta\Psi m$ or long-lasting or permanent $\Delta\Psi m$ dissipation can lead to cell death [80]. MOMP causes the release of toxic proteins from the mitochondria to the cytosol as mentioned above. Pro-apoptotic Bcl-2 proteins appear to cause the release of Cyt *c*, Smac/Diablo, and HtrA2/Omi but not EndoG and AIF [81]. On the other hand, BH3-only protein Bid cleavage by caspase-8 serves to engage a mitochondrial amplification loop during extrinsic apoptosis. Caspase-8 cleaves Bid, generating a truncated fragment known as truncated Bid (tBid) that can permeabilize the mitochondrion, resulting in MOMP [82].

Inhibitors of apoptosis proteins (IAPs) play an important role in the regulation of apoptosis. Eight human IAPs have been identified consisting of X-IAP, IAP-like protein-2 (ILP-2), cIAP-1, cIAP-2, melanoma inhibitor of apoptosis protein (ML-IAP), neuronal apoptosis inhibitory protein (NAIP), survivin, and apollon [83]. Human IAP family members such as X-IAP, cIAP-1, and cIAP-2 are potent caspase inhibitors [84, 85]. X-IAP, cIAP-1, and cIAP-2 block Cyt *c*-induced activation of caspase-9, thus preventing the activation of caspase-3, caspase-6, and caspase-7. Furthermore, these IAPs bind to and inhibit the enzymatic activity of caspase-3 following its activation by caspase-8, thereby arresting the proteolytic cascade initiated by the initiator caspase [86]. X-IAP primarily inhibits caspase by disrupting the conformation of the active caspase and masking the substrate-binding active site [83].

Smac/Diablo and HtrA2/Omi inhibit the anti-apoptotic function of several members of the IAP family [87, 88]. Smac/Diablo and HtrA2/Omi are two nuclear-encoded mitochondrial proteins

functioning as IAP antagonists, identified in mammals [69, 89–92]. After their release into the cytosol stimulated by apoptotic triggers, Smac/Diablo and HtrA2/Omi competitively bind to the BIR domains of IAPs via the IAP-binding motif, so that the BIR-bound caspases are released and reactivated [93–95]. Smac/Diablo and HtrA2/Omi manifest distinct physical characteristics and biochemical activities, of which the active Smac/Diablo is a homodimer, whereas HtrA2/Omi is a homotrimer [87, 96]. Despite Smac/Diablo, HtrA2/Omi is a mitochondrial serine protease [97, 98]. HtrA2/Omi has diverse roles, including maintenance of mitochondrial homeostasis and regulation of cellular apoptosis [99]. A comprehensive proteome-wide analysis of Jurkat cell lysates leads to the identification of potential HtrA2/Omi substrates, for example, the cytoskeleton-associated proteins such as actin, α- and β-tubulin, and vimentin, further suggest its role in the caspase-independent pathway [100].

AIF and EndoG function in a caspase-independent manner, by relocating to the nucleus, where they mediate large-scale DNA fragmentation, independent of caspases [101, 102]. Mammalian EndoG is a nuclear-encoded protein targeted to mitochondria and compartmentalized in the intermembrane space (IMS) and is known to possess DNase/RNase activity [103]. It is implicated in the mitochondrial DNA replication and is shown to be involved in apoptotic DNA degradation [102]. In isolated non-apoptotic nuclei, EndoG first generates large fragments of DNA (>50 kb) and then cleaves at inter- and intra-nucleosomal sites [104]. Although EndoG apoptotic activity appears to occur in the absence of caspase activation, the pathway leading to EndoG-dependent DNA damage remains controversial [105, 106].

AIF was originally discovered as an IMS component capable of inducing chromatin condensation and DNA loss in the nuclei isolated from healthy cells [104, 107]. AIF is a flavoprotein which was first proposed to act as a protease or protease activator [108]; notably, its apoptogenic activity is not affected by z-VAD-fmk [109]. Contribution of AIF to apoptosis depends on the cell types and death triggers [104]. Both endogenous and recombinant AIF are found to trigger peripheral chromatin condensation and large-scale DNA fragmentation in a caspase-independent manner [110, 111]. AIF is not known to possess nuclease activity; therefore, AIF is postulated to directly interact with DNA and disrupt/collapse chromatin structure by displacing chromatin-associated proteins and/or by recruiting proteases and nucleases to form DNA-degrading complexes or degradosomes [104, 112].

Another important signaling pathway affecting the regulation of apoptosis worth mention is the nuclear factor-kappa B (NF-κB). NF-κB is a sequence-specific transcription factor known to be involved in the inflammatory and innate immune responses. Under normal conditions, NF-κB becomes activated only upon stimulation and subsequently upregulates the transcription of its target genes. NF-κB is activated by many divergent stimuli, including proinflammatory cytokines such as TNF-α, TRAIL, interleukin-1β (IL-1β), epidermal growth factor (EGF), T- and B-cell mitogens, bacteria and lipopolysaccharides (LPS), viral proteins, double-stranded RNA, drugs, and a variety of physical and chemical stresses [113]. However, in tumor cells, molecular alterations result in impaired regulation of NF-κB and become constitutively activated in such cases, leading to deregulated expression of NF-κB-controlled genes [114]. Some genes targeted by NF-κB include cytokines/chemokines and their modulators, immunoreceptors, transcription factors, and regulators of apoptosis such as Bcl-X_L, Fas, FasL, and IAPs [113].

NF-κB is also known to play a pro-apoptotic role, in addition to its more common anti-apoptotic role. Examples of its pro-apoptotic effects in cells include those found in B cells [115], T cells [116, 117], and neuronal cells [118, 119]. On the other hand, the anti-apoptotic effects of NF-κB appeared to be cell-type specific and/or dependent on the inducing signal. Normally, NF-κB is transcriptionally inactive in the cytoplasm of most cells as it is bound to its cytoplasmic inhibitor IκBα. Upon stimulation with proinflammatory cytokines, such as TNF-α or IL-1, IκBα protein is phosphorylated, ubiquitinated, and subsequently degraded by the

proteasome (the role of proteasome is further discussed under proteasome inhibitors). This process exposes the previously masked nuclear localization signal of NF-κB, allowing it to translocate into the nucleus upon IκBα proteolysis and subsequently activate the expression of important target genes involved in cell growth, survival, and adhesion [120, 121]. Activated NF-κB leads to the activation of A1/Bfl-1, a member of the Bcl-2 family, which suppresses Cyt *c* release from the mitochondria [122]. NF-κB activation blocks caspase-8 cleavage and Cyt *c* release, indicating that NF-κB suppresses the earliest signaling components of the caspase cascade. The IAP family genes (*cIAP-1* and *cIAP-2*) and TRAF family genes (*TRAF1* and *TRAF2*) are positively regulated by NF-κB with rapid kinetics following TNF addition [123, 124]. Another member of the IAP family, X-IAP, has been shown to be activated by NF-κB in endothelial cells [125, 126]. Thus, NF-κB activation functions to suppress apoptosis at multiple levels.

The Nomenclature Committee on Cell Death (NCCD) suggests to define "intrinsic apoptosis" as cell death mediated by MOMP and associated with generalized and irreversible ΔΨm dissipation, release of IMS proteins, and respiratory chain inhibition [19]. On the other hand, differentiation between caspase-dependent and caspase-independent intrinsic apoptosis pathways is based on the extent of cytoprotection as conferred by inhibition of caspases. The caspase-independent mechanisms mediated by AIF, EndoG, or ATP depletion tend to prevail over caspase inhibition and kill cells in conditions that would have been rapidly executed by the caspase cascade [19]. However, the caspase-independent signaling pathway is still vague, and the exact mechanisms remain to be investigated. Figure 13.4 illustrates the caspase-dependent and caspase-independent intrinsic apoptosis pathway.

13.3 Apoptosis and Cancer

Apoptosis is an essential developmental process to maintain tissue homeostasis. Therefore, defect in apoptosis regulation plays an important role in cancer development. Deregulation in the apoptosis pathway is one of the reasons why neoplastic cells gain extended lifespan, develop genetic mutations capable of growth under stress conditions, and undergo angiogenesis [12]. Several key pathways controlling apoptosis are commonly altered in cancer [127]. Tumor resistance to apoptotic cell death is often a hallmark of cancer and contributes to chemoresistance [12]. Alteration of many proteins involved in both intrinsic and extrinsic signaling pathways has been described, and many more to be discovered in near future. For example, overexpression of certain anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Akt, NF-κB, and IAP protein family, is found in various human tumors [128].

The apoptotic pathway of Fas, one of the TNF receptor family members, is frequently blocked by several mechanisms in cancer, one of which is *Fas* gene mutation [129–131]. *Fas* mutations have been detected in several types of human cancers with frequent allelic losses of chromosome 10q24 where the gene resides [130–132]. Both *TRAIL-R1* and *TRAIL-R2* genes are mapped on chromosome 8p21-22 [133, 134]. Allelic losses of the chromosome 8p21-22 have been reported as a frequent event in several cancers, including non-Hodgkin lymphoma (NHL), lung cancer, breast cancer, colon cancer, prostate cancer, hepatocellular carcinoma, and head and neck cancer [135–141]. Mutations of *TRAIL-R2* gene have been reported in head and neck cancer [142] and non-small cell lung cancer (NSCLC) [143]. In addition, somatic mutations of *TRAIL-R1* and *TRAIL-R2* genes are found in NHL [144] and breast cancer [145]. The number of pancreatic tumor tissues with positive membrane staining for TRAIL-R1 and TRAIL-R2 is lower than non-tumor tissues [146]. Loss of *TRAIL-R2* expression is associated with poorer prognosis in patients [146]. A significant association is also observed between lower expression of *TNF* gene and poor prognosis in childhood adrenocortical tumors [147].

On the other hand, PP2A inactivation in cancer occurs frequently through the upregulation of CIP2A, a PR65 interactor and PP2A inhibitor [148]. PR65β, a scaffold protein which interacts

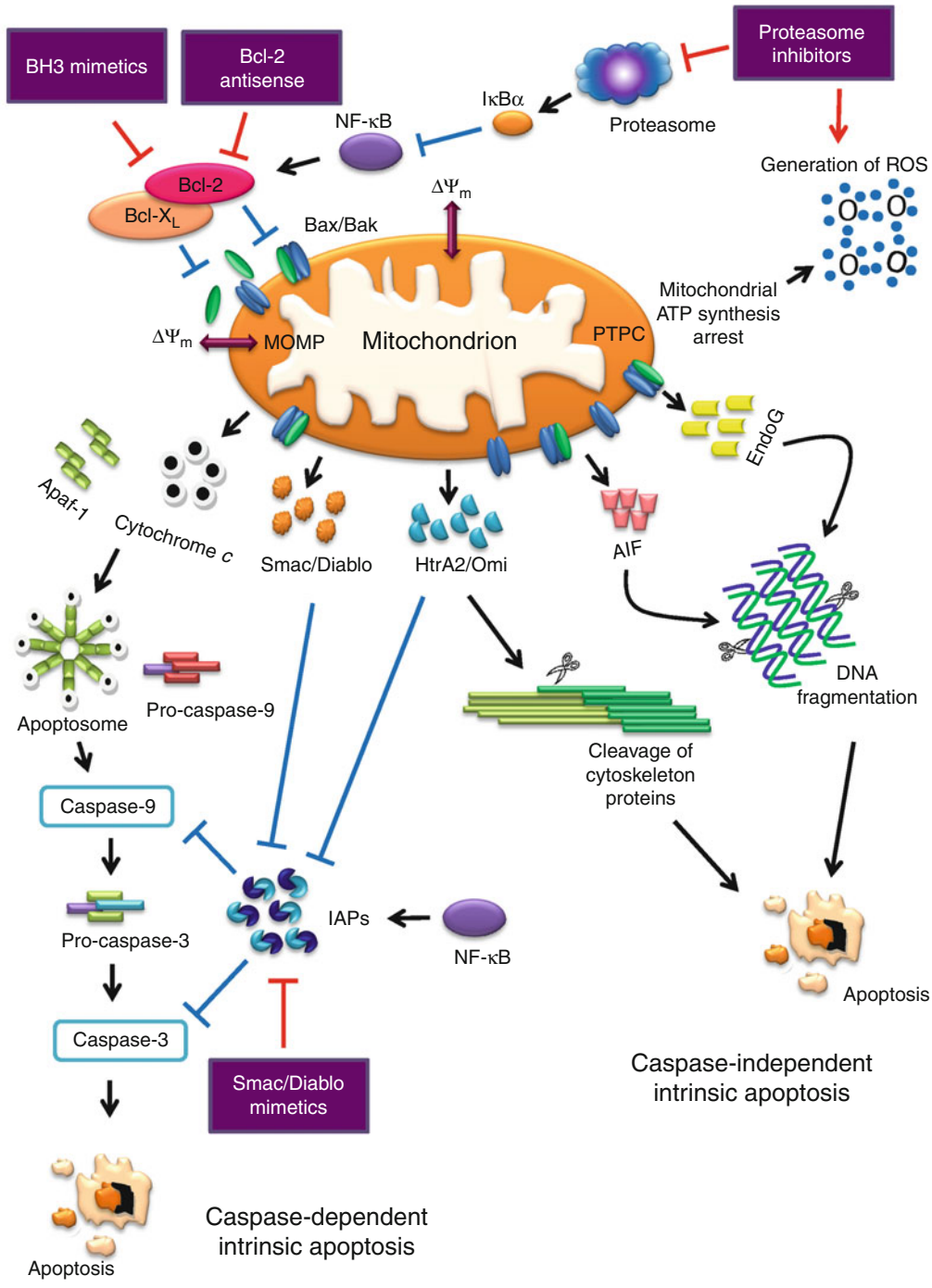

Fig. 13.4 Caspase-dependent and caspase-independent intrinsic apoptosis signaling pathway and antitumor targets

with the catalytic subunit of PP2A, appears to play a key regulatory role in cancer. This scaffold protein is decreased or mutated in a large fraction of human cancers and has been recently linked to cancer development [149]. On the other hand, *Ptc* is a tumor suppressor and mutations of *Ptc* are associated with neoplasia, especially in basal cell carcinoma and medulloblastoma [39, 40]. DCC expression is shown to be markedly reduced in more than 50 % of colorectal tumors. The loss of DCC is not restricted to colon carcinoma, but has been observed in other tumor types, including carcinoma of the stomach, pancreas, esophagus, prostate, bladder, and breast, male germ tumors, neuroblastomas, gliomas, and some leukemias [36, 150, 151].

Members of the Bcl-2 family of proteins as prominent regulators of apoptosis signaling are often deregulated in many cancers, including lung carcinoma, lymphoma, and glioblastoma [152–156]. Aberrant expression of *Bcl-2* is common in chronic lymphocytic leukemia (CLL) and is associated with poor response to chemotherapy and decreased overall survival [157]. *Bcl-2* gene amplification is reported in diffuse large B-cell lymphomas (DLBCL) and overexpression of Bcl-2 protein has been associated with poor prognosis in some forms of NHL [158–160]. *Myc/Bcl-2* co-expression in DLBCL is associated with aggressiveness, is more common in the unfavorable activated B-cell-like subtypes, and contributes to the overall inferior prognosis of patients with activated B cell-DLBCL [161]. Single-nucleotide polymorphisms in *Bcl-2* are found to have an association with survival in advanced-stage NSCLC patients who received chemotherapy [162]. Furthermore, mutations that inactivate the pro-apoptotic *Bax* gene have been observed in solid tumors and hematologic malignancies [163, 164]. Higher *Bcl-2* to *Bax* ratios has been associated with progression of CLL, shorter remission duration, and shorter survival [165, 166]. Therefore, cancer therapeutics that specifically inhibit the anti-apoptotic proteins or activate the pro-apoptotic members of the Bcl-2 family proteins are an attractive strategy to reverse the intrinsic or acquired resistance of cancer cells to apoptosis [167].

Studies have reported that polymorphic variants of the *caspase-8* gene are associated with the risk of multiple cancers [168–172]. For example, a six-nucleotide insertion-deletion variant polymorphism (6 N ins/del) of *caspase-8* promoter is linked to a significant decreased risk of bladder and lung cancer in Chinese populations [171, 172]. Since cancer cells are highly dependent on these genetic changes in the apoptotic pathways for survival, designing novel anticancer drugs that selectively kill cancer cells while sparing normal cells seem appealing [173]. Survivin, a member of the IAP family, is undetectable in terminally differentiated adult tissues, but abundantly expressed in human cancers such as lung, colon, pancreas, prostate, and breast [167]. Increased survivin mRNA is associated with decreased overall survival in colon cancer patients [174]. Furthermore, increased levels of cIAPs in malignant cells are associated with a shorter relapse-free survival in patients with prostate cancer [175]. Livin or ML-IAP, another member of the IAP family of proteins, is found to be expressed in tumor cells [176, 177]. Thus, the possibility of IAP inactivation through therapeutic intervention is rather attractive and has gained much interest over the years.

Another important pathway linked to the apoptotic cell death is the p53 pathway, which is often inactivated and deregulated in human cancers [178, 179]. The p53 protein is a transcription factor with tumor suppressor activities. Its role in tumor suppression relies partly on its ability to regulate the transcription of genes important in cell cycle arrest and in apoptosis. The p53 protein upregulates the expression of a number of genes in response to genotoxic stress, including the pro-apoptotic Bax [180]. It is also found to inhibit the expression of the *Bcl-2* gene [181]. Studies have also shown that Bid is a p53-responsive chemosensitivity gene which may enhance the cell death response to chemotherapy [182]. The fact that a majority of human cancers harbor mutations in the *p53* gene suggests that such mutations would have contributed to the apoptosis-resistant environment. However, the p53 network and the mechanism by which p53 determines the fate of cells remain to be explored.

13.4 Apoptosis Signaling Pathways and Therapeutic Targets in Cancer

13.4.1 TRAIL (TRAIL Ligands, Monoclonal Antibodies Against TRAIL-R1 and TRAIL-R2)

TRAIL (Apo2 ligand) induces cell death via the extrinsic pathway by recruiting and activating caspase-8 and caspase-10 to its R1 and R2 receptors [183]. It activates the intrinsic pathway via the TRAIL-caspase-8-tBid-Bax cascade, through the cleavage of Bid, which promotes Bax and Bak oligomerization, leading to Cyt *c* release and activation of caspase-9 [184]. These processes collectively amplify the activities of the related executioner caspases. TRAIL is a promising cancer therapeutic agent, known to induce apoptosis in a wide variety of tumor cells while sparing normal cells [185, 186]. TRAIL activity is also known to be independent of the p53 status, making it potentially effective against chemotherapy-resistant tumors [187]. Early clinical trials have been initiated in cancer patients, using soluble recombinant TRAIL (rhApo2L, codeveloped by Genentech and Amgen) [188, 189] and monoclonal antibodies (mAbs) (agonists) targeting TRAIL-R1, such as mapatumumab [HGS-ERT1 is developed by Human Genome Sciences (HGS)], and anti-TRAIL-R2 agents, such as lexatumumab (HGS-ETR2 is developed by HGS), conatumumab (developed by Amgen), and apomab (developed by Genentech) [190].

In an early phase I safety and pharmacokinetic trial of rhApo2L used as a single agent in patients with advanced solid tumors and NHL, of 32 patients with post-baseline tumor assessment, 17 (53 %) had stable disease and 13 (41 %) proceeded with disease progression. Only a single patient was reported to have a partial response to the drug [188]. Phase I/Ib trials of rhApo2L in advanced cancer [191], advanced NSCLC [192], and NHL [193] reported that this drug was well tolerated by patients and no anti-rhApo2L Abs were detected. Promising outcome in phase Ib trial of rhApo2L in combination with cytotoxic chemotherapy (paclitaxel and carboplatin) and targeted anti-angiogenesis agent (bevacizumab) in advanced NSCLC has led to a randomized phase II study [192]. Despite the encouraging phase Ib results, the addition of rhApo2L to paclitaxel/carboplatin or paclitaxel/carboplatin/bevacizumab combination did not improve the outcome and produced a higher incidence of treatment-related adverse effects [194]. Similarly, the addition of rhApo2L to rituximab did not improve the objective outcome in phase II NHL study despite its promising activity in phase Ib study [193, 195]. Adverse effects commonly associated with rhApo2L include neutropenia and serum lipase elevation [194, 195]. Phase I trials of rhApo2L in colorectal cancer are ongoing (Table 13.1).

Mapatumumab, a fully human agonistic mAb targeting TRAIL-R1, either used alone or in combination with other chemotherapy drugs in phase I or phase II trials, has yet to produce impressive trial outcomes, as in most cases, few patients ended with partial response or stable disease [260–263]. Despite its favorable safety profile, mapatumumab demonstrated limited or no clinical activity in phase I and II trials in advanced solid malignancies [196, 197], NHL [199], NSCLC [201], refractory colorectal cancer [200], and advanced hepatocellular carcinoma [198]. Additional trials of mapatumumab in advanced hepatocellular carcinoma and advanced cervical cancer may provide additional data on the usefulness of this drug (Table 13.1).

Lexatumumab, apomab, and conatumumab are agonistic human mAbs against TRAIL-R2. Generally, the percentage of patients who developed partial response or stable disease in several early phase I trials involving these novel drugs is low, despite being well tolerated by patients. For example, no objective activity of apomab was demonstrated in a phase II study among patients with NHL [206], despite some evidence of activity in phase I study in patients with advanced malignancies [204]. The effects of apomab in phase II NSCLC trial coincide with rhApo2L, where addition of apomab to paclitaxel/carboplatin/bevacizumab combination did not improve the efficacy, while increasing the rate of some adverse effects [194, 205].

Table 13.1 Current therapeutic targets in the apoptosis signaling pathway and clinical trial stages

Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
TRAIL-R1 and TRAIL-R2	rhApo2L (dulanermin)	Phase I: advanced cancer	–	[191]
		Phase Ib: NHL	Rituximab	[193]
		Phase Ib: advanced NSCLC	Paclitaxel, carboplatin, and bevacizumab	[192]
		Phase II: NHL	Rituximab	[195]
		Phase II: advanced NSCLC	Paclitaxel, carboplatin, and bevacizumab	[194]
		Ongoing phase I: colorectal cancer	Cetuximab and irinotecan or FOLFIRI regimen and bevacizumab	http://clinicaltrials.gov/show/NCT00671372
		Ongoing phase I: colorectal cancer	FOLFOX regimen and bevacizumab	http://clinicaltrials.gov/show/NCT00873756
TRAIL-R1	Mapatumumab	Phase I: advanced solid tumor	Gemcitabine and cisplatin	[196]
		Phase I: advanced solid tumor	Paclitaxel and carboplatin	[197]
		Phase Ib: advanced hepatocellular carcinoma	Sorafenib	[198]
		Phase Ib/II: NHL	–	[199]
		Phase II: colorectal cancer	–	[200]
		Phase II: advanced NSCLC	Paclitaxel and carboplatin	[201]
		Ongoing phase I/II: advanced cervical cancer	Cisplatin and radiotherapy	http://clinicaltrials.gov/show/NCT01088347
		Ongoing phase II: advanced hepatocellular carcinoma	Sorafenib	http://clinicaltrials.gov/show/NCT01258608
TRAIL-R2	Lexatumumab	Phase I: advanced solid tumor	–	[201] [203]
	Apomab (drozitumab; PRO95780)	Phase I: solid tumor Phase I: advanced cancer	– –	[204] [205] [206]
	Conatumumab (AMG 655)	Phase II: NSCLC	Paclitaxel, carboplatin, and bevacizumab	[207]
		Phase II: NHL	Rituximab	[208]
		Phase I: advanced solid tumor	–	[209]
		Phase I: advanced solid tumor	–	[210]
		Phase Ib: pancreatic cancer	Gemcitabine	[211]
		Phase Ib: advanced NSCLC	Paclitaxel and carboplatin	[212]
		Phase Ib: colorectal cancer	mFOLFOX6 and bevacizumab	[213]
		Phase I/II: soft tissue sarcoma	Doxorubicin	[214]

(continued)

Table 13.1 (continued)

Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
		Phase Ib/II: colorectal cancer	Panitumumab	[215]
		Phase II: pancreatic cancer	Gemcitabine	[216]
		Phase II: colorectal cancer	FOLFIRI	
		Phase II: advanced NSCLC	Paclitaxel and carboplatin	
		Ongoing phase I/II: colorectal cancer	mFOLFOX6 and bevacizumab	http://clinicaltrials.gov/show/NCT00625651
		Ongoing phase II: advanced solid tumors	AMG 479	http://clinicaltrials.gov/show/NCT01327612
Anti-apoptotic Bcl-2 family members	AT-101	Phase I: CLL	–	[217]
		Phase I/II: SCLC	Topotecan	[218]
		Phase II: SCLC	–	[219]
		Phase II: NSCLC	Docetaxel	[220]
		Ongoing phase II: advanced laryngeal cancer	Docetaxel and cisplatin/carboplatin	http://clinicaltrials.gov/show/NCT01633541
		Ongoing phase II: SCCHN	Docetaxel	http://clinicaltrials.gov/show/NCT01285635
	Obatoclax mesylate (GX15-070)	Phase I: leukemia and myelodysplasia	–	[221]
		Phase I: advanced CLL	–	[222]
		Phase I: solid tumor	Topotecan	[223]
		Phase I: advanced solid tumor or lymphoma	–	[224]
		Phase II: SCLC	Topotecan	[225]
		Phase II: Hodgkin lymphoma	–	[226]
		Ongoing phase I: CLL	Fludarabine and rituximab	http://clinicaltrials.gov/show/NCT00612612
		Ongoing phase I: solid tumor, lymphoma, or leukemia	Vincristine sulfate, doxorubicin, and dexrazoxane hydrochloride	http://clinicaltrials.gov/show/NCT00933985
		Ongoing phase I/II: SCLC or advanced solid tumor	Topotecan	http://clinicaltrials.gov/show/NCT00521144
	ABT-263 (navitoclax)	Phase I: lymphoid tumor	–	[227]
		Phase II: SCLC	–	[228]
		Ongoing phase I: solid tumor	Erlotinib or irinotecan	http://clinicaltrials.gov/show/NCT01009073
		Ongoing phase I/IIa: CLL	–	http://clinicaltrials.gov/show/NCT00481091
		Ongoing phase II: lymphoid cancer	–	http://clinicaltrials.gov/show/NCT00406809

Bcl-2 mRNA	Oblimersen sodium	Phase I: advanced melanoma	Temozolomide and albumin-bound paclitaxel	[229]
		Phase II: CML	Imatinib	[230]
		Phase II: SCLC	Carboplatin and etoposide	[231]
		Phase III: advanced melanoma	Dacarbazine	[232]
		Phase III: CLL	Fludarabine and cyclophosphamide	[233]
		Phase III: CLL	Fludarabine and cyclophosphamide	[234]
		Phase III: MM	Dexamethasone	[235]
		Ongoing phase I: AML	Cytarabine and daunorubicin hydrochloride	http://clinicaltrials.gov/show/NCT00039117
		Ongoing phase I: advanced melanoma	Abraxane and Temodar	http://clinicaltrials.gov/show/NCT00409383
Proteasome	Bortezomib	Approved by FDA for multiple myeloma (2005)	–	[236]
		Approved by FDA for mantle cell lymphoma (2006)	–	[237]
		Phase I: acute leukemia	–	[238]
		Phase I: advanced solid tumor	Paclitaxel	[239]
		Phase I/II: MM	Cyclophosphamide and prednisone	[240]
		Phase II: MM	Dexamethasone	[241]
		Phase II: mantle cell lymphoma	–	[242]
		Phase II: Hodgkin lymphoma	Gemcitabine	[243]
		Phase II: esophageal, gastric, and gastroesophageal cancer	Paclitaxel and carboplatin	[244]
		Phase II: mantle cell lymphoma	–	[245]
		Phase II: MM	–	[246]
		Phase III: MM	Dexamethasone	[247]
		Ongoing phase I: prostate cancer	Mitoxantrone	http://clinicaltrials.gov/show/NCT00059631
		Ongoing phase I: neuroblastoma	Irinotecan	http://clinicaltrials.gov/show/NCT00644696
		Ongoing phase I: T-cell lymphoma	Azacitidine	http://clinicaltrials.gov/show/NCT01129180
		Ongoing phase I: acute leukemia or myelodysplastic syndrome	Belinostat	http://clinicaltrials.gov/show/NCT01075425
		Ongoing phase I/II: mantle cell lymphoma	Rituximab and lenalidomide	http://clinicaltrials.gov/show/NCT00633594
		Ongoing phase I/II: Waldenstrom's macroglobulinemia	Everolimus and rituximab	http://clinicaltrials.gov/show/NCT01125293

(continued)

Table 13.1 (continued)

Therapeutic targets	Current drugs	Clinical trial stages (published reports)/type of cancer	Combined with	References
		Ongoing phase II: plasma cell leukemia	Liposome doxorubicin and dexamethasone	http://clinicaltrials.gov/show/NCT01328236
		Ongoing phase III: MM	Panobinostat and dexamethasone	http://clinicaltrials.gov/show/NCT01023308
		Ongoing phase III: MM	Vorinostat	http://clinicaltrials.gov/show/NCT00773747
	Carfilzomib (PR-171)	Phase I: hematologic malignancies	–	[248]
		Ongoing phase I: AML or ALL	–	http://clinicaltrials.gov/show/NCT01137747
		Ongoing phase I/II: MM	Immunomodulatory drugs	http://clinicaltrials.gov/show/NCT01365559
		Ongoing phase II: MM	–	http://clinicaltrials.gov/show/NCT00884312
		Ongoing phase III: MM	Dexamethasone	http://clinicaltrials.gov/show/NCT01568866
X-IAP mRNA	AEG35156	Phase I: advanced cancer	–	[249]
		Phase I/II: AML	Idarubicin and cytarabine	[250]
		Phase II: AML	Idarubicin and cytarabine	[251]
Pan-IAP	LCL161	Phase I: advanced cancer	–	[252]
		Phase I: advanced solid tumor	Paclitaxel	[253]
		Ongoing phase II: breast cancer	Paclitaxel	http://clinicaltrials.gov/show/NCT01617668
	HGS1029	Phase I: advanced solid tumor	–	[254]
	TL32711	Phase I: advanced solid tumor and lymphoma	–	[255]
		Ongoing phase I: advanced solid tumor	Gemcitabine	http://clinicaltrials.gov/show/NCT01573780
		Ongoing phase I/II: myelodysplastic syndrome	Azacitidine	http://clinicaltrials.gov/show/NCT01828346
		Ongoing phase I/II: AML, ALL, and myelodysplastic syndrome	–	http://clinicaltrials.gov/show/NCT01486784
		Ongoing phase I/II: solid tumor	–	http://clinicaltrials.gov/show/NCT01188499
		Ongoing phase II: ovarian, fallopian tube, and peritoneal cancer	–	http://clinicaltrials.gov/show/NCT01681368
Survivin	YM155	Phase I: advanced solid tumor or lymphoma	–	[256]
		Phase I: advanced solid tumor	–	[257]
		Phase II: NSCLC	–	[258]
		Phase II: melanoma	–	[259]
		Ongoing phase II: breast cancer	Docetaxel	http://clinicaltrials.gov/show/NCT01038804
		Ongoing phase II: NHL	Rituximab	http://clinicaltrials.gov/show/NCT01007292

As for conatumumab, a phase I study in advanced solid tumors showed that this drug is generally well tolerated [207, 208]. Conatumumab in combination with gemcitabine shows evidence of an improved 6-month survival rate and tolerable toxicity in phase Ib and II metastatic pancreatic cancer trials [209, 214]. In metastatic colorectal cancer, conatumumab improves progression-free survival (PFS) when combined with FOLFIRI [215], but limited activity when combined with modified FOLFOX6 and bevacizumab [211], and no activity when combined with panitumumab [213]. The effect of conatumumab in NSCLC is similar as compared with rhApo2L [192, 194], of which combination of this drug with paclitaxel and carboplatin did not produce promising results [210, 216]. Combination of conatumumab with other chemotherapy drugs also produces no evidence of activity in soft tissue sarcomas [212]. The common adverse effects with this drug are neutropenia and thrombocytopenia [214–216]. Generally, these early trials lacked data on the correlation between patient's TRAIL status and response to therapy. Preferential TRAIL sensitivity and presence of TRAIL-R1 and TRAIL-R2 expression in certain cancers are considered factors in patient's response. Therefore, rhApo2L and agonistic anti-TRAIL-R therapies may be limited to patients with TRAIL-sensitive tumors. The efficacy of TRAIL targeting therapies may be improved if diagnostic methods determining TRAIL sensitivity of clinically detectable human cancers are available [190]. Trials are still ongoing, especially those involving the combination of these agents with current chemotherapy drugs.

13.4.2 Bcl-2 Family Proteins (BH3 Mimetics and Bcl-2 Antisense)

Bcl-2 family proteins can regulate apoptosis both positively and negatively. The Bcl-2 family members consist of anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-W, Bag-1, Mcl-1, and A1/Bfl-1) as well as pro-apoptotic (Bad, Bax, Bak, Bcl-xs, Bid, Bik, and Hrk) molecules [264, 265]. The balance and interaction between Bcl-2 gene family members and posttranslational modifications of Bcl-2-related proteins have been demonstrated to play important roles in regulating cell survival and death.

The Bcl-2 family is characterized by specific regions of homology termed Bcl-2 homology (BH1, BH2, BH3, and BH4) domains. Anti-apoptotic proteins have BH1–BH4 domains (e.g., Bcl-2 and Bcl-X_L). Pro-apoptotic proteins have either BH1–BH3 domains (e.g., Bax and Bak) or BH3-only domains (e.g., Bid, Bim, Puma, Bad, Noxa, Hrk, Bik) [77, 266, 267]. These domains are critical to the function of these proteins, especially their impact on cell survival and cell death and their ability to interact with other family members and regulatory proteins. The molecular surface of the multidomain anti-apoptotic Bcl-2 protein contains a BH3-binding groove, which accommodates BH3 domain from pro-apoptotic Bcl-2 protein family members. The BH3-only proteins are known to function as antagonists of anti-apoptotic Bcl-2 family proteins and act as tumor suppressors [77]. This forms the basis or platform for subsequent drug discovery strategies based on mimicking BH3 peptides with chemical compounds that bind in the same groove [268].

The earlier observation that apoptosis deregulation in cancer cells primarily affects the upstream of the signaling pathways of Bax/Bak and mitochondria, leaving the downstream core of the apoptotic machinery mostly intact, has led to a therapeutic strategy of which manipulation of the equilibrium between the pro- and anti-apoptotic Bcl-2 family members could possibly restore apoptosis [128, 173]. Since pro-apoptotic BH3 domains directly bind to the hydrophobic grooves of pro-survival proteins with high affinity, and are necessary and sufficient for initiation of apoptosis, agents mimicking the BH3 domains may provide some degree of selectivity against cancer cells. This is mainly because cancer cells are postulated to be more sensitive to inhibition of pro-survival proteins compared with their normal counterparts [12]. Cancer cells often express high levels of Bcl-2-like anti-apoptotic proteins to evade the apoptotic fate imposed by aberrant cell proliferation, activation of oncogenes, or DNA damage [269]. Therefore, it is possible to

design BH3 mimetics to target specific anti-apoptotic proteins that are overexpressed in a particular type of cancer for improved specificity [173]. Several chemicals mimicking BH3 peptides exclusively targeting the Bcl-2 anti-apoptotic proteins have since been described [268, 270, 271]. Another antitumor strategy is direct inhibition of Bcl-2 mRNA, in the form of antisense.

One of the earliest small-molecule BH3 mimetics or more accurately Bcl-2 and Bcl-X_L inhibitor that went through several phase I/II clinical trials is gossypol, an orally available compound derived from cottonseed extracts [272]. It binds to the BH3-binding grooves of Bcl-2, Bcl-X_L, and Mcl-1 [273]. However, several past clinical trials have not indicated this compound as an effective anticancer agent. Either used alone or in combination, patients treated with gossypol failed to show evidence of tumor regression or any therapeutic responses in several clinical trials [274–276]. A derivative of R-(-)-gossypol (AT-101) is found to be well tolerated in a phase I trial involving CLL patients [217]. However, later studies showed that either AT-101 is not active in patients or the response rates are too low that it did not meet the criteria for additional enrolment in further trials for small cell lung cancer (SCLC) [218, 219]. In NSCLC, patients did not meet the primary endpoint of improved PFS when given a combination of AT-101 and docetaxel [220]. Current trials to evaluate the potency of this drug in other forms of cancer are still ongoing, for example, as a combination therapy in squamous cell carcinoma of the head and neck (SCCHN) and advanced laryngeal cancer. A semisynthetic analog of gossypol with improved pharmacologic properties, such as apogossypolone (ApoG2), was found to inhibit the growth of diffuse large cell lymphoma cells *in vitro* and *in vivo* [277]. However, this compound has yet to proceed to clinical trials.

GX15-070 (obatoclax mesylate) is an indole derivative and a broad-spectrum inhibitor of pro-survival Bcl-2 family proteins; it has been extensively evaluated in clinical trials. A phase I clinical trial of obatoclax mesylate in 44 patients with refractory leukemia and myelodysplasia has demonstrated that the drug is well tolerated up to the highest dose. However, only a single patient with acute myeloid leukemia (AML) with mixed lineage leukemia t(9;11) rearrangement achieved complete remission, which lasted 8 months, and of 14 patients with myelodysplasia, only three showed hematologic improvement [221]. In another phase I trial, where obatoclax was administered to patients with advanced CLL, activation of Bax and Bak was demonstrated in peripheral blood mononuclear cells, and induction of apoptosis was related to overall obatoclax exposure, as monitored by the plasma concentration of oligonucleosomal DNA/histone complexes. Obatoclax is noted to have some biological activity and modest single-agent activity in heavily pretreated patients with advanced CLL [222]. In advanced solid tumor and lymphoma, of 35 patients given obatoclax infusions, only one patient with relapsed NHL achieved partial response of 2 months duration, and one patient had stable disease for 18 months [224]. In a phase II study in patients with relapsed SCLC, obatoclax added to topotecan produced no difference in response rates as compared to topotecan alone, even though the drug was generally well tolerated [223, 225]. Obatoclax has also showed limited clinical activity in heavily pretreated patients with classic Hodgkin lymphoma (HL) [226]. Neurological symptoms are reported as the most common adverse effects in patients. Obatoclax appears to have limited efficacy as a single agent or even in combination with some of the more common anticancer drugs. Clinical trials using obatoclax in combination with other chemotherapy drugs in solid tumor, leukemia, and SCLC are currently ongoing (Table 13.1).

Another BH3 mimetic, ABT-737, possesses greater binding affinity to BH3-only proteins, enhances the effect of death signal, and is synergistic with cytotoxic agents and radiation [268]. To overcome the low solubility and oral bioavailability of ABT-737, the ABT-263 analog (navitoclax) was developed for clinical investigation. Preclinical studies confirmed that navitoclax has high affinity for the anti-apoptotic Bcl-2 family proteins and kills cancer cells in a Bax/Bak-dependent manner [227]. In a phase II clinical

study, navitoclax exhibits limited single-agent activity against advanced and recurrent SCLC [228]. Clinical trials of navitoclax as a single agent or as combination therapy in a variety of cancers such as lymphoid, leukemia, and other solid tumors are ongoing.

A nuclease-resistant phosphorothioate antisense oligonucleotide targeting Bcl-2 mRNA (oblimersen sodium) has shown promising activity for CLL and malignant melanoma in randomized phase III trials [232–234]. It is an 18-mer phosphorothioate antisense oligonucleotide designed to bind to the first six codons of the human Bcl-2 mRNA [278]. The use of oblimersen in combination with chemotherapy in a variety of cancers has shown diverse response rates with good tolerability. In the Oblimersen Melanoma Study Group, the addition of oblimersen to dacarbazine improved the multiple clinical outcomes in patients with advanced melanoma and increased overall patient's survival [232]. In another phase III trial, the addition of oblimersen to fludarabine and cyclophosphamide significantly increased the complete response/nodular partial response rate in patients with relapsed or refractory CLL [233]. In the same study, a significant 5-year survival benefit was observed with oblimersen in combination with fludarabine and cyclophosphamide. Among patients with fludarabine-sensitive disease who had previously demonstrated maximum benefit with the same treatment, a 50 % reduction in the risk of death was observed [234].

However, not all combination therapies produce desirable outcomes. In the Cancer and Leukemia Group B Study 10107 (CALGB), although the combination of oblimersen and imatinib was safe and feasible, no clinical benefits were observed in imatinib-resistant chronic myeloid leukemia (CML) patients [230]. In a randomized phase II study of carboplatin and etoposide with or without oblimersen for extensive-stage SCLC (CALGB 30103), the addition of oblimersen to a standard regimen did not improve any clinical outcome measure [231]. A randomized study of dexamethasone with or without oblimersen sodium in patients with advanced multiple myeloma (MM) demonstrated no significant differences in time to tumor progression or objective response rate [235]. Interestingly, in a recent phase I study, the combination of oblimersen, temozolomide, and albumin-bound paclitaxel was well tolerated and demonstrated encouraging activity in patients with advanced melanoma, with objective response rate and disease control rate at 40.6 % and 75 %, respectively [229]. Some of the common adverse effects associated with oblimersen sodium administration include fatigue, transaminase elevation [279, 280], and hematologic disorders [231–233]. There are a number of trials listed in the NIH ClinicalTrials.gov website; some trials are terminated, and some are completed, while the outcome of trials involving some other tumor types is unknown at this point of time.

13.4.3 Proteasome Inhibitors

The proteasome is a multicatalytic enzyme complex that degrades intracellular proteins by a targeted and controlled mechanism. The 26S proteasome, a large protein complex, composes approximately 50 subunits that function as a highly specific molecular shredder by hydrolyzing ubiquitinated proteins into small peptides [281]. The 26S proteasome can be further divided into two sub-complexes, a central 20S proteolytic core particle (CP) that is capped at either end by one or two 19S regulatory particles (19S RP). The 20S CP is the degradation unit and contains the active sites required to hydrolyze proteins into peptides [281]. On the other hand, the 19S RP controls the degradation of ubiquitin-tagged substrates by acting as a receptor for polyubiquitinated proteins and facilitating their ATP-dependent translocation into the catalytic chamber of the 20S CP [281].

The ubiquitin-proteasome pathway (UPP) is responsible for proteolytic degradation of the majority of damaged and misfolded proteins within the eukaryotic cell. The UPP is essentially important for controlled degradation of key regulatory proteins involved in a wide variety of cellular functions such as apoptosis [282], cell cycle control, proliferation [283], and transcriptional regulation [284]. However, overactivity of the

UPP results in an accelerated turnover of proteins that regulate the cell cycle, leading to a deregulated mitosis, thereby supporting cancer growth [285]. A defect in the proteasome function is associated with the development of different diseases such as neurodegenerative disorders, cardiovascular and rheumatoid diseases, and cachexia, but not cancer, suggesting that cancer cells use the proteasome for their survival [286]. In humans, three deubiquitinases (DUBs) are associated with the 19S RP. Two of these (UCHL5/Uch37 and USP14/Ubp6) are cysteine proteases and members of the ubiquitin C-terminal hydrolases (UCH) and ubiquitin-specific proteases (USP) families, respectively. The expression of the cysteine DUBs UCHL5 and USP14 is also deregulated in cancer. Activities of UCHL5 (along with several other DUBs) are found to be enhanced in tumor biopsies of cervical carcinoma when compared to adjacent normal tissues [287].

The transcription factor NF-κB is inactive in the cytoplasm under normal conditions and is activated when its binding partner, IκBα, is degraded by the proteasome. Constitutive NF-κB activity has been observed in a variety of tumors including MM; sustained activity of NF-κB may lead to aberrant expression of target genes promoting tumor cell proliferation and survival [288]. Bcl-2 is identified as a key target of NF-κB in B-cell lymphoma [289]. NF-κB, a centrally important transcription factor involved in immune and inflammatory cellular responses affecting both cell growth and survival, appears to be pivotally involved in the pathogenesis of aggressive lymphoid malignancies [290]. As a result, the inhibition of proteasome function serves as an important mechanism in anticancer therapy. Proteasome inhibitors have recently emerged as an interesting and potentially new group of chemotherapeutic agents for various human cancers, including breast, prostate, and lung carcinomas, that function in part by stabilizing the IκBα protein and, finally, inhibiting NF-κB activation [121, 291]. Preclinical studies have shown that the proteasome inhibitor, bortezomib, decreases proliferation, induces apoptosis, enhances the activity of chemotherapy and radiation, and reverses chemoresistance in a variety of hematologic and solid malignancy models *in vitro* and *in vivo* [292]. Bortezomib is a novel synthetic dipeptide boronic acid that reversibly inhibits the chymotryptic-like activity and, to a lesser extent, the caspase-like activity of the β5- and β1-subunits of the 20S CP [293].

However, the role of NF-κB as a key determinant of bortezomib-induced cytotoxicity is rather controversial, as several studies have shown that direct inhibition of NF-κB signaling is insufficient to induce apoptosis in bortezomib-sensitive cells [294–296]. Recent studies also found that bortezomib exerts no inhibition of constitutive NF-κB activity in MM or mantle cell lymphoma cells [297, 298]. Results of the genome-wide siRNA screen performed by Chen and co-workers showed that bortezomib induces cell death by interfering with ribosome function and DNA damage pathways and through deregulation of Myc signaling [299]. A separate screen by Zhu and co-workers demonstrates that knockdown of cyclin-dependent kinase 5 (CDK5), as well as a number of other genes, potentiated bortezomib-induced cytotoxicity in MM cells [300]. In addition, proteasome inhibitors are also potent inducers of endoplasmic reticulum (ER) stress [295, 301]. Acute ER stress response caused by proteasome inhibition results in apoptosis [301]. In addition to ER stress, several reports indicate that proteasome inhibitors induce the rapid production of ROS, known to be involved in apoptotic signaling [295, 302, 303].

Bortezomib (Velcade®, Millennium Pharmaceuticals, Inc., Cambridge, MA and Johnson & Johnson Pharmaceutical Research and Development, L.L.C.) is the first proteasome inhibitor approved by the US Food and Drug Administration (FDA) in 2005 for the treatment of progressive MM in patients who have received at least one prior therapy [236]. The drug is later approved for the treatment of mantle cell lymphoma, a lymphoid malignancy derived from mature B cells [237, 242, 245]. Bortezomib has undergone a series of successful preclinical and clinical studies. Phase I and II trial results produced encouraging prospects. In a retrospective study [based on data from phase II (SUMMIT or

CREST) or phase III (APEX) registration studies] to clarify the utility of bortezomib as a repeat therapy, bortezomib retreatment appeared to be safe and effective in patients with relapsed MM [304]. In a separate phase I/II trial, weekly bortezomib plus oral cyclophosphamide and prednisone produced more than 50 % complete response rate and an encouraging 1-year survival in relapsed/refractory patients with MM [240]. The regulatory approval of bortezomib was based on its efficacy and safety in a large, international, multicenter phase III prospective study. This randomized, open-label trial compared single-agent bortezomib with single-agent, high-dose dexamethasone in patients with progressive MM after at least one prior therapy [236, 247]. Bortezomib manifested significant efficacy and safety, supported by an improved response rate, including achieving near-complete responses [236, 247]. Updated results of a multicenter phase II PINNACLE study of bortezomib in patients with relapsed or refractory mantle cell lymphoma indicate that single-agent bortezomib is associated with lengthy responses and notable survival in these patients [245]. However, clinical trials using bortezomib in combination with other chemotherapy drugs in cancers such as HL [243]; advanced solid tumors such as breast, ovarian, and prostate [239]; and metastatic gastroesophageal cancer [244] lacked favorable outcomes.

It is clear that although bortezomib has potent anti-multiple myeloma activity, not all patients respond to bortezomib, and most responders ultimately relapsed [305, 306]. To date, however, no marker has been identified and validated in a manner that would allow clinical use and to distinguish patients likely to respond to bortezomib treatment from those who would not [305]. The most common adverse events are gastrointestinal symptoms, fatigue, thrombocytopenia, and sensory neuropathy, which comprised a major reason of treatment discontinuation [241]. Despite the clinical success of bortezomib in MM and mantle cell lymphoma, resistance to this drug remains a clinically significant problem. For example, in studies of bortezomib in relapsed refractory patients [241, 306], almost all responding patients ultimately experienced disease progression. Even when bortezomib was used as a single agent in newly diagnosed patients, 52 % did not achieve a partial response or a better outcome [246]. Furthermore, the clinical response to bortezomib in other hematologic malignancies and solid tumors remains low [238, 306].

Resistance to proteasome inhibitors has been examined in cell-based studies, and potential clinical mechanisms of bortezomib resistance have been highlighted. Understanding the molecular basis of resistance to proteasome inhibitors in patients with myeloma and other malignancies will aid in the development of therapeutic strategies to overcome bortezomib resistance. In an effort to overcome bortezomib resistance, novel proteasome inhibitors have been developed that act through mechanisms distinct from bortezomib. These newer proteasome inhibitors may also possess side effect profiles distinct from that of bortezomib. Second-generation proteasome inhibitors with novel properties, such as NPI-0052 and carfilzomib, are currently evaluated in clinical trials and have shown evidence of anti-myeloma activity. Carfilzomib (previously known as PR-171) is a tetrapeptide epoxyketone-based, irreversible proteasome inhibitor, more potent and selective, and produces more sustained inhibition of the proteasome [307, 308].

Although a recent phase I study of carfilzomib revealed tolerability and some clinical activity in patients with multiple hematologic malignancies, the response rates were rather low [248]. Currently, various trials are ongoing for carfilzomib, either as a single agent or in combination with other chemotherapy drugs for MM patients who have relapsed or are refractory to bortezomib-containing treatments. Other clinical studies are currently exploring the potential benefit of this drug in patients with relapsed AML or acute lymphoblastic leukemia (ALL) (Table 13.1).

13.4.4 Inhibitor of Apoptosis Protein (IAP) Antagonists

During apoptosis, natural IAP antagonists such as Smac/Diablo and HtrA2/Omi translocate from the mitochondria and inactivate IAPs to facilitate

caspase activation and cell death. Smac/Diablo and HtrA2/Omi promote apoptosis by antagonizing the IAPs, such as X-IAP, cIAP-1, and cIAP-2, which are often upregulated in many cancer cells [309]. X-IAP is a potent direct inhibitor of caspase-3, caspase-7, and caspase-9 [310]. Smac/Diablo contains an IAP-binding motif which forms the basis for the design of the novel class of anticancer drugs named Smac mimetics [311]. Peptides that mimic Smac/Diablo functions are capable of inducing death or increasing the apoptotic effect of chemotherapeutic agents [64, 309]. In a preclinical study, the synthesized Smac/DIABLO-N7 peptides are found to increase the apoptosis-inducing potential of chemotherapeutic drugs (paclitaxel, doxorubicin, and tamoxifen) and irradiation; in addition, they sensitize TRAIL-resistant cells to undergo apoptosis [312]. Smac mimetic such as AEG40730 triggers the autoubiquitination of cIAP-1 and cIAP-2 and targets them for proteasomal degradation. Loss of cIAPs leads to TNF-dependent cell death in some cell types [313].

AEG35156, an X-IAP antisense oligonucleotide, is the first IAP antagonist that has advanced to human clinical trial. A phase I trial of AEG35156 in advanced refractory cancer produced reduction in X-IAP mRNA level; however, the suppression was not preserved. Nonetheless, it is well tolerated in patients after intravenous infusion and some clinical evidence of antitumor activities are observed [249]. However, in a randomized phase II trial of patients with primary refractory AML, the addition of AEG35156 to idarubicin and cytarabine did not improve the rate of remission as compared with the control arm consisting of cytarabine and idarubicin alone [250, 251]. The mRNA level of X-IAP was not determined in this study, therefore, whether efficient knockdown of X-IAP mRNA was achieved in this phase II trial remains unknown [251].

A phase I report of another novel IAP antagonist, LCL161, indicated that this orally bioavailable agent is well tolerated in patients with advanced cancer. However, no objective responses were observed, despite the fact that LCL161 treatment results in target inhibition, as shown by cIAP-1 degradation and cytokine induction [252]. Phase Ib study of LCL161 in combination with paclitaxel in advanced solid tumors is currently underway. Early report shows that this combination is well tolerated and demonstrates preliminary antitumor activity in breast cancer patients [253]. Two other small-molecule IAP antagonists, HGS1029 and TL32711, are also reported to be well tolerated in phase I studies and have produced some evidence of antitumor activity as well as suppression of cIAP-1 level [254, 255]. YM155, a small-molecule inhibitor of survivin, another human IAP, has also demonstrated to be safe and to possess antitumor activity in phase I studies [256, 257]. However, a phase II trial reported modest single-agent activity of YM155 in NSCLC [258]. In patients with stage III or IV melanoma, prespecified primary endpoint was not achieved in a phase II trial [259]. LY2181308, a survivin antisense oligonucleotide, has been reported to be safe in the first-in-human phase I study, although further studies would be needed to assess its activities [314]. The overall efficacy of IAP antagonists remains uncertain at this point of time. Table 13.1 summarizes the various drugs targeting the apoptosis pathways and clinical trial stages based on published reports as well as ongoing trials listed in the NIH ClinicalTrials.gov website.

The crosstalk between apoptosis, autophagy, and necroptosis signaling pathways and future directions of cancer therapeutic targets will be discussed in Chap. 14.

13.5 Concluding Remarks

Resistance to cell death induction has long been recognized as a hallmark of cancer. Therefore, increased understanding of the underlying molecular events regulating different cell death mechanisms such as apoptosis, necroptosis, and autophagy has provided new possibilities for targeted interference of these pathways. Various phases of clinical trials have been conducted which interfere with these pathways.

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Autophagy and Necroptosis in Cancer

14

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Contents

14.1 Introduction

The ubiquitin-proteasome system (UPS) and lysosomes are two primary intracellular protein degradation pathways recognized in eukaryotic cells. Differences between these two major protein degradation systems depend on their functional significance and the type of substrates taken in for degradation [1]. The UPS catalyzes the rapid degradation of abnormal proteins and short-lived regulatory proteins, leading to a control of a diversity of essential cellular processes [2]. In the lysosomal protein degradation pathway, degradation of extracellular materials is mediated by endocytosis, whereas degradation of intracellular long-lived cytoplasmic proteins and damaged organelles is mediated by three types of

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autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which are classified based on their transport of cytoplasmic materials into the lysosome for degradation [3, 4].

Autophagy literally means self-digestion in Greek [5]. Macroautophagy, usually referred to autophagy, is responsible for the turnover of unnecessary or dysfunctional organelles and proteins, such as damaged mitochondria [6]. These processes are important to maintain a well-controlled balance between anabolism and catabolism to facilitate normal cell growth and development. It is also a survival pathway, required during starvation or growth factor deprivation as it provides an alternative energy source [7, 8]. Autophagy process provides catabolic intermediates for intracellular production of ATP when energy supplies are limited. It plays an essential role during starvation, cellular differentiation, cell death, cell survival, aging, and tumor prevention [4, 6, 9].

Autophagy is a multistep process characterized by induction, vesicle nucleation, extension, and completion of an isolation membrane to form an organelle called autophagosome [10]. Briefly, the autophagy process begins with the formation of a pre-autophagosomal structure known as isolation membrane or phagophore [11]. The isolation membrane engulfs and elongates to form the autophagosome, surrounding the components destined to be recycled. The autophagosome, which is a double membrane-bounded structure, undergoes maturation and fuses with both endosomal and lysosomal vesicles to form autolysosome [11–13]. The sequestered contents are subsequently degraded by lysosomal hydrolases and are recycled. Based on morphological features, the term "autophagic cell death" has been described in instances of cell death that are accompanied by massive cytoplasmic vacuolization. The morphology characteristics of cells undergoing autophagic cell death are depicted in Chap. 13 (Fig. 13.2).

The core autophagy machinery composes of four major functional groups: (1) the Atg1-Atg13-Atg17 kinase complex; (2) the Class III phosphoinositide-3-kinase (PI3K) complex I, including Class III PI3K (the mammalian ortholog of vascular protein sorting 34; Vps34), p150 (the mammalian ortholog of Vps15), Beclin-1 (the mammalian ortholog of Atg6, also called Vps30), and Atg14; (3) two ubiquitin-like conjugation systems, Atg12 and Atg8; and (4) Atg9 and its cycling system [14]. The unc-51-like kinases (ULKs; the mammalian orthologs of Atg1), which exist in a large complex with mammalian Atg13 (mAtg13), focal adhesion kinase family interacting protein of 200 kDa (FIP200; the mammalian homolog of Atg17), and the recently identified Atg101, plays a crucial role in autophagy induction [15–19]. Phosphorylation of Atg13 and FIP200 by ULK1 is an important step in the initiation of autophagy, although the exact role of phosphorylation in generating autophagosomes is currently unclear.

The early stages of the phagophore membrane nucleation are dependent on the Class III PI3K complex which consists of the Class III PI3K protein, its regulatory protein kinase p150, and Beclin-1 [20]. Beclin-1 is a 60 kDa tumor suppressor protein and is identified from a yeast two-hybrid screen as a Bcl-2 interacting protein [21]. Recent studies have demonstrated that several binding molecules positively regulate Beclin-1 activity and autophagosome formation and maturation. Ultraviolet (UV) radiation resistance-associated gene (UVRAG), Atg14L, and autophagy/Beclin-1 regulator 1 (Ambra1) associate with Beclin-1 to activate autophagy [22–26].

The next stage of phagophore membrane elongation (expansion and closure of the autophagosome) requires two ubiquitin-like systems [27]. The ubiquitin-like protein Atg12 conjugates with Atg5 in an Atg7- and Atg10-dependent manner [1]. The Atg5-Atg12 complex interacts with Atg16 to form a stable and large multimeric complex called the Atg16L complex, which localizes on the outer surface of the extending autophagosomal membrane [10]. This complex is important in the stimulation and localization of the microtubule-associated protein 1 light chain 3 (LC3) conjugation reactions. LC3 is first cleaved by Atg4 to expose a C-terminal glycine residue required for subsequent activation and conjugation reactions [28]. It is then conjugated to the

lipid phosphatidylethanolamine (PE), also via Atg7 and E2-like Atg3, and is subsequently recruited to both outer and inner surfaces of the autophagosomal membrane [27, 29]. Actually, two forms of LC3 are produced posttranslationally in various cells; the unconjugated form (LC3-I) is in the cytosol, while the conjugated form (LC3-II) targets to the autophagosomal membrane with the assistance of the Atg16L complex [29, 30]. The Atg5-Atg12-Atg16 complex is recycled, while the LC3 complex stays on the membrane until it is degraded by the lysosome [1]. In mammalian autophagy, LC3-II protein is used as an index of autophagosome formation or as an autophagosomal marker [31].

Atg16L complex is a ubiquitin-protein ligase (E3)-like enzyme that functions as a scaffold for LC3-II lipidation by localizing to the source membranes for autophagosome formation [30, 32]. The association of LC3-II to the autophagosome is crucial for membrane elongation of the autophagosome and the final limitation of the membrane to form the vacuoles [1]. These conjugation systems are considered to be uniquely important to the autophagosome formation and have been identified as possible drug targets in cancer [33]. On the other hand, Atg9 provides lipids to the isolation membrane by cycling between distinct subcellular compartments. The cycling of Atg9 requires Atg1/ULK1 and the kinase activity of Vps34 [34]. However, the role of Atg9 is currently not completely understood.

The completed autophagosome membrane subsequently fuses with lysosome via the actions of the lysosomal proteins including the lysosomal-associated membrane protein 1 (LAMP1), LAMP2, member RAS oncogene family (Rab7), and UVRAG [35]. The eventual autolysosome is a single-membrane-bound acidic vesicle where the contents are digested and recycled by lysosomal hydrolases such as cathepsins (CTS), and its nutrient and energy are recycled [36]. These single-membrane autolysosomes, filled with degraded cytoplasmic materials, can be easily observed using transmission electron microscopy (TEM) [10]. As a precautionary note, the Nomenclature Committee on Cell Death (NCCD) recommends that the term "autophagic cell death" to be used based on some biochemical and functional considerations, before indicating that a cell death is mediated by autophagy. Some of the considerations include making sure that the investigated cell death can be suppressed by the inhibition of the autophagic pathway using chemicals and/or genetic means (e.g., gene knockout or RNAi silencing of essential autophagy modulators such as AMBRA1, Atg5, Atg12, or Beclin-1) [37].

One of the most studied and important pathways involved in autophagy regulation is the PI3K-Akt-mTOR signaling pathway. The mammalian target of rapamycin, commonly known as mTOR, is a serine/threonine kinase which belongs to the family of phosphatidylinositol-3-kinase-related kinases. It regulates translation and cell growth by its ability to phosphorylate both binding protein of eukaryotic translation initiation factor 4E (4E-BP1) and p70 ribosomal S6 kinase (p70S6k). Upon stimulation by a variety of signals including cytokines, growth factors, cellular stress such as heat shock, hypoxia, and oxidative stress, PI3K is recruited to the inner cell membrane via phosphorylated receptor tyrosine kinases and catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The recruitment of inactive Akt from the cytosol to the plasma membrane requires that the pleckstrin homology (PH) domain of Akt binds to PIP3 synthesized at the plasma membrane by PI3K. Akt is then phosphorylated at Thr 308 by phosphatidylinositol-dependent kinase 1 (PDK1) [38, 39]. PTEN phosphatase antagonizes PI3K-Akt signaling by converting PIP3 back to PIP2 [38].

Upstream PI3K and Akt activation by growth factors leads to the activation of mTOR and subsequently phosphorylation of downstream substrates. Phosphorylation of p70S6k promotes ribosome biogenesis and increases the capacity of the translational machinery for protein synthesis [40]. Phosphorylation of 4E-BP1 initiates the transcription of a subset of mRNAs important for cell growth and proliferation [40–42]. The mTOR kinase is a key regulatory component that controls the induction of autophagy [43]. Inhibition of mTOR (by nutrient depletion, starvation, or

rapamycin) leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression, and induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process, resulting in an increase in cell growth and tumor development [13]. Rapamycin, a specific mTOR inhibitor, complexes with the cytosolic receptor FK506-binding protein (FKBP12) and subsequently binds to a distinct region of mTOR upstream of the catalytic domain [44]. It induces autophagy and inhibits the proliferation of a variety of cells [45].

In eukaryotic cells, mTOR exists in two different complexes: mTORC1, a rapamycin-sensitive complex defined by its interaction with the supplementary protein Raptor (regulatory-associated protein of mTOR), and mTORC2, a rapamycin-insensitive complex defined by its interaction with Rictor (rapamycin-insensitive companion of mTOR) [46–48]. mTORC1 and mTORC2 accessorial complexes consist of mTOR, mammalian lethal with SEC13 protein 8 (mLST8) (also known as GßL), and DEP domain containing mTOR-interacting protein (Deptor) [49]. mLST8 binds to the kinase domain of mTOR and stabilizes the interaction of Raptor with mTOR in a rapamycin-sensitive pathway [50]. Raptor is the first protein shown to bind directly to mTOR that is required to mediate mTOR regulation of p70S6k and 4E-BP1 activities [47, 51]. On the other hand, PRAS40 and Deptor play roles as distinct negative regulators of mTORC1 [52, 53].

In a rapamycin-sensitive mTOR signaling pathway, much of the knowledge about mTORC1 function comes from the use of rapamycin, a bacterial macrolide antibiotic [54]. Upon entering the cell, rapamycin binds FKBP12, its intracellular receptor, which subsequently binds to the FKBP12-rapamycin-binding domain (FRB) of mTOR, thus inhibiting the mTORC1 functions [55, 56]. Rapamycin weakens the interaction between mTOR and Raptor [57]. However, the exact mechanism of how rapamycin and several rapamycin derivatives bind to FKBP12 to inhibit mTORC1 signaling is not completely understood [58]. Various conditions including starvation or lack of nutrients such as amino acids and/or glucose mimic rapamycin treatment, hence inhibit mTOR function in cultured cells, as indicated by rapid inactivation of p70S6k and hypophosphorylation of the 4E-BP1 [59].

Studies have shown that mTORC1 controls autophagy through the regulation of a protein complex consisting of ULK1, mAtg13, and FIP200 [16, 18, 60]. The ULK complex is directly controlled by mTOR, leading to maintenance of the mAtg13 hyperphosphorylation state and suppression of autophagy induction [61]. A recent study has demonstrated that inhibition of mTOR by rapamycin leads to dephosphorylation of ULK1, ULK2, and mAtg13 and activates ULKs to phosphorylate FIP200. These results suggested that the ULK-Atg13-FIP200 complexes are direct targets of mTOR and important regulators of autophagy in response to mTOR signaling [18]. One of the most important proteins involved in the regulation of mTORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer of two proteins, TSC1 (also known as hamartin) and TSC2 (also known as tuberin) [56]. TSC1 and TSC2 function as a GAP (GTPase-activating protein) that negatively regulates a small GTPase called Rheb (Ras homolog enriched in brain). TSC1 and TSC2 inhibit mTORC1 signaling by transforming Rheb into its inactive GDP-bound state [62, 63].

In contrast to mTORC1, relatively little is known about the regulatory pathway influencing mTORC2 (mTOR-Rictor) [64]. mTORC2 consists of mTOR, mLST8, Rictor, Deptor, mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1), and the recently identified protein observed with Rictor (PROTOR) [49, 65]. Rictor is defined as a novel mTOR-interacting protein which is Raptor-independent [46, 66]. Unlike mTOR-Raptor, the mTOR-Rictor complex does not bind to FKBP12-rapamycin and is insensitive to rapamycin treatment [46, 48]. Therefore, rapamycin treatment does not represent a complete inhibition of mTOR function [67]. mTORC2 stimulates cell signaling through activation and phosphorylation of the pro-proliferative and pro-survival kinase Akt [68]. Inhibitors of the mTOR kinase domain have been developed to suppress the activity of both mTOR complexes (mTORC1 and mTORC2) [69, 70]. Figure 14.1 illustrates the simplified autophagy signaling pathways.

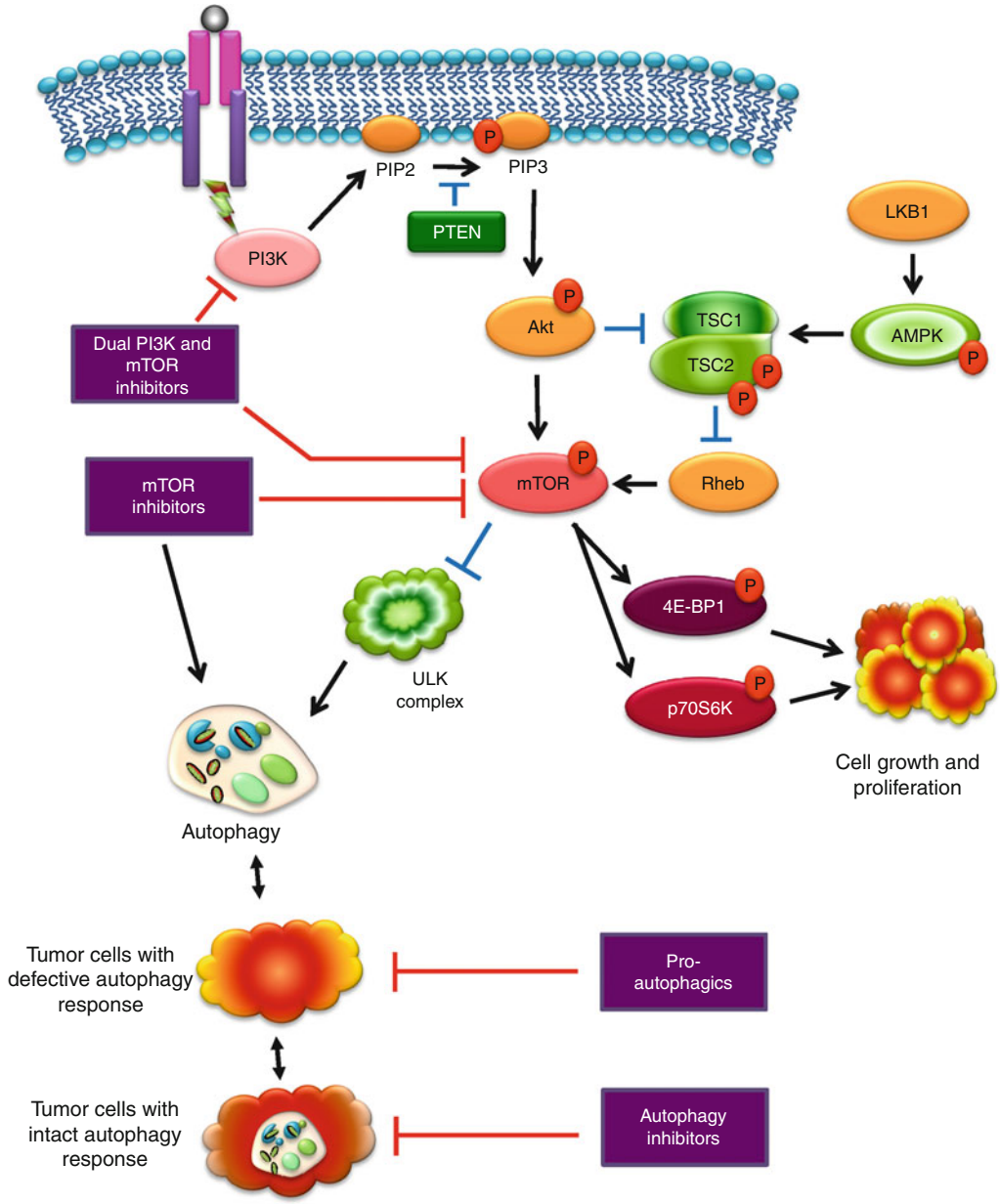

Fig. 14.1 Autophagy signaling pathway and antitumor therapeutic targets

14.2 Autophagy and Cancer

The role of autophagy in cancer is rather perplexing. It is widely postulated that the autophagic pathway is deregulated in tumor cells. Several proteins and pathways related to autophagy signaling are deregulated during cancer development [25, 71]. Cell lines derived from hepatic, pancreatic, and breast carcinoma exhibit low autophagic activity, as compared with normal cells from the same origin [25, 72]. Autophagic capacity is known to increase during the premalignant stages of pancreatic carcinogenesis and then decreases during the transition of pancreatic adenoma into adenocarcinoma, suggesting that a decreased autophagic activity

possibly contributing to the malignancy of pancreatic cancer [73, 74]. A decrease in autophagic capacity is also observed during animal experimental carcinogenesis, where cells from preneoplastic liver nodules or primary hepatocellular carcinomas induced by chemical carcinogens showed a decreased autophagic capacity as compared to normal liver cells [74, 75]. In addition, *Beclin-1* is found to be mono-allelically deleted in a high percentage of ovarian, breast, and prostate cancers (based on the 17q21 and gene mapping studies). It has been demonstrated to have a direct link between tumorigenesis and the disruption of autophagy [25]. *PTEN* deletions as well as the amplifications of both *Class III PI3K* and *Akt* are found in several cancers [76, 77].

The mTOR signaling pathway is constitutively activated in many tumor types. For example, the mTOR pathway is frequently found to be hyperactive in cancers such as breast cancer, suggesting that mTOR is an attractive target for cancer drug development and therapy [78–80]. The mTOR signaling network contains a number of tumor suppressor genes which includes *PTEN*, *LKB1* (liver kinase B1), *TSC1/2*, and a number of proto-oncogenes such as *PI3K*, *Akt*, and *eIF4E* genes [81]. Cancer-related changes in pathways at the downstream of mTOR such as p70S6k and eIF4E are reported in breast carcinoma [82, 83]. In addition, malignant cell types undergo autophagic cell death when responding to anticancer agents and traditional herbs, indicating the potential utility of autophagic cell death induction in cancer therapy [13, 84, 85]. Autophagic cell death characterized by an increase in the number of autophagic vacuoles in the cytoplasm, followed by cell demise, has been observed in various diseases such as Alzheimer's disease [86], Huntington's disease [87–90], and Parkinson's disease [91]. Therefore, manipulation of autophagy may provide an attractive strategy to increase the efficacy of cancer treatments, prevent cancer development, and limit tumor progression.

However, autophagy is divergent in nature in both tumor suppression and tumor progression [92]. Although the argument supports that if cells cannot activate autophagy, protein synthesis will predominate over protein degradation, and cellular growth continues (typical characteristic of tumor cells), there are some exceptional cases. For example, a study in human epidermoid lung carcinoma cells revealed that the autophagic pathway in response to nutrient deprivation is not downregulated when compared to their normal counterparts [93]. Human colon cancer cells which are able to survive for long period of time in the absence of nutrients have a high rate of autophagy activity [94]. Studies in colorectal cancer cells revealed that these cancerous cells harbor functional autophagic machinery to prolong cell survival during shortages of nutrients [95]. A recent study by Fuji and co-workers has also showed that strong LC3 expression in the peripheral area of pancreatic cancer tissue is correlated with poor outcome and short disease-free period [96]. Activated autophagy observed in pancreatic cancer cells is thought to be a response to factors in the cancer microenvironment, such as hypoxia and poor nutrient supply.

Autophagy has been identified as the key mechanism of cell survival in estrogen receptor-positive (ER^+) breast cancer cells undergoing treatment with 4-hydroxytamoxifen (4-OHT) [97]. Antiestrogen therapy is the standard treatment for ER^+ breast cancers which improves overall survival and provides chemoprevention [98, 99]. Unfortunately, approximately half of the women treated with antiestrogen therapy either do not respond or their breast cancer ultimately acquires resistance during treatment [100, 101]. Studies have shown that autophagy activity reduces the efficacy of chemotherapy and tamoxifen therapy in ER^+ breast cancer cells [97, 102, 103], supporting the thesis that blocking autophagy signaling pathways may provide a new mechanism of anticancer therapy for resistant tumors.

In another example, electron microscopy examination of autophagic vesicles in melanoma tumors from 12 patients enrolled in a Phase II clinical trial of temozolomide and sorafenib therapy revealed that autophagic index (mean number of autophagic vacuoles per cell) is significantly higher in patients who derived little or no clinical benefit from the combination of temozolomide and sorafenib treatment. Patients who had stable

disease or responded to therapy had low levels of autophagy in their tumors. These findings validate the emerging preclinical evidence that autophagy plays a critical role in resistance to chemotherapy. Results of this study indicate that pretreatment levels of autophagy can predict resistance to therapy. Patients with aggressive melanoma are more likely to have higher levels of autophagy in their tumor and therefore may respond to autophagy inhibition as a therapeutic strategy [104]. Hence, the divergent nature of autophagy has resulted in strategies for using pro-autophagics or autophagy inhibitors depending on the inherent nature of the cancer involved.

14.3 Autophagy Signaling Pathways and Therapeutic Strategies in Cancer

14.3.1 mTOR Signaling Pathway Inhibitors

Rapamycin, as the first prototype of an mTOR inhibitor, has a strong immunosuppressive property but poor aqueous solubility. Therefore, its utilization at doses capable of exerting anticancer effects is rather limited [105]. Nevertheless, trials utilizing rapamycin as a single-agent or combination therapy are still being carried out. In a recent Phase I study of rapamycin and sunitinib in patients with advanced non-small-cell lung cancer (NSCLC), combination of rapamycin and sunitinib is reported to be well tolerated and has warranted further investigation in Phase II trials [106].

Various rapamycin analogs have since been developed. Temsirolimus (CCI-779) is the first mTOR inhibitor approved by the US FDA for cancer treatment and is considered a first-line treatment for patients with advanced renal cell carcinoma (RCC) with poor prognostic features [107]. A great deal of clinical trials was carried out for this drug, mainly as combination therapy with other chemotherapy drugs. Generally, clinical activity is observed in patients with bone and soft tissue sarcoma given a combination of temsirolimus and cixutumumab [108]; in patients with metastatic adrenocortical carcinoma, the same combination therapy results in 40 % of patients achieving prolonged stable disease [109]. However, Phase I and II clinical trials with temsirolimus and sorafenib carried out in patients with metastatic melanoma did not produce sufficient activity to justify further use [110, 111]. In Phase II trial for metastatic colorectal cancer, temsirolimus has limited efficacy in chemotherapy-resistant KRAS-mutant disease [112].

Everolimus is another rapamycin analog which is already approved as an anticancer agent. Everolimus (RAD001; rapamycin derivative 001) is a hydroxyethyl ether derivative of rapamycin that has been developed for oral administration [113]. This drug was approved by FDA for use in a variety of cancers, including advanced RCC, advanced pancreatic neuroendocrine tumors, renal angiomyolipoma, and HER2-negative breast cancer. Everolimus, a derivative of rapamycin, is structurally similar to temsirolimus and binds to an intracellular protein, FKBP-12, forming a complex that inhibits the mTOR kinase. A recent Phase II trial showed that everolimus demonstrates efficacy and acceptable tolerability in patients with advanced endometrial cancer [114]. A randomized Phase II study indicates that combination therapy of everolimus with tamoxifen increases the clinical benefit rate (defined as the percentage of all patients with complete or partial response or stable disease at 6 months), time to progression (TTP), and overall survival compared with tamoxifen in postmenopausal women with aromatase inhibitor-resistant metastatic breast cancer [115]. In patients with advanced NSCLC, Phase I study showed that combination therapy with everolimus and erlotinib provides acceptable tolerability and disease control [116].

Ridaforolimus (deforolimus or AP23573) has been tested in Phase I and II clinical trials and has shown promising results in several tumor types including sarcoma [105, 117]. In a Phase II clinical study of ridaforolimus in 216 patients with advanced bone and soft tissue sarcomas, the overall clinical benefit response (CBR) was 28.8 % with a median progression-free survival of 15.3 weeks. Interestingly, the archival tumor

protein markers analyzed were not correlated with CBR [118]. Ridaforolimus receives fast track and orphan drug status from the US FDA, as well as orphan status from the European Medicines Agency. The FDA is currently reviewing its registration for maintenance therapy in patients with sarcoma [119]. In another Phase II trial study on the efficacy and safety of single-agent ridaforolimus in patients with relapsed or refractory hematologic malignancies, results were unremarkable. Of the 52 patients evaluated, partial responses were noted in five subjects, while hematologic improvement/stable disease was observed in less than half of the patients [120].

PI3K-Akt-mTOR pathway is often constitutively activated in human tumor cells and thus has been considered as a promising drug target. NVP-BEZ235 is a potent imidazo(4,5-c)quinoline derivative that inhibits PI3K and mTOR kinase activities by binding to the ATP-binding cleft of these enzymes and induces G1 arrest [121]. Preclinical studies have suggested that NVP-BEZ235 is a potent dual PI3K/mTOR modulator with favorable pharmaceutical properties. For example, it inhibits VEGF-induced HUVEC cell proliferation and survival *in vitro* and VEGF-induced angiogenesis *in vivo* [122]. The compound also inhibits microvessel permeability in BN472 mammary carcinoma grown orthotopically in syngeneic rats, suggesting that this compound is potentially anti-angiogenic [122]. Deregulated angiogenesis and high tumor vasculature permeability are known VEGF-mediated characteristics of human tumors. In addition, NVP-BEZ235 is found to produce significant tumor growth inhibition in xenograft models of pancreatic cancers and breast cancer cells [123, 124]. Phase I/II clinical trials of NVP-BEZ235 in patients with advanced solid malignancies and breast cancer were completed, but reports on the safety and efficacy of this drug have yet to be published. Other ongoing trials either as a single-agent or combination therapy with other chemotherapy drugs in breast cancer, prostate cancer, leukemia, and other advanced solid tumors are listed in the NIH ClinicalTrials.gov website.

14.3.2 Pro-autophagics

Temozolomide is the first pro-autophagic cytotoxic drug used to overcome apoptosis resistance in cancer cells and was approved for use in glioblastoma multiforme (GBM) [125]. It demonstrates therapeutic benefits in patients with glioblastoma and has been evaluated for several types of apoptosis-resistant cancers [126]. Temozolomide is a prodrug, is a monofunctional alkylating agent, and is chemically related to dacarbazine. It is the 3-methyl derivative of the experimental anticancer drug, mitozolomide. The ability of temozolomide in inducing autophagic cell death is reported in various preclinical studies [127–130]. In addition, temozolomide also demonstrates pro-apoptotic activities in malignant melanoma cells [131]. In a systematic assessment of three randomized controlled trials addressing whether temozolomide holds any advantage over conventional therapy for high-grade gliomas, it was shown that temozolomide is an effective therapy for GBM. The drug prolongs survival, delays disease progression, and has a low incidence of early adverse events [132]. Similar outcomes were observed in a Phase II study involving erlotinib in combination with radiation therapy and temozolomide to treat GBM and gliosarcoma. Patients treated with the combination of erlotinib and temozolomide during and following radiotherapy have better survival than historical controls [133].

In a recent Phase II trial, patients with unresectable or multifocal glioblastoma, an upfront regimen of temozolomide and bevacizumab was well tolerated and provided a significant level of disease stabilization [134]. In patients with recurrent glioblastoma, either used as a single agent in a dose-intense schedule or in combination with other chemotherapeutic agents, temozolomide is proven to be well tolerated and safe [135–137]. In pediatric patients with recurrent solid tumors or brain tumors, low-dose temozolomide improves tolerability and is convenient as outpatient therapy [138]. Temozolomide in combination with vorinostat is also well tolerated in children with recurrent central nervous system

(CNS) malignancies with myelosuppression [139]. However, good therapeutic effects are not observed in patients with NSCLC. In a current efficacy and safety study of temozolomide in a total of 31 pretreated patients with NSCLC, only two patients achieved partial response, and three had stable disease [140]. Moreover, the researchers pointed out that prolonged low daily doses of temozolomide produces minimal activity in patients with advanced NSCLC. Hence, more Phase II and III studies to characterize the efficacy of this drug in various cancers are definitely warranted.

Arsenic trioxide (ATO) has recently been introduced as part of a regimen in the therapy and management of acute promyelocytic leukemia (APL) [141]. It is now considered to be "the most biologically active single drug in APL" by a panel of international leukemia experts for the European LeukemiaNet. The combination of ATO and all-trans retinoic acid (ATRA) holds the promise to "replace conventional approaches for most, if not all, patients in the very near future" [142]. ATO is known to induce both autophagy and apoptosis depending on cell types; therefore, its role as an autophagy inducer remains largely uncertain. In some preclinical trials, ATO has induced the autophagic pathway in ovarian carcinoma cells and has synergized with everolimus to induce the cytotoxicity of ovarian cancer cells. The enhanced cytotoxicity is accompanied by the upregulation of Atg5-Atg12 conjugate and LC3-II, a hallmark of autophagy [143]. In another recent study, ATO induced the autophagic degradation of the BCR-ABL1 oncoprotein, known to cause chronic myeloid leukemia (CML) and Ph^+ acute lymphoblastic leukemia (ALL) [144]. However, in other studies, in the presence or absence of ionizing radiation and in specific low concentrations, ATO induced apoptosis in MTLn3 cells, known to be highly malignant and resistant to both radio- and chemotherapy [145]. Interestingly, in human glioma cells, ATO induces both autophagy and apoptosis *in vitro* and *in vivo*, mediated by the inhibition of PI3K/Akt and activation of MAPK signaling pathway [146].

In a Phase I clinical study, ATO given concomitantly with radiation therapy in children with newly diagnosed anaplastic astrocytoma, glioblastoma, or diffuse intrinsic pontine glioma was safe and well tolerated by patients throughout the entire dose escalation [147]. ATO is also reported to be well tolerated when used in combination with temozolomide and radiotherapy in malignant gliomas [148] or when used in combination with bortezomib, high-dose melphalan, and ascorbic acid in multiple myeloma (MM) patients [149]. A Phase II study to evaluate the efficacy and feasibility of a sequential treatment consisting of induction and consolidation with ATO followed by autologous hematopoietic cell transplantation for relapsed APL revealed that ATO demonstrates outstanding efficacy. Of the 23 patients who underwent autologous hematopoietic cell transplantation with PML-RARα-negative PBSC graft, posttransplant relapse occurred only in three patients, and there was no transplant-related mortality. The 5-year event-free and overall survival rates were 65 % and 77 %, respectively [150]. Phase I/II/III clinical trials using ATO mostly as combination therapy with other chemotherapy drugs are currently ongoing for CML and APL.

14.3.3 Autophagy Inhibitors

The knowledge that autophagy plays a role as a cell survival pathway in response to therapeutic and cellular stresses in the tumor microenvironment (which is highly acidic and hypoxic) implies that autophagy may work in favor of cancer cells. Therefore, inhibition of protective autophagy may break the resistance mechanism for survival of the harsh tumor microenvironment and lead to cell death [151]. Since autophagy activities are known to differ according to stages of cancer, modulation of autophagy is postulated to enhance the efficacy of anticancer therapy. In a preclinical study, effects of imatinib, with or without different types of autophagy inhibitors, on human malignant glioma cells were carried out [152]. It is demonstrated that suppression of imatinib-induced

autophagy by 3-methyladenine (3-MA) or siRNA against Atg5 (which inhibits autophagy at an early stage) attenuates the imatinib-induced cytotoxicity. On the other hand, inhibition of autophagy at a late stage by bafilomycin A1 or RTA 203 enhanced imatinib-induced cytotoxicity through the induction of apoptosis [152]. Thus, the authors have even suggested that therapeutic efficiency of imatinib for malignant glioma may be augmented by inhibition of autophagy at a late stage, which could help sensitize tumor cells to anticancer therapy [152].

The current autophagy inhibitors used in trials for human cancer are chloroquine (CQ) and hydroxychloroquine. Both drugs are widely used as antimalarials and have recently received attention as potential chemosensitizers in treating tumors when used in combination with cytotoxic chemotherapeutic agents [153–155]. CQ inhibits lysosomal acidification and therefore prevents autophagy by blocking autophagosome fusion and degradation [154, 156, 157]. A number of clinical trials are now revealing the promising role of CQ, an autophagy inhibitor, as a novel antitumor drug. For example, adding chloroquine to conventional treatment for GBM improves midterm survival of patients [158]. In a Phase I study involving patients with advanced NSCLC, hydroxychloroquine, with or without erlotinib, was safe and well tolerated, although the overall response rate was as low as 5 % [159]. Other trials on metastatic breast cancer, pancreatic cancer, RCC, NSCLC, and MM are currently ongoing. Table 14.1 summarizes the various drugs targeting the autophagy pathways and clinical trial stages based on published reports as well as ongoing trials listed in the NIH ClinicalTrials.gov website.

14.4 Mechanisms of Necroptosis

Necrosis is initially known as a passive and uncontrolled death process usually caused by physical or chemical insult. An irreversible drop in intracellular ATP and energy insufficiency lead to the morphological characteristics of organelle swelling, plasma membrane rupture, and spillage of cytoplasmic content [160, 161]. DNA in necrotic cells is usually degraded randomly, giving rise to a smear of DNA [162]. The cellular content leaks into the extracellular environment and is usually associated with inflammation [163, 164]. Release of cytokines and other factors from the necrotic cells and the secretion of pro-inflammatory cytokines from activated macrophages triggered by necrotic cells are thought to be responsible for the inflammatory response [165, 166]. Interestingly, in the past decade, studies have revealed necrosis as a form of regulated cell death, executed through a mechanism termed necroptosis or programmed necrosis [167, 168]. Necroptosis can be stimulated via a class of death receptors including TNFR1, TNFR2, TRAIL-R, and Fas. Upon binding to their agonists, these death receptors can induce cells toward either survival or death. Depending on the circumstances, the induction of cell death may be either apoptosis or necroptosis. However, the exact mechanisms that dictate the cellular decision to undergo apoptosis or necroptosis remained largely unknown.

TNF-α can be massively generated during hyperinflammatory shock, accumulated upon infection or produced primarily by macrophages. It induces apoptosis in many cells, while triggering necrosis in some [161, 169]. In necroptosis, TNF-α binds to the extracellular portion of the death receptors and triggers downstream signaling pathway by forming complex I with proteins containing a death domain, such as TNF-receptor-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIP1), and several E3 ubiquitin ligases, such as TNF-receptor-associated factor 2/5 (TRAF2/5), cIAP-1, and cIAP-2. Ubiquitination of these proteins is important for the regulation of the activity of complex I and impacts the outcome of the cell survival [170]. The ubiquitination and phosphorylation states of RIP1 determine whether it functions as a pro-survival molecule or a kinase promoting cell death. RIP1 is a member of the RIP family exhibiting a homologous N-terminal kinase domain and has recently emerged as an essential mediator of cellular stress and cell death. RIP1 is polyubiquitinated by TRAF2/5,

Table 14.1 Current therapeutic targets in the autophagy signaling pathways and clinical trial stages

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/ type of cancer	Combined with	References
mTOR signaling pathway	mTOR	Rapamycin (sirolimus)	Phase I: advanced NSCLC	Sunitinib	[106]
			Ongoing Phase I: NSCLC	BIBW 2992	http://clinicaltrials.gov/show/NCT00993499
			Ongoing Phase I: solid tumor	–	http://clinicaltrials.gov/show/NCT01331135
			Ongoing Phase II: hepatocellular carcinoma	–	http://clinicaltrials.gov/show/NCT01374750
		Temsirolimus (CCI-779)	Approved by FDA for advanced RCC (2007)	–	The US Food and Drug Administration[a]
			Phase I: melanoma	Sorafenib	[111]
			Phase I: advanced adrenocortical carcinoma	Cixutumumab	[109]
			Phase II: melanoma	Sorafenib	[110]
			Phase II: bone and soft tissue sarcoma	Cixutumumab	[108]
			Phase II: colorectal cancer	Irinotecan	[112]
			Ongoing Phase I: advanced cancer	Vemurafenib	http://clinicaltrials.gov/show/NCT01596140
			Ongoing Phase I: solid tumor	Erlotinib	http://clinicaltrials.gov/show/NCT00770263
			Ongoing Phase I: advanced cancer	Bevacizumab and valproic acid	http://clinicaltrials.gov/show/NCT01552434
			Ongoing Phase I: Hodgkin lymphoma	–	http://clinicaltrials.gov/show/NCT00838955
			Ongoing Phase II: diffuse large B-cell lymphoma	Rituximab, cytarabine, cisplatin, and dexamethasone	http://clinicaltrials.gov/show/NCT01653067
			Ongoing Phase II: head and neck cancer	–	http://clinicaltrials.gov/show/NCT01172769
		Everolimus (RAD001)	Approved by FDA for advanced RCC (2009), advanced pancreatic neuroendocrine tumor (2011), renal angiomyolipoma (2012), and hormone receptor-positive, HER2-negative breast cancer (2012)	–	The US Food and Drug Administration[b]
			Phase I: advanced NSCLC	Erlotinib	[116]
			Phase II: advanced breast cancer	Tamoxifen	[115]
			Phase II: advanced endometrial cancer	–	[114]
			Ongoing Phase II: advanced breast cancer	Exemestane	http://clinicaltrials.gov/show/NCT01783444
		Ridaforolimus (deforolimus; AP23573)	Phase II: hematologic malignancies	–	[120]
			Phase II: advanced bone and soft tissue sarcoma	–	[118]

(continued)

Table 14.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages (published reports)/ type of cancer	Combined with	References
	PI3K/mTOR	NVP-BEZ235 (BEZ235)	Ongoing Phase I: advanced solid tumor, breast cancer, or renal cell carcinoma	Everolimus	http://clinicaltrials.gov/show/NCT01482156
			Ongoing Phase I: advanced solid tumor	–	http://clinicaltrials.gov/show/NCT01343498
			Ongoing Phase I: acute leukemia	–	http://clinicaltrials.gov/show/NCT01756118
			Ongoing Phase I: breast cancer	Capecitabine	http://clinicaltrials.gov/show/NCT01300962
			Ongoing Phase I: advanced solid tumor	–	http://clinicaltrials.gov/show/NCT01195376
			Ongoing Phase Ib: castration-resistant prostate cancer	Abiraterone acetate	http://clinicaltrials.gov/show/NCT01634061
			Ongoing Phase I/II: castration-resistant prostate cancer	Abiraterone acetate and prednisone	http://clinicaltrials.gov/show/NCT01717898
			Ongoing Phase I/II: advanced solid tumor	Everolimus	http://clinicaltrials.gov/show/NCT01508104
			Ongoing Phase Ib/II: breast cancer	Paclitaxel	http://clinicaltrials.gov/show/NCT01495247
			Ongoing Phase II: advanced pancreatic neuroendocrine tumor	Everolimus	http://clinicaltrials.gov/show/NCT01628913
			Ongoing Phase II: perivascular epithelioid cell tumor	–	http://clinicaltrials.gov/show/NCT01690871
			Ongoing Phase II: advanced pancreatic neuroendocrine tumor	–	http://clinicaltrials.gov/show/NCT01658436
Pro-autophagics		Temozolomide	Approved by FDA for GBM (2005)	Radiotherapy	[125]
			Phase I: glioblastoma	Tipifarnib and radiotherapy	[137]
			Phase I: primary brain or spinal cord tumor	Vorinostat	[139]
			Phase I: solid tumor or brain tumor	Bevacizumab, vincristine, and irinotecan	[138]
			Phase I/Ib: glioblastoma	Lonafarnib	[136]
			Phase II: advanced NSCLC	–	[140]
			Phase II: GBM or gliosarcoma	Erlotinib and radiotherapy	[133]
			Phase II: glioblastoma	Bevacizumab	[134]
			Phase II: glioblastoma	–	[135]
			Ongoing Phase I: glioblastoma	BKM120	http://clinicaltrials.gov/show/NCT01473901
			Ongoing Phase I: acute leukemia	Veliparib	http://clinicaltrials.gov/show/NCT01139970
			Ongoing Phase I/II: melanoma	Doxycycline and ipilimumab	http://clinicaltrials.gov/show/NCT01590082
			Ongoing Phase I/II: melanoma	Decitabine and panobinostat	http://clinicaltrials.gov/show/NCT00925132

			Ongoing Phase II: hepatocellular carcinoma	Veliparib	http://clinicaltrials.gov/show/NCT01205828
			Ongoing Phase II: neuroblastoma	Bevacizumab and irinotecan	http://clinicaltrials.gov/show/NCT01114555
			Ongoing Phase II: breast cancer	Veliparib	http://clinicaltrials.gov/show/NCT01506609
			Ongoing Phase II: small cell lung cancer	Veliparib	http://clinicaltrials.gov/show/NCT01638546
			Ongoing Phase III: glioblastoma	Lomustine	http://clinicaltrials.gov/show/NCT01149109
			Ongoing Phase III: high-grade glioma	Interferon-α	http://clinicaltrials.gov/show/NCT01765088
		Arsenic trioxide	Phase I: malignant glioma	Temozolomide and radiotherapy	[148]
			Phase I: infiltrating astrocytomas of childhood	Radiotherapy	[147]
			Phase II: multiple myeloma	Bortezomib, melphalan, and ascorbic acid	[149]
			Phase II: APL	–	[150]
			Ongoing Phase I: CML	Tyrosine kinase inhibitors	http://clinicaltrials.gov/show/NCT01397734
			Ongoing Phase II: small cell lung cancer	–	http://clinicaltrials.gov/show/NCT01470248
			Ongoing Phase II: APL	Tretinoin	http://clinicaltrials.gov/show/NCT01404949
			Ongoing Phase II: APL	Gemtuzumab ozogamicin and ATRA	http://clinicaltrials.gov/show/NCT01409161
			Ongoing Phase III: APL	ATRA	http://clinicaltrials.gov/show/NCT00378365
			Ongoing Phase IV: APL	–	http://clinicaltrials.gov/show/NCT00504764
Autophagy inhibitors		Chloroquine	Ongoing Phase I: pancreatic cancer	Gemcitabine	http://clinicaltrials.gov/show/NCT01777477
			Ongoing Phase I/II: ductal carcinoma in situ	–	http://clinicaltrials.gov/show/NCT01023477
			Ongoing Phase II: multiple myeloma	Bortezomib and cyclophosphamide	http://clinicaltrials.gov/show/NCT01438177
			Ongoing Phase II: breast cancer	Taxane or taxane-like drugs	http://clinicaltrials.gov/show/NCT01446016
		Hydroxychloroquine	Phase I: advanced NSCLC	Erlotinib	[159]
			Ongoing Phase I/II: RCC	Everolimus	http://clinicaltrials.gov/show/NCT01510119
			Ongoing Phase I/II: NSCLC	Gefitinib	http://clinicaltrials.gov/show/NCT00809237
			Ongoing Phase I/II: pancreatic cancer	Gemcitabine	http://clinicaltrials.gov/show/NCT01506973
			Ongoing Phase I/II: colorectal cancer	FOLFOX and bevacizumab	http://clinicaltrials.gov/show/NCT01206530

[a]http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ucm129247.htm
[b]http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm317385.htm

cIAP-1, and cIAP-2 at the 63rd position of lysine (K63) [171, 172]. K63-linked ubiquitination of RIP1 by cIAP-1/cIAP-2 is known to inhibit TNF-α-induced apoptosis [173, 174].

Complex I is crucial for activating the NF-κB and mitogen-activated protein kinase (MAPK) pathways. cIAPs direct the formation of polyubiquitin chains on RIP1, allowing it to interact with TGF (transforming growth factor)-β-activated kinase 1/TAK-1-binding protein 2/3 (TAK1/TAB2/3) complex. TAK1 activates the IκB kinases (IKK) complex and, in turn, phosphorylates IκBα. When IκBα is degraded by the proteasome, it allows NF-κB to translocate to the nucleus and activate its target genes in a pro-survival manner. Inhibitors of NF-κB are known to facilitate TNF-α-induced necrotic cell death, suggesting that NF-κB suppresses the necrotic cell death pathway [175]. On the other hand, deubiquitination of RIP1 could inhibit NF-κB pathway, leading to cell death pathways. Cylindromatosis (CYLD) blocks the activation of NF-κB by cleaving K63-linked polyubiquitin chains, and its deubiquitinating activity on RIP1 facilitates the direct interaction of RIP1 with caspase-8 and initiation of cell death [176, 177]. Knockdown of CYLD inhibits TNF-α-induced necroptosis, which indicates that the deubiquitination of RIP1 is an important step in TNF-α-induced necroptosis [178]. CYLD also interacts directly with TRAF2, an adaptor molecule involved in signaling of the TNF/nerve growth factor family receptors. TRAF2, an E3 ligase, has been demonstrated to be essential for TNF-α-induced necroptosis, as TRAF2−/−cells are resistant to TNF-α-induced necroptosis [175].

When RIP1 is deubiquitilitized by CYLD, RIP1 can dissociate from complex I and is released into the cytoplasm, forming complex II with FADD, RIP3, and caspase-8. If the conditions are apoptotic competent, TNF-α stimulation induces the sequential protein complexes, complex I and complex IIa, leading to the activation of NF-κB and apoptosis, respectively [168, 179]. However, proteolytical cleavage of RIP1 by caspase-8 during TNF-induced apoptosis abolishes NF-κB activation and enhances pro-apoptotic signaling through the TRADD-FADD interaction [180]. Cleavage of RIP3 by caspase-8 induces caspase-dependent apoptosis [181]. However, if the apoptotic machinery is deficient or when the apoptosis pathway is blocked by pan-caspase inhibitors such as Z-VAD-FMK, or caspase-specific inhibitors such as cytokine response modifier A (CrmA) or in cells deficient in FADD or caspase-8, triggering TNFR1 results in necrosis [182–186]. This process involves the formation of complex IIb, consisting of mainly RIP1 and RIP3. It appears that FLICE-inhibitory protein (FLIP), together with caspase-8, is recruited to FADD and the formation of this complex is dominant for inhibiting apoptosis [187]. Cellular FLICE-inhibitory protein (c-FLIP) is known as a crucial inhibitor of death receptor-mediated apoptosis by interfering with caspase-8 activation at the death-inducing signaling complex (DISC) signaling [188]. Due to its structural similarity to caspase-8 and caspase-10, c-FLIP can bind to FADD and inhibit complete caspase-8 processing and activation [189]. However, the involvement of FADD, caspase-8, and FLIP in complex IIb remains unclear.

RIP3 has an N-terminal kinase domain and a C-terminus lacking a death domain or CARD motif. RIP3 binds RIP1 through this unique C-terminal segment to inhibit RIP1- and TNF-receptor-1-mediated NF-κB activation [190]. However, the interaction between RIP1 and RIP3 via the RIP homotypic interaction motif (RHIM) domain is required for necroptosis [191]. RHIMs of RIP1 and RIP3 mediate the assembly of heterodimeric functional amyloid signaling complex which is ultrastable [192]. Mutations in the RHIMs of RIP1 and RIP3 which render them defective in interactions compromise kinase activation and necroptosis *in vivo*, indicating the crucial role of RHIM in necroptosis [192]. Mutations of RHIMs in RIP1 or RIP3 block the formation of necrosomes and protect cells from necroptosis [190]. RIP3 acts upstream to phosphorylate RIP1, which in turn mediates downstream RIP3 phosphorylation. Phosphorylation of RIP3 is essential in necroptosis, but the exact mechanism remains unclear [191]. Both RIP3 and the kinase activity of RIP1 are essential for stable formation of the RIP1-RIP3 pro-necrotic complex, which critically controls downstream ROS production [191].

RIP3 is essential in necroptosis induced by various stimuli, and RIP3 knockdown leads to a notable inhibition of necroptosis [191, 193]. Cells with low levels of RIP3 expression are resistant to necroptosis, but transfection of these cells with the *RIP3* gene enables them to undergo necroptosis when the apoptotic pathway is blocked, clearly highlighting RIP3 as an essential mediator in TNF-α-induced necroptosis [194]. In addition to TNF-α, IFN-γ also induces an NF-κB-dependent transcriptional response that is cytoprotective. However, in mammalian cells deficient in NF-κB signaling, IFN-γ promotes mitochondrial ROS accumulation, loss of mitochondrial membrane potential, and necroptosis [195]. The necroptosis signaling pathway is illustrated in Fig. 14.2.

Necroptosis shows identical subcellular events with necrosis and secondary necrosis. The cellular disintegration phases are characterized by lysosomal membrane permeabilization, mitochondrial hyperpolarization, oxidative burst, and eventually plasma membrane permeabilization; however, the kinetics and timing may be different [196]. A number of events have been implicated and proposed to contribute to the downstream events in necroptosis. One important downstream event is the production of ROS which acts as an executioner of necroptosis [168]. The ROS is implicated to play an important role in necroptosis; in addition, RIP, TRAF2, and FADD are crucial in mediating ROS accumulation in TNF-induced necroptotic cell death [175]. This was based on the observation that in TNF-induced necroptosis, the cellular ROS level was significantly elevated in wild type, but not in $RIP^{(-/-)}$, $TRAF2^{(-/-)}$, and $FADD^{(-/-)}$ cells [175].

Interestingly, RIP3 has been reported to interact with several metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) [197]. PYGL plays a key role in using reserved glycogen as an energy source and catalyzes the rate-limiting step in the degradation of glycogen by releasing glucose-1-phosphate. On the other hand, GLUL is a cytosolic enzyme catalyzing the condensation of glutamate (Glu) and ammonia to form glutamine (Gln). Gln transfers into the mitochondria to function as an energy substrate. GLUD1 is a mitochondrial matrix enzyme that converts Glu to α-ketoglutarate. GLUL and GLUD1 are essential for the use of amino acid Glu or Gln as substrates for adenosine triphosphate (ATP) production by means of oxidative phosphorylation [197]. Taken together, these enzymes increase substrates for oxidative phosphorylation, which is a major source of ROS in the cell. RIP3-deficient cells have reduced ROS production downstream of TNF-α signaling [191]. Zhang and co-workers postulated that RIP3 activation of all these enzymes results in an increased energy metabolism and subsequent ROS production [197].

Nicotinamide adenine dinucleotide phosphate oxidases (NADPH) are a family of enzymes specifically important in ROS production and have been implicated in TNF-α-induced necroptosis. For example, TNF treatment induces the formation of a signaling complex containing TRADD, RIP1, Nox1, and the small GTPase Rac1. RIP1 is shown to be essential for Nox1 recruitment, and activation of Nox1 is implicated in ROS production [198]. Other NADPH oxidases, such as Nox1, Nox2, Nox3, and Nox4, are also shown to be upregulated in the presence of TNF-α [198–200]. In addition, riboflavin kinase (RFK), a TNFR1-binding protein, functionally couples TNFR1 to NADPH oxidase. RFK binds to both the TNFR1 death domain and $p22^{phox}$, the common subunit of NADPH oxidase isoforms and triggers TNF-induced ROS production [201]. Both RFK and the NADPH oxidases are found to be crucial for downstream ROS production [198, 201].

ROS are thought to act by oxidizing MAP kinase phosphatases (MKPs) whose normal function is to downregulate the c-Jun N-terminal kinase (JNK) signaling pathway [202]. Sustained JNK activation is required for Cyt *c* release and caspase-3 cleavage in apoptosis as well as necroptosis [202]. In necroptosis, JNK is activated in the downstream of RIP1 and TRAF2 [203, 204]. ROS and JNK appear to form a loop to enhance necroptosis as JNK also affects the mitochondrial function and produces ROS [205]. JNK activation is required for mitochondrial depolarization, AIF translocation, and subsequent cell death in PARP-1-hyperactivated cells [204].

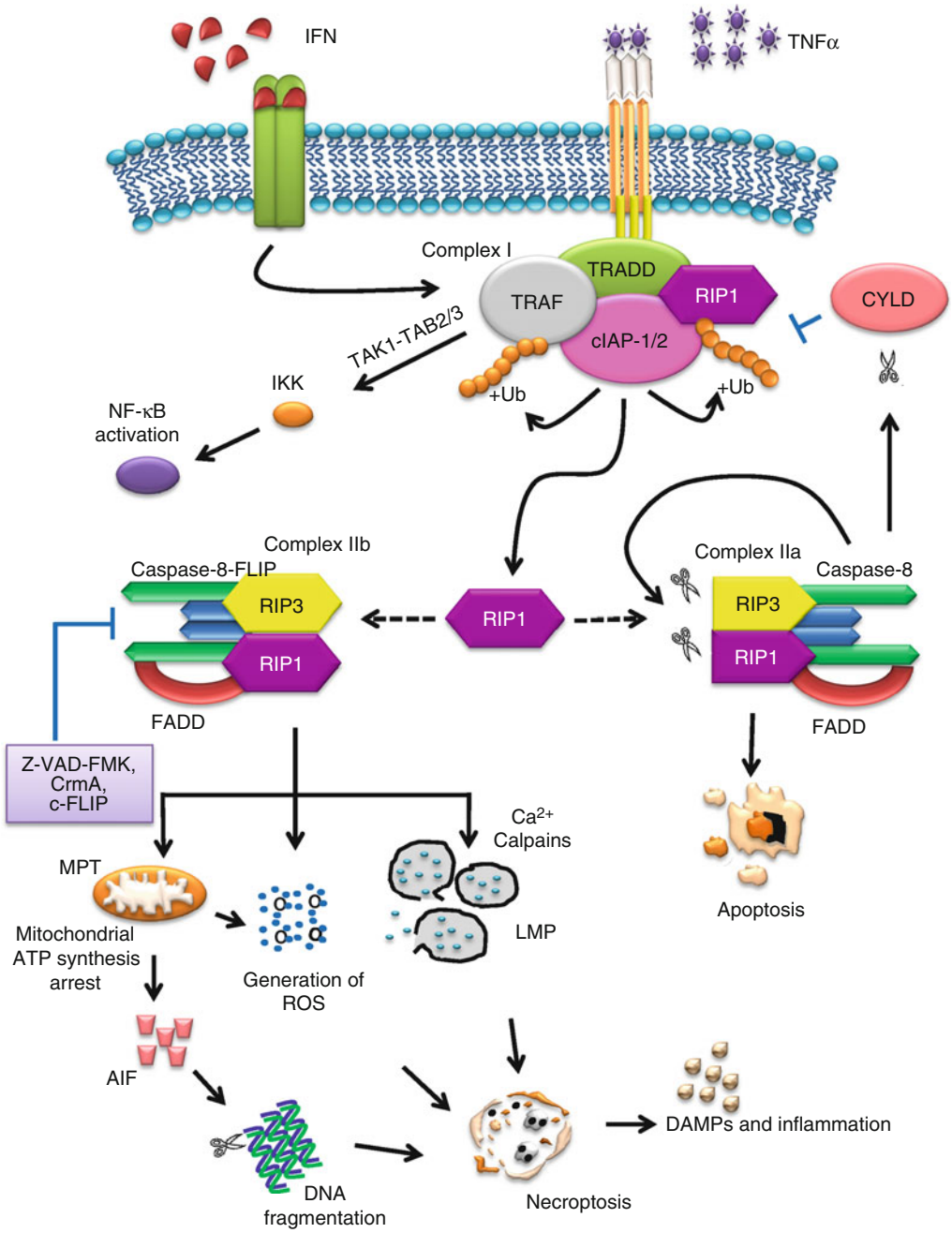

Fig. 14.2 Necroptosis signaling pathways

Activation of phospholipase A_2 (PLA_2) and lipoxygenase pathways are also known to contribute to the TNF-α-induced necrotic death [206]. Cytosolic PLA_2 ($cPLA_2$), a subfamily of PLA_2, is an intracellular enzyme that hydrolyzes arachidonate-containing phospholipids and facilitates the release of arachidonic acid. Arachidonic acid is the main substrate for lipoxygenase, which further catalyzes the conversion of the fatty acids into hydroperoxides [163]. Overexpression of

$cPLA_2$ sensitizes TNF-α-resistant cells to TNF-α-induced necrosis, emphasizing the role of $cPLA_2$ in necroptosis [207, 208]. Lipid peroxidation leading to disruption of organelle and plasma membranes are key features of necrosis.

The mitochondrion has been implicated downstream of RIP1. Mitochondrial synthesis of ATP requires ADP transport from cytosol into mitochondria by the inner mitochondrial membrane ADP/ATP carrier adenine nucleotide translocase (ANT) [209]. ADP/ATP exchange depends on transition between two conformational states of ANT. In the cytosolic state (c-state), the hydrophilic loop of the ANT nucleotide-binding site faces the cytosol, while in the matrix state (m-state), this binding site faces the matrix [209, 210]. The interaction of ANT with cyclophilin D (CYPD) and voltage-dependent anion channel (VDAC) is important in regulating the mitochondrial permeability transition pore (MPTP); in addition, CYPD is an important regulator of MPTP [211]. Z-VAD-FMK is found to block the ability of ANT to transport cytoplasmic ADP, thereby inducing a massive ATP depletion in the mitochondria [212]. The inhibition of ADP/ATP exchange coincides with the loss of interaction between ANT and CYPD as well as with the inability of ANT to adopt the cytosolic conformational state, which prevents Cyt *c* release and subsequently necroptosis [212].

The release of cytosolic Ca^{2+} and overactivation of calpains are also thought to play important roles in necroptosis. Yamashima and co-workers postulated that excessive Ca^{2+} overload leads to calpain-mediated lysosomal disruption with releases of cathepsins B and L [213]. Cathepsin B is shown to be involved in caspase-independent cell death induced by death receptor ligands [213]. Lysosomal membrane permeability (LMP) is associated with activation of PLA_2, causing the production of ROS [214]. LMP is also known to activate mitochondrial permeability transition (MPT), leading to cell death. The opening of MPTP accounts for the MPT resulting in disruption of the inner mitochondrial transmembrane potential ($\Delta\psi m$) during cell death [215–217]. Oxidative stress can serve as a facultative inducer of MPTP opening; moreover, ROS are potent inducers of MPTP opening [218–220]. The induction of MPT, which increases mitochondrial membrane permeability, causes the mitochondria to become further depolarized, resulting in the abolishment of $\Delta\psi m$ as well as allowing the release of ROS and other molecules such as AIF and necrotic danger-associated molecular patterns (DAMPs) into the cytosol.

AIF is a FAD-dependent oxidoreductase that has a vital role in oxidative phosphorylation [221]. After a caspase-independent cell death insult, AIF is cleaved by calpains and/or cathepsins to yield truncated AIF (tAIF), the pro-apoptotic AIF form (~57 kDa) [222, 223]. The tAIF relocates from the mitochondria to the cytosol and nucleus, where it associates with histone H2AX in the nucleus, through its C-terminal proline-rich-binding domain (PBD, residues 543–559). This interaction generates an active DNA-degrading complex with cyclophilin A, leading to chromatin condensation and DNA fragmentation, as observed in necroptotic cells [224].

Interestingly, necroptosis induced by high doses of the alkylating DNA-damaging agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is found to be regulated by the kinase RIP1 and executed by the activation of PARP-1, Ca^{2+}-dependent calpain Cys proteases, and the pro-apoptotic Bcl-2 member Bax [225]. MNNG treatment induces a PARP-1 hyperactivity that leads to calpain activation. Calpains generate tBid, which redistributes from the cytosol to mitochondria, where it regulates Bax activation. Once activated, Bax provokes mitochondrial damage and tAIF mitochondrial release. The tAIF relocalizes to the nucleus, associates with H2AX and cyclophilin A, and subsequently induces chromatinolysis [226]. PARP-1 is a nuclear enzyme activated by DNA strand breaks and plays a key role in repairing DNA damage. PARP-2, the closest homolog to PARP-1, has been identified as one of the essential regulators of necroptosis by a genome-wide siRNA screen study [178]. PARP activation is also found to play a critical role in glutamate-induced necroptosis [227]. The mechanisms of PARP-induced mitochondria dysfunction in necroptosis remain to be explored.

Necroptotic cells spill their contents which contain DAMPs. DAMPs can trigger inflammation by activating pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG-1)-like receptors [228]. DAMPs are intracellular molecules that have inflammation-inducing capacities when released from cells, resulting in the activation of macrophages and subsequently the inflammation processes [229].

14.5 Necroptosis and Possible Therapeutic Targets in Cancer

Necroptosis is found to occur during the early phases of T-cell clonal expansion, indicating that this mode of cell death may be involved in the regulation of the immune system [230]. In addition, virus-infected cells, which are resistant to apoptosis, are found to be highly sensitive to necroptosis, indicating that it may serve as an alternative mechanism of cell death other than apoptosis [231]. In acute lymphoblastic leukemia (ALL), necroptosis cell death can be induced to overcome the glucocorticoid resistance of ALL cells. Resistance to the initial phase of chemotherapy, in particular poor response to glucocorticoids, is a strong predictor of adverse outcome for childhood ALL. In a clinical study using primary leukemia cells from patients with very high-risk disease, obatoclax mesylate, a Bcl-2 antagonist, and rapamycin increased RIP1 activity and restored the response to dexamethasone by inducing a type of cell death morphologically consistent with necroptosis [232]. In addition, necroptosis appears to cause cancer cell death as a response to several anticancer treatment strategies, clearly indicating the role of this cell death in cancer [233–236].

Defects in necroptosis or variations of necroptosis-related genes may contribute to the pathological process of human malignancies, based on several observations and studies. Deubiquitination of RIP1 by CYLD is important for the formation of complex II, leading to either apoptosis or necroptosis. Tumors carrying the mutated CYLD(C/S) [catalytically inactive form of CYLD that mimics the identified mutations of CYLD in human tumors] exhibit a faster growth, are poorly differentiated, have robust angiogenesis activity, and are aggressive tumors [237]. Both cIAP-1 and cIAP-2 may promote cancer cell survival by functioning as E3 ubiquitin ligases that maintain constitutive ubiquitination of the RIP1 adaptor protein [174]. cIAP-1 and cIAP-2 directly ubiquitinate RIP1 and induce constitutive RIP1 ubiquitination in cancer cells which then associates with the pro-survival kinase TAK1 and suppresses apoptosis [174]. Immunohistochemical analysis of cIAP-1 and cIAP-2 in archival bladder specimens revealed that both cIAP-1 and cIAP-2 expression are significantly increased in bladder cancer compared with normal bladder urothelium. Nuclear cIAP-1 expression is strongly correlated to bladder cancer stage, tumor grade, and tumor recurrence suggesting the possibility of using cIAP-1 as a marker in bladder cancer prognosis [238]. Furthermore, X-IAP, cIAP-1, and cIAP-2 are found to be highly expressed in chronic lymphocytic leukemia samples [239–242]. Thus, the IAPs should be an attractive antitumor strategy. In addition, RIP1 polyubiquitination by TRAF2/TRAF5 at the position of K63 inhibits TNF-α-induced apoptosis [171, 172] and TRAF2 knockdown by siRNA radiosensitizes cancer cells via a reduced NF-κB activation, suggesting that TRAF2 may also be an attractive target for anticancer activity [243].

Necroptosis in cancer cells is induced by various approaches including administration of alkylating DNA-damaging agents [244] and the application of photodynamic therapy through which photosensitizing compounds accumulated in tumor cells generate ROS following excitation with light from various spectra [245, 246]. Necroptosis induction is speculated to be useful in cancers which are resistant to the apoptotic effects of chemotherapy. One preclinical study on shikonin, a naturally occurring naphthoquinone, has demonstrated that induction of necroptosis by the compound is able to overcome resistance to cancer drugs mediated by P-glycoprotein, Bcl-2, and Bcl-X_L in cancer cell lines [236]. All these results indicate that

necroptosis could be a potent therapeutic strategy for the treatment of cancer. The further exploration of the necroptosis signaling pathway will be important to identify strategies and novel antitumor drugs which can be brought forward to the human clinical trials.

14.6 Crosstalk in Apoptosis, Autophagy, and Necroptosis

Functional relationships between apoptosis and autophagy are gaining much interest, as both cell deaths are not mutually exclusive. Perturbations in the apoptotic machinery, such as caspase inhibition, have been reported to induce both autophagic cell death and necroptosis [247, 248]. Inhibition of autophagy in cancer cells results in an accelerated cell death that manifests the hallmarks of apoptosis including chromatin condensation, MOMP, and activation of caspases [249]. In some cases, mixed phenotypes of both autophagy and apoptosis are detected in response to common stimuli [156, 249]. Studies in a variety of experimental systems indicate that autophagy cell death is likely to be cell type dependent. Autophagy can delay the onset of apoptosis, following starvation, DNA damage, and hemodynamic stress [13]. For example, 1-day fasting causes liver autophagy in rats, but when starvation is prolonged for a few days, hepatocytes succumb to apoptosis [250]. Similarly, hematopoietic cell lines withdrawn from growth factor first activate autophagy and eventually apoptosis [7]. Studies have also demonstrated that certain compounds have the ability to trigger both apoptosis and autophagy cell deaths simultaneously in cancer cells [251, 252]. Blocking of one pathway will trigger the activation of another [253]. Researchers have also hypothesized that there are factors (either external or internal) that may affect the preferential shunting into either biochemical cascades that will ultimately result in either apoptosis or autophagic cell death [254].

Crosstalks between autophagy and apoptosis exist at multiple levels because both pathways share mediators and pathway regulators. Several signals and pathways involved in autophagy are in common with apoptosis. Starvation and oxidative stress can trigger both apoptosis and autophagy. Bcl-2 proteins function to inhibit both apoptosis and autophagy, providing another clue to the interplay between both processes. Beclin-1, the essential autophagy protein and haploinsufficient tumor suppressor, interacts with several cofactors such as Ambra1, Bif-1, and UVRAG to activate the lipid kinase Class III PI3K and induce autophagy [255]. In normal conditions, Beclin-1 is bound to and inhibited by Bcl-2 or the Bcl-2 homolog Bcl-X_L, well-characterized apoptosis regulators, which involve an interaction between the BH3 domain in Beclin-1 and the BH3-binding groove of Bcl-2/Bcl-X_L. BH3-only proteins can competitively disrupt the interaction between Beclin-1 and Bcl-2/Bcl-X_L to induce autophagy. Nutrient starvation can stimulate the dissociation of Beclin-1 from its inhibitors, either by activating BH3-only proteins (such as Bad) or by posttranslational modifications of Bcl-2 (such as phosphorylation) that may reduce its affinity for Beclin-1 and BH3-only proteins [255]. Anti-apoptotic Bcl-2 family members participate in the inhibition of autophagy, whereas the pro-apoptotic BH3-only proteins participate in the induction of autophagy.

A recent finding suggests a link between autophagy and the extrinsic apoptotic pathway mediated by p62/SQSTM1. Autophagy is recently known to be responsible in selective degradation of polyubiquitinated proteins via sequestosome-1 (SQSTM1), which encodes for p62 protein. p62 interacts with LC3 via its LC3 interacting region (LIR). Recent studies indicate that p62 is recruited to damaged mitochondria via binding to ubiquitinated outer mitochondrial membrane proteins, suggesting that p62 may serve as an autophagy receptor for ubiquitinated proteins and damaged mitochondria [256–258]. In addition to its role in autophagy, p62 mediates a cell's decision to undergo apoptosis or survival through its organization of signaling complexes in the cytoplasm [257, 259, 260]. Upon cytokine stimulation, p62 activates the NF-κB pathway, which subsequently induces the pro-survival genes, such as anti-apoptotic and cell proliferation genes and induces the

expression of inflammatory genes such as cytokines, chemokines, and adhesion molecules [260–263]. However, p62 is also found to activate caspase-8 in the extrinsic apoptosis pathway, resulting in the initiation of apoptosis and cell death [259].

The expression of Ptc induces apoptosis, but this activity is suppressed by its ligand, sonic hedgehog (Shh). Interestingly, hedgehog inhibition is found to induce autophagy through upregulation of Bnip3 and is also found to increase apoptosis in hepatocellular carcinoma cells at the same time [264]. In a very recent study, apoptosis suppressed by the knocking down of PP2A can be reversed by the administration of 3-MA, a known autophagy inhibitor. The elevated accumulation of LC3-II and the decline of the autophagy substrate p62 are also observed in PP2Ac-small interfering RNA transfected cells. However, overexpression of *PP2Ac* suppresses the accumulation of LC3-II and restores p62 [265]. Interestingly, 3-MA increases cell death induced by diamindichloridoplatin (DDP), which suggests the protective function of autophagy in DDP-induced cell death [265].

The relationship between autophagy and necroptosis is said to be complex, at least at this point of time. There is increasing evidence suggesting that necroptosis is associated with autophagy [232], is suppressed by autophagy [266], or is not associated with autophagy at all [230]. For example, in ALL cells, reversal of glucocorticoid resistance occurred through rapid activation of autophagy-dependent necroptosis, and the effect was associated with dissociation of the autophagy inducer Beclin-1 from the anti-apoptotic Bcl-2 family member Mcl-1, as well as a marked decrease in mTOR activity. Combination of rapamycin with the glucocorticoid dexamethasone triggered autophagy-dependent cell death, with characteristic features of necroptosis [232].

In addition, necroptosis signaling appears to activate autophagy process as a cleanup mechanism for cell death. Experiments using proliferating T cells have shown that caspase-8-deficient T cells exhibit RIP1-dependent necroptosis [267, 268]. On the other hand, caspase-8 is known to inhibit autophagy [269], probably through direct cleavage of RIP1 [180]. Cleavage of RIP1 by active caspase-8 constitutes a negative feedback loop to limit autophagic induction. In T cells, autophagy is induced in response to energetic demands, resulting in formation of a DISC-like complex including Atg5–12/Atg16L, FADD, caspase-8, and RIP1. In addition, blocking of necroptosis by necroptosis inhibitor necrostatin-1 (Nec-1) was sufficient to rescue the hyperactive autophagy and restored the cell cycle profile and survival capacity of actively dividing FADDdd (cells expressing a dominantly interfering form of FADD) and caspase-$8^{-/-}$ T cells. When autophagy is inhibited with 3-MA, Nec-1 reduces LC3 processing, suggesting that RIP1-dependent necroptotic signaling, or perhaps necroptosis itself, promotes autophagy. Since autophagy is directly induced by RIP1 activity, RIP1 may influence autophagic signaling either directly or perhaps indirectly as a response to necroptotic stress [267, 269]. While autophagy is necessary for rapid T-cell proliferation, studies suggest that FADD and caspase-8 form a feedback loop to limit autophagy and prevent this salvage pathway from inducing RIP1-dependent necroptosis. Thus, the linkage of FADD and caspase-8 to autophagic signaling intermediates is essential for rapid T-cell clonal expansion and may serve to promote caspase-dependent apoptosis under hyperautophagic conditions, thereby averting necroptosis and inflammation *in vivo* [267].

However, other reports tend to demonstrate that inhibition of autophagy promotes necroptosis in various human cancer cells. For example, TNF-α significantly induces necroptosis and autophagy in murine fibrosarcoma L929 cells. Nec-1 completely blocks TNF-α-induced necroptosis and autophagy, but inhibition of autophagy with 3-MA or Beclin-1 siRNA promotes necroptosis, indicating that autophagy acts as a negative regulator of TNF-α-induced necroptosis [270]. In other studies, T-cell receptor-induced necroptosis is found to be death receptor and autophagy independent, indicating the existence of an alternate RIP1-dependent necroptotic pathway downstream of T-cell receptor signaling [230]. The molecular link between necroptosis and autophagy remains elusive.

14.7 Future Directions

There is increasing evidence that the three major cell deaths, i.e., apoptosis, necroptosis, and autophagic cell death, share overlapping molecular pathways and can occur in parallel under similar conditions. Fundamental knowledge in apoptosis, necroptosis, and autophagy has also generated a great deal of insight into the pathogenesis of cancer and has provided important considerations in strategizing cancer pharmacotherapy. Much effort and investment has been devoted to experimental drugs modulating autophagy or apoptosis, and scientists are beginning to look at necroptosis in a different light. A number of drugs have proven to be promising during preclinical studies and experimental anticancer therapies, but these drugs appear to be effective in one type of cancer and not the other. The percentage of patients who totally responded or partially responded to these treatments, either as single-agent or in combination therapies, is relatively low, even though the outcome of these trials suggests some potential. These unforeseen effects are probably due to the specific-targeted nature of the therapy, in addition to the interconnected relationships between these cell death pathways. The contradictory role of autophagy and the status of autophagy in the human tumors concerned remain speculative and further complicate the response to conventional anticancer treatment.

Thus, modulating apoptosis, necroptosis, and autophagy by various means may be an important strategy to fight against the disease. Cancers, which are resistant to the apoptotic effects of certain chemotherapy drugs, may be sensitive to drugs that evoke necroptosis or autophagic cell deaths. An intact autophagy pathway has a role in promoting carcinogenesis as well as suppressing it. It also has a role in the development of resistance to treatment. Therefore, if autophagy response and activity are normal in tumors, combining standard chemotherapy drugs with autophagy inhibitors may sensitize tumor cells to anticancer agents. Cancer cells which present defects in the autophagy pathway may be managed by replacement of autophagy-inducing signals, e.g., pro-autophagics, or by inhibiting mTOR kinase. In some other cases, utilizing both autophagy and apoptosis inducers may present a deadly strategy against highly resistant tumors. Devising personalized pharmacotherapeutic strategy based on the autophagy status of the tumors has become an attractive option and offers significant potential to be translated into the clinic.

So far, targeted drugs like oblimersen, bortezomib, and mTOR inhibitors such as everolimus and ridaforolimus have shown to be useful in some clinical trials. These novel classes of drugs appear to work synergistically in combination with other chemotherapeutics and have also showed specific activities against certain cancers. Since these drugs are specifically targeted against certain molecules or receptors in the pathway, further unveiling of the tumor's characteristics such as receptor or protein status may be critical in assessing patient's response and clinical trial success. Furthermore, a number of known genes that play a role in these cell death pathways are either activated or inactivated in several cancers. This will certainly affect not only the promotion and progression of cancer but also their response to treatment. Therefore, to optimize and personalize treatment strategies, the genetic profile of the tumors is important. This may provide information on the optimal point in the pathway to be targeted and can be identified as prognostic markers. At the same time, the development of both robust tissue markers and relevant techniques that can be used in the clinical context needs to occur along with novel treatments, which will be another challenge.

14.8 Concluding Remarks

Although recent studies have incorporated some predictive biomarkers by examining tumor status, the utility of such practice remains non-conclusive. For example, the expression of peptidyl O-glycosyltransferase GaLNT14 has been proposed to be a potential marker of dulanermin or Apo2L/TRAIL activity in NSCLC as high GaLNT14 mRNA and protein expression in tumor cell lines are associated with Apo2L/TRAIL sensitivity [271]. An increase in progression-free survival and overall survival was

observed in GaLNT14-positive patients with advanced NSCLC in the dulanermin arm, indicating the potential predictive response biomarker for Apo2L/TRAIL-based cancer therapy [272]. On the other hand, in a Phase Ib/II trial on mapatumumab, a humanized mAb against TRAIL-R1, strong expression of TRAIL-R1 (indicated by immunohistochemical staining), did not appear to be a prerequisite for the effectiveness of mapatumumab in patients with replased or refractory follicular lymphoma [273]. Noteworthy, in the two patients who experienced a partial or complete response, the TRAIL-R1 staining was either undetected or weak [273]. However, this could be an isolated case, and trials with bigger sample size should be carried out. Tumor profiling would be a good strategy to identify patients who may respond to the relevant treatment.

Fundamental knowledge of cell death pathways remain an area of major interest among scientists in the field of cancer. More studies to characterize these pathways and identify potential targets and further evaluation of the efficacy of the current drugs in various cancers are certainly warranted.

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Prognostic Value of Innate and Adaptive Immunity in Cancers

15

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15.1 Introduction

It is now accepted that human carcinogenesis is a dynamic process depending on multiple variables and is regulated at multiple spatial and temporal scales [1–4]. According to the theory of multistep carcinogenesis, cancer cells accumulate a number of molecular changes to eventually become fully malignant. The "reductionist" view of cancer expressed in myriads of molecular biology-based investigations stated that all the information necessary for a cell to transform itself into a neoplastic cell can be attributed to changes at the genomic level [5]. This "certainty" is based on the fact that the genome carries all of the information related to any cell process and that any cellular transformation is due to a specific genomic change [6]. Today, cancer is recognized as a highly heterogeneous disease: more than 100 distinct types of human cancer have been described, and various tumor subtypes can be found within specific organs. In addition, tumors have somatic mutations and epigenetic changes, many of which are specific to the individual neoplasm [7]. It is now recognized that this genetic and phenotypical variability primarily determines the self-progressive growth, invasiveness, and metastatic potential of neoplastic disease and its response or resistance to therapy. It seems that the multilevel complexity of cancer explains the clinical diversity of histologically similar neoplasia [8, 9]. In simple mathematical terms, carcinogenesis is a nonlinear process, and the behavior

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of which does not follow clearly predictable and repeatable pathways. In linear systems, the behavior of a system changes linearly in response to an environmental factor. In contrast, the behavior of nonlinear complex systems may be perceived as surprising and unpredictable. Periods of inactivity may be punctuated by sudden change, apparent patterns of behavior may disappear, and new patterns may unexpectedly emerge [2]. Moreover, nonlinear systems do not react proportionally to the magnitude of their inputs and depend on their initial conditions, i.e., small changes in the initial conditions may generate significantly different end points. These characteristics are commonly highlighted by the frequency with which differences in progression or therapeutic response are seen in the same tumor type and by the fact that cancer morphology does not always reveal a similar underlying biology [10]. It is now ascertained that tumors grow in a complex network of epithelial, mesenchymal, inflammatory, and immune cells, as well as vascular and lymphatic vessels [11–13]. Neoplastic cells take advantage from their surrounding microenvironment, as they are supplied by nutrients supplied by the blood stream and growth factors produced by inflammatory and stromal cells, in addition to fighting for space to expand and escape the immune attack [14]. When tumor cells metastasize to distant organs, the same crosstalk is established at the new site. Therefore, these complex interactions determine the overall tumor aggressiveness and the clinical outcome.

15.2 Immune Infiltration as a Major Player of the Tumor Microenvironment

Among the various factors influencing tumor establishment, growth, local invasion, and metastasis, the impact of immunity has been debated for a long time [15]. While inflammation is known to contribute to cancer progression [16], the immune system is programmed to recognize tumors from their inception. Immunosurveillance against the tumor is stimulated by the presence of tumor-associated antigens (TAA) and by stress-induced molecules. However, only recent murine models have unraveled the role of the immune system in cancer progression, a process termed cancer immunoediting [17]. Immunoediting is a dynamic process composed of three phases: first, the elimination of tumor cells by immunosurveillance; then an equilibrium phase, during which the tumor is subjected to immune-mediated latency, and the immune system is in balance with the tumor; and the last phase, during which tumor cells escape immune restraints and co-opt the immune system to promote malignancy. Tumor cells employ diverse mechanisms to escape from immunosurveillance, as well as to manipulate the immune system and their microenvironment in order to facilitate the development of a malignant phenotype. These include mechanisms that promote escape, such as the downregulation of TAA and the decrease in expression/secretion of proinflammatory cytokines, as well as mechanisms that induce immune suppression, such as the production of immunosuppressive cytokines, metabolites, and immune checkpoint molecules. Immunoediting enables tumor cells to evade immune system detection, disseminate from the initial niche, survive in the circulation, and settle at new metastatic sites.

Histopathological analyses of solid tumors reveal that they are infiltrated by cells of the innate and adaptive immunity [18–20]. Macrophages represent a significant portion of the tumor mass, where they are commonly termed tumor-associated macrophages (TAMs) [21]. These cells are generated from blood monocytes [22], which differentiate into two distinct macrophage types, identified as M1 (or classically activated) and M2 (or alternatively activated). M1- and M2-polarized macrophages are endowed with opposite functional roles in terms of tumor suppression and immune stimulation, M1 cells enhance immune responses, and restrain tumor progression through eliciting the Toll-Like Receptor (TLR) pathway, whereas M2 macrophages switch-off the immune system and promote tumor development. Mast cells, myeloid-derived suppressor cells (i.e., the most abundant type of hematopoietic cells in the immune system) [23] and neutrophils [24] have

also been reported to invade the intra-tumoral space. Dendritic cells (DCs) are found in different locations within a tumor, most immature Langerhans cell-type DCs home in the tumor nests, and are tightly linked to malignant cells, whereas both immature interstitial DCs and plasmacytoid DCs are located in the stroma [25]. Mature DCs concentrate in lymphoid islets adjacent to the tumor nests and some draining lymph nodes. NK cells are usually found in the stroma of most tumors [26, 27] but can also be found in close contact with tumor cells in renal cell carcinoma. The distribution of lymphocytes may be differently orchestrated depending on the tumoral architecture [28]. T lymphocytes are mainly located in the core, often referred to as the center of the tumor, its invasive margin and in adjacent lymphoid islets. Among T lymphocytes, most have a memory phenotype, with naïve cells being found mostly in adjacent lymphoid aggregates [29]. Some $CD8^+$ T lymphocytes contact malignant cells, whereas others are dispersed in the stromal compartment. Forkhead/winged helix transcription factor $(FoxP3)^+$ T lymphocytes, T lymphocytes helper 17 (Th17), T follicular helper (TFH) cells, and B lymphocytes concentrate in the stromal tissue and in lymphoid islets. A similar organization is found in metastatic sites, as in the primary tumors; however, their organization may vary among tumors and between patients. Significant correlations between the level of immune cell infiltration in tumors and their clinical outcome have been investigated in several cancers of unrelated histological origin [30–33]. A strong lymphocytic infiltration is found to be associated with good clinical outcome in different tumor types and subtypes, including melanoma, head and neck, breast, bladder, ovarian, colorectal, renal, prostatic, and lung cancer [33–35, 30, 36, 31, 37, 38]. The role of other T lymphocyte infiltrates has also yielded apparently contradictory results. It is reported that Th17 cell infiltration is associated with poor prognosis in colorectal, lung, and hepatocellular carcinoma, whereas it is considered as a predictor of better survival in some esophageal and gastric cancers. The effect of intra-tumoral B lymphocytes in cancer is far from clear; B cells have recently been appreciated as paracrine mediators of solid tumor development [39]. However, their capability to enhance T cell activation might have a positive impact on the organization of the antitumor immune response [40]. Here, the roles played by innate and adaptive immune system in the local progression and metastasis of human cancers of unrelated histologic origin are discussed; in addition their prognostic roles understood and exploited to date are pointed out.

15.3 Cellular Players of the Innate Immunity in Cancer

Rudolf Virchow (1821–1902) observed infiltrating leukocytes in tumors for the first time and proposed the inflammatory microenvironment as a primary site of cancer occurrence [41]. Later, epidemiological and experimental studies have associated chronic infections to about 15–20 % of tumors [42, 43] and linked inflammation to tumorigenesis by modulation of a variety of complex processes, including the increased cell proliferation, rate of mutagenesis, angiogenesis, and inhibition of apoptosis. Therefore, inflammation has been acknowledged as a critical element in cancer occurrence and has been included as a new "hallmark of cancer" [16].

15.3.1 Tumor-Associated Macrophages (TAM)

A number of studies appraised tumor-associated macrophages (TAM) as crucial mediators of the connection between inflammation and cancer occurrence [44, 45]. TAMs secrete a plethora of cytokines and chemokine, which are the soluble mediators of inflammation and are mainly responsible to mediate such processes [46]. It is widely accepted that in the majority of cancers TAMs have a pro-tumoral effect [47]. However, these cells are intrinsically "plastic" in their functions, and they were shown to acquire antagonistic properties ranging from immunosuppressive to immune-stimulatory properties in the complexity

of tumor microenvironment. While the antitumor role of TAM has been previously linked to the orchestration of T lymphocyte antitumor immune response, recent findings have shown that tumor immunosurveillance can be firmly directed by TAMs when "educated" by specific treatments, in a T cell independent fashion [48]. The functional plasticity of macrophages is regulated by environmental stimuli, thus their immune profile results in the identification of two distinct polarized functions, schematically simplified as M1/M2 classification. Macrophages are recruited at peripheral sites by locally secreted chemotactic factors and cytokines, including inflammatory chemokines and growth factors [i.e., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and macrophage colony-stimulating factor (M-CSF)] [49]. These cytokines can also promote macrophage survival and polarization. Although mobilization of the circulating pool of monocytes is the main mechanism of macrophage recruitment, local proliferation can contribute to macrophage accumulation at the tumor site [50]. In the tumor context, both tumor and stromal cells secrete a variety of chemoattractants for blood-circulating monocytes, including CCL-2, originally discovered as a tumor-derived chemotactic factor [51]. Molecular profiling analyses of both human and murine TAMs have evidenced a profile closer to that of M2 macrophages [52, 53], whose remodeling, immunosuppressive activities, and production of trophic factors for tumor and stromal cells functionally correlate to important pro-tumor activities [54], including proteolytic activity [55], remodeling of the extracellular matrix [56], and induction of angiogenesis [57]. Liu et al. have shown that M2-polarized TAMs increased fibroblastic morphology, upregulated mesenchymal markers (i.e., vimentin and Snail) at the mRNA and protein levels, and increased proliferation, migration, and metalloproteinase MMP2 and MMP9 proteolytic activity in pancreatic cancer cells [58]. In addition, it has been shown that the MMP-9 inhibitor is associated with decreased survival in breast cancer [59]. Leifler et al. identified MMP-9 as a potent player in modulating the innate immune response into antitumor activities [59]. Notably, TAMs exert their pro-tumor functions both directly, by acting on tumor cells, and indirectly, by orchestrating suppression of the adaptive immune response. Macrophages, when adequately activated, have the capability to both directly kill tumor cells [60, 61], a property mediated by contact-dependent [62] as well as independent mechanisms [48], and to orchestrate an antitumor adaptive immune response, through the activation of cytotoxic lymphocytes.

15.3.2 Tumor-Associated Neutrophils (TAN)

Although TAMs are the most prevalent innate cellular components of the tumor microenvironment, the role of tumor-associated neutrophils (TANs) on tumor progression has been reconsidered [63, 64]. Accordingly, TANs have been recognized as a source of cytokines and chemokine, as well as anti-inflammatory mediators in different settings, thus likely to mediate a dual effect on tumor progression depending on their polarization state, i.e., N1 and N2 [65, 66]. TAMs and TANs functional polarization and prognostic value reflect the intrinsic plasticity as it varies along with the tumor type, location in the tumor tissue (i.e., necrotic and hypoxic areas), and the tumor stage. Studies have demonstrated specific examples of tumor-mediated signals (such as transforming growth factor-β, TGF-β) that induce the formation of a pro-tumorigenic N2 phenotype capable of supporting tumor growth and suppressing the antitumor immune response. However, there are evidences showing that TAN can also have an anti-tumorigenic N1 phenotype [67].

15.4 Cellular Players of the Adaptive Immunity in Cancer

It has been accepted that immune cells infiltrate the tumor stroma and are essential players of the tumor microenvironment. Cells of the adaptive immune system are mainly represented by $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$

T-helper lymphocytes. The main function of $CD4^+$ T lymphocytes is to sustain activation of other cells, including macrophages, B cells, and CTLs, by the release of several cytokines, such as interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and interferon gamma (INF-γ). Identification and specific elimination of tumor cells are mediated by CTLs $CD8^+$ T cells [68, 69], which produce perforin and granzyme B [70]. Antigen (Ag) recognition by lymphocytes after the first encounter is kept at a higher activation level compared to baseline. Activated T lymphocytes have a long life, are more reactive to stimulation than naïve T lymphocytes, and are detectable by specific surface molecules, suggesting that their presence in the context of solid tumors has important implications. Accordingly, antigen-experienced CTLs phenotypically switch CD45 isoform from CD45RA to CD45RO when activated [71].

T lymphocyte activation is also modulated by a subpopulation of T lymphocytes indicated as Tregs, which suppress immune responses [72]. The transcription factor FOXP3 is a specific Treg cell marker [73, 72]. Treg lymphocytes include different subpopulations, although the most investigated are $CD4^+$ $CD25^+$ [72, 74]. However, these markers are not completely specific for Tregs as CD25 and FOXP3 might also be expressed by activated CTLs [71]. Moreover, it is not clear whether regulatory cells are capable to suppress T lymphocytes with tumor antigen specificity. The identification and targeting of Tregs selectively suppressing tumor-specific T cells would avoid unwanted depletion of regulatory cells involved in peripheral immune regulation and generation of autoimmunity. Tregs may exert different functions according to the tumor contexture, i.e., they might block antitumor immunity or decrease chronic pro-tumor inflammation [71].

In the clinical setting of some human cancers, lymphocytic reaction can comprise different components beside dispersed tumor infiltrating lymphocytes (TILs) and include discrete "lymphoid aggregates," resembling lymph-node-like structures. These aggregates are similar to those observed in chronic inflammatory conditions, where tissues harboring target Ags are infiltrated by cellular effectors of the adaptive immune system, which are organized anatomically and functionally as in secondary lymphoid organs, with recruitment of B cells and T cells, follicular dendritic cells with germinal centers, and specialized vessels suited to mediate traffic of immune cells [75, 76]. Those structures are named tertiary lymphoid tissue (TLT) and might be involved in the organization of the immune response. Few studies have reported the presence of TLTs in cancer [77, 78]. Moreover, the concept of ectopic lymphoid structures within solid tumors has only recently become appreciated, and it is still unclear whether these structures retain functional immune activities to mediate recruitment and activation of TILs.

15.5 Prognostic Value of Innate and Adaptive Cells of the Immune System in Cancer

The stromal compartment of solid tumors is infiltrated by immune and inflammatory cells expressing a wide array of specific markers and exerting critical effects on tumor outcome depending on their specific subset, density, spatial location [79], and the staging of tumor at diagnosis [80–82]. It is widely accepted that in preclinical studies cellular mediators of the innate immunity favor tumor progression [16, 54, 83]. Accordingly, the quantification of the number of $CD68^+$ TAMs was linked to a poor prognosis in pancreatic cancer and Hodgkin lymphoma [84, 85]. In the case of pancreatic cancer, expression of M1 markers of macrophage polarization was associated with better prognosis, while M2 markers were linked to worst prognosis [85]. In lung cancer, $IL10^+$-$CD68^+$ TAMs were associated with worst prognosis in patients with late-stage disease at diagnosis [86], while in a subsequent study a high ratio of M1/M2 macrophages was a feature of patients with good outcome [87]. Thus, according to the simplified view of macrophage polarization provided by Mantovani et al., in clinical studies macrophages infiltrate tumor nest

as a heterogeneous population, which seem to retain different functional and molecular properties that may vary according to the instructions provided by the tumor milieu. On the contrary, a meaningful correlation between high number of TAMs and better prognosis has been described in colorectal cancer [62, 88]; in addition, this correlation held true regardless of TAM polarization in another study [89]. Discrepancies among clinical studies on prognostic abilities of innate immune cells underline the importance of the tumor type when trying to determine the influence of TAMs on tumor progression. Further clinical data are warranted to determine whether the effect of TAM differs along tumor progression, as well as in response to chemotherapy treatments in a clinical relevant scenario. Several retrospective clinical studies on colorectal, melanoma, ovarian, breast, and non-small-cell lung tumors have generally underlined tumor infiltration of the adaptive immune cells as a prognostic indicator of good prognosis [90–92, 79, 93, 77, 94]. Variability with respect to prognostic potential of the markers employed relies on the specific population of T lymphocytes and the type of tumor settings investigated. In this view, colorectal cancer represents a paradigm since its milieu is highly permeated by adaptive immune cells with potential antitumor abilities. A seminal paper by Galon et al. claimed that concomitant local infiltration of $CD3^+$ lymphocytes at the tumor invasive margin and in the intra-tumoral location was a better predictor of survival compared to the tumor-node-metastasis (TNM) staging system [79]. However, TNM is still the gold standard predictor of CRC patient prognosis, while TILs have not been employed in clinical practice to date. A subsequent study by Laghi et al. raised doubts on previous claim and showed that while $CD3^+$ T-infiltrating lymphocytes (TILs) were not independent from TNM staging in predicting patient's prognosis, TILs were a strong prognostic factor only among lymph-node-negative but not among lymph-node-positive CRCs [80]. Later Mlecnik et al. showed that an immune score was re-proposed, although represented by partly overlapping subpopulations of TILs (i.e., $CD8^+$ and $CD45RO^+$), which had to be concomitantly located at the tumor invasive margin and intra-tumoral region in each CRCs specimen [95]. By these means, these immune features identified a benchmarking population with a dismal prognosis and devoid of TILs representing only 6.5 % of the CRCs (stages I–III) [95]. This strategy fostered statistical analysis, but might not provide proper clinical prognostic relevance when addressing surveillance strategies and allocation to chemotherapy in the overall population of CRC. The biological relevance of tumor lymph node infiltration in the context of TILs prognostic abilities was previously shown in ovarian cancer in a study suggesting a negative interaction of nodal status with antitumor immunity [81]. In CRC, the density of activated $CD8^+$ TILs decreased in patients with metastatic lymph nodes and advanced tumor staging, suggesting that immune escape might occur along CRC disease progression [96]. Accordingly, in a different study, the expression of eumesodermin, a transcription factor critically involved in the production of perforin, was inversely associated with tumor lymph node involvement [97]. In melanoma, these observations were supported by the fact that a primary tumor devoid of TILs was shown to predict sentinel lymph node metastasis. These studies underline that the plasticity of TILs with regard to their recruitment and antitumor activity seems to differ along the clinical progression of different solid cancers [82]. Therefore, future design of clinical trials aimed to employ TILs as diagnostic tools or novel immunotherapeutic strategies should take these considerations into account. Recruitment of Treg cells into the tumor milieu is another mechanism of tumor immune evasion. In ovarian cancer, recruitment of Tregs decreased specific antitumor TILs and was associated with a worst prognosis [98]. In hepatocellular, renal cell, and breast carcinomas, the number of $CD4^+CD25^+Foxp3^+$ cells was associated with worst patients outcome [99–101], although not independently by other histopathological features in the case of breast cancer. Counterintuitively, different CRC studies showed that a high density of $Foxp3^+$ cells was independently associated with better prognosis [102–104]. This discrepancy might be explained

by hypothesizing that Foxp3$^+$ cells instead of inhibiting antitumor immunity decrease chronic pro-tumor inflammation. However, the biological basis explaining differing roles of Treg cells in tumor progression with respect to the tumor type is still unknown. New experimental models properly simulating tumor development will be helpful in better understanding Tregs activity in tumor.

15.6 Concluding Remarks

Solid tumors contain a heterogeneous mixture of malignant and nonmalignant cells within an extracellular matrix supported by an irregular vascular network [105, 106]. The cancer microenvironment makes up the stroma of the neoplasm and is the tissue that determines tumor growth, progression, and ability to initiate metastases. Due to the role played by cancer microenvironment in each stage of tumor development, better knowledge about the interactions of the tumor with its microenvironment would seem to be of utmost importance for developing new treatment strategies [107, 108]. It has been ascertained that cancerous stroma coevolves alongside tumor progression, thereby promoting the malignant conversion of epithelial carcinoma cells [109]. However, tumor stroma is infiltrated by a variety of immune cells with the ability to influence tumor development and with a relevant impact on prognosis. The understanding that the immune system plays an important role in cancer progression has led to the recent development of targeted immunotherapies [110]. Moreover, the recognition that immune cells are key determinant of cancer progression has reinforced the idea that immune elements might represent new biomarkers of outcome or response to therapy.

It is indubitable that the analysis of the type, quantity, location, and the functions of the immune infiltrate becomes a primary step in understanding the history of cancer in a clinical relevant perspective. A comprehensive analysis of all components of the lymphocytic infiltrates in the context of their localization, organization, and impact at various steps of tumor progression remains largely, if not entirely, to be addressed in prospective studies [111, 112]. In parallel, understanding the mechanisms of efficient immune reactions, the place where they are initiated, the cellular and molecular mediators involved, and their impact at different stages of the disease should provide new tools and goals for more effective and less toxic targeted therapies.

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Epigenetics and microRNAs in Cancer

16

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16.1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs) which regulate gene expression by directly binding mostly, but not exclusively, to the 3′-untranslated region (3′-UTR) of target mRNAs [1]. In 1993, Victor Ambros first identified a small ncRNA, called lin-4, able to regulate the expression of a gene called lin-14 involved in the development of *C. elegans* [2]. In 2001, Lagos-Quintana M. et al. showed for the first time that many of these small ncRNAs (in the meantime called microRNAs) are present not only in invertebrates but also in vertebrates [3]. In 2002, Croce's group provided the first evidence of miRNA involvement in cancer by showing that a specific cluster of miRNAs (namely, the miR-15a/16-1 cluster) is located in the frequently deleted chromosomal region 13q14 in chronic lymphocytic leukemia (CLL) [4]. In 2005 Frank Slack supported this molecular evidence of miRNA involvement by demonstrating that let-7 directly targets the *RAS* oncogene in lung cancer [5]. In the same year, Cimmino et al. found that the miR-15a/16-1 cluster directly targets the anti-apoptotic *BCL2* gene in human CLL [6]. From this time on, we assist at a plethora of studies identifying dysregulation of miRNAs in almost all types of human cancers and unraveling their contribution to human carcinogenesis by identifying which genes are modulated by the dysregulated miRNAs. Overall, these studies clearly state that aberrancies of the miRNome (defined as the

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full spectrum of miRNAs in a specific genome) contribute to human cancer development and can be therapeutically targeted to restore miRNA expression to normal [7]. Moreover, it has become clearer that miRNA involvement goes beyond cancer, since they are involved in a variety of biological processes, spanning from development, differentiation, apoptosis, and proliferation to senescence and metabolism [8–13].

MiRNAs are genes, like any other *protein coding gene (PCG)*, transcribed by RNA polymerase II into a capped and polyadenylated precursor, called pri-miRNA [14, 15]. A double-stranded RNA-specific ribonuclease called Drosha, in conjunction with its binding partner DGCR8 (DiGeorge syndrome critical region gene 8, or Pasha), cleaves the pri-miRNA into a hairpin-shaped RNA precursor (pre-miRNA), about 70–100 nucleotides (nt) long [16]. Transferred to the cytoplasm by Exportin 5, the pre-miRNA is cleaved into an 18–24 nt duplex by a ribonucleoproteic complex, composed of a ribonuclease III (Dicer), and TRBP (HIV-1 transactivating response RNA binding protein). Finally, the duplex interacts with a large protein complex called RISC (RNA-induced silencing complex), which includes proteins of the Argonaute family (Ago1-4 in humans), which drives one strand of the duplex (the so-called mature miRNA) mainly, but not exclusively, to the 3′-UTR of the target mRNAs. Overall, miRNAs exert its effect by modulating the expression of the target mRNAs either by mRNA cleavage or by translational repression. In 2007, Vasudevan et al. discovered that miRNAs can also increase the expression of target mRNAs [17]. Each miRNA can target several different transcripts. For instance, it has been demonstrated that a cluster of two miRNAs (namely, miR-15a and miR-16) can affect the expression of about 14 % of the human genome in a leukemic cell line [18]. In addition, the same mRNA can be targeted by several miRNAs [19].

Epigenetics is defined as all heritable changes in gene expression not associated with concomitant alterations in the DNA sequence. In a traditional sense, gene epigenetic regulation usually includes DNA promoter methylation and chromatin histone modifications which are catalyzed by specific enzymes, overall indicated as effectors of the epigenetic machinery. However, if we consider the above definition, also *miRNA* gene regulation sensu stricto represents a component of epigenetics. Interestingly, it has been discovered that there is a two-way correlation between miRNAs and other epigenetic mechanisms: miRNAs can regulate the expression of effectors of the epigenetic machinery and miRNA genes undergo the same epigenetic regulatory mechanisms of any other *PCG*. These two main aspects of miRNome-epigenome cross-regulation and their implications in human carcinogenesis will be the main focus of this chapter.

16.2 MiRNAs Regulate Effectors of the Epigenetic Machinery

In 2007, Fabbri et al. provided the first evidence that miRNAs can affect the expression of epigenetically regulated *PCG* in cancer by directly targeting key effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs) [20]. The miR-29 family (composed of miR-29a, miR-29b, and miR-29c) can directly silence the expression of de novo *DNMT3A* and *DNMT3B* in non-small cell lung cancer (NSCLC), leading to a global hypomethylation status of cancer cells and re-expression of *tumor suppressor genes (TSGs)* such as *FHIT* and *WWOX*, whose expression is silenced in NSCLC by promoter hypermethylation. As a result of the re-expression of these TSGs, NSCLC cells undergo apoptosis both *in vitro* and in an *in vivo* xenograft model [20]. Subsequently, Garzon et al. showed that in addition to directly targeting de novo *DNMTs*, miR-29b is also capable of targeting the *maintenance DNMT1*, even though in an indirect way: by directly silencing Sp1, a transactivator of *DNMT1* [21]. These combined effects of miR-29s on all three major *DNMTs* highlight their relevance for epigenetic processes and explain the profound effects of their restoration on the global methylation status of cells. MiRNAs such as the miR-29 family, able to directly target effectors of the

epigenetic machinery, have been called "epi-miRNAs." In mouse embryonic stem (ES) cells, two independent groups have shown that members of miR-290 cluster directly target RBL2, an inhibitor of *DNMT3* genes [22, 23]. ES Dicer null cells are characterized by no expression of the miR-290 cluster, overexpression *of RBL2*, and disruption of de novo methylation pathway, leading to increased telomere recombination and aberrant telomere elongation. Restoration of the miRNA cluster reverted this phenotype [23, 22]. Interestingly, the regulatory effect of miR-290 cluster on de novo *DNMTs* was not observed in human embryonic kidney 293 cells following Dicer knockdown, suggesting that miR-290 targeting effect on *DNMT3s* might be cell- and/or species-specific [22].

Another important family of epi-miRNAs is the miR-148a/b-152 family. In 2008, Duursma et al. showed that miR-148a and miR-148b can indeed bind to the coding region (not the 3′-UTR) of DNMT3b mRNA, affecting the expression of this gene [24]. This seminal study also concluded that by binding to this unusual site, miR-148 family might be responsible for the several different splice variants of DNMT3b [24]. A role for the miR-148a/b-152 family was further confirmed in cholangiocarcinoma, where it was shown that these miRNAs, in addition to miR-301, can directly target DNMT1, and their expression is silenced by IL-6, which is involved in cholangio-cancerogenesis [25]. This paper provided the first evidence of a correlation between epi-miRNAs, inflammation, and cancer. In 2010, Das et al. showed that all-trans-retinoic acid (ATRA)-treated neuroblastoma cells undergo downregulation of MYCN, hence leading to overexpression of MYCN repressed miRNAs such as miR-152, miR-26a/b, and miR-125a/b [26]. They also showed that these miRNAs are epi-miRNAs in this model, since they downregulate DNMT1 and DNMT3B expression, leading to re-expression of epigenetically silenced NOS1, which promotes neural cell differentiation. Also, the expression of miR-152 was normally downregulated with concurrent increase of *DNMT1* expression in HBV-induced HCCs [27]. More recently, Wang et al. identified miR-342 as another epi-miRNA involved in colon carcinogenesis [28]. They showed that the expression of *miR-342* is inversely correlated to DNMT1 levels in colorectal cancer (CRC) tissues and cell lines, and that this miRNA targets DNMT1, leading to reactivation of epigenetically silenced TSGs such as *ADAM23*, *Hint1*, *RASSF1A*, and *RECKS*. Functionally, restoration of miR-342 resulted in a reduction of *DNMT1* expression, reduced cell proliferation, and invasiveness in CRC cells and inhibition of tumor growth and lung metastasis formation in nude mice [28]. In 2010, viral epi-miRNAs have been shown to control the epigenetic machinery of host cells through DNMTs [29]. MiR-K12-4-5p, a Kaposi sarcoma-associated herpesvirus (KSHV) miRNA, was found to regulate the expression of DNMT1, 3A, and 3B indirectly, by targeting the expression of Rbl2, a known repressor of DNMT1, 3A, and 3B transcription. Ectopic expression of miR-K12-4-5p reduces Rbl2 protein expression and increases DNMT1, 3A, and 3B mRNA levels in 293 cells, thus affecting the overall epigenetic reprogramming of the host cell [29].

Epi-miRNAs are also involved in regulating the expression of histone deacetylases (*HDACs*) and Polycomb Repressive Complex (*PRC*) genes. For instance, HDAC4 is a direct target of both miR-1 and miR-140 [30, 31], while miR-449a binds to the 3′-UTR region of *HDAC1* [32]. *HDAC1* is upregulated in several kind of cancers, and *miR-449a* re-expression in prostate cancer cells induces cell-cycle arrest, apoptosis, and a senescent-like phenotype by reducing the levels of HDAC1 [32]. Recently, Jeon et al. showed that miR-449a,b regulate *HDAC1* expression by directly targeting its 3′UTR transcript, indicating that this might be one of the reasons for the low miR-449a, b expression and the high expression of *HDAC1* in lung cancer [33]. MiR-140 has also been shown to be involved in chemoresistance mechanisms by targeting *HDAC4* [34]. Inhibition of endogenous miR-140 by locked nucleic acid (LNA)-modified anti-miRNAs partially sensitized resistant colon cancer stemlike cells to 5-FU treatment by increasing HDAC4 levels, leading to a G_1 and G_2 phase arrest [34]. Low expression of miR-9 along with high expression levels of *HDACs* (*HDAC4* and *5*) were discovered

in Waldenstrom macroglobulinemia (WM) [35]. Mir-9 targets HDAC4 and HDAC5 in WM cells. Overexpression of miR-9 causes downregulation of HDAC4, 5, leading to an upregulation of acetylated-histone-H3 and acetylated-histone-H4. This provides evidence that the loss of miR-9 might be responsible for upregulation of HDAC4 and HDAC5 in WM cells, contributing to the pathogenesis of WM disease [35].

EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) and is responsible for heterochromatin formation by trimethylating histone H3 lysine 27 (H3K27me3), leading to the silencing of several TSGs. Varambally et al. showed that in prostate cancer cell lines and primary tumors, the expression of miR-101 decreases during cancer progression, inversely correlating with an increase of EZH2. These findings are suggestive of a role as epi-miRNA for miR-101, a hypothesis which was tested and confirmed by showing that miR-101 directly targets EZH2 both in prostate and in bladder cancer models [36, 37]. Moreover, miR-101-mediated suppression of EZH2 inhibits cancer cell proliferation and colony formation, revealing a TSG role for miR-101, mediated by its modulatory effects on cancer epigenome [37]. The inverse correlation between *miR-101* and *EZH2* was also observed in glioblastoma [38], gastric cancer [39], and NSCLC [40]. In prostate cancer it has been shown that *miR-101* can be inhibited by androgen receptor and HIF-1α/HIF-1β [41]. Ectopic expression of miR-26a targets *EZH2* in Burkitt's lymphoma, leading to reduced cell proliferation, increased percentage of cells in G_1-phase, and increased apoptosis in Raji and Namalwa cells [42]. Intriguingly, the authors also found that c-Myc negatively regulates miR-26a, therefore maintaining high *EZH2* expression levels in cells and significantly contributing to c-Myc-induced tumorigenesis [42]. In 2009, Juan et al. analyzed a regulatory double-negative feedback loop between miR-214 and EZH2 in controlling PcG-dependent gene expression during differentiation [43]. PcG proteins suppress the transcription of miR-214 in undifferentiated skeletal muscle cells (SMC). Ectopic expression of miR-214 directly targets *EZH2*, increases myogenin expression, and promotes muscle differentiation [43]. *EZH2* is also highly expressed in nasopharyngeal carcinoma (NPC) patients and correlates with a higher risk of relapse [44]. *MiR-26a*, *miR-98*, and *miR-101*, whose expression is consistently downregulated in human NPC specimens when compared to normal nasopharyngeal epithelial tissue samples, have been shown to directly target *EZH2* [44], suggesting a prognostic role for these three miRNAs in NPC. Recently, there has been an extensive series of studies unraveling the central role of miR-101 in the regulation of *EZH2*, in several types of cancer. In hepatoma tissues, it was shown that miR-101 and miR-29c are downregulated, but their expression can be restored (leading to reduced levels of EZH2, EED, and H3K27me3 proteins) after treatment with TPA (12-O-tetradecanoylphorbol 13-acetate), which is dependent on protein kinase C (PKC) and ERK pathways in HepG2 cells [45]. Also, Smiths et al. have established a pro-angiogenic effect of miRNA-101 working together with EZH2 and VEGF during the process of angiogenesis [46]. The group analyzed the expression of miR-101 in endothelial cells derived from glioma patients and found it to be low. VEGF downregulates the expression of miR-101 resulting in increased protein expression of EZH2 and induces the elongation of endothelial cells leading to a pro-angiogenic response. Transfection with pre-miR-101, or EZH2 siRNA, or treatments with DZNep, a small inhibitor of EZH2 methyltransferase activity, reverses this process in HBMVECs controls, providing a network between VEGF/miR-101/EZH2 proteins toward pro-angiogenic response in endothelial cells [46]. A summary of the described epi-miRNAs is provided.

Overall, these studies indicate that epi-miRNAs can modulate several key effectors of the epigenetic machinery, which indirectly affects the expression of epigenetically regulated genes. Considering that inactivation of *TSGs* by epigenetic mechanisms represents one of the main strategies adopted by cancer cells to promote their oncogenic phenotype, it is of the utmost importance to completely dissect these mechanisms,

since they could provide new molecular targets for anticancer treatments.

16.3 MiRNAs Are Epigenetically Regulated in Several Types of Human Cancers

As previously anticipated, the relationship between miRNome and epigenome is bidirectional. Not only do miRNAs regulate the expression of effectors of the epigenetic machinery, but they also undergo the same epigenetic regulation of any other *PCG*.

By treating bladder cancer cell lines with both a DNA demethylating agent (5-aza-2′-deoxycytidine, 5-AZA) and an HDAC inhibitor (4-phenylbutyric acid), Saito et al. found that about 5 % of all human miRNAs increased their expression levels [47]. MiR-127 was the most upregulated after this treatment, and its re-expression led to direct targeting and downregulation of the oncogene *BCL-6*, inducing a tumor suppressor function. MiR-127 is part of a cluster which includes miR-136, miR-431, miR-432, and miR-433 and is embedded in a CpG island region; however, *miR-127* is the only member of the cluster whose expression increases upon treatment with the two epigenetic drugs [47]. Moreover, when each drug was used alone, no variation in *miR-127* expression was observed [47], suggesting that both DNA methylation and histone modifications affect the epigenetic regulation of *miR-127*. This seminal work shows that indeed miRNAs undergo epigenetic regulation, that it is a complex epigenetic regulation (involving both methylation and histone modifications), and that there are differences among miRNAs which even belong to the same cluster. Lujambio et al. created a double knockout (DKO) for DNMT1 and DNMT3B in the CRC cell line HCT-116 and compared miRNA expression profile of DKO and wild-type cells. About 6 % analyzed miRNAs were re-expressed in the DKO cells [48]. Among them, miR-124a (embedded in a CpG island heavily methylated in this cell line) was re-expressed, reducing the levels of its direct target gene *CDK6* and impacting on the phosphorylation status of CDK6-downstream effector Rb protein [48]. Prosper's work has identified a signature of 13 miRNAs embedded in CpG islands, with high heterochromatic markers (such as high levels of K9H3me2 and/or low levels of K4H3me3) in acute lymphoblastic leukemia (ALL) patients [49, 50]. Among these, miR-124a was methylated in 59 % of ALLs, and its promoter hypermethylation was associated with higher relapse rate and mortality rate *vs*. non-hypermethylated cases; hence, miR-124a promoter methylation status was an independent prognostic factor for disease-free and overall survival [50]. Finally, supporting Lujambio's results, also in ALL the impact of miR-124a in the CDK6-Rb pathway was confirmed by showing that miR-124a directly silences *CDK6* [50]. Hypermethylation of miR-124a promoter is also involved in the formation of epigenetic field defect which is a gastric cancer predisposing condition characterized by accumulation of abnormal DNA methylation in normal-appearing gastric mucosa, mostly induced by *H. pylori* infection [51]. These findings also suggest that miR-124a promoter hypermethylation is an early event in gastric carcinogenesis. MiR-107, another epigenetically controlled miRNA, targets *CDK6* in pancreatic cancer as well and impacts this oncogenic pathway [52]. In HCT-116 cells, deficient for DNMT1 and DNMT3B, Bruckner et al. showed increased expression of let-7a-3, an miRNA normally silenced by promoter hypermethylation in the wild-type cell line [53]. In lung adenocarcinoma, primary tumors let-7a-3 promoter was found hypomethylated with respect to the normal counterpart [53], whereas hypermethylation of let-7a-3 promoter was described in epithelial ovarian cancer, paralleled the low expression of insulin-like growth factor-II expression, and was associated with a good prognosis [54]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNA with oncogenic function. Also, the miRNA-200 family participates in the maintenance of an epithelial phenotype, and loss of its expression can result in epithelial to mesenchymal transition (EMT). Furthermore, the loss of expression of miR-200 family members is associated with an

aggressive cancer phenotype. Vrba et al. found that hypermethylation of the miR-200c/141 CpG island is closely linked to their inappropriate silencing in cancer cells, and the epigenetic regulation of this cluster appears evolutionarily conserved, since similar results were obtained in mouse [55]. Interestingly, no variation in miRNA expression was observed in lung cancer cells treated with either demethylating agents or HDAC inhibitors or their combination [56]. Another miRNA which is under epigenetic control is miR-1. In hepatocarcinoma, miR-1 is frequently silenced by promoter hypermethylation [57]. However, in DNMT1 null HCT-116 cells (but not in DNMT3B null cells), hypomethylation and re-expression of miR-1-1 were observed [57], revealing a key role for the maintenance DNMT in the regulation of this miRNA. Han et al. observed that neither *5-AZA* nor *DNMT1* deletion alone can recapitulate miRNA expression profile of DKO DNMT1/DNMT3B HCT-116 cells [58]. Also, Lehmann et al. found that in breast cancer cell lines, 5-AZA re-activates miR-9-1 (hypermethylated in up to 86 % of primary tumors), but not miR-124a-3, miR-148, miR-152, or miR-663 (hypermethylated as well) [59]. Previously, Meng et al. observed that in malignant, but not in normal cholangiocytes, 5-AZA induces re-expression of miR-370 [60]. Overall, these results indicate that the epigenetic control of miRNAs is both cancer specific and miRNA specific. More recently, Chang and Sharan reported that *BRCA1* recruits the *HDAC2* complex to the miR-155 promoter, which is consequently silenced epigenetically through the deacetylation of H2A and H3 histones [61]. The study also showed the upregulation of miR-155 in BRCA1-deficient or BRCA1-mutant human tumors. The knockdown of miR-155 in a BRCA1 mutant tumor cell line attenuates *in vivo* tumor growth. However, a knockdown of BRCA1 results in a two- to threefold increase in miR-155 levels *in vitro*. In contrast, a 50 to 150-fold increase in miR-155 in human breast cancer cell lines or tumor samples was observed, suggesting that this increase may not be caused only by *BRCA1* loss; other transcription factors may activate the miR-155 promoter after it is epigenetically activated due to the loss of BRCA1 [61]. Mazar et al. studied which miRNAs were re-expressed upon treatment of a melanoma cell line with demethylating agents [62]. Among the 15 re-expressed miRNAs, miR-375 and miR-34b were also involved in melanoma progression [62]. Liu et al. [63] found that miR-182 was significantly upregulated in human melanoma cells after combined treatment with 5-AZA and trichostatin A. Genome sequence analysis revealed the presence of a prominent CpG island 8–10 kb upstream of miR-182, but methylation analysis showed that this genomic region was exclusively methylated in melanoma cells, not in normal human melanocytes. Since miR-182 has been shown to harbor oncogenic properties, this finding raises a possible concern for melanoma patients treated with epigenetic drugs [63]. MiR-31 maps at 9p21, a genomic region frequently deleted in solid cancers including melanoma. Asangani et al. [64] found recurrent downregulation of miR-31 in melanoma primary tumors and was associated with genomic loss or epigenetic silencing by DNA methylation and EZH2-mediated histone methylation. Moreover, miR-31 overexpression resulted in downregulation of EZH2 and a derepression of its target gene rap-1GAP. The increased expression of EZH2 was associated with melanoma progression and poor overall survival [64].

Nickel (Ni) compounds are well described human carcinogens. Recently an important regulatory double-negative feedback loop has been discovered between *miR-152* and *DNMT1* in nickel sulfide (NiS)-transformed human bronchial epithelial (16HBE) cells [65]. Expression of *miR-152* was specifically downregulated by promoter hypermethylation, whereas ectopic expression of *miR-152* resulted in a remarkable reduction of *DNMT1* expression in transformed cells. Interestingly, treatment with 5-AZA or knock down of DNMT1 reversed this process. Further, inhibition of *miR-152* expression in 16HBE cells was found to increase *DNMT1* expression and DNA methylation. Moreover, ectopic expression of *miR-152* caused a significant decrease of cell growth, whereas inhibition of *miR-152* reversed this process in 16HBE cells, suggesting the

existence of an important functional negative feedback loop between *miR-152* and *DNMT1*, likely to play an important role in NiS-induced lung carcinogenesis [65]. The relationship between miRNA and cognate host gene epigenetic regulation was addressed by Grady et al. by studying miR-342, located in an intron of the *EVL* (*Ena/Vasp-like*) gene [66]. EVL promoter hypermethylation occurs in 86 % of colorectal cancers and is already present in 67 % of adenomas, suggesting that it is an early event in colon carcinogenesis. The combined treatment with 5-AZA and the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342. The *EGFL7* gene, frequently downregulated in several cancer cell lines and in primary bladder and prostate tumors, hosts miR-126 in one of its introns. While the mature miR-126 can be encoded by three different transcripts of the cognate host gene, each of them with its own promoter, *miR-126* is concomitantly upregulated with one of *EGFL7* transcripts which has a CpG island promoter, when cancer cell lines are treated with inhibitors of DNA methylation and histone deacetylation, indicating that silencing of intronic miRNAs in cancer may occur by means of epigenetic changes of cognate host genes [67]. In summary, miRNAs are encoded by either *ncRNA* genes with their own promoters or by noncoding sequences in introns of *PCGs*. In the latter case, miRNA expression is usually driven by the same promoters of the corresponding *PCGs*.

The role of miRNA epigenetic modifications in the metastatic process has also been investigated by several groups. Lujambio et al. treated three lymph-node metastatic cell lines with 5-AZA and identified 3 miRNAs which showed cancer-specific CpG island hypermethylation: miR-148a, miR-34b/c, and miR-9 [68]. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited cell motility and their metastatic potential in xenograft models and was associated with downregulation of miRNA oncogenic target genes, such as *c-MYC, E2F3, CDK6*, and *TGIF2* [68]. Finally, promoter hypermethylation of these three miRNAs was significantly associated with metastasis formation also in human malignancies [68]. MiR-34b/c cluster is also epigenetically regulated in CRC (promoter hypermethylation in 90 % of primary CRC samples *vs* normal colon mucosa) [69], whereas epigenetic silencing of miR-9 and miR-148a (together with miR-152, miR-124a, and miR-663) was described also in breast cancer [59].

Finally, Fazi et al. showed that transcription factors can recruit epigenetic effectors at miRNA promoter regions and contribute to the regulation of their expression. The AML1/ETO fusion oncoprotein is the aberrant product of t(8;21) translocation in acute myeloid leukemia (AML) and can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG in close proximity to the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [70]. In SkBr3 breast cancer cell line, Scott et al. were able to demonstrate that 27 miRNA expression levels are rapidly modified (5 up- and 22 downregulated) by a treatment with the HDAC inhibitor LAQ824 [71], indicating that some miRNAs are mainly silenced by histone modifications. In A549 lung cancer cell line, the HDAC inhibitor SAHA deregulates 64 miRNA (>2 fold change) targeting genes involved in angiogenesis, apoptosis, chromatin modification, cell proliferation, and differentiation [72]. A list of the discussed epigenetically regulated miRNAs is provided.

In summary, these studies convincingly support an epigenetic regulation of miRNAs, and the fact that cancer cells adopt epigenetic mechanisms to silence/re-express key miRNAs modulating relevant PCGs for the development of their oncogenic phenotype. The metastatic process also seems to be driven, at least in part, by the selected epigenetic regulation of miRNAs, in addition to the well-known epigenetic regulation of relevant PCGs.

16.4 Concluding Remarks

The series of studies listed in this chapter should have convinced the readers that a tight connection relates miRNAs and epigenetics, and this

relationship harbors significant implications in the development and spreading of malignancies. Aberrancies of the miRNome can effectively be reversed by overexpressing miRNAs that are downregulated in cancer and/or by silencing miRNAs overexpressed by cancer cells. Synthetically generated miRNA-mimic molecules can be effectively delivered to cancer cells. Conversely, miRNAs can be administered as anti-miRNA molecules in case the silencing of a miRNA needs to be achieved. Most commonly, anti-miRNAs can be administered as antagomiRs [73], or LNA anti-miRNAs [74], which are oligonucleotides complementary to the sequence of the targeted mature miRNA, but biochemically modified to reduce the risk of degradation by cellular RNAses, and are conjugated with cholesterol to facilitate their entrance in the cells. By designing mimics and/or anti-miRNAs of epi-miRNAs, a profound modulation of several epigenetically regulated PCGs is anticipated. Similarly, epigenetic drugs such as 5-AZA and histone active drugs will directly affect the expression of several epigenetically regulated miRNAs, as well as indirectly the expression of those mRNAs modulated by these epigenetically regulated miRNAs. The overall effect on cell phenotype is the combination of these modifications in the transcriptome and miRNome. Therefore, a clear and deep understanding of these basic mechanisms is necessary in order to avoid re-expression of oncogenes and/or onco-miRNAs. Despite the complexity suggested by these interactions, an increasing number of excellent works is bringing us on the right track by dissecting the complexity of such mechanisms and supporting a general optimistic view: that in a future not too far to come, we will be able to effectively translate these discoveries into new strategies to fight cancer, resulting in decreased mortality.

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Immunogenetics of Cancer

17

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17.1 Introduction

The influence of genes in the development of cancers can be very high, very well depicted in numerous hereditary cancers or very low in some

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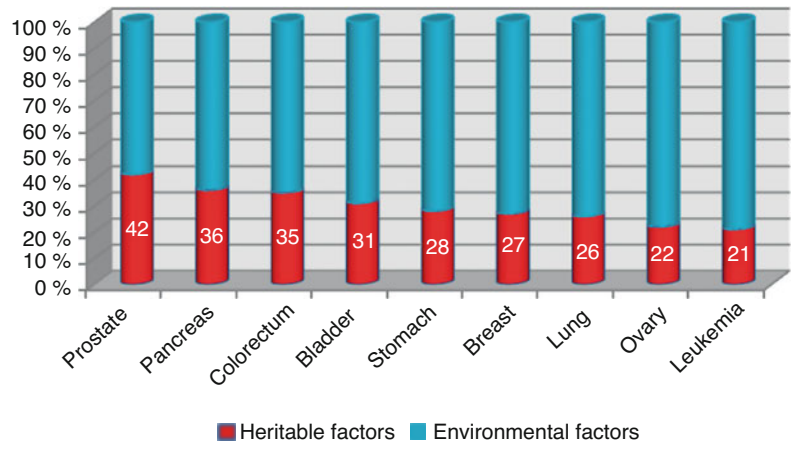


Fig. 17.1 Heritability of cancers in different sites based on the information available from twin studies

cancers. Although the roles played by genes in the pathophysiology and prognosis of the malignant transformation are highly variable in different cancers, their role cannot be ignored. For sure, polymorphisms in immune-related genes, known as immune polymorphisms, have an undeniable role in shaping undeniable but complex interactions between the immune system and malignancies which can significantly influence the face of malignancy with respect to predisposition, nature, prognosis and response to treatment in each individual.

17.2 Cancers: Why Are There Different Faces?

It has been long observed that individuals are different with respect to predisposition nature, prognosis, and response to treatment in cancer [1, 2]. Since the first observations, scientific minds have been preoccupied with the question that, what is the reason for this high inter-individual variation. Nowadays, it is obvious that behind the ugly scene of cancers, there is a complex interplay between genes and environment and this question can be answered straightforwardly by the high variability of genetic and environmental factors for each individual [1]. Although it is estimated that less than 0.1 % of the genome is different between any two individuals, this variability is equal to at least several million nucleotide differences per individual [3, 4]. The influence of genes in the development of cancers can be very high, very well depicted in numerous hereditary cancers like familial adenomatous polyposis, or very low in some cancers like cancer of the cervix (Fig. 17.1) [5]. Although the roles played by genes in the pathophysiology and prognosis of the malignant transformation are highly variable in different cancers, their role cannot be ignored [6, 7]. Malignant transformation is not just a result of a cell-autonomous process and is shaped by intrinsic properties, but also its cross talk with microenvironment governed by the immune and endocrine systems, stroma, vascular system, and other systems [6]. Therefore, this heritability results from additive effects of low-penetrance genetic factors, each one contributing a small amount of risk [6].

17.3 Immune Polymorphism

The role of immune system in defense against malignancies was proposed in the early 1990s by Paul Ehrlich [8]. So far this book, page by page, has tried to show the undeniable but complex interactions between the immune system and malignancies. This complex interaction mostly results from the manipulation of the immune system by cancer cells evoluting to prevent self-destruction [8]. Four phenomena contribute to the escape of malignant cells from the immunosurveillance:

1. *Immunoedition*: Natural selection of malignant cells which are most successful in deceiving

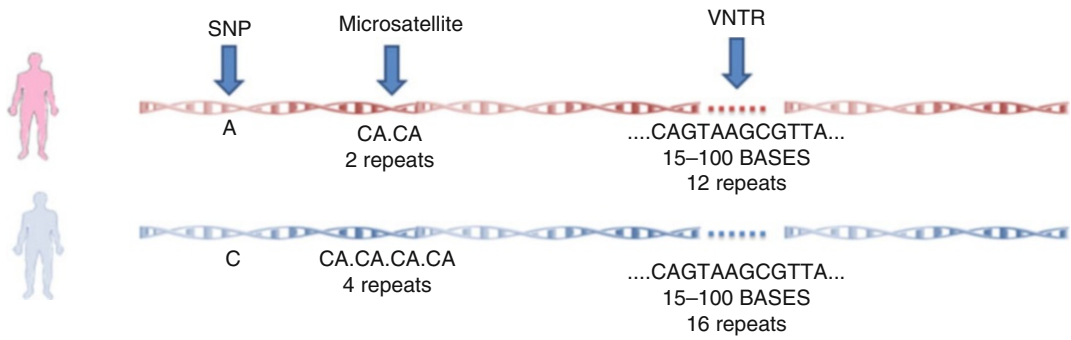


Fig. 17.2 Different types of polymorphisms in the human genome

the immune system occurs by the pressure of the immune system itself. This pressure leads a somatic evolution toward variants proficient in immune escape in primary tumor lesions [9, 10].

Down regulation of the local immune system: Several tumors can manipulate the local defense by producing inhibitory molecules such as indoleamine 2,3-dioxygenase (IDO) and different cytokines or expression of apoptose-inducing ligands such as Fas-ligand [9, 11]

2. *Tolerance induction and losing immunogenicity*: The absence of co-stimulatory molecules, localization in natural environment of healthy cells and therefore absence of danger signals, losing human leukocyte antigen (HLA) class I molecules, and aberrant expression of immunomodulatory non-classical HLA class I antigen (Ag) can all induce tolerance in the immune system [9, 11, 12].
3. *Host immunodeficiency*: Any deficiency in the immune status of individuals can predispose them to various malignancies.

In addition, once the immune escape occurred, the immune system can profoundly influence the prognosis, natural history, and response to different therapies either by direct effects on malignant cells or indirect effects on angiogenesis and inflammation [9, 11–13].

The immune system of each individual is subject to variability due to different environments, different diets and nutritional status, and different antigenic exposures and most importantly due to an uncountable number of polymorphisms in genes governing the immune system elements and cells [14, 15].

Genetic polymorphisms are defined as variations in human genome present in at least 1 % of the population [16]. These polymorphisms were beneficiary either in their cross talk with certain environmental factors alone or in combination with their associated polymorphisms, or they were at least neutral enough not to compromise the life of the individual bearing them; therefore they were not erased by the evolutionary pressure [14, 16, 17]. Immune response-associated genes are not an exception, and they have an uncountable number of polymorphisms [14]. For example, HLA region includes the most polymorphic genes in the human genome [14]. This high variety in immune-associated genes is a product of a long interaction with an environment consisting of numerous ever-evolving pathogens [14]. In this context, majority of polymorphisms had the chance to be beneficiary in defense against some pathogens [15, 18, 19].

Single nucleotide polymorphisms (SNP), variable number of tandem repeats (VNTRs) (a repeat unit includes 15–100 nucleotides) and microsatellites are three important types of polymorphisms [20].

SNP is defined as a difference in a single nucleotide in the DNA sequence and is estimated to account for 90 % of the human genome variations. Microsatellites, scattered through the genome with an average density of one in every 2,000 pb, are variable tandem repeats of 2–8 bp, most commonly CA dinucleotide, and their alleles are differentiated by the number of repeats (Fig. 17.2) [20, 21].

Polymorphisms are able to change the immune function at several levels from expression patterns to posttranslation modifications:

1. Some polymorphisms might change DNA methylation and consequently chromatin structure and expression patterns [22, 23].
2. Some polymorphisms may disrupt transcription factor binding sites (TFBSs) and consequently influence the expression [20, 24, 25].
3. mRNAs splicing patterns can be modified by polymorphisms as a result of deletion of a splice site, creation of a new splice site, or modification of exon-splicing enhancers and silencers [24].
4. MicroRNAs (miRNAs) are important elements in gene regulation with various actions. Their binding sites might be disrupted as a result of polymorphisms [24].
5. Some polymorphisms can cause mRNA instability and its early destruction [20, 26].
6. Polymorphisms may create premature termination codons [24].
7. Exonic polymorphisms can substitute an amino acid in protein sequence, change protein structure, and consequently alter protein function [20, 25, 26].
8. Some polymorphisms may change posttranslational modification (PTM) site and consequently influence posttranslational modifications [24].

Therefore, it seems that this high genetic variability in immune response associated genes known as immune polymorphism contributes to the observed interindividual differences [14, 19].

17.4 Immunogenetics

17.4.1 Background

Immunogenetics, as the meeting point of two exciting fields of immunology and genetics, is a new but rapidly expanding field of science studying this immune polymorphism in order to understand the governance of genetics on the immune system [14, 27, 28].

Although the term "immunogenetics" was used earlier [29], the first milestone in the history of immunogenetics was coincident with the failed study of blood transfusion in 1952 [30]. This failure resulted in the discovery of HLA system [14, 31], which attracted the attention of biomedical researchers to interindividual differences in the immune system. From that point on, for decades, investigators tried to associate different complex diseases with various HLA types using serological methods [32, 33]. However, modern immunogenetics required more than one century of biomedical advances remarked by Mendel's laws of heredity in 1865 [16, 34], discovery of chromosomes as the cellular basis of heredity in 1902, discovery of DNA double helix as the molecular basis of heredity in 1953 [35], decoding the genetic codes, and last but not least the completion of Human Genome Project in April 2003 [16, 36, 37]. Human Genome Project not only contributed to the discovery of genetic polymorphisms but also provided a infrastructure for other large-scale projects like International HapMap Project and "1,000 Genomes Project" [38]. Discovery of approximately 25–35 % of estimated nine to ten million SNPs is just one of the uncountable achievements of such projects [14, 37–39]. Genetic polymorphisms in the immune system contribute to a large part of the interindividual variation in immune response and today, immunogenetic studies have provided a vast knowledge of the effects of immune polymorphism on the host defense. However, just the estimation that there is one SNP per every 290 bp shows that there is much more to be brought to light [38, 39].

17.4.2 Immunogenetic Tools

Along with the concert of conceptual advancements, tools employed in this field have changed in order to gather immunogenetic information more accurately, in less time and less cost [14]. Twin studies recruit twins in order to remark the importance of genetic component in susceptibility to traits and diseases [16, 40]. The result of

such studies provides a rough estimation of genetic contribution to interindividual differences in immune system by comparison of concordance rates of immune traits between monozygotic and dizygotic twins [16, 33, 40]. The higher the concordance difference is, the greater the heritability [7, 16].

Upon introduction of immune polymorphism, several association studies tried to show the contribution of specific genes using the candidate gene approach or hypothesis-driven approach [16, 41]. This approach includes looking into the differences between patients and controls in allele frequencies of SNPs in genes selected based on the known pathophysiologic pathways of the disease. These studies at first employed restriction enzymes to identify specific SNPs called restriction fragment length polymorphisms (RFLPs) in the restriction site of the enzyme [42]. This approach is also known as a reductionist approach, since studies employing this approach investigate only a few genes and polymorphisms at a time [16, 41, 43].

In the early 1990s, discovery of hundreds of informative microsatellites provided the possibility of a dramatic change in the approach of immunogenetic studies from a hypothesis-driven approach to positional approach [4, 16, 44]. In this approach, studies known as genome-wide association studies (GWAS) mainly aim to identify the genome regions bearing disease-associated genes and to localize causal genetic variants of disease as accurately as possible [44, 45]. Therefore, in this approach, new hypothesis are generated after making thousands of unbiased observations [4, 33, 41, 44]. They are especially helpful in order to find unexpected genes as representatives of unknown disease-related pathway [4, 16]. In mid-1990s, early GWASs employed informative microsatellite markers distributed evenly in the 23 chromosomes and investigated their aggregation in multi-case families and large pedigrees identified major susceptibility loci for complex diseases [4, 42, 44].

By introduction of linkage disequilibrium (LD) defined as the coinheritance of alleles of a block of neighboring SNPs; in 2002, the International HapMap Project, as a global movement, began to identify these blocks (known as haplotypes) and pattern of LD in the human genome [38, 39]. LD results in organization of genetic variation in haplotype blocks with strong LD separated by recombination hotspots [16, 39]. The information from this project provided the immunogenetic scientists with the most suitable SNPs for genotyping in order to indirectly gather as much as information about the genome variation of an individual [16, 46]. These SNPs, which are representative of a block of SNPs, are known as tagSNPs. The extent of LD in a region determines the number of tagSNPs required to cover a region. The lower the LD is in a region, the higher number of tagSNPs are needed and therefore the higher the cost of genotyping the region is [47]. Nowadays, availability of high-throughput gene technologies such as gene chips or microarrays has enabled investigators to genotype cost-effectively, rapidly, and almost effortless hundreds of thousands to millions of SNPs at the same time [4, 33, 41, 44]; therefore this approach is also known as "nonreductionist" approach [4]. These technological advancements were employed in community-based and large-scale GWASs in order to identify trait-associated regions with higher resolution. The results of such studies is a trait-associated SNP (TAS) as a representative of the true casual variant which might be each of the known and unknown variants in whole TAS block. The TAS block is defined as all known and unknown polymorphisms in strong LD with the tagSNP [4, 16, 48]. Therefore, LD along with technological advances turned SNPs, the most common and more importantly the most stable genetic variations in human DNA, into application [49].

However, there are major limitations in GWASs to be overcome.

1. Generally, the genetic component of complex diseases originates from several major susceptibility loci and a component of as many as a dozen minor susceptibility loci known as

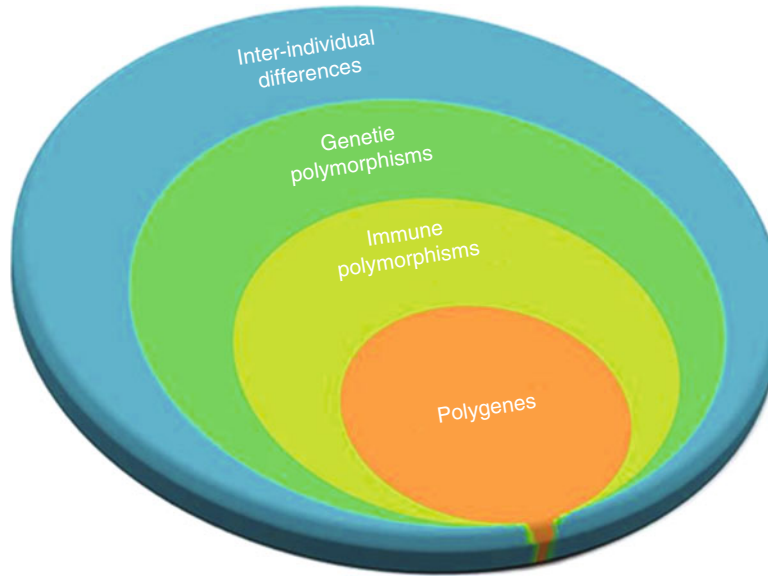


Fig. 17.3 Immune polymorphism component in inter-individual differences

polygenes (Fig. 17.3). These polygenes individually have small to medium impact on the overall genetic component; therefore, GWASs require a large study sample with homogenous ethnicity and phenotype to have enough high power to identify these polygenes [4, 21, 48, 50]. This is a major problem in immunogenetic studies of cancers as patients with cancers present with highly variable phenotypes. As a result, the odds ratio for each allele is typically below 1.5, and the *P* value should be less than 10^{-6} to show a significant association [6, 51].

2. The genetic component and therefore effect of any risk allele decreases by increased exposure of populations to environmental risk factors which is the reason why some results could not be replicated in different populations [6]. For example, increased prevalence of acquired immune deficiency syndrome (AIDS) in some African populations predisposes population to different cancers disregarding their genetic background [52, 53]. This is also the case in regard to some extreme dietary patterns, smoking habits, and other environmental factors [54, 55].
3. Some cancer susceptibility variants have nonadditive interactions with other genetic and environmental factors. It is possible that the effect of one variant depends on the presence of one or several specific alleles in another locus or even certain environmental risk factors. Therefore, such susceptibility variants can be detected only in GWASs with samples of patients with particular genetic and environmental background [6].
4. At least 10 % of SNPs within a range of 1 kpb of hotspots are untaggable which means they don't have any LD with tagSNPs [47]. The presence of these numerous untaggable SNPs always limits the power of GWASs in finding all possible genetic associations [39]. Therefore, GWASs should employ additional sequencing within known recombination hotspots [39].
5. GWASs are less effective in some old population like African countries, since LD is generally lower in these populations due to the longer duration being affected by genetic recombination [4, 16, 48, 49].
6. The different LD, hotspots, and haplotype patterns in different populations might complicate replication studies in different populations [49]. For example, in some population, the causal variant may be separated from the associated TAS block by a hotspot.

7. Sometimes the associated TAS block does not include a causative allele but an allele beneficiary for the affected individuals with the disease, and therefore the natural selection has selected them instead of those affected individuals without the allele [16].
8. Population stratification is another source of bias in such studies as the association of the trait and TAS block may be due to an ancient branching of the population bearing both causal trait alleles and the TAS block; however, this bias can be minimized by the careful selection of the control group or by assessing population structure and correcting for it [16, 49, 56].
9. If certain alleles are associated with a more aggressive disease and lower survival, they are less presented in patients and may not be detected as a susceptibility allele [57].

After identification of associated TAS blocks by GWASs, the actual functional variant in the associated TAS block can be found by further genetic association studies employing more accurate low-throughput technologies and other SNP markers in order to finely map the associated genes and alleles in the associated TAS block [44]. In these studies, allele frequencies of polymorphisms are compared in groups of cases and controls. However, results of such association studies are often contradictory due to the heterogeneous nature of the cancers, numerous gene–gene and gene–environment interactions [58, 59]. In addition, another source of discrepancy between these studies is the limitation in study design. For example, using hospital-based controls can result in a serious selection bias since polymorphisms under investigation might have association with the diseases that hospital-based controls may have [60, 61]. Moreover, some association studies failed to consider other genetic and environmental risk factors such as socioeconomic status, nutritional statues, smoking patterns, etc. [60]. Lacking such information may cause serious confounding bias [62]. Therefore, in order to get the most benefit from results of genetic association studies and to systematize their findings, employing meta-analyses as a powerful statistical method is essential [26, 63]. Meta-analysis by pooling the results of old studies allows us to see the whole picture of the effect of a certain polymorphism [26].

Regardless of interspecies differences, there are similarities in cancer development between humans and rodents, and therefore mouse studies are a complementary tool for genetic association studies within human population [6, 64, 65]. Numerous genetically engineered mouse (GEM) models provide a simplified model of various cancers with controllable genetic and environmental background in which the effects of a unique polymorphism on the malignancy can be studied [6, 66].

Exact mechanism of action of polymorphisms can be identified using different bioinformatic tools and *in vitro* studies [24]. Numerous bioinformatic online and offline tools are available which can predict the effect of polymorphisms by considering amino acid biophysical properties, active site residues, metal and lipid binding sites of gene product, TFBSs, splice sites and its regulatory motifs, miRNA binding sites, and PTM sites (Table 17.1) [24]. However, bioinformatics is limited by the extent of our knowledge [22, 24].

Different *in vitro* methods are developed to identify functional polymorphisms. The most important ones are reporter gene assay and electrophoretic mobility shift assay (EMSA) (Figs. 17.4 and 17.5) [22]. The reporter gene assay employs a reporter gene with a quantifiable product and clones the promoter of interest in its upstream [22, 67, 68]. Therefore, quantification of reporter gene product can provide information about the promoter strength [22, 67, 68]. On the other hand, EMSA can measure the effect of different polymorphisms on the affinity of TFBS sequence for different transcription factors. In these studies, double-stranded oligonucleotide containing the polymorphism of interest is mixed with nuclear extract with various transcription factors [22, 69, 70]. Higher affinity for these factors results in the formation of more protein–DNA complex resulting in retardation of mobility in electrophoresis [22, 69, 70].

Table 17.1 A small example of different bioinformatics tool

Title	Address	Description
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	A database for SNP information
Ensembl	http://www.ensembl.org/	A database for genome information, comparative genomics, variation, and regulatory data
HapMap consortium	http://www.hapmap.org/	A database for haplotype blocks
SNPper	http://snpper.chip.org/	Online tool available for SNP analysis
SNP3D	http://www.snps3d.org/	Online tool available for functional analysis of SNPs based on structure and sequence analysis
SNPeffect	http://snpeffect.vib.be/index.php	A database for phenotyping human SNPs and for finding information regarding SNPs effect on structure stability functional sites, structural features, and PTM sites
MutDB	http://www.mutdb.org/	Online database for human variation data with protein structural information and other functionally relevant information
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	A database for SNP information
Ensembl	http://www.ensembl.org/	A database for genome information, comparative genomics, variation, and regulatory data
HapMap consortium	http://www.hapmap.org/	A database for haplotype blocks
SNPper	http://snpper.chip.org/	Online tool available for SNP analysis
SNP3D	http://www.snps3d.org/	Online tool available for functional analysis of SNPs based on structure and sequence analysis
SNPeffect	http://snpeffect.vib.be/index.php	A database for phenotyping human SNPs and for finding information regarding SNPs effect on structure stability functional sites, structural features, and PTM sites
MutDB	http://www.mutdb.org/	Online database for human variation data with protein structural information and other functionally relevant information

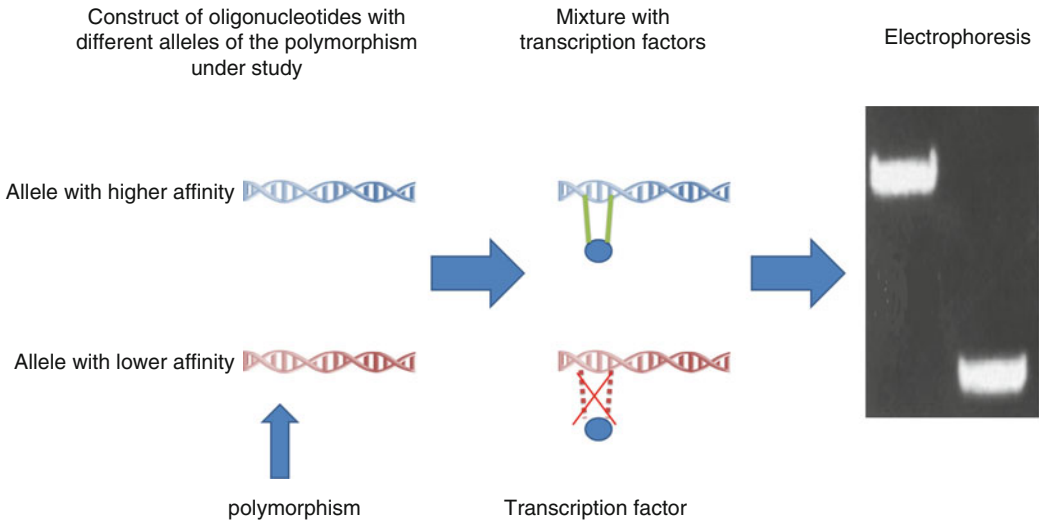


Fig. 17.4 EMSA, an *in vitro* experiment to measure binding affinities of different TFBS for transcription factors

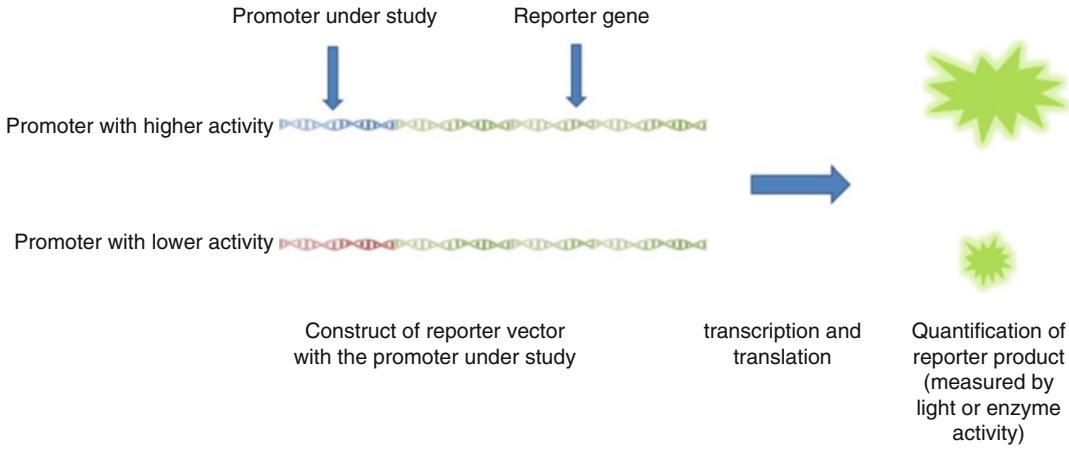


Fig. 17.5 Reporter gene assay, an *in vitro* tool to measure strength of different promoters

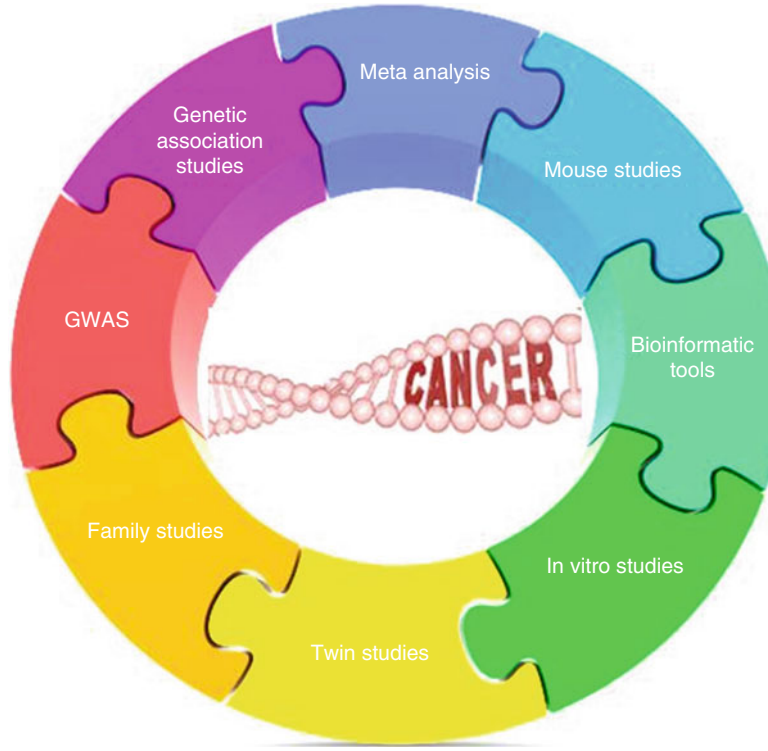


Fig. 17.6 Different methods in immunogenetic studies are pieces of a complex puzzle

The results from immunogenetic studies should always be interpreted with consideration of information from immunogenomics and immunoproteomics [33]. It should be noted that information from each type of study i.e., GWASs, genetic association studies, *in vitro* and mouse studies and bioinformatics, are just pieces of the complex puzzle of immunogenetics and cancer. No individual method is precise enough to see the final picture (Fig. 17.6).

17.5 Immunogenetics: A Champion in Fighting the Losing Battle Against Cancer

The application of immunogenetics in cancer is more than promising. Some variations in immune polymorphism reduce the immune capacity in clearing either malignant transformations or cancer-inducing

infectious agents and predispose bearing individuals to various cancers as exaggerated in case of most primary immunodeficiency diseases [4, 16, 19, 33]. Although each individual variant has a little informative potential for clinical application, understanding their interactions and therefore their cumulative effect is of high clinical importance [6].

Immunogenetic studies not only can help clinicians in risk assessment of individuals for susceptibility to certain cancers in order to employ preventive strategies but also may open new windows for treatment [4, 16, 19, 33, 48, 71–73]. GWASs might result in the identification of unexpected genes which in turn result in identification of new pathways in pathophysiology of cancers [48]. These new pathways not only provide a broader insight into how and why of the cancers but also may suggest new molecular targets for prevention and immunopharmacology and immunotherapy [4, 16, 33, 42, 48]. Keeping in mind that immune system provides the only antineoplastic reaction completely specific to cancer cells, it is vital to completely understand the genetic factors governing the immune system–cancer interactions and employ this knowledge in eliminating the cancers [4, 74]. In addition, this knowledge might begin a post-genomic era in individualized medicine [4, 33]. The presence of some variants in immune associated genes might affect the success or failure in applying a particular therapy and immunogenetic information provides a way to predict toxicity and clinical effectiveness of different immune-based therapies [4, 14, 20, 33]. Therefore, employing the knowledge from immune polymorphism in prediction of treatment outcome may justify the application of an expensive partly effective treatment option [4, 14, 33, 75].

17.6 Human Leukocyte Antigen

17.6.1 Background

Human leukocyte antigens are specialized elements of the immune system in recognition of self from non-self. HLA is responsible for presenting Ags to T cells and therefore serves as a door to the specific immune system. HLA class 1 Ags are on the surface of almost all nucleated cells and generally present processed endogenous antigens to $CD8^+$ cells [13, 76]. Presentation of abnormal Ags derived from intracellular pathogens or malignant transformations potentially initiate a cytotoxic T lymphocyte (CTL) response and consequently target cell lysis [77]. By their interaction with killer cell immunoglobulin-like receptors (KIRs) on the surface of natural killer (NK) cells, HLA class 1 antigens regulate lytic activity of NK cells. Therefore, any change in either in expression or structure of HLA class 1 profoundly influence T and NK cell-mediated immunity [10].

On the other hand, HLA class 2 Ags are exclusively expressed on the surface of professional antigen-presenting cells (APC) and present processed exogenous Ags to T helper (Th) cells. Following presentation of unfamiliar Ags and in the presence of appropriate costimulatory molecules, Th cells activate effector elements of the immune system [13, 77].

Both classes of Ags comprise an intracellular, transmembrane, and an extracellular part which includes highly polymorphic antigen binding groove. From the evolutionary view, this high variety favors the chance of heterozygosity and consequently Ag presenting potential for each individual along with a significant increase in the general repertoire of the whole specie for Ag presentation [14, 77].

17.6.2 Genes Behind HLA

HLA loci, located in 6p21.3 region, occupy only a small part of major histocompatibility complex (MHC) genetic system which is home to at least 220 genes [78, 79] (Fig. 17.7). MHC is divided into three classes of genes distributed from centromere to telomere. Class 2 with 0.9 mb is the nearest one to the centromere; class I with 1·9 Mb is near telomere, and class 3 with 0·7 Mb lies in between [80]. The first two classes encode for HLA class 1 and 2 and the third class consists of a group of genes encoding some members of the complement system, some cytokines like tumor necrosis factor alpha (TNF-α), heat shock proteins (HSP) and an enzyme called 21-OH hydroxylase [31, 80].

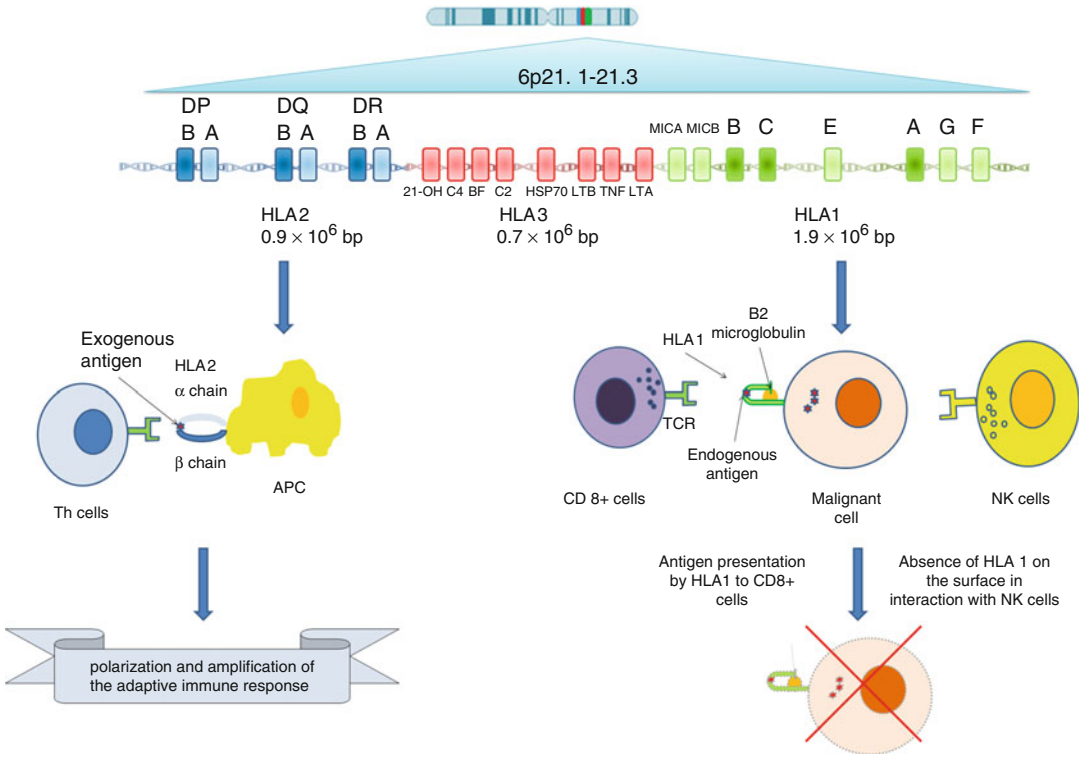


Fig. 17.7 HLA as the gate of adaptive immunity from genes to function

In class 1, there are three highly polymorphic classic genes known as *HLA-A*, *HLA-B* and *HLA-C*, while there are numbers of nonclassical genes known as *HLA-E*, *HLA-F* and *HLA-G* [81, 82]. Class 1 genes encode the highly polymorphic heavy chain of HLA class 1 (45 kDa) which later joins the non-polymorphic B2 microglobulin encoded by chromosome 15 [81, 82]. Classic genes consist of eight exons, but the most important exons are exons 2 and 3 encoding for peptide binding groove. Other exons encode for transmembrane region and cytoplasmic tail [31, 83]. Beside these highly polymorphic classic HLA class 1 genes, there are three other HLA genes in class 1 known as *HLA-E*, *HLA-F* and *HLA-G* which are more conserved. Most probably, they are not involved in Ag presentation but in interaction with more conserved parts of the immune system. For example, *HLA-E*, which is minimally polymorphic, regulates cytotoxic activity of NK cells by interacting with CD94/NKG2 lectin-like receptors. The conservation within this gene guarantees that there is a constant protection for healthy cells in most people and provides a minimum safeguard for autoimmunity [32, 84, 85]. Some of them like HLA-G are expressed on trophoblastic cells and placental chorionic endothelium and induce immune tolerance during pregnancy [81, 86–90].

Class 2 consists of classic genes called *DP*, *DQ* and *DR* and nonclassic genes known as *DM* and *DO*. Classic genes encode for one highly polymorphic beta chain (26–28 kDa) and a less polymorphic alpha chain (33–35 kDa) [80]. Therefore, there are six classic *D* genes in this region. Genes for alpha chain consist of five exons, while beta chains are encoded by six exons. The exons 2 and 3 in both set of genes are responsible for encoding peptide binding domains [31].

HLA class 1 and *2* genes are the most polymorphic genes in the human genome with 2,365, 3,005 and 1,848 alleles for *HLA-A*, *HLA-B* and *HLA-C*, respectively, and 2,156 alleles for class 2 genes (based on IMGT/HLA database, release

3.13 on July 2013) [91]. This high polymorphism is mostly clustered in several hypervariable blocks in exons 2 and 3 which are responsible for encoding antigen binding groove. Therefore, a unique combination of sequence motifs in these hypervariable regions determine each allele [13]. This genetic structure is accompanied by high LD not only between *HLA* genes but also non-HLA genes constituting extended haplotypes [92]. The majority of polymorphisms in hypervariable regions result in amino acid substitutions in peptidebinding grooves, which in turn dramatically changes Ag binding affinity of the final product [13]; on the other hand, variants in noncoding regions, influence transcription, translation, and splicing and thereby expression levels [77].

Nowadays, with a few exceptions, HLA alleles are named by six or even eight digits. The first two digits are representative of the serological family the allele belongs to, while the third and fourth digits distinguish between different sequences affecting amino acid sequences. The next two digits are identifiers of synonymous polymorphisms, and seventh and eighth digits are used to distinguish intronic polymorphisms or ones located into untranslated regions [93].

17.6.3 From Polymorphisms to Clinic

HLAs are involved in cancer immunity and therefore in susceptibility and prognosis mainly by presenting certain Ags known as tumor-associated antigens (TAA). TAA are the first contact of malignant cells with adaptive immunity. Since introduction of the first TAA in melanoma patients in 1991, a broad heterogeneous group of Ags were discovered and associated with different malignancies. This heterogeneous group can be divided in to four classes of Ags [8, 94]:

1. Cancer–testis Ags are a result of epigenetic alterations leading to reactivation of silence genes. One of the famous examples is Ags from MAGE family. These Ags are not exclusive to just one type of cancer. The reason for this naming is that they are normally expressed in MHC-negative testicular germ cells and placental trophoblasts.
2. Differentiation Ags are normally expressed in the tissue of origin of the tumor, like Melan-A, and tyrosinase in melanomas
3. Unique tumor Ags are products of mutated tumor suppressor genes and oncogenes like abnormal product of RAS or p53. Fusion proteins as a result of chromosomal aberrations are also included in this group.
4. Infectious tumor Ags are expressed by oncogenic viruses associated with some malignancies. The examples are latent membrane proteins 1 and 2 (LMP-1 and LMP-2) in Epstein-Barr Virus (EBV)-associated Hodgkin lymphoma (HL) and E6 and E7 associated with human papillomavirus (HPV)-associated cervical cancer.

Nowadays, hundreds of HLA association studies prove that HLA alleles are important elements in predisposition to cancer. Seven mechanisms are suggested for complex relationship of HLA genotypes and susceptibility, prognosis, recurrence, and clinical response to immunotherapy and tumor vaccines:

1. *Efficiency in TAA presentation*: One of the major factors in Ag presenting ability of different HLA is the affinity of their Ag binding grooves to different epitopes. This affinity is highly dependent on the amino acid sequence in the hypervariable regions. Even one change in this sequence due to polymorphisms profoundly influences binding affinities to TAAs and Ags used in tumor vaccines and therefore susceptibility prognosis and response to tumor vaccines [32, 82, 95–97]. For instance, *HLA-A*0207* is associated with susceptibility to EBV-associated lymphoma in East Asian population, while *HLA-A*0201* is a protective factor; however, this huge difference at the clinical level is a result of a single amino acid change (Y99 to C) at the protein level [98, 99].
2. *Interaction with T cells and NK cells*: Change in variable regions and constant regions involved in interaction with T cells and NK cells can change HLA potential for inducing an effective immune response [96, 100].
3. *Efficiency in inducing immune response to infectious agents*: Antigen binding abilities of different HLA alleles influence immune reaction to infectious agents associated with malignant

transformation. For example, EBV is frequently emphasized as an important environmental factor in the pathogenesis of HL and nasopharyngeal carcinoma (NPC) [101]. Latent membrane protein-1 (LMP-1) and Epstein-Barr virus nuclear antigen (EBNA-4 and EBNA-6) proteins produced during latent infection by EBV, are efficiently presented by A*0201 and A*1101 respectively [83]. Therefore, these alleles can induce a strong immune response which consequently results in resolving the infection and lower chance of malignant transformation. Another example is the protective effect of DQB1*0301 allele on hepatitis C virus (HCV) infection, HCV-associated liver cirrhosis, and HCV-associated Hepatocellular carcinoma (HCC). This allele can efficiently present majority of immunodominant epitopes of HCV [102].

4. *Change in HLA expression patterns*: In some malignancies like melanoma, Burkitt's lymphoma, and carcinoma of the cervix and lung, HLA expression and Ag processing machinery are disturbed in order to prevent TAA presentation and consequently immune recognition of malignant cells. This mechanism is one of the major pathways for the immune escape of tumoral cells [10]. Some polymorphisms within the noncoding regions can influence expression levels [32]. In addition, some HLA alleles are specifically lost during malignant transformation [103]. Loss of HLA-A2 in colorectal cancers, breast cancer, and cervical cancer or lower expression levels of HLA-DR4 and HLA-DR6 in melanoma is a good example for these phenomena [104, 105]. On the contrary, some alleles like HLA-B*4405 are not dependent on some elements of the regular Ag processing machinery like transporter associated with Ag presentation (TAP) and therefore, can present antigens without susceptibility to viral-induced diminished TAP function [106].
5. *Increased susceptibility to chronic infections or autoimmunity*: Some HLA haplotypes and alleles are associated with various chronic inflammatory diseases which in turn predispose individuals to various cancers [75, 107]. Excess growth factors and prolonged proliferation in the background of chronic destruction increase the risk of malignant transformation [107].

In addition, chronic immune stimulation of B cells and prolonged and repeated DNA double-strand breaks associated with somatic hypermutation (SHM) and class switch recombination (CSR) significantly increase the chance of malignant transformation, and therefore, autoimmunity and chronic infection are important risk factors for some hematological malignancies like non-Hodgkin lymphoma (NHL) [107]. In these cases, HLA alleles can affect the extent of immune reaction and stimulation of B cells [107]. For instance, *HLA-DRB1*0301*, *HLA-B*0801 HLA-DRB1*0101,* and *HLA-DRB1*0401*, the susceptibility alleles of NHL is associated with autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome, and celiac disease [97, 102, 108]. The more prominent example is the paradoxical relationship of DQB1*0301 with HCV infection and HCV-related B-cell lymphoma. While DQB1*0301 is associated with a better immunologic control of HCV and a self-limiting infection, it is a susceptibility factor for HCV-related NHL. In this case, efficient presentation of viral antigens by DQB1*0301 in the context of persistent HCV infection results in $CD4^+$-dependent chronic stimulation of B cells [102].

6. *Sensitivity to mutation*: It is suggested that some HLA alleles are more susceptible to mutations like rearrangements of the DNA material and crossover. Such dramatic alterations might influence the function of oncogenes or tumor suppressors in the proximity of HLA genes. An example of such an oncogene is Waf1/p21 gene, located in 6p21.1 [100].
7. *Linkage disequilibrium*: LD with non-HLA genes of class 3 or even nonclassical HLA in the form of extended haplotypes can justify some of the founded associations. Some classical genes are in LD with certain HLA-G and HLA-E alleles which are both involved in suppression of NK cell-mediated immunity against tumors [73]. LD with non-HLA genes like *TNF-α*, in context with extended haplotype, can influence the relationship between toxicity of immunotherapy and HLA alleles. For example, high TNF-α increases the IL-2 toxicity in patients with melanoma [109, 110].

17.6.4 HLA Typing and HLA Association Studies: Lessons from the Past

HLA has a history as long as immmunogenetics itself. An observation of transfusion failures in 1952 paved the road to the discovery of the first HLA allele by Jean Dausset in 1958 [111]. Since 1958, there was a continuous international effort in order to share experimental data and HLA typing technologies, identify new HLA alleles and serotypes, and uncover the role of HLA system in pathogenesis of numerous diseases [31]. The result of such effort was the identification of over 9,500 alleles for HLA class 1 and 2 over a short period of four decades [31]. Along with the discovery of new alleles, the first nomenclature committee was held in 1987 followed by several nomenclature committees to unify the nomenclature and classification [31].

Early studies employed low-resolution serological methods which detected HLA on T cells or B cells [112]. Although these serological methods were subject to huge development in detection methods from complement-dependent cytotoxicity test to ELISA method, flow cytometry, and Luminex technique, the real breakthrough in HLA association studies was the introduction of PCR and high resolution DNA-based typing methods [31]. This technology allowed not only detection of high HLA polymorphisms with higher sensitivity and specificity but also the detection of new alleles with more flexibility by simply adding new probes to the old panels [113]. Nowadays, the old DNA-based method employing PCR-RFLP has been replaced by more rapid tests [113]. Generally, they either identify PCR products containing hypervariable regions by hybridization with sequence-specific probes (SSO) or employ sequence-specific primers (SSP) to identify variants as part of PCR process itself [13, 31, 114]. The latter was extensively used back in mid-1990 [13, 31, 114]. Even though aberrant typing as a sign of new allele can be followed by direct DNA sequencing, both methods are ineffective in case there is a new allele [13]. Later this limitation was overcome by polymerase chain reaction-sequence-based typing which can directly detect the sequence of alleles. In this method which is based on dye terminator chemistry, dye bounded 2,3 dideoxynucleotides are used as substrates for PCR process. Randomly addition of labeled dideoxynucleotides, and consequently, a stop in elongation of DNA chain result in the development of numerous DNA fragments with different sizes. These DNA fragments can easily be separated by capillary electrophoresis, and the ending dideoxynucleotides can be identified by specific fluorescence emitted from the related dye.

In parallel, huge efforts were made to understand the role of these alleles in etiology and natural history of several diseases. In oncology, the first association was found in HL in 1967 [32]. This finding triggered a series of HLA association studies on different cancers worldwide. The fruit of this global movement was finding association between HLA alleles and susceptibility to several hematological malignancy including HL, NHL, childhood acute lymphoblastic leukemia, Kaposi's sarcoma, chronic myeloid leukemia (CML), and also non-hematological malignancies including nasopharyngeal carcinoma, thyroid cancer, renal cell carcinoma (RCC), cervical cancer, and both melanoma and non-melanoma skin cancers [13, 115]. Moreover, investigations on natural history of cancers showed relationship of several alleles from both classes with mortality in ovarian cancer, non-small cell lung carcinoma, head and neck squamous carcinoma, and local recurrence in melanoma [73, 96, 100]. Several studies showed importance of HLA context in the outcome of immunotherapy and tumor vaccines in melanoma, RCC, cervical carcinoma and CML [73, 95, 110, 116].

Although the result of such studies was inconsistent in some cases, most studies pointed to the undeniable role of HLA polymorphism in susceptibility, prognosis, natural history, and response to immunotherapy in different cancers [32]. These past experiences emphasize that a prestigious HLA association study is a complex art rather than a simple case-control study and several factors should be considered in interpreting their results. In this regard, results of meta-analysis of these association studies are more reliable (Table 17.2).

Table 17.2 Significant results from published meta-analysis of HLA associations with cancers

Alleles	Cancer site	Total number of cases	Total number of controls	OR ±95 % CI	Population included	Reference
DQB1*03	Hepatocellular carcinoma	398	593	0.65 (0.48–0.89)	China, Italy, Spain, Egypt	Xin et al. [117]
DQB 1*02	Hepatocellular carcinoma	398	593	1.78 (1.05–3.03)	China, Italy, Spain, Egypt	Xin et al. [117]
DQB1*0502	Hepatocellular carcinoma	257	349	1.82 (1.14–2.92)	China, Spain	Xin et al. [117]
DQB1*0602	Hepatocellular carcinoma	173	226	0.58 (0.36–0.95)	China, Spain	Xin et al. [117]
HLA-DRB1*07	Hepatocellular carcinoma	281	466	1.65 (1.08–2.51)	China, Italy, Spain, Egypt	Lin et al. [118]
		156	224	2.1 (1.06–4.14)	China	Lin et al. [118]
		125	242	1.41 (0.83–2.42)	Italy, Spain, Egypt	Lin et al. [118]
HLA-DRB1*12	Hepatocellular carcinoma	281	516	1.59 (1.09–2.32)	China, Italy, Spain, Thailand	Lin et al. [118]
		206	324	1.73 (1.17–2.57)	China, Taiwan	Lin et al. [118]
		75	192	0.3 (0.04–2.47)	Spain, Italy	Lin et al. [118]
HLA-DRB1*15	Hepatocellular carcinoma	281	466	1.7 (0.8–3.59)	China, Italy, Spain, Egypt	Lin et al. [118]
		156	224	3.22 (1.63–6.37)	China	Lin et al. [118]
		125	242	0.8 (0.34–1.89)	Spain, Egypt, Italy	Lin et al. [118]
HLA-DRB1* 0701	Cervical squamous cell carcinoma	1,445	2,206	1.59 (1.09–2.35)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
		1,083	1,248	1.29 (1.02–1.63)	Caucasians	Yang et al. [119]
HLA-DRB1* 1301	Cervical squamous cell carcinoma	2,743	3,904	0.63 (0.52–0.78)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
		2,013	2,360	0.61 (0.48–0.77)	Caucasians	Yang et al. [119]
HLA-DRB1* 1302	Cervical squamous cell carcinoma	1,877	2,966	0.49 (0.36–0.68)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
		2,013	2,360	0.75 (0.57–0.98)	Caucasians	Yang et al. [119]
HLA-DRB1* 1501	Cervical squamous cell carcinoma	1,915	2,628	1.42 (1.23–1.65)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
		2,191	2,628	1.22 (1.01–1.47)		Yang et al. [119]
HLA-DRB1* 1502	Cervical squamous cell carcinoma	1,424	2,184	1.87 (1.08–3.26)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]

(continued)

Table 17.2 (continued)

Alleles	Cancer site	Total number of cases	Total number of controls	OR ±95 % CI	Population included	Reference
HLA-DRB1* 1503	Cervical squamous cell carcinoma	432	894	3.4 (1.69–6.87)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
HLA-DRB1* 1602	Cervical squamous cell carcinoma	1,314	2,234	0.61 (0.38–0.98)	Iran, USA, England, Sweden, France, Brazil	Yang et al. [119]
HLA-DRB1* 0403	Cervical squamous cell carcinoma	1,796	2,050	2.05 (1.02–4.12)	Caucasians	Yang et al. [119]
HLA-DRB1* 0405	Cervical squamous cell carcinoma	1,496	1,700	6.13 (1.03–36.33)	Caucasians	Yang et al. [119]
HLA-DRB1* 0407	Cervical squamous cell carcinoma	1,796	2,050	2.71 (1.11–6.61)	Caucasians	Yang et al. [119]
HLA-DRB1* 0901	Cervical squamous cell carcinoma	1,796	2,050	0.58 (0.34–0.99)	Caucasians	Yang et al. [119]

17.6.5 Typing Methods

Indeed, immunogenetic studies are deeply influenced by technological advances. Low-resolution serologic HLA typing was one of the major limitations in early studies [83]. Serologic typing is only enabled to identify the family of alleles. This family often comprises a heterogeneous group of alleles with different affinities and different potential for Ag presentation. Since distribution of alleles belonging to the same serotype is different in various populations, such studies often obtained conflicting results in different populations. One of the best historical examples is HLA association studies in nasopharyngeal carcinoma (NPC).

NPC, as an epithelial carcinoma of the head and neck origin, was one of the main focuses of early HLA association studies. Early serological studies showed an association between HLA-A2 and NPC-in Chinese population, while studies in Caucasians found HLA-A2 as a protective allele for both NPC and EBV-associated HLA [106, 120–124]. Later, higher-resolution studies showed HLA-A*02:07, a common allele in Chinese population but rare among Caucasians, as the main risk factor, while HLA-A*02:01, a common allele in Caucasians, was shown to be the actual protective factor in this population [125, 126]. It is possible that future studies employing higher-resolution methods reveal even new causal variants within the current associations.

17.6.6 Environmental Factors

Various environmental and genetic factors play roles behind scenario of cancer, and malignant transformation is the result of a complex interaction between these factors. It is often the case that certain genetic factors need certain environmental factors to play their role in pathogenesis of cancer. The role of environmental factors in HLA association studies is more prominent in virus-associated malignancies like HL, NPC and cervical cancer. Each virus has different strains with different Ags and the prevalence of these strains is not the same in different populations. Each strain is best presented by certain HLA alleles. Therefore, one HLA allele efficient for presenting Ags of one population's prevalent strain may not present Ags of another population's prevalent strain efficiently [83]. Such a phenomenon might be extended to other environmental factors like virus prevalence, viral load, diet, cigarette smoking, and socioeconomic status, all of which are highly dependent on the population under study [74, 127]. For instance, pathogenesis of cervical cancer is dependent on persistent infection with high-risk human papillomavirus (HPV) and this risk factor itself is highly related to socioeconomic status, sexual relationship, and prevalence of high risk variants in the region [127, 128].

17.6.7 Linkage Disequilibrium

MHC region is home to more than 200 genes beside classic HLA genes. Due to the low recombination rates, these genes are often in strong linkage disequilibrium together [78]. This strong LD can complicate finding the actual causal allele. The problem gets worse when the causal allele is an unknown allele in strong LD with the associated allele. This limitation can be overcome by whole genome sequencing (WGS) of the region in close proximity of the associated allele [101]. One example is the association of NPC with HLA-A*0207 and HLA-B*4601 which are in strong LD. In this case, either allele, both of them, or even a third allele in LD with both of them might influence the pathogenesis of NPC [126].

Some studies reported extraordinary LD in MHC region between alleles from one class and alleles of other classes and even non-HLA genes. This extraordinary haplotypes are known as extended haplotypes [83]. Thus, in interpreting results of HLA association studies or design of one, non-HLA genes such as the *transporter associated with Ag processing (TAP) MHC class I chain-related A (MIC-A), heat shock proteins (HSP)*, and *TNF-α* which are located nearby or within the classic HLA genes should be considered [78, 83]. These extended haplotypes are

especially of importance in immunogenetic studies of cancers, since numerous elements of the immune system are in the front line of defense against cancer.

For instance, the ancestral haplotype 8.1 (AH 8.1: HLA-A*01-B*08-Cw*07-DRB1*03-TNF-G308A), in which HLA alleles are in LD with TNF-α, is the most frequent extended MHC haplotype in Caucasian populations [109]. Primarily, this extended haplotype was associated with clinical course of NHL [75, 109]; however, later studies showed that polymorphism in *TNF-α* gene has a more prominent effect in this association compared to Cw*07 and DRB1*03 alleles [8, 75]. In this case, polymorphisms in TNF-α promoter influence TNF-α expression levels. TNF-α level consequently affects the extent of immune activation upon tumor challenge. In addition, increased TNF-α impairs Ag presentation potential of APCs and by its effect on cytokine profile results in a bias toward Th2 immune responses [75]. All these factors can contribute to the exacerbation of systemic symptoms, anemia, hypoalbuminemia, and poor outcome [8].

Another example is the association of HLA-A*03 and chronic myeloid leukemia (CML) [78]. A translocation between t(9;22)(q34;q11) creating a truncated chromosome 22 known as Philadelphia chromosome is present in majority of patients with CML [129]. Depending on the precise location of the fusion, different fusion proteins are encoded. Keeping this in mind and the absence of costimulatory molecules on CML cells, it is improbable that the association of HLA-A*03 is due to its efficiency in presenting fusion proteins and its ability to induce an effective immune response [78]. However, this allele is in with the C282Y mutation of the *hemochromatosis gene*, a susceptibility marker for CML [78].

In some cases, an optimal immune response is dependent on optimal Ag presentation by both HLA classes and the presence of certain alleles in non-HLA genes. An absence of one of these optimal alleles may result in anergy and immune escape. In some populations, these alleles might be in LD in form of an unknown extended haplotype, while in other populations this haplotype might be absent [57]. One of such associations has been reported between cervical squamous cell carcinoma and multi-locus haplotype of B*4402-Cw*0501-DRB1*0401-DQB1*0301 [57].

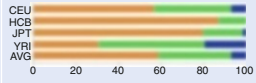
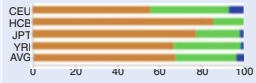
17.7 The Cytokine Network

17.7.1 Background

Cytokines are a group of soluble regulatory factors by which the immune system controls and modulates different activities of its cells. Each cytokine triggers certain cascade of events in their target cells by binding to their receptors and activating intracellular signal transduction pathway [14, 20]. Cytokine network is responsible for coordination of effector actions of different elements of the immune system, as well as the differentiation and proliferation of different immune cells. In addition, secretion of antibodies and inflammation is tightly regulated by complex interaction between these cytokines [13, 23, 26].

Chronic inflammation, by inducing chronic tissue damage and compensatory cell proliferation, is a considered a major promoter of malignant transformations. As an example, nitric oxide, produced during inflammation, might damage DNA structure in different tumor suppressor genes and oncogenes [130]. Therefore, any dysregulation in cytokine network can result in excessive production of tumor-inducing factors, DNA damage, angiogenesis, and dysplasia and consequent development of various inflammatory diseases including different cancers [26, 131]. Cytokine network is a determinant factor in the development of metastasis and natural history of cancers [26]. In some cancers, malignant cells can manipulate cytokine network in order to escape immunosurveillance or promote their own proliferation [130, 132]. In addition, cytokine network can influence the outcome and toxicity of different immunotherapy methods [13, 20, 133]. Several cancers including hepatocellular carcinoma (HCC), oral squamous cell carcinoma, melanoma, the gastric, pancreatic, and prostate cancer were associated with high

Table 17.3 Genotype details for SNPS of IL-1

SNP	GMAF[a] [137]	Population diversity[b] [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800587	*T*=0.253		−889 C>T	NA[c]	T allele: ↑
rs17561	*T*=0.203		+4845 G>T	Ala114Ser	T allele: ↑

[a]GMAF: the minor allele frequency in 1,094 worldwide individuals provided from 1,000 genome phase 1 genotype data
[b]*CEU* European, *CHB* Han Chinese, *JPT* Japanese Tokyo, *YRI* Yoruba African, *AVG* Mathematical average of all samples
[c]*NA* not applicable

levels of certain proinflammatory or antiinflammatory cytokines [26].

Cytokine levels are not the same in all individuals. Interindividual differences in cytokine levels in both baseline and stimulated phases are a result of both genetic and environmental factors [133]. Since there is no an intracellular storage for cytokines, their secretion is dependent on the transcriptional and translational rates of their genes [14, 26]. Not surprisingly, genes responsible for encoding cytokines and their receptors are relatively polymorphic [13, 20, 23]. Several polymorphisms in their gene can affect their expression, structure, and activity [20, 23, 26, 130, 134]. Most of these polymorphisms are in non-coding regions including promoter or intronic sequences and exonic regions are usually highly conserved [13, 14]. So far, numerous genetic association studies have been suggested as associations of these SNPs with various cancers in different populations. However, results of such studies were often inconsistent, and the reported associations varied not only in different populations but also in different cancers and even in their different subtypes [131]. Therefore, a meta-analysis of these studies can show some more conclusive evidence of these associations.

In addition to polymorphisms of cytokine genes, there are other polymorphic elements such as various transcription factors and cytokine-specific receptors which are involved in actions of cytokine network [20, 26]. For instance, polymorphisms in the *NF-κB* nuclear factor-kappa B gene, one of the most important transcription factors, can result in extensive changes in the cytokine network by altering transcription of TNF-a, IL-1, IL-6, and IL-8 [20]. Although the exact roles of these polymorphisms in tumor immunology are less clear, the relevance of this role is becoming more and more apparent in recent years [20].

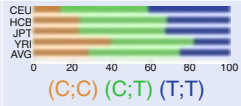
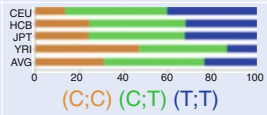
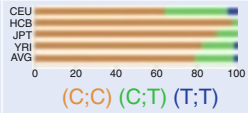
17.7.2 Interleukin-1 Superfamily

IL-1α and IL-1β and their antagonist IL-1Ra are members of this superfamily with pleiotropic effects on inflammation, immunity, and hemopoietic system. High levels of IL-1 are found in tumor sites, however IL-1 family plays an ambivalent role in tumor immunity. IL-1 induces cytokine secretion from T cells to potentiate the differentiation and function of immunosurveillance cells. On the other hand, IL-1 induces the expression of adhesion molecules, matrix metalloproteinases, growth factors, and angiogenic factors and promotes invasiveness and metastasis of malignant cells [135, 136].

17.7.2.1 Interleukin-1 α

IL-1α is encoded by seven exons of a gene located in 2q14. Variant−889 C>T (rs1800587) is one of the common promoter variants of IL-1α gene (Table 17.3). Although, the promoter containing T allele has been shown to result in a marginally

Table 17.4 Genotype details for SNPS of IL-1β

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs16944	*C*=0.465		−598 T>C	NA	C allele: ↑
rs1143627	*C*=0.4808		−31 C>T	NA	T allele: ↑
rs1143634	*T*=0.146		+3954 C>T	NA	UA[a]

[a]*UA* unavailable

higher level of expression, at the protein level, T allele was associated with significantly increased IL-1α levels which could not be justified by only different expression patterns. Further studies showed that this SNP has high LD with an exonic SNP in +4845 G>T (rs17561) resulting in substitution of alanine with serine at the position of 114 which results in more efficient process of pre-IL-1α comparing to Ala114 and consequently higher release of IL-1α [23].

17.7.2.2 Interleukin-1 β

High levels of IL-1β have been shown to be associated with increased risk of most human cancers and also poor prognosis in cancer patients [130, 132, 139]. IL-1β is encoded by a 7.5 kb gene with seven exons located on 2q14. Its expression is regulated by two distal and proximal promoter elements [140, 141]. So far, several polymorphisms have been identified in this gene. −598T>C (rs16944) and −31 C>T (rs1143627) are two common variants in the promoter region, and +3954 C>T (rs1143634) is a common synonymous polymorphism in coding region of IL-1β gene (Table 17.4) [26].

In northern and western European ancestry (CEU), −598T>C (rs16944) and −31 C>T (rs1143627) had strong LD (r^2=0.94) [26, 132]. *In vivo*, −598C/−31T haplotype has been associated with higher IL-1β levels in the lungs and gastric mucosa. It is suggested that −31 C>T (rs1143627) is the causal variant of this haplotype [23, 141]. In the same line, *in vitro* studies like luciferase reporter assay showed higher expression of luciferase gene with promoter containing T allele in −31 C>T (rs1143627) [23]. Results of EMSA studies suggested that this higher expression is a result of higher affinity for several transcription factors as a result of a change in a TATA-box motif [23].

T allele in rs1143634 was associated with increased IL-1β secretion and several inflammatory diseases [132]. However, no evidence on the functionality of +3954 C>T (rs1143634) is available, and it seems that +3954 C>T (rs1143634) is just a marker for a functional polymorphism such as −31 T>C (rs1143627) [23, 26].

One recent meta-analysis of 81 case-control studies with 19,547 patients with HCC, gastric, lung, blood, cervical, esophageal, prostate, breast, and skin cancers and 23,935 controls showed that, overall, −598T>C (rs16944) has no significant association with cancers [132], while another meta-analysis of 26 studies with 8,083 patients with cancer and 9,183 controls showed a significant association of +3954 C>T (rs1143634) with increased risk of cancers in a dominant model which is in accordance with the results of another metaanalysis of 33 studies (Table 17.5) [132, 145].

A meta-analysis of studies on associations between *IL-1β* gene polymorphisms and gastric cancer published from January 2000 to December 2009 (including 18 studies with 4,111

Table 17.5 Significant results from published meta-analysis of associations of IL-1β polymorphisms with cancers

Alleles	Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
rs16944	Gastric cancer	2,041	2,441	TT + CT *vs.* CC	1.23 (1.09–1.37)	Italy, Japan, China, Korea, Portugal, UK, mixed Asian	Vincenzi et al. [142]
	Cervical cancer	836	980	TT *vs.* CC	1.74 (1.28–2.36)	Egypt, Korea, India, China	Xu et al. [132]
				CT *vs.* CC	1.71 (1.32–2.23)		
				TT + CT *vs.* CC	1.74 (1.35–2.23)		
	Hepatocellular carcinoma	890	821	CT *vs.* CC	0.75 (0.60–0.94)	Japan, Taiwan, Thailand	Xu et al. [132]
				TT + CT *vs.* CC	0.68 (0.47–0.99)		
	Blood cancers	3,839	3,762	CC + CT *vs.* TT	1.19 (1.04–1.37)	Italy, Spain, Germany, USA, Canada, Greece	Xu et al. [132]
rs1143627	Lung cancer	3,435	4,719	TT + TC *vs.* CC	1.23 (1.06–1.43)	China, Italy, mixed European, Denmark	Peng et al. [143]
	Gastric cancer	1,535	2,585	TT + TC *vs.* CC	1.16 (1.01–1.33)	Korea, Mexico, China, Brazil, Italy, USA	Vincenzi et al. [142]
	Hepatocellular carcinoma	1,039	1,588	CC + CT *vs.* TT	1.31 (1.09–1.57)	Japan, Taiwan, Morocco	Jin et al. [144]
rs1143634	Malignancy	8,083	9,183	TT + CT *vs.* CC	1.15 (1.01–1.30)	Sweden, Poland, China, UK, Germany, Tunisia, Costa Rica, Oman, USA, Greece, Netherlands, Norway, Japan	Xu et al. [132]
	Gastric cancer	2,359	3,613	CT *vs.* CC	1.16 (1.03–1.32)	USA, China, UK, Germany, Italy, Japan, India, Sweden, Oman	Zhang et al. [145]
	Oral cancer	346	417	CT *vs.* CC	0.65 (0.45–0.94)	Greece, China	Zhang et al. [145]
				TT + CT *vs.* CC	0.69 (0.49–0.98)		

controls and 3,295 cases for −598T>C (rs16944), 21 studies with 5,883 controls and 3,786 cases for −31 T>C (rs1143627) polymorphism, 10 studies with 3,610 controls and 1,559 cases for +3954 C>T (rs1143634)) showed significantly increased risk of cancer in individuals with IL-1β −598T allele. In stratified analysis for different ethnicities, such an association was present in Caucasians but not in Asians or in Hispanics. This study also showed such an association for intestinal-subtype and noncardia gastric cancer [146, 147]. However, this study didn't show any significant associations between gastric cancer risk and −31 T>C (rs1143627) and +3954 C>T (rs1143634) [146]. Older studies conducted on 2005 and 2007 more or less showed such pattern for this SNP [142, 147]. However, a meta-analysis of five studies published up to September 2008 showed association of +3954 C>T (rs1143634) and gastric cancer risk in Chinese and Japanese population [148].

Another systematic review evaluating associations of HCC with polymorphisms of IL-1 gene (reported up to September 2010) and a meta-analysis of 1,279 patients with lung cancer and 2,248 controls failed to support any significant increased risk for −598T>C (rs16944) and −31 C>T (rs1143627) [143, 149].

17.7.2.3 Interleukin-1Ra (IL-1Ra)

IL-1RA has antiinflammatory properties by competing with IL-1 cytokines in binding to their receptors. This cytokine is encoded by *IL-1RN* gene located on 2q14.2. Its transcript may contain six, five, or four exons [23, 130]. There is an 86-bp variable number tandem repeat (VNTR) in intron 2 of this gene [23]. The short allele of this VNTR contain only two repeats (IL-1RN*2), while long alleles may have three to six repeats (IL-1RN L) [58, 146]. The more prevalent allele containing four repeats is named IL1RN*1 [150]. *In vitro* and *in vivo* studies have shown extensive associations of this variant with the members of IL-1 superfamily. IL-1RN*2 was associated with not only higher IL-1RA levels but also enhanced IL-1β production and decreased IL-1α production [151]. However, the final result of IL-1RN*2 was decreased IL-1RA/IL-1β ratio, followed by prolonged proinflammatory immune response [23]. Although, intronic VNTR contains potential binding sites for an interferon-α silencer, an interferon-β silencer, and an acute-phase response element, all leading to its functional importance, these associations are suggested to be a result of LD with other variants [140, 152]. Some authors suggested that the enhancing effect of IL-1RN*2 on IL-1RA levels is dependent on the presence of −511T allele or the absence of +3954T in IL-1β [23].

A meta-analysis of 71 case-control studies (including 37 studies on gastric cancer, six studies on HCC, four on cervical cancer, four on breast cancer, four on lung cancer, and 16 studies on other cancers) with 14,854 cases and 19,337 controls showed that overall carriers of IL-1RN*2 are significantly more susceptible to cancer (Table 17.6) [145].

17.7.3 Interleukin-4

Interleukin-4 (IL-4) is a pleiotropic cytokine with major roles in regulation of humoral immunity by its various effects on production of several other cytokines and dedifferentiation of B cells and promoting expression of class II MHC Ags [26, 130]. It also has potent antitumor activity against various tumors by its inhibitory effect on the growth of tumor cells and its growth stimulatory effect on lymphocytes [153, 154].

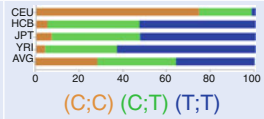
IL-4 gene is located on the long arm of chromosome 5 (5q31.1), and through recent years, many variants identified on this gene. Among these variants, −589 C>T (rs2243250) is a promoter SNP of which T allele is associated with increased production of IL-4 in *in-vivo* studies [26, 155]. The other variant of this gene is a 70-bp VNTR at intron 3 (Table 17.7) [155].

A meta-analysis of 8,715 patients with various cancers and 9,532 controls presented in 23 case-control studies found no significant association between this SNP and overall cancer susceptibility. This study also didn't find any significant relationship in stratified analysis for ethnicity or different cancer types [156]. However, another meta-analysis of 14 studies involving 3,562

Table 17.6 Significant results from published meta-analysis of associations of IL-1RN VNTR with cancers

Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
Malignancy	14,854	19,337	22 *vs.* LL 2 L *vs.* LL 22 + 2 L *vs.* LL 2 *vs.* L	1.37 (1.07–1.75) 1.19 (1.07–1.32) 1.25 (1.12–1.41) 1.23 (1.10–1.38)	40 studies of Asian descendents, 29 of Caucasian descendents, and two with mixed ethnicity	Zhang et al. [145]
Breast cancer	1,145	1,102	2 L *vs.* LL 22 + 2 L *vs.* LL	0.74 (0.58–0.93) 0.78 (0.62–0.97)	Japan, Germany, Korea, India	Zhang et al. [145]
Gastric cancer	3,209	4,856	2 L *vs.* LL 22 + 2 L *vs.* LL 2 *vs.* L	1.22 (1.05–1.41) 1.25 (1.09–1.43) 1.20 (1.05–1.38)	Portugal, China, Germany, Brazil, Taiwan, Thailand, UK, Italy	Zhang et al. [145]
	3,418	5,789	22 + 2 L *vs.* LL	1.26 (1.06–1.51)	Arab, Brazil, Netherland, Korea, USA, China, Italy, Mexico, South Korea, Germany, Taiwan, Portugal, Poland	Xue et al. [146]

Table 17.7 Genotype details for SNPS of IL-4

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs2243250	$T = 0.484$		−589C>T	NA	T allele: ↑

cancer cases found that T allele was significantly associated with decreased oral cancer risk and increased risk of RCC (for oral cancer, TT *vs.* CC: OR = 0.40, 95 % CI = 0.19–0.84; TT + CT *vs.* CC: OR = 0.45, 95 % CI 0.22–0.94; and for renal cell carcinoma, TT *vs.* CC: OR = 1.98, 95 % CI = 1.06–3.69; TT *vs.* CC + CT OR = 1.43, 95 % CI = 1.05–1.95) [157].

17.7.4 Interleukin-6 (IL-6)

IL-6, a 23.7 kD proinflammatory cytokine, is involved in inducing acute-phase response, differentiation of monocytes to macrophages, proliferation of T cells and Th2 cytokine production [158]. It has been previously shown to be of importance in susceptibility, natural history, and prognosis of several malignancies including prostate cancer, colorectal carcinoma, and breast cancer [23, 26]. This cytokine is encoded by a gene on chromosome 7p21 with five exons [159]. Two common promoter variants of IL-6, −174G>C (rs1800795) and −572G>C (rs1800796), were extensively studied in different inflammatory diseases (Table 17.8). −174G>C (rs1800795) is the first identified common promoter variant of IL-6 [23]. C allele in both of these variants was associated with lower IL-6 levels in several studies [134, 138, 160–165]. However, such an effect on IL-6 levels was not confirmed by some studies on −174G>C (rs1800795) [134, 160–164]; therefore, this inconsistency might be the result of partial LD between this SNP and an actual functional SNP [23]. EMSA studies showed that −572G>C (rs1800796) is not in a TFBS; therefore, its influence on IL-6 serum levels probably results from strong LD with a functional variant such as −6331 T>C (rs10499563) [160]. C allele in −572G>C (rs1800796) is

Table 17.8 Genotype details for SNPS of IL-6

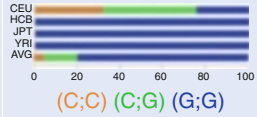
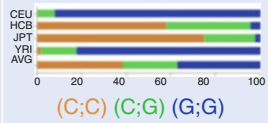
SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800795	*C* = 0.185		−174G>C	NA	C allele: ↑
rs1800796	*C* = 0.290		−572G>C	NA	C allele: ↑

Table 17.9 Significant results from published meta-analysis of associations of −174G>C (rs1800795) in IL-6 gene with cancers

Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
Colorectal cancer	3,061	4,024	GC/CC *vs.* GG	0.75 (0.64–0.88)	Individuals from Denmark, USA, and Spain who regularly or currently took NSAIDs	Yu et al. [166]

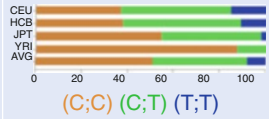
highly associated with T allele in −6331 T>C (rs10499563) [160]. Interestingly, T allele in this SNP is associated with higher expression of IL-6 gene [160]. −6331 T>C (rs10499563) is near the distal promoter of IL-6 located between −5202 and −5307. EMSA studies showed that T allele in −6331 T>C (rs10499563) resulted in more affinity for Oct-1 of which binding changes the chromatin structure and locates the distal promoter to the transcription start site [23].

A systematic review of 12 case-control studies on breast cancer (published till December 2009) with 10,137 cases and 15,566 controls found no significant association between −174G>C (rs1800795) and susceptibility to breast cancer [134]. Similarly, another meta-analysis of 7,210 patients with colorectal cancer and 9,467 controls did not show any significant association in any genetic model between −174G>C (rs1800795) and colorectal cancer [166]. However, in stratified analysis in a subgroup of patients with the history of current or habitual use of NSAIDs (3,061 cases and 4,024 controls), carriers of C allele in −174G>C (rs1800795) had significantly lower risk for colorectal cancer (Table 17.9) [166]. This study didn't show any significant association between colorectal cancer and −572G>C (rs1800796) in 2,574 cases and 3,344 controls [166]. In line with this, two recent meta-analyses on gastric cancer patients did not confirm any effect of these two SNPs on susceptibility to cancer [167, 168]. The most recent one evaluated 13 studies reporting associations of −174G>C (rs1800795) (1,581 gastric cancer patients and 2,563 controls) and four studies on −572G>C (rs1800796) [167]. In addition, a systematic review of 2,949 patients with lung cancer and 3,375 controls did not show any significant association between −174G>C (rs1800795) and lung cancer [143].

17.7.5 Interleukin-8

IL-8, a member of human α-chemokine subfamily, has a major influence on tumor invasion and metastasis by its stimulatory properties on angiogenesis and inflammation [23, 26, 59, 169, 170]. A gene located on chromosome 4q13–q21 with four exons is responsible for encoding this

Table 17.10 Genotype details for SNPS of IL-8

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs4073	$T=0.497$	UA	–251A/T	NA	A allele: ↑
rs2227306	$T=0.294$		+781C/T	NA	T allele: ↑
rs2227307	$G=0.422$	UA	+396T/G	NA	UA

cytokine [169]. Fifteen functional SNPs have been identified within this gene including –251A>T (rs4073), +396T>G (rs2227307), and +781C>T (rs2227306) (Table 17.10) [26]. –251A>T (rs4073), located in the promoter region, was identified in 2000. Although there was little evidence on the functionality of this SNP *in vitro*, several *in vivo* studies showed higher levels of IL-8 in carriers of A allele [23]. On the contrary, one study showed higher transcription for T allele in gastric carcinoma cell line [131, 171]. EMSA studies showed that T allele in +781 C>T allele (rs2227306) is associated with higher binding ability for a transcription factor (C/EBPb) [23]. Several studies showed associations of –251 A>T (rs4073) with lung, gastric, colorectal, bladder, and prostate cancer in different populations (Table 17.11) [155]. A meta-analysis of 13,189 patients with lung, prostate, breast, colorectal, and nasopharyngeal cancers and 16,828 controls showed that carriers of A allele in –251 A>T (rs4073) which were more susceptible to different cancers [131]. Another study reviewed results of 45 studies including 14,876 cases and 18,465 controls and showed such an association only among hospital-based studies and surprisingly showed significantly decreased risk of cancers for AA genotype among population-based studies [171]. It should be noted that hospital-based studies have an increased chance of a selection bias since hospital-based controls might have disease conditions under the influence of the studied polymorphism [169].

Another systematic review of ten papers including 2,195 gastric cancer patients and 3,505 controls confirmed that AA genotype was a risk factor for gastric cancer in whole population and in Asian population. In stratified analysis for tumor location and histology, this association remained significant only in the cardia gastric cancer and diffused type [59]. A more recent meta-analysis evaluating papers on gastric cancer published from January 2000 to January 2011 (18 papers including 6,554 controls and 4,163 cases) also found such an association in Asians but not in Caucasians. However, unlike the previous study, when stratifying for pathology types, the association remained significant only in intestinal-type cancer but not in the diffused type [174].

A systematic review of 1,324 patients with oral cancer and 1,879 controls reported in six studies (published till October 2012) also showed higher risks of oral cancer in carriers of A allele in –251A>T (rs4073). In subgroup analysis for ethnicity, there were only significant associations among Caucasians but not in Asians [172].

On the contrary, T allele in this SNP was associated with an increased risk of breast cancer in Asian and African populations. However, this study showed no significant associations between this SNP and breast cancer in 1,880 breast cancer patients and 2,013 controls [173]. There were not any significant associations between this SNP and colorectal cancer in a meta-analysis of nine case-control studies with 3,019 cases and 3,984 controls [175].

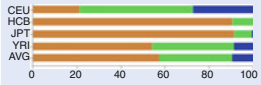
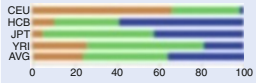
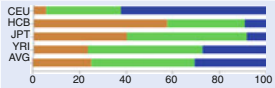
17.7.6 Interleukin-10

IL-10 is a pleiotropic, immunoregulatory cytokine which can affect both the innate and adaptive immune systems [176]. IL-10 has pleiotropic effects on tumor immunology. It

Table 17.11 Significant results from published meta-analysis of associations of −251T/A (rs4073) in IL-8 gene with cancers

Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
Malignancy	13,189	16,828	AA *vs*. TT	1.21 (1.08–1.36)	Tunisia, Iran, Denmark, UK, Croatia, Germany, USA, Greece, China, Japan, Portugal, Spain, Mexico, Finland, France, Norway, Poland, Korea, India, Netherlands	Wang et al. [131]
			(AA + TA) *vs*. TT	1.12 (1.03–1.23)		
	5,633	8,240	(AA + TA) *vs*. TT	0.90 (0.83–0.97)	(Population-based studies)	Gao et al. [171]
Gastric cancer	3,036	3,082	AA *vs*. TT	1.48 (1.13–1.95	(Asian population)	Wang et al. [131]
			TA *vs*. TT	1.20 (1.04–1.40)		
			(AA + TA) *vs*. TT	1.27 (1.08–1.48)		
	4,274	6,498	AA *vs* TT	1.28 (1.02–1.62)	Japan, Iran, China, Korea, Finland, Spain, Mexico, Poland	Wang et al. [131]
			(AA + TA) *vs*. TT	1.17 (1.01–1.36)		
Nasopharyngeal cancer	440	459	AA *vs*. TT	2.04 (1.38–2.99)	Tunisia, China	Wang et al. [131]
			TA *vs*. TT	1.59 (1.19–2.13)		
			(AA + TA) *vs*. TT	1.70 (1.30–2.24)		
	545	568	(AA + TA) *vs*. TT	1.48 (1.16–1.89)	Tunisia, China	Gao et al. [171]
Oral cancer	1,324	1,879	AA *vs*. (AA + TA)	1.23 (1.03–1.46)	China, Taiwan, Thailand, Greece, Japan, France	Wang et al. [172]
			AT *vs*. TT	1.25 (1.07–1.47)		
Breast cancer	683	880	TA *vs*. AA	1.444 (1.092–1.908)	Iran, China	Xue et al. [173]
			AA *vs*. (AA + TA)	1.435 (1.107–1.861)		
	717	537	TA *vs*. AA	0.541 (0.396–0.741)	Tunisia	Xue et al. [173]
			AA *vs*. (AA + TA)	0.737 (0.570–0.953)		
	1,262	1,419	TT *vs*. (AA + TA)	0.692 (0.566–0.861)	(Population-based) Tunisia, China, UK	Xue et al. [173]

Table 17.12 Genotype details for SNPS of IL-10

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800896	$G=0.303$		−1082 A>G	NA	G allele: ↑
rs1800871	$T=0.409$		−819 C>T	NA	UA
rs1800872	$C=0.409$		−592 A>C	NA	UA

plays an antiinflammatory role by inhibiting production of proinflammatory mediators such as IL-1α, IL-1β, IL-6, IL-8, IL-12, TNF-α and IFN-γ [23, 63]; in addition, IL-10 inhibits presentation of tumor Ags by suppressing the expression of HLA molecules [130, 133]. On the other hand, IL-10 induces proliferation in B cells and T cells and regulates angiogenesis in various cancers [26, 177].

Twin studies demonstrated that IL-10 levels are significantly influenced by genetic factors with a heritability of 74 % [23, 178]. IL-10 is encoded by five exons of a gene located on 1q31-1q32. At least 40 SNPs have been identified in this gene [62, 63, 179]. Several common variants including −1082 A>G (rs1800896), −819 C>T (rs1800871), and −592 A>C (also called −571 rs1800872) have been identified within the promoter region of this gene (Table 17.12) [177].

In vivo studies showed higher levels of IL-10 in individuals with GCC haplotype of these three SNPs, while ATA haplotype was associated with the lowest levels of IL-10 [23, 133]. It is suggested that −1082 A>G (rs1800896) is the most functional SNP of these three variants and G allele in this SNP results in higher IL-10 levels [23]. EMSA studies showed different affinities of alleles of this SNP for a nuclear protein identified as poly ADP-ribose polymerase1 (PARP-1) which acts as a transcription repressor [23, 62]. So far, several studies have evaluated the associations of different IL-10 polymorphisms with various cancers including lung cancer, breast cancer, cervical cancer, gastric cancer, melanoma and nasopharyngeal cancer, and prostate cancer [62, 63]. A systematic review evaluated associations of −1082 A>G (rs1800896) with risk of malignancy by reviewing results of 61 articles (published up to September 2010) with a total of 14,499 cancer patients and 16,967 controls. This study found no significant association between alleles of this SNP and overall susceptibility to cancers. However, carriers of G allele in Asian population had significantly more susceptibility to various cancers. In stratified analysis for cancer types, there was increased risk of lung cancer and NHL in carriers of G allele (Table 17.13) [62]. The first systematic reviews of gastric cancer studies showed significant associations between −1082 A>G (rs1800896) and gastric cancer not in overall population but only when the analysis was limited to the Asian populations [184]. However, a more recent systematic review of 22 studies with 4,289 patients and 5,965 controls evaluated the association of −1082 A>G (rs1800896) with susceptibility to gastric cancer. This meta-analysis showed that carriers of G allele has significantly increased the risk for gastric cancer especially in Caucasian populations [177]. Another meta-analysis with 3,631 patients and 6,431 controls showed similar results; nonetheless, results remained significant in Asian population but not in Caucasians. This study, in stratified analysis, showed that this association is

Table 17.13 Significant results from published meta-analysis of associations of polymorphisms of IL-10 gene with cancers

Alleles	Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
rs1800872	HCC	354	1,683	CC *vs.* (AA + AC)	1.68 (1.25–2.26)	Taiwan, Korea, China, Japan	Wei et al. [63]
				C *vs.* A	1.29 (1.12–1.49)		
	Cervical cancer	2,396	1,388	A *vs.* C	1.16 (1.04–1.31)	Korea, Netherlands, Sweden, India, China	Ni et al. [180]
				(AA + AC) *vs.* CC	1.18 (1.01–1.39)		
	Gastric cancer	1,526	2,538	AA *vs.* (CC + AC)	0.81 (0.68–0.97)	(Asians) China, Korea, Japan	Xue et al. [181]
	Lung cancer	601	1,008	(CC + AC) *vs.* AA	1.8 (1.28–2.54)	Chinese, Denmark, Germany	Xue et al. [143]
				CC *vs.* AA	2 (1.24–3.23)		
rs1800871	Gastric cancer	989	2,350	TT *vs.* (CT + CC)	0.82 (0.7–0.96)	(Asians) China, Korea, Japan	Xue et al. [182]
	Lung cancer	311	507	C *vs.* T	1.27 (1.01–1.58)	China, Denmark, Germany	Peng et al. [143]
				CC *vs.* TT	2.27 (1.32–3.89)		
rs1800896	Gastric cancer	4,289	5,965	A *vs.* G	0.489 (0.335–0.713)	China, USA, Italy, Korea, Costa Rica, Honduras, Finland, Japan, Spain	Pan et al. [177]
		3,631	6,431	(GG + GA) *vs.* AA	1.41 (1.13–1.76)	China, USA, Italy, Korea, Costa Rica, Honduras, Finland, Japan, Spain, mixed European	Ni et al. [183]
	Lung cancer	315	507	GA *vs.* AA	3.16 (1.16–8.63)	Taiwan, Germany, Turkey	Peng et al. [143]
				GG *vs.* AA	2.07 (1.16–3.70)		
				(GG + GA) *vs.* AA	3.17 (1.31–7.68)		
	Non-Hodgkin lymphoma	2,338	1,999	GA *vs.* AA	1.18 (1.02–1.36)	Australia, Maryland, Sweden, France, Athens, Germany	Wang et al. [62]
				(GG + GA) *vs.* AA	1.17 (1.02–1.35)		
	Malignancy	1,733	2,003	GA *vs.* AA	1.80 (1.17–2.76)	(Asian) China, Taiwan, Kentucky, Korea, Japan	Wang et al. [62]
				GG *vs.* AA	3.32 (1.62–6.82)		
				(GG + GA) *vs.* AA	1.67 (1.07–2.60)		
				GG *vs.* (AA + GA)	2.93 (1.43–6.03)		

significant in cardiac subtype and intestinal-type but not in noncardia subtype or diffuse-type cancer [183]. Regarding −819 C>T (rs1800871), a systematic review based on 11 studies and 4,008 controls and 1,490 cases showed significantly increased risk for carriers of C allele among Asians but not Caucasians. Such increased risk was also noted for diffuse-subtype cancer but not for intestinal-subtype [182].

A systematic review of studies on −592 A>C (rs1800872) found significantly increased risk of gastric cancer in carriers of C allele only in Asian populations but not in Caucasians and Latinos. In stratified analysis for non-cardia and cardia subtypes or intestinal, diffuse, or mixed subtypes, no significant association was found [181].

A meta-analysis of seven articles published on association of −1082 A>G (rs1800896) and HCC with 1,012 HCC cases and 2,308 controls showed no association between this SNP and susceptibility to HCC. The same systematic review based on the results of four studies showed carriers of C allele in −592 A>C (rs1800872) had an increased risk of HCC. This study also showed no significant association between −819 C>T (rs1800871) and HCC based on results of three studies [63].

A meta-analysis reviewed the results of 13 studies with 9,692 patients with prostate cancer and 10,488 healthy individuals as controls. However, this review did not show any significant association for the three SNPs which was in accordance with the results of an older review on the basis of ten studies [179, 185]. Another review, which analyzed results of eight studies with 1,636 breast cancer patients and 1,670 controls did not show any altered risk of breast cancer for different alleles of −1082 A>G (rs1800896). This review also showed no significant associations between −592 A>C (rs1800872) and breast cancer in any genetic model [186].

In addition to its regulating effects on the immune system, IL-10 can induce transcription of one of the promoters of HPV [180]. Therefore, polymorphisms of this cytokine were under focus of researchers in the field of cervical cancer. However, no significant association was found between −1082 G>A (rs1800896) and susceptibility to cervical cancer in a meta-analysis of studies published up to June 2012. The same review indicated significant increased susceptibility to cervical cancer in carriers of A allele in −592 A>C (rs1800872) [180].

17.7.7 Interleukin-12

Interleukin-12 (IL-12) is a proinflammatory cytokine with several functions including differentiation of Th1 pathway, the critical pathway involved in protection against malignancy [23]. It can also induce IFN-γ production by T and NK cells and therefore suppress angiogenesis. In addition, IL-12 has a major role in the reactivation and survival of memory $CD4^+$ T cells which results in repolarization of $CD4^+$ T cells from dysfunctional antitumor Th2 into Th1 cells [187, 188].

IL-12 is composed of two parts, a p35 unit which is encoded by *IL-12a* on 3q25.33 and a p40 unit encoded by *IL12b* on 5q33.3 [23]. One common variant in IL-12b gene, including +1188A>C (rs3212227) in 3′ UTR, and three common variants of IL-12a including +277 G>A (rs568408) in 3′ UTR, IVS2 T>A (798 T>A; rs582054), and −564 T>G (rs2243115) in 5′UTR have been extensively studied previously (Table 17.14) [189]. *In vitro* and *in vivo* studies showed that A allele in +1188A>C (rs3212227) was associated with higher expression and greater mRNA stability [23, 190]. It is suggested that +277 G>A (rs568408) may disrupt exon-splicing enhancers and miRNAs binding and therefore results in an unstable IL-12 mRNA and lower IL-12 secretion [191].

One meta-analysis of ten studies involving 2,954 cancer patients and 3,276 controls showed significant associations between +1188A>C (rs3212227) and susceptibility to cancer (Table 17.15). In addition, by stratified analysis for cancer type, this study showed significant increased susceptibility to cervical cancer and nasopharyngeal cancer in C allele carriers [190].

A recent meta-analysis of 18 studies evaluated the associations of polymorphisms of both *IL-12* genes and cancer susceptibility. This study reviewed results of 13 studies on +1188A>C (rs3212227), including nine studies in Asians,

Table 17.14 Genotype details for SNPS of IL-12

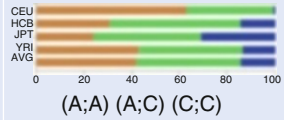
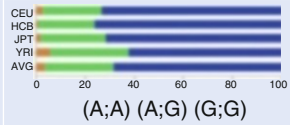
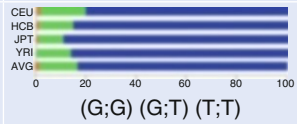
SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs3212227	*C* = 0.338		+1188A>C	NA	A allele: ↑
rs568408	*A* = 0.128		+277 G>A	NA	G allele: ↑
rs582054	*A* = 0.489	UA	+798 T>A	NA	UA
rs2243115	*G* = 0.107		−564 T>G	NA	UA

Table 17.15 Significant results from published meta-analysis of associations of 1188A>C (rs3212227) in IL-12b with cancers

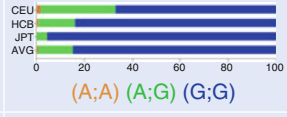
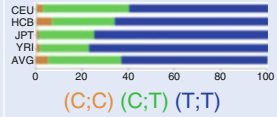
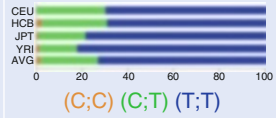
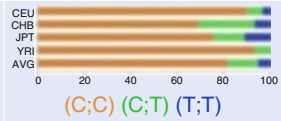
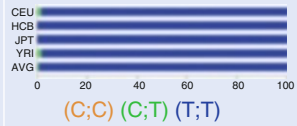
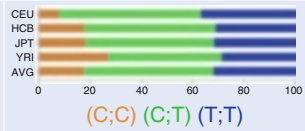
Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
Malignancy	2,954	3,276	(CC + AC) *vs.* AA AC *vs.* AA CC *vs.* AA CC *vs.* AC + AA	1.32 (1.06–1.63) 1.30 (1.07–1.57) 1.39 (1.05–1.86) 1.17 (1.02–1.33)	UK, Bulgaria, China, France	Chen et al. [190]
	10,404	10,861	C *vs.* A (AC + CC) *vs.* AA	1.14 (1.02–1.27) 1.20 (1.01–1.15)	UK, USA, Italy, China, Russia, Korea, Bulgaria, Tunisia	Zhou et al. [189]

three studies in Caucasians, and one in Africans, and showed increased risk of all cancers in C allele carriers. This association remained significant in Asian population but not in Caucasians [189]. This study like the previous one showed increased susceptibility to cervical and nasopharyngeal cancer in carriers of C allele. However, no significant association was found between cancer susceptibility and +277 G>A (rs568408). Also, there was no significant association for +564 T>G (rs2243115) and IVS2 T>A (rs582054) of IL-12a [189].

17.7.8 Tumor Necrosis Factor-α and Lymphotoxin-α

Tumor necrosis factor-α (TNF-α), by its triggering effect on the cytokine cascade of IL-1, IL-6 and other mediators, is one of the most important pro-inflammatory cytokines in the maintenance and homeostasis of the immune system, inflammation, and host defense [192]. TNF-α has both procarcinogenic and anticarcinogenic properties, and its importance in cancer is evidenced by previous studies which repeatedly reported high levels of TNF-α in cancer patients [193–195]. Some tumor cells can even produce TNF-α in an autocrine manner [130]. Consistent with its name, high levels of TNF-α result in tumor necrosis, but low levels of this cytokine impair antitumor immune response and induce tumor angiogenesis and therefore is associated with increased tumor growth, progression, invasion, and metastasis of tumor cells [193–196]. In addition, TNF-α levels can influence weight loss cachexia, and anemia in the host and also its response to treatment [197].

Table 17.16 Genotype details for SNPS of TNF-α and Lymphotoxin-α

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800629	*A* = 0.096		−308G>A	NA	A allele: ↑
rs361525	*A* = 0.051	UA	−238 G>A	NA	G allele: ↑
rs1799964	*C* = 0.200		−1031 C>T	NA	C allele: ↑
rs1800630	*A* = 0.145		−863 C>A	NA	A allele: ↑
rs1799724	*T* = 0.097		−857 C>T	NA	T allele: ↑
rs1800610	*A* = 0.102	UA	IVS1 + 123G>A	NA	UA
rs1800750	*A* = 0.013		−376 G>A	NA	A allele: ↑
rs909253	*C* = 0.398		+252 A>G	NA	G allele: ↑

Lymphotoxin-α (LTA), another cytokine of the TNF family, is similar to TNF-α with respect to amino acid sequence, receptors, and biologic activities [193–196].

TNF-α is encoded by a gene located on chromosome 6 (region p21.3) and is a member of HLA class 3. −308G>A (rs1800629) and −238 G>A (rs361525) are two common promoter variants of *TNF-α* gene [23]. Other variants include −1031 C>T (rs1799964), −863 C>A (rs1800630) and −857 C>T (rs1799724), −376 G>A (rs1800750), and IVS1 + 123G>A (rs1800610) (Table 17.16) [23]. The *LTA* gene is located in the same region and has an *Nco*I restriction fragment length polymorphism (+252 A>G) in its first intron (rs909253).

A allele of −308G>A (rs1800629) is associated with higher levels of TNF-α [198]. While several *in vitro* studies did not show any functionality for this SNP, some authors suggested that this allele had more affinity for a transcriptional activator and another study showed that A allele disrupts a 10-bp binding region for activator protein-2 (AP-2) (a repressor protein) [23, 197]. Of interest, −308G>A (rs1800629) is in high LD with +252G>A, a functional SNP in *lymphotoxin alpha* gene, and other *HLA* genes within ancestral haplotype, *HLA A1-B8-DR3-DQ2-TNF_308A-LT_252A* [197, 199, 200].

An allele of −238 G>A (rs361525) was associated with lower levels of TNF-α in peripheral blood mononuclear cells carrying TNF-α-238A allele [193]. However, several *in vitro* studies did not provide any evidence on the functionality of this SNP [23].

A Japanese *in vitro* study showed that C allele in rs1799964 is associated with higher

production of TNF-α by concanavalin A (Con A)-activated peripheral blood mononuclear cells [201]. Reporter assays showed increased promoter activity for A allele of −376 G>A (rs1800750), and EMSA studies showed more affinity of this allele for Oct-1 transcription factor comparing to other allele [20, 23]. *In vivo* studies showed that individuals carrying at least one allele out of three (−1031C, −863A, −857T) had higher TNF-α production and higher transcriptional activity [20, 23, 202]. In the same line, minor alleles of −863 C>A (rs1800630) and −857 C>T (rs1799724) were associated with higher promoter activity and more affinity for oct-1 transcription factor [20, 23, 202]. On the contrary, one study showed that −863A allele had less affinity for NF-κB [20, 23, 203].

In vitro studies showed that phytohemagglutinin-activated mononuclear cells having +252G allele (rs909253) produce more LTA and interestingly TNF-α [204, 205].

Previously, several associations have been reported between TNF-α polymorphisms and susceptibility to NHL, gastric carcinoma, breast cancers, prostate, uterine endometrium, lung, cervix, and nasopharynx. However, a meta-analysis reviewed 34 studies (published up to March 2011) including 34,679 cancer patients and 41,186 controls and found no significant association between −238 G>A (rs361525) polymorphism and susceptibility to cancer [206]. In line with this, a meta-analysis of 30,000 breast cancer cases and 30,000 controls from 30 studies of the breast cancer association consortium could not find any significant association between −238 G>A (rs361525) and susceptibility to breast cancer [207].

A review of 18 studies with 11,320 breast cancer patients and 14,112 controls found a significant relationship between −308G>A (rs1800629) polymorphism and breast cancer only in Caucasian population (Table 17.17) [192]. In addition, after excluding hospital-based studies a significant decreased risk in carriers of A allele was found. This study also reviewed 33,112 patients and 35,814 (reported in 35 studies) and found no significant association for −238 G>A (rs361525). This study also did not find any significant association between breast cancer and −863 C>A (rs1800630) and −857 C>T (rs1799724), −1,031 C>T (rs1799964) polymorphisms, which may be due to the fact that the overall sample analyzed for these polymorphisms was very small [192]. Consistent with the previous study, a meta-analysis of 11 studies on 10,184 patients with breast cancer and 12,911 controls found that G allele in −308G>A (rs1800629) is associated with significantly increased risk of breast cancer [196]. Another meta-analysis evaluated 10,236 breast cancer cases and 13,143 controls presented in 13 studies [212]. This study could confirm such a decreased breast cancer risk in carriers of −308A allele only in Caucasians [212]. However, no significant association between breast cancer susceptibility and other polymorphisms of TNF-α was found [212]. A meta-analysis of 4,625 breast cancer patients and 4,373 controls for LTA-252 A>G (results from seven studies published up to January 2012) did not find any significant association between genotypes of this polymorphism and breast cancer. However, in stratified analysis for ethnicity, carriers of G allele had significantly increased risk of breast cancer in Asian population [213]. A systematic review of 11 studies with 3,094 cervical cancer cases and 3,037 controls found that carriers of AA genotype for −308G>A (rs1800629) had 39 % increased risk of cervical cancer compared with −308GA/GG genotypes [195]. In addition, in stratified analysis, such an association remained significant in Asian population [195]. This meta-analysis by its review on 1,190 cases and 1,784 controls showed decreased risk of cervical cancer in carriers of A allele in −238 G>A (rs361525) [195]. In a meta-analysis of 13 studies reported up to October 2011 which involved 3,294 cervical cancer patients and 3,468 controls, no association was found between −308G>A (rs1800629) and cervical cancer [189]. However, in Caucasian and African population, significantly increased risk of cervical cancer was observed in carriers of A allele in this SNP. This study also meta-analyzed results of six studies on −238 G>A (rs361525) (2,416 cases and 2,010 controls) and found that carriers of −238A allele had lower risk of cervical cancer

Table 17.17 Significant results from published meta-analysis of associations of polymorphisms of TNF-α with cancers

Alleles	Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
rs1800629	Cervical cancer	2,710	2,877	AA *vs*. GG	2.09 (1.34–3.25)	(Caucasian) India, USA, Portugal, Costa Rica, Sweden	Zhou et al. [189]
				AA *vs*. GA + GG	2.09 (1.35–3.25)		
		3,094	3,037	AA *vs*. GG	1.41 (1.03–1.92)	Sweden, India, Costa Rica, South Africa, Mexico, Portugal, Zimbabwe, USA, South Korea	Liu et al. [195]
				AA *vs*. GA + GG	1.39 (1.02–1.90)		
	Breast cancer	10,184	12,911	G *vs*. A	1.08 (1.02–1.14)	Italy, Tunisia, UK, Iran, USA, Poland, Croatia, Russia, Germany	Fang et al. [196]
				AA *vs*. GA + GG	1.10 (1.04–1.17)		
		10,254	12,926	AA + AG *vs*. GG	0.91 (0.85–0.97)	(Caucasians) Italy, USA, Poland, UK, Russian, Croatia, Germany	Yang et al. [192]
	HCC	2,357	3,161	AA *vs*. GG	1.97 (1.01–3.83)	USA, Turkey, China, Japan, Thailand, Italy	Wei et al. [203]
				AG *vs*. GG	1.88 (1.23–2.88)		
				AA + AG *vs*. GG	1.80 (1.19–2.72)		
		1,665	2,177	AA + AG *vs*. GG	1.74 (1.12–2.72)	Israel, Turkey, China, Italy, Thailand, USA, Japan	Yang et al. [149]
	Gastric cancer	4,399	6,855	AA *vs*. GG	1.49 (1.11–1.99)	South Korea, Taiwan, USA, Portugal, Colombia, China, Germany, Japan, Mexico, Brazil, Italy, Honduras, Poland, Finland, Spain	Gorouhi et al. [208]
				GA *vs*. GG	1.14 (1.02–1.27)		
		3,335	5,286	A *vs*. G	1.23 (1.11, 1.36)	USA, Spain, Korea, China, Finland, Germany, Mexico, Portugal, Honduras, Italy, Brazil, Japan	
				AA *vs*. GG	1.78 (1.28, 2.48)		
				AA *vs*. GG + GA	1.65 (1.21, 2.25)		
				AA + GA *vs*. GG	1.21 (1.08, 1.36)		
	UADT cancer	1,751	3,345	AA *vs*. GA + GG	1.54 (1.07–2.21)	China, India, USA, Australia	Wang et al. [209]
	Oropharynx	944	1,712	AA *vs*. GA + GG	2.68 (1.34–5.35)	China, India, USA	Wang et al. [209]
				AA *vs*. GG	2.70 (1.35–5.36)		
				AA *vs*. GA	2.59 (1.23–5.46)		

(continued)

Table 17.17 (continued)

Alleles	Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
rs361525	Cervical cancer	2,416	2,010	A *vs*. G	0.61 (0.47–0.78)	South Korea, USA, India, Sweden, Costa Rica	Zhou et al. [189]
				GA *vs*. GG	0.59 (0.45–0.77)		
				GA + AA *vs*. GG	0.59 (0.46–0.77)		
		1,190	1,784	GA *vs*. GG	0.54 (0.40–0.73)	Costa Rica, Mexico, USA, India, Korea	Liu et al. [195]
				GA + AA *vs*. GG	0.55 (0.41–0.74)		
	HCC	1,572	1,875	A *vs*. G	1.32 (1.04–1.69)	China, Thailand, Italy, Taiwan, South Korea, China	Cheng et al. [210]
				AG *vs*. GG	1.32 (1.02–1.71)		
				AA + AG *vs*. GG	1.33 (1.03–1.72)		
		938	1,370	AG *vs*. GG	1.63 (1.17–2.26)	China, Korea, Thailand, Italy, Japan	Wei et al. [203]
				AA + AG *vs*. GG	1.62 (1.18–2.22)		
rs1800630	HCC	627	1,004	AC *vs*. CC	1.72 (1.03–2.88)	China, Korea, Thailand, Italy, Japan	Wei et al. [203]
				AA + AC *vs*. CC	1.65 (1.06–2.57)		
rs1799724	Gastric cancer	1,118	1,591	T *vs*. C	1.17 (1.01–1.35)	China, Japan	Zhang et al. [211]

which remained significant in Caucasian populations [189]. A recent meta-analysis reviewed results of 12 case-control studies including 1,751 cases with upper aerodigestive tract (UADT) cancer and 3,345 controls [209]. Oropharynx cancer was investigated in six of these studies, while five studies investigated esophagus cancer and one investigated larynx cancer. Squamous cell carcinoma and adenocarcinoma were investigated in nine and two studies, respectively, and one study investigated both cancer types. This study overall found a significant increased risk of UADT cancer in carriers of AA genotype in −308G>A (rs1800629) compared to individuals who had GA or GG genotypes [209]. In addition, significantly increased risks were found in oropharynx cancer but not in esophagus cancer or larynx cancer. In the subgroup analysis for histologic type, this association remained significant only for squamous cell carcinoma, but not for adenocarcinoma [209].

The most recent meta-analysis on gastric cancer and −308G>A (rs1800629) reviewed 5,225 patients and 8,473 controls in 26 papers. This study found a significant increased risk of gastric cancer in carriers of A allele in comparison with G allele [214]. Another meta-analysis on gastric cancer evaluated 4,399 cases and 6,855 controls presented in 24 studies published up to October 2007 [208]. This study found a significant increased risk of gastric cancer in carriers of AA genotype in −308G>A (rs1800629) polymorphism. In stratified analysis, AA genotype was significantly associated with an increased risk of noncardia cancers and intestinal type of gastric cancer compared to the GG genotype [208]. Another meta-analysis on gastric cancer and −308G>A (rs1800629) polymorphism included 19 studies with 3,335 GC patients and 5,286 controls [211]. In addition, this study included five studies with 1,118 GC patients and 1,591 controls for −857 C>T (rs1799724). This study also found a significant increased risk of gastric cancer in carriers of A allele and AA genotype in −308G>A (rs1800629) compared with G allele in the whole population and in Caucasians but not in East Asian [211]. This study also found a weak but significant association between T allele of −857 C>T (rs1799724) and GC risk compared with the C allele [211].

Several systematic reviews have been published on the associations of TNF-α polymorphisms and susceptibility to HCC. The most recent one evaluated results of 11 case-control studies (reported up to July, 2012) with a total of 1,572 HCC cases and 1,875 controls revealed an increased risk of HCC in carriers of A allele in −238 G>A (rs361525) [210]. In stratified analysis, this association remained significant only in Asian populations [210]. Another meta-analysis included 2,357 cases and 3,161 controls presented in 17 studies published till November 2010 [203]. This study showed that A allele in both −238 G>A (rs361525) and −308G>A (rs1800629) was associated with an increased risk of HCC. In stratified analysis for ethnicity, these associations remained significant in Asians but not in Caucasians [203]. AA and AC genotypes in −863 C>A (rs1800630) were also associated with increased HCC risk compared to CC genotype. However, this study did not find any significant association for −857 C>T (rs1799724) and −1031 C>T (rs1799964) polymorphisms [203]. The pattern for −238 G>A (rs361525) and −308G>A (rs1800629) was also repeated in other systematic reviews [149, 215, 216].

A meta-analysis of seven case-control studies with 1,311 bladder cancer cases and 1,436 controls found that carriers of A allele in −308G>A (rs1800629) had an increased risk of bladder cancer [217]. A multicenter study investigated associations between six polymorphisms of TNF-α (rs1799964, rs1800630, rs1799724, rs1800629, rs361525, rs1800610) and prostate cancer risk in 2,321 cases and 2,560 controls from two nested case-control studies within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trials and the Cancer Prevention Study II Nutrition Cohort for [218]. Overall, this study found no significant association between these polymorphisms and prostate cancer risk. But this study found a significant decreased risk in carriers of T-C-T-G-A haplotype in rs1799964, rs1800630, rs1799724, rs1800629, and rs1800610 comparing to the most frequent haplotype (T-C-C-G-G) [218]. In subgroup analysis, T allele in −1036

C>T (rs1799724) in individuals who did not regularly use NSAID was associated with significantly less susceptibility to prostate cancer compared to the CC genotype. In addition, when limiting analysis to non-advanced tumors, carriers of −1036T or A allele in IVS1 + 123G>A (rs1800610) had a significantly decreased chance for prostate cancer [218].

Another multicenter study evaluated associations of −308G>A (rs1800629) with NHL in 7,999 cases and 8,452 controls from participating studies from the InterLymph Consortium. Carriers of −308A allele had increased risk for NHL, B-cell NHL, diffuse large B-cell lymphoma (DLBCL), and other marginal zone lymphoma. However, no significant associations was found between −308G>A (rs1800629) and chronic small lymphocytic lymphoma CLL/SLL or T-cell NHL [219].

Although this study also did not find any significant association between LTA +252 A>G (rs909253) and NHL, carriers of G allele in this SNP had increased risk to DLBCL and mycosis fungoides [219].

In a meta-analysis of 33 studies with 14,435 cancer patients and 10,583 healthy controls, statistically significant increased risk of malignant transformation was found in carriers of G allele in +252 A>G (rs909253) which remained significant in both Asian population and Caucasians [220]. A recent study performed a meta-analysis on 11 individual case-control studies with 2,270 cases and 4,404 controls and found that G allele of +252 A>G (rs909253) is associated with a significant increased risk of gastric cancer, but this risk was significant only in Asians, but not Caucasians [221]. An older study also showed such a risk only in Asians especially those with *H. pylori* infection [222].

17.7.9 Interferon Gamma (IFN-γ)

Interferon gamma (IFN-γ) is a proinflammatory cytokine of Th1 subset with major roles in antitumor immune response. This cytokine enhances differentiation of lymphocytes and their function and Ag presentation through inducing expression of HLA molecules [130]. In addition, it inhibits angiogenesis in various tumors [61, 223].

IFN-γ gene with four exons and a length of 5.4 kb is located on chromosome 12q24 [223]. Two common SNPs including an intronic SNP (+874 T>A (rs2430561)) and a promoter variant in (−179 T>G (rs2069707)) have been previously identified [23, 61, 223]. This promoter variant is adjacent to a HSF-binding motif. In addition, there is a CA repeat microsatellite within the first intron of the gene ranging from 12 to 15 repeats [23, 223]. It was shown that allele 2 of the microsatellite and T allele in +874 T>A (rs2430561) are in complete LD [23].

In vitro studies showed that T allele of +874 T>A (rs2430561) is associated with higher IFN-γ production. EMSA studies showed that this allele has higher affinity for NF-κB which is in accordance with the location of this SNP in the first intron of the gene, a region related to binding of NF-κB [61, 223].

A meta-analysis of 17 studies with 1,929 cancer cases and 2,830 controls showed a nonsignificant increased risk of cancer in the presence of AA genotype for +874 T>A (rs2430561). However, this study showed significant increased susceptibility in individuals with AT genotype compared with TT genotype (Table 17.18) [223]. Another meta-analysis with 32 studies and 4,524 cases and 5,684 controls did not find a significant association either [61]. Interestingly, in stratified meta-analysis for ethnicity, carriers of T allele had significant increased susceptibility to cancer in European and African population but not in Asian population [61]. This study also found that TT genotype significantly contributes to the risk of breast cancer in all ethnicities [61].

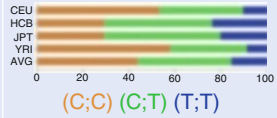
17.7.10 Transforming Growth Factor-β (TGF-β)

Transforming growth factor-β (TGF-β) is a functional mediator of epithelial and fibroblast cell proliferation and a regulator of immune cell populations [224]. In early stages of tumor progression, it acts as a tumor suppressor; however, in advanced cancers, TGF-β induces many activities

Table 17.18 Significant results from published meta-analysis of associations of (+874 T>A (rs2430561) in IFN-γ gene with cancers

Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
Cervical cancer	661	835	AT *vs*. TT	1.10 (1.02–1.19)	India, South Africa	Mi et al. [223]
Breast cancer	527	715	TT *vs*. AA TT *vs*. AT/AA	1.58 (1.10–2.27) 1.53 (1.14–2.06)	Iran, Italy, Turkey, China, USA	Liu et al. [61]

Table 17.19 Genotype details for SNPS of TGF-β

SNP	GMAF [137]	Population diversity [138]	Change at DNA level	Change at protein level	Effect on cytokine level
rs1800470	*G* = 0.444	UA	+29 T>C	Pro10Leu	C allele: ↑
rs1800471	*G* = 0.046	UA	+74G>C	Arg25Pro	G allele: ↑
rs1800469	*T* = 0.359		−509 C>T	NA	T allele: ↑

that lead to growth, invasion, and metastasis of cancer cells [224–226].

TGF-β family consists of three isoforms with pleiotropic roles in cancer immunity [227–229]. TGF-β1 as the most common isoform of this family has enhancing effects on angiogenesis and its regulatory role in growth, differentiation, and apoptosis of different cells [60, 133, 229]. It also results in escape of malignant cells from immunosurveillance by suppressing expression of HLA molecules [130, 133, 228, 229].

TGF-β1 gene is located in the long arm of chromosome 19 (19q13.1). +869 T>C (rs1800470; also called +29 T>C, or rs1982037) is a common variant in the first exonic region of *TGF-β1* which results in substitution of leucine to proline at codon 10 in signal sequence [227]. +915 G>C (also called +74 or rs1800471) is another exonic variant resulting in an arginine-to-proline substitution at codon 25. −509C>T (rs1800469) and −800G>A are two promoter variants in a proximal negative regulatory region (Table 17.19) [230, 231]. *In vivo* studies showed that T allele in −509 C>T (rs1800469) was associated with higher levels of TGF-β1 in plasma and also higher expression [23, 71]. Despite some contrary results, C allele in +869 T>C (rs1800470) was associated with higher secretion of TGF-β1 in *in vitro* studies [23, 228]. Arginine in +915 G>C (rs1800471) was also associated with higher levels of TGF-β1 in *in vivo* studies [23]. *In vitro* studies showed that A allele in −1287 G>A (rs11466314), another variant of this gene, is associated with higher expression of TGF-β1 [23]. EMSA studies showed that C allele in −387 C>T (rs11466315) had greater affinity for Sp1 and Sp3 complexes [23].

Results of 40 case-control studies (including three studies with African population, 14 on Asian descendants, and 23 studies with European population) with 16,166 patients with various cancers and 19,126 controls were analyzed in a systematic review. Although this meta-analysis did not find any significant association with overall risk of cancer, its result suggested that individuals with C allele in +869 T>C (rs1800470) have significantly greater risk for prostate cancer. In addition, in Asian populations, this allele was significantly associated with susceptibility to cancers (Table 17.20) [229].

A meta-analysis of 30 studies including 20,401 patients with breast cancer and 27,416 controls showed increased risk of breast cancer in individuals with C allele in +869 T>C (rs1800470). In stratified analysis, this association remained significant in Caucasian population and

Table 17.20 Significant results from published meta-analysis of associations of SNPs of TGF-β gene with cancers

Alleles	Cancer site	Total number of cases	Total number of controls	Analysis type	OR ± 95 % CI	Population included	Reference
rs1800470	Malignancy	5,183	6,524	CC *vs*. TT	1.26 (1.03–1.53)	(Asian) Korea, China, Japan	Wei et al. [229]
				CT *vs*. TT	1.20 (1.01–1.43)		
	Prostate cancer	2,605	3,129	CT *vs*. TT	1.28 (1.01–1.61)	USA, Germany, Brazil, Japan	Wei et al. [229]
				(CC + CT) *vs*. TT	1.24 (1.02–1.52)		
	Breast cancer	20,401	27,416	CT *vs*. TT	1.046 (1.003–1.090)	Mixed from Asian, Caucasian, and African	Qiu et al. [60]
				(CC + CT) *vs*. TT	1.052 (1.012–1.095)		
rs1800469	Gastric cancer	2,130	2,374	TT *vs*.(CC + CT)	1.35 (1.1–1.65)	India, China	Li et al. [71]
	Colorectal cancer	994	2,335	CC *vs*. TT	1.62 (1.30–2.02)	Iran, Germany, Korea, China	Fang et al. [232]
				(TC + CC) *vs*. TT	1.30 (1.08–1.58)		
				CC *vs*. (TC + TT)	1.48 (1.26–1.75)		
		4,440	6,785	(CC + CT) *vs*. TT	1.18 (1.06–1.32)	USA, UK, Iran, China, Korea, Germany	Wang et al. [233]
	Colon cancer	1,760	2,454	(CC + CT) *vs*. TT	1.31 (1.05–1.63)	UK, USA, China	Wang et al. [233]

population-based studies [60, 234]. However, older meta-analysis on breast cancer with almost half of this sample could not find such an association [234, 235]. Another recent meta-analysis of 20,022 cases and 24,423 controls could find this increased risk for C allele just in Caucasians [231]. This study also reviewed results of 8 studies with 10,633 cases and 13,648 controls for −509 C>T (rs1800469) and did not find any significant association between alleles of this polymorphism and risk of breast cancer in accordance with another meta-analysis (including 10,197 patients with breast cancer and 13,382 healthy controls) [231, 236, 237]. Some authors suggested that the effect of TGF-β1 is different according to expression of estrogen receptor and progesterone receptor in breast cancer tumors [230].

A systematic review analyzed results of 55 studies with a total number of 21,639 cancer patients and 28,460 controls for associations of −509 C>T (rs1800469) and susceptibility to different cancers. Although there was no a significant association between overall risk of cancer and genotypes of this SNP, this study found increased susceptibility of carriers of C allele to colorectal cancer particularly in Caucasians [238]. In addition, a meta-analysis of five studies with 994 colorectal cancer patients and 2,335 controls found increased risk of colorectal cancer for C allele of −509C>T (rs1800469) which remained significant only in Asian population but not Caucasians in stratified analysis [232]. On the other hand, a systematic review of seven original articles with a total of 2,130 patients with gastric cancer and 2,374 controls found significant increased susceptibility to gastric cancer in carriers of T allele in −509C>T (rs1800469) in a recessive model [71]. Another meta-analysis pooled the results of 29 case-controls studies with 8,664 patients with digestive tract cancers and 12,532 controls. This study did not show any significant association with overall risk of digestive tract cancers. However, this study found that C allele in −509 C>T (rs1800469) is significantly contributed to the risk of digestive tract cancers in Caucasians. In addition, carriers of C allele in the whole study sample had increased risk for colorectal cancer [239]. Another systematic review of 12 studies with 4,440 colorectal cancer patients and 6,785 controls could find such an association only in colon cancer [233]. Regarding HCC, a review of 11 studies including 2,577 HCC cases and 4,107 controls revealed a significant association between this SNP and the risk of HCC only in Caucasians [240].

17.8 Concluding Remarks

In the recent decades, a great scientific effort has uncovered the importance of immune polymorphisms in cancers. However, this uncovered part, although is promising, only reminds us that there is much more to reveal in this field. There comes a day that gathering immunogenetic data becomes one main part of every clinical trial in cancer. This information will help understand more about subgroups of patients, natural history of the cancers, responsiveness of cancer to treatment, or toxicity of treatment, all in relation to immune polymorphism [14]. One day, it might be possible to assess the degree of predisposition to different cancers for each individual and to employ preventive measurement, and in case of suffering from cancers, to efficiently choose between treatment options and predict their clinical effectiveness [26]. Although it seems a vague dream in the far future, it is becoming closer to reality everyday considering the pace of scientific advancements.

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Primary Immunodeficiencies and Cancers

18

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18.1 Introduction

Immunodeficiency disorders are classified as either primary (genetic) or secondary (acquired). Primary immunodeficiencies (PIDs) are a heterogeneous group of disorders that predispose to frequent and severe infections, autoimmunity, and, in certain diseases, malignancies. According to the updated classification of PIDs by the International Union of Immunological Societies Expert Committee in 2011 [1], over 175 PIDs have been identified, with a total incidence of 1 in 10,000 births [2]. The overall risk for developing malignancies in children with PIDs is 4–25 % [3], with lymphomas representing up to 60 % of all cancer types [4]. Considering the improved therapeutic options and increasing life expectancy of PID patients, it is possible that the incidence of malignancies may increase as patients live longer lives. Increasing evidence suggests that defective immunosurveillance mechanisms, interacting with oncogenic viruses, chronic antigen stimulation, defective DNA damage response, and genetic alterations of oncogenic and tumor suppressor genes, are the major factors driving the development of cancer in patients with PIDs [5–9]. While further elucidation of the precise molecular pathogenesis of malignancies in the context of immunodeficiency syndromes offers an exciting prospect for the development of targeted cancer therapies, we report here the most recent clinical observations on the incidence and types of malignancies, which should alert clinicians to the potential importance of more vigilant screening in immunodeficient patients. It should be noted, however, that surveillance protocols should be applied judiciously, without indiscriminate and frequent use of certain radiological procedures, due to increased risk of radiosensitivity in some syndromes [10]. Furthermore, early intervention with hematopoietic cell transplantation, which is indicated in certain PIDs, may decrease not only the infection but also the cancer risk [11].

18.2 Primary Antibody Deficiencies

18.2.1 Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) is the second most common PID (second to selective IgA deficiency), which is estimated to affect as many as 1 in 25,000 individuals [12]. CVID is a clinically and genetically heterogeneous group of diseases characterized by hypogammaglobulinemia of two or more isotypes (IgG, IgA, or IgM), impaired functional antibody responses, and consequently increased susceptibility to chronic recurrent bacterial infections [13]. Furthermore, affected individuals are predisposed to autoimmune and granulomatous diseases as well as hematological and certain solid malignancies in up to 15 % of subjects [13, 14]. Non-Hodgkin lymphomas (NHLs) represent the most common malignancies with up to a 259-fold increase in risk compared to the general population [15–17]. NHLs in CVID are mostly extranodal, well differentiated, and of B-cell origin [15]. In older studies, there was an increased risk of gastric cancer (up to 47-fold) [16, 18], probably related to the increased frequency of pernicious anemia or *Helicobacter pylori* infection [19]. However, a 2010 study of 476 patients revealed that gastric cancer was diagnosed in only 0.6 % of patients, suggesting a potential downward trend. In this study, 6.7 % of patients developed NHL and 0.8 % developed Hodgkin lymphoma (HL). Other solid malignancies, including breast, colon, oral, and other cancers, collectively accounted for cancer in up to 4 % of patients [14].

Defects in genes encoding the inducible costimulator (*ICOS*; OMIM*604558) [20], tumor necrosis factor (*TNF*) receptor superfamily members 13B (*TNFRSF13B* or *TACI*; OMIM*604907) [21, 22] and 13C (TNFRSF13C or *BAFF-R*; OMIM*606269) [23], *CD19* (OMIM*107265) [24], *CD20* (OMIM*112210) [25], *CD81* (OMIM*186845) [26], *CD21* (CR2;

OMIM*120650) [27], and *LRBA* (OMIM*606453) [28] have thus far been identified in patients with CVID.

The immunologic defects in CVID are multifaceted. Despite normal numbers of B cells in the majority of affected individuals, their inability to undergo terminal differentiation into immunoglobulin-secreting plasma cells forms the core common defect [15, 29]. T-cell abnormalities are also frequently encountered in patients with CVID, including impaired T-cell proliferative responses, partly due to defects in T-cell receptor signaling [30, 31]; decreased numbers of CD_4^+ T cells in conjunction with normal to increased numbers of CD_8^+ T cells, giving rise to reversed CD_4:CD_8 ratio [32, 33]; imbalanced T-helper cell responses, representing a shift toward a Th1 phenotype [34–36]; increased suppressor T-cell activity [34]; and diminished expression of the costimulatory molecule CD40 ligand [37]. Moreover, the absolute and relative NK, invariant NKT, and plasmacytoid dendritic cell numbers are reported to be decreased in patients with CVID [38–40].

The complex derangement in numerical and functional characteristics of B, T, NK, and dendritic cells results in impaired humoral and cellular immune responses. As a result, patients often develop chronic inflammatory and autoimmune diseases, as well as recurrent bacterial infections. These factors, along with persistent antigenic stimulation, mainly from chronic *Helicobacter pylori* [41, 42], human herpesvirus 8 [43], and cytomegalovirus [44] infections, may ultimately drive tumorigenesis; however, their relative contribution and the precise underlying mechanisms remain to be elucidated [13]. Furthermore, given the possible role of an autocrine B-cell activating factor (BAFF) signaling circuit in promoting tumor cell survival and proliferation [45, 46], it is possible that aberrant BAFF-R signal transduction resulting from CVID-related mutations might enhance malignant transformation [13]. Finally, defective DNA repair, as evident by enhanced radiosensitivity, has been reported in patients with CVID [47, 48], with those having the highest rate of chromosomal aberration developing lymphoma [48].

18.2.2 X-Linked Agammaglobulinemia

X-linked agammaglobulinemia (XLA) is the prototypic humoral immunodeficiency arising from a defect in B-cell maturation, affecting the transition of B-cell progenitors into mature B lymphocytes and leading to the consequent failure of immunoglobulin production. It is estimated to afflict three to six out of every million males of all racial and ethnic groups. As the maternally derived antibodies (Abs) are degraded, most patients with XLA begin to experience recurrent infections by the end of the first year of life [18, 49]. Approximately 10–15 % of individuals with XLA have higher concentrations of serum immunoglobulin than expected or are not recognized to have immunodeficiency until after the age of 5 years. XLA is mainly characterized by recurrent bacterial infections, in particular with extracellular encapsulated bacteria, most commonly localized in the respiratory tract. Diarrhea and skin infections are also frequently seen [18, 49, 50]. Despite general resistance to viral infections, affected individuals are susceptible to severe and chronic enteroviral infections [51].

The gene defective in XLA, Bruton's tyrosine kinase (*BTK*; OMIM*300300), encodes a cytoplasmic tyrosine kinase of the Btk/Tec family [52]. The crucial role of BTK in B-cell growth and differentiation has been documented by a developmental block at the pro-B-cell to pre-B-cell transition with a reduction in mature B cells [50], whereas T-lymphocyte subsets are normal and may show a relative increase. In B cells, B-cell antigen receptor (BCR) cross-linking activates BTK downstream of the Src family kinases [53, 54], where it is a critical component in BCR-coupled

calcium signaling cascade [55, 56]. BTK also acts as a mediator of oxidative stress-induced apoptosis of irradiated neoplastic B cells and B-cell precursors [57], probably via the negative regulation of the antiapoptotic signal transducer and activator of transcription 3 (STAT3) function [58]. BTK also interacts with and functions downstream of Toll-like receptor (TLR)-8 and TLR9. These latter functions might explain the susceptibility to enteroviral infections in XLA patients [59, 60].

Although the overall chance of developing malignancies in XLA is low, there are reports of a 30-fold increased risk of colorectal cancer in patients with XLA [61, 62]. Aberrant immunological function and/or persistent asymptomatic inflammation in the colon is generally thought to contribute to the increased risk of colorectal cancer. However, it has been shown that *BTK* loss of function is associated with excessive Wnt-β-catenin signaling [63], which is known as a major contributor to the development of colorectal carcinoma [64]. In addition to colorectal cancer, cases of pituitary adenomas [18], gastric adenocarcinoma [65], and squamous lung cancer [66] have been reported.

18.2.3 Selective IgA Deficiency

Selective IgA deficiency (IgAD) is the most common PID with a prevalence that varies from 1 in 143 to 1 in 18,550 in different ethnic groups [67, 68]. It is defined as occurring when serum IgA levels are equal to or below 0.07 g/L with normal IgM and IgG levels in individuals 4 years of age or older in whom other causes of hypogammaglobulinemia have been excluded [69]. As many as 85–90 % of patients with IgAD are asymptomatic, which could be explained by a compensatory increase in IgM production and subsequent increase in secretary IgM in the mucosal lumen [70]. However, IgAD can present with a broad spectrum of clinical manifestations, including recurrent sinopulmonary and gastrointestinal infections, allergic disorders, GI diseases (especially celiac disease), progressive neurodegenerative disorders, autoimmunity, and malignancy, with gastric carcinomas and lymphomas being frequently associated with the disease [70–75].

In IgAD, the common finding is a defect in the maturation of B cells producing IgA [73]. The genetic basis of IgAD is complex and has remained unclear. Autosomal recessive, autosomal dominant, and sporadic transmission patterns have all been observed. In view of the lack of an identified primary genetic defect and the variation in the inheritance patterns, it is likely that IgAD represents a heterogeneous group of genetic abnormalities such as CVID. In support of this notion is the observation that mutations in transmembrane activator and calcium modulator and cyclophilin ligand interactor (*TACI*) gene (*TNFRSF13B*; OMIM*604907), which appear to act as a disease-modifying mutation, have been found in IgA deficiency and CVID [22]. Moreover, a novel shared risk locus associated with lower inducible costimulator (ICOS) and higher cytotoxic T-lymphocyte-associated protein-4 (CTLA4) expression has been recently defined in both diseases [76]. The presence of specific major histocompatibility complex (MHC) haplotypes, in particular the ancestral HLA-A1, B8, DR3, and DQ2 (8.1), have been associated with susceptibility to IgAD [77].

The association of malignancy, especially of the lymphoreticular and gastrointestinal systems, with IgAD has been documented mainly in adults [78, 79] with an estimated two fold increased risk compared to general population [80]. However, in a combined Danish and Swedish study of 386 patients with IgAD, the incidence of cancer was not increased. Yet, the investigators in the same study found that relatives of the same patients had slightly elevated cancer rates. In contrast to adults, children with IgAD appear not to be at risk of malignancy [81, 82], which has only been reported in case reports [83–85].

18.3 Combined Immunodeficiencies

18.3.1 IL-2-Inducible T-Cell Kinase Deficiency

IL-2-inducible T-cell kinase (ITK) deficiency is a novel PID characterized by severe EVB-associated immune dysregulation, with a clinical picture similar to that seen in X-linked lymphoproliferative

disease (XLP) [86, 87]. ITK deficiency was originally described in 2009, where two ITK-deficient female siblings from a consanguineous Turkish family developed uncontrolled Epstein–Barr virus (EBV) infection resembling hemophagocytic lymphohistiocytosis (HLH) with eventual progression to HL [86]. In a report of three cases from a single Arab family, the first presentation was HL, whereas fulminant hemophagocytosis and severe mononucleosis appeared after remission of lymphoma [87]. Adding to the complexity of the disease, seven additional ITK-deficient patients, of whom four developed HL, were identified following the screen of patients with autoimmune lymphoproliferative syndrome or suspicion of congenital forms of HLH [88, 89]. More recently, the clinical spectrum of ITK deficiency has been further extended to include late-onset isolated involvement of the lungs and the mediastinal lymph nodes with a polyclonal proliferation of small B cells not suggestive of any malignant lymphoma [90].

In ITK deficiency, germ line loss-of-function mutations in the *ITK* gene (OMIM*186973) result in pronounced instability or truncation of the ITK protein [86, 87]. ITK, a member of the Tec family tyrosine kinases, is expressed in T as well as NK cells, invariant NKT cells, and mast cells [91–93]. ITK plays a critical modulatory role in the T-cell receptor (TCR) signaling cascade. In mice, it functions in the positive/negative selection of thymocyte development, as well as regulation of conventional *vs*. innate-type $CD8^+$ T-cell development [94, 95]. Moreover, $Itk^{-/-}$ $CD8^+$ T cells fail to mount effective primary or memory immune responses to a variety of viral infections [95–97]. Itk is also crucial for invariant NKT-cell development and function in mice [93]. Similarly, a characteristic reduction in naive $CD45RA^+$ T cells and NKT cells has been reported in ITK-deficient patients [86–88]. Moreover, ITK has been shown to differentially regulate NK-cell-mediated cytotoxicity, which might be impaired in the absence of ITK protein [98].

The development of LPDs in ITK-deficient patients almost always follows primary EBV infection and is diagnosed as HL, as opposed to Burkitt's lymphoma or other NHL seen in XLP. It is speculated that perturbed innate and adaptive antitumor immunosurveillance, including lack of NKT cells and impaired NK- and T-cell-mediated cytotoxicity, plays contributory roles in the development of EBV-associated LPDs in ITK-deficient patients [99].

18.3.2 Purine Nucleoside Phosphorylase Deficiency

Purine nucleoside phosphorylase (PNP) deficiency is a rare, autosomal recessive, combined immunodeficiency disorder, with an estimated frequency of 4 % among patients with SCID [100]. The disease usually manifests during the first year of life; however, the onset of symptoms may vary, with some patients having no apparent clinical immunodeficiency until later in childhood [101–104]. Common clinical manifestations in patients with PNP deficiency include recurrent, bacterial, viral, and opportunistic infections; prolonged diarrhea; failure to thrive; neurologic abnormalities, including nonprogressive cerebral palsy, ataxia diplegia, or disequilibrium; and autoimmune disorders, including autoimmune hemolytic anemia, idiopathic thrombocytopenia, autoimmune neutropenia, lupus, and central nervous system vasculitis [100–102, 105–108]. Due to profound T-cell abnormalities, patients are extremely susceptible to viral infections and may develop disseminated or even fatal disease [100, 103]. A high frequency of malignancy is also noted, including pharyngeal tumors, lymphoma, and lymphosarcoma [100, 109, 110]. In a report of 33 patients with PNP deficiency, four had developed lymphoma or lymphosarcoma and one had a pharyngeal tumor [100]. Immunological evaluations of patients with PNP deficiency revealed marked T-cell lymphopenia, with decreased T-cell proliferative responses and abnormal humoral immunity in most cases, as assessed by B-cell number, total immunoglobulin levels, and specific antibody formation. NK numbers may be variable.

Several disease-causing mutations have been identified in the *PNP* gene (OMIM*164010), producing proteins with differing degrees of enzymatic activity that inversely correlate with clinical severity (i.e., more functional proteins are associated with milder forms of disease, while less functional proteins lead to severe

phenotypes) [104, 111, 112]. PNP is an enzyme in the purine salvage pathway that reversibly converts inosine to hypoxanthine and guanosine to guanine. Of all accumulated PNP substrates, only deoxyguanosine can be phosphorylated further in the mammalian cells. Thus, in PNP deficiency, there is accumulation of abnormally high levels of lymphotoxic dGTP [113, 114]. This, in turn, inhibits ribonucleotidase reductase activity, depletes dCTP, and inhibits DNA synthesis and repair [113, 114]. Moreover, mitochondrial dGTP is also likely to inhibit mitochondrial DNA repair and initiate the apoptotic protease cascade triggered by cytochrome C release [115–117]. The most characteristic immune abnormality is thus a profound defect in T-cell number and function; however, abnormal B-cell functions, including defective Ab production, are common and in part due to abnormal T-cell help [100, 118]. However, an intrinsic defect in B-cell function has not been excluded. The T-cell specificity of PNP lies in the high deoxyguanosine phosphorylating activity in the T lymphocytes, as compared with B lymphocytes or other tissues [119, 120], and the inherent susceptibility of immature thymocytes to apoptosis during T-cell selection [121, 122].

18.3.3 Dedicator of Cytokinesis 8 Deficiency

Dedicator of cytokinesis 8 (DOCK8) deficiency, initially described as a form of autosomal recessive hyper-IgE syndrome [123], is now regarded as a combined immunodeficiency disorder [1], presenting early in life with: (1) recurrent sinopulmonary infections; (2) cutaneous viral, bacterial, and fungal infections; (3) severe atopy, asthma, and allergies; (4) immune-mediated pathologies including autoimmune hemolytic anemia and vasculitis; (5) neurological complications; (6) malignancies; and (7) extremely high serum IgE levels and eosinophilia [123–127]. Cutaneous viral infections are the most distinctive clinical feature and often identified as recalcitrant, extensive lesions caused by herpes simplex (HS), human papilloma (HP), molluscum contagiosum (MC), and varicella zoster (VZ) viruses [124–127]. Moreover, EBV and/or cytomegalovirus infections are documented in up to 40 % of patients [126, 127]. Increased frequencies of malignancies, including squamous cell carcinoma (SCC), cutaneous T-cell lymphoma/leukemia, Burkitt's lymphoma, anaplastic B-cell lymphoma, as well as adrenal leiomyoma and microcytic adnexal carcinoma, have been reported in up to 17 % of DOCK8-deficient patients [124, 125, 127, 128].

The disease is due to biallelic mutations in the *DOCK8* gene (OMIM*611432), which encodes DOCK8, a member of the DOCK180-related family of atypical guanine nucleotide exchange factors (GEFs) [129]. DOCK8 was shown to bind to the Rho GTPases Cdc42, Rac1, RHOJ, and RHOQ in a yeast two-hybrid system but not in GST pulldown assay [130]. Following the generation of DOCK8-knockout mice, it has been documented that DOCK8 is a Cdc42-specific GEF [131] and that DOCK8 exists in a macromolecular complex with the Wiskott–Aldrich syndrome protein (WASP), an actin nucleation-promoting factor activated by Cdc42, as well as with talin, a protein required for integrin-mediated adhesion [132]. These findings further support the role of DOCK8 in the regulation of actin dynamics and formation of the immunologic synapse, which are required for full T-cell activation, proliferation, and acquisition of effector functions.

Immunological features of DOCK8 deficiency, besides high serum IgE levels and eosinophilia, include lymphopenia (progressive with age) affecting $CD4^+$ and $CD8^+$ T cells (especially the $CD4^+$ T cells) and, to a lesser extent, NK and B cells [124–127], plus a virtual lack of circulating $CD19^+CD27^+$ memory B cells [133]. Studies in DOCK8-deficient patients have demonstrated decreased T-cell activation and proliferation in response to mitogens [124–127], but not to specific antigens [126]; however, these functional studies are inconclusive due to the difficulty in isolating naive T cells from the peripheral blood. In murine models of DOCK8 deficiency, the defect has been localized to normal survival of $CD8^+$ memory T cells [134]. DOCK8-deficient humans and/or mice also exhibit abnormalities in cytokine secretion associated with a T-helper 2-biased immune response [124, 126, 134],

low serum IgM levels and impaired Ab responses [133, 135], decreased $CD4^+$ T-helper type 17 cells, and impaired NK-cell cytotoxicity [124, 125, 132, 136].

Increased susceptibility to malignancy in DOCK8-deficient patients can be explained by failure of $CD8^+$ T- and NK-cell-mediated tumor immunosurveillance, as well as chronic antigenic stimulation. Moreover, there is evidence that DOCK8 itself might have direct tumor suppressor activity [137–140], and that loss of DOCK8 expression might contribute to carcinogenesis [141]. Reduced DOCK8 expression has been demonstrated in the vast majority of primary lung cancers, irrespective of the histological type, compared with normal lung tissue. Epigenetic mechanisms, including DNA methylation and histone deacetylation, were indicated to be involved in DOCK8 downregulation in lung cancer cells [137], as with other candidate tumor suppressor genes, such as p16, RASSF1A, and MYO18B [142–145]. Moreover, homozygous deletions of the *DOCK8* gene has been shown in breast and gastric cancer cell lines. These results suggest that genetic and epigenetic inactivation of DOCK8 is involved in the development and/or progression of lung cancers and other cancers by disturbing the regulatory functions of DOCK8 in cell migration, morphology, adhesion, and growth of cells [137].

18.3.4 RHOH Deficiency

Ras homolog family member H (RHOH) deficiency is a novel form of PID recently identified by genome-wide linkage analysis in two young adult siblings born to consanguineous French parents [146]. Since childhood, both patients displayed a phenotype resembling epidermodysplasia verruciformis (EV), characterized by persistent cutaneous infections with EV-specific HPV (EV-HPV) genotypes. The older sibling had also developed Burkitt's lymphoma in childhood, granulomatous lung disease, and psoriatic-like lesions, whereas the younger sibling had molluscum contagiosum, psoriatic lesions, and gingivostomatitis, indicating that the phenotypic spectrum of the disease is not restricted to susceptibility to HPV [146].

RHOH deficiency results from homozygous loss-of-expression mutations (Y38X) in the *RHOH* gene (OMIM*602037) located on chromosome 4p13, which encodes an atypical Rho GTPase (RHOH) expressed predominantly in hematopoietic cells. RHOH is GTPase deficient and remains constitutively in the active, GTP-bound state, suggesting that its activity is likely regulated by the level of the protein expressed in the cells rather than guanine nucleotide cycling [147]. It has been shown to counteract Rac GTPase activities in lymphoid cell lines and cytokine-stimulated hematopoietic progenitor cells, resulting in reduced proliferation, increased apoptosis, and defective actin polymerization [147–150].

Immunologic evaluation of RHOH-deficient patients revealed no major abnormality in the frequencies of B-cell subsets, NK cells, NKT cells, monocytes, and polymorphonuclear cells and in Ab production. Despite maintaining normal T-cell counts, both patients displayed a restricted T-cell repertoire, lack of circulating naive T cells consistent with the defect in thymic T-cell development observed in *Rhoh*-/- mice [149], expansion of effector memory T cells (more likely to be consequences of chronic infection), altered expression of T-cell tissue-homing markers with strikingly lower than normal proportion of skin-homing $\beta7^+$ T cells, and impaired T-cell proliferative responses to anti-CD3 but variable responses to mitogens and recall antigens (Ags) [146]. It is evident that on TCR stimulation, murine RHOH undergoes tyrosine phosphorylation and mediates recruitment of ZAP70 and Lck to the TCR/linker of activation in T-cell (LAT) signalosome [151]. This finding has been confirmed in RHOH-deficient T cells of patients, showing little or no ZAP70 phosphorylation in the presence or absence of CD3 stimulation [146]. The combination of T-cell defects common to both mice and humans, including impaired T-cell responses, a lack of naive cells, and smaller than normal proportion of $\beta7^+$ T cells, might explain the pathogenesis of susceptibility to cutaneous EV-HPVs.

The *RhoH/TTF* (translocation three four) gene was first identified by fusion to the *BCL6/LAZ3* oncogene resulting from t(3;4)(q27;p11) translocation in an NHL cell line [152–154]. Another chromosomal alteration involving the *RhoH/TTF* gene in a patient with multiple myeloma and t(4;14)(p13;q32) translocation has also been identified [154]. Moreover, aberrant somatic hypermutations in *RHOH* gene have been previously reported in various B-cell malignancies, including diffuse large B-cell lymphomas [155], AIDS-related NHL [156], primary central nervous system lymphomas [157], and, rarely, Burkitt's lymphoma [155]. However, it remains unclear whether these mutations translate into abnormal levels of RhoH expression in lymphomas and what pathophysiological contribution hypermutation in the RhoH gene plays in lymphomagenesis.

18.3.5 MAGT1 Deficiency

A novel X-linked immunodeficiency has been recently identified in seven male patients (two of which were siblings) with mutations in the magnesium transporter 1 (*MAGT1*) gene [158, 159]. The clinical phenotype of MAGT1 deficiency is characterized by chronic viral infections, EBV in particular, which led to the development of EBV-related lymphomas or related lymphoproliferative disorders in four patients. Other clinical features include recurrent upper respiratory tract infections, viral pneumonia, HSV-1 infections, recurrent shingles, molluscum contagiosum, and chronic diarrhea. [158, 159]

MAGT1 deficiency (OMIM*300715), named X-linked immunodeficiency with Mg^{2+} defect, EBV infection, and neoplasia (XMEN) disease, has been reported to be caused by null mutations in the *MAGT1* gene [158]. *MAGT1* encodes a membrane-associated transporter that selectively conducts Mg^{2+} across the membrane, with almost no permeability to other cations including Ca^{2+} [160, 161]. Despite the well-known essential roles of Mg^{2+} as a cofactor for ATP, nucleic acids, and numerous metabolic enzymes, its critical role as a second messenger in intracellular signaling has only begun to be unraveled [158, 162–165].

Immunological investigations in patients with MAGT1 deficiency revealed CD4 lymphopenia, leading to an inverted CD4:CD8 ratio and reduced number of recent thymic emigrant T cells, indicating that impaired thymopoiesis may contribute to CD4 lymphopenia. No major disturbance was observed in other lymphocyte populations. MAGT1-deficient T cells showed impaired proliferation and activation upon *in vitro* stimulation with anti-CD3 Ab. In contrast, T-cell activation in response to phorbol myristate acetate and ionomycin was intact, showing that the patients had a proximal TCR signaling defect prior to the induction of the Ca^{2+} flux. MAGT1-deficint B cells showed normal activation upon BCR stimulation [158]. Recapitulating the patients' phenotype by knocking down MGAT1 in normal T cells, as well as rescuing patients' T cells with ectopic expression of MAGT1, established that MAGT1 is required for TCR-stimulated Mg^{2+} influx that transiently raises free $[Mg^{2+}]_i$ in order to temporarily coordinate T-cell activation [158, 166].

Individuals with genetic deficiencies in MAGT1 have uncontrolled EBV infection and a predisposition to lymphoma. This has been attributed to a selective loss of NKG2D expression (posttranscriptional, accelerated protein turnover) and the resultant impaired cytolytic responses of NK and cytotoxic $CD8^+$ T lymphocytes [159], which are essential for control of viral infections and tumor immunosurveillance [167]. Hence, MAGT1 not only mediates TCR-induced Mg^{2+} flux but also regulates the basal-free $[Mg^{2+}]_i$ homeostasis required for NKG2D cytolytic activity. This has been verified by cultivation of NK and cytotoxic $CD8^+$ T lymphocytes from XMEN patients in Mg^{2+}-supplemented medium, causing a dose-dependent increase in free $[Mg^{2+}]_i$, which did recover the cytotoxicity defect partially in cytotoxic $CD8^+$ T lymphocytes and almost completely in NK cells [159]. Most notably, magnesium supplementation *in vivo* concurrently reduced EBV-infected cells, which

may provide an adjunctive treatment to prevent early lymphoma development and mortality in XMEN patients.

18.4 Phagocyte Defects

The underlying mechanism of cancer development in PIDs caused by defects of phagocytic cells is quite different from that observed in other immunodeficiency disorders. Here the implicated genes are important for proper myeloid cell development; thus cancers form due to dysregulated myelopoiesis. This is distinct from cancers that occur in some other conditions including impaired immunosurveillance and presence of specific viruses.

18.4.1 Severe Congenital Neutropenia (Kostmann Syndrome)

Severe congenital neutropenia (SCN) is a rare PID characterized by a maturation arrest of myelopoiesis at the level of the promyelocyte/myelocyte stage with peripheral blood absolute neutrophil counts (ANCs) below 0.5×10^9/L, in addition to early-onset superficial and systemic bacterial infections [168, 169]. The skin and mucous membranes are usually affected by ulceration, gingival hyperplasia, periodontitis, and abscess formation [170]. Patients may also suffer from neurological disorders including developmental delay, mental retardation, epilepsy, and decreased cognitive function [171, 172].

SCN follows an autosomal dominant or recessive pattern of inheritance or can occur sporadically. It is a genetically heterogeneous disorder caused by a variety of mutations in several different genes. Nonetheless, the different genetic forms of SCN share a rather similar clinical phenotype. Mutations in the neutrophil elastase (*ELA2*) gene (OMIM*130130) are found in approximately 50 % of all cases, i.e., those with dominant autosomal or sporadic SCN [170, 173]. ELA2 is a serine protease, exclusively expressed in neutrophils and monocytes, and is stored in the primary granules of neutrophils [174]. Interestingly, mutations in the *ELA2* gene are also responsible for the clinical phenotype of cyclic neutropenia. The pathophysiological mechanisms responsible for the development of different phenotypes, congenital or cyclic neutropenia, are not yet understood [175]. Most patients with autosomal recessive disease, which comprises approximately 30 % of SCN, have mutations in the HS-1-associated protein X (*HAX1*) gene (OMIM*605998) [176]. HAX-1, a mitochondria-targeted protein containing Bcl-2 homology domains, is an apoptosis-regulating protein [176]. Mutations in the glucose-6-phosphatase catalytic subunit 3 (*G6PC3*) gene (OMIM*611045) have recently been identified in a group of autosomal recessive SCN patients with additional syndromic features including cardiac and urogenital anomalies and increased venous marking [177]. Patients with X-linked SCN harbor activating mutations in Wiskott–Aldrich syndrome (*WAS*) gene (OMIM*300392), leading to a constitutively active form of the WAS protein and unregulated actin polymerization [178]. Inactivating mutations in the proto-oncogene growth factor-independent 1 (*GFI1*) gene (OMIM*600871) are also associated with SCN [179]. In addition, SCN without a maturation arrest has recently been associated with p14 protein deficiency [180]. Finally, acquired nonsense mutations in colony-stimulating factor 3 receptor (*CSF3R*) gene (OMIM*138971) have also been found to affect 20 % of SCN patients [181].

SCN patients are at an increased risk of myelodysplasia (MDS) and acute myeloid leukemia (AML) development with a cumulative incidence of leukemia of 22 % after 15 years of G-CSF treatment [182, 183]. Independent of the genetic subtype, conversion to leukemia in patients with SCN is often associated with one or more somatic cellular genetic abnormalities (e.g., monosomy 7, *RAS* mutations, trisomy 21, or *CSF3R* mutations), which may be diagnostically useful to identify subgroups of patients at high risk of developing leukemia [175]. Other risk factors for progression to MDS and/or AML are

the severity of neutropenia, younger age at diagnosis, and prior exposure to G-CSF. [184] Interestingly, marrow cells from nearly 80 % of patients with SCN who transform to leukemia show point mutations in *CSF3R*, suggesting that these mutations play an important role in leukemogenesis [185].

Hematopoietic stem-cell transplantation (HSCT) is the only definitive treatment for patients with bone marrow failure, MDS, or leukemia; however, it seems that patients with SCN may be at increased risk of transplant-related mortality for unknown reasons. As a result, there is no clear consensus on when a patient with SCN should undergo HSCT [186].

18.4.2 Shwachman–Diamond Syndrome

Shwachman–Diamond syndrome (SDS) is a rare autosomal recessive, systemic disease characterized by exocrine pancreatic insufficiency, impaired hematopoiesis, and leukemia predisposition [187]. Other clinical features include skeletal, immunologic, hepatic, and cardiac disorders [186]. There is considerable phenotypic variability between individuals, and making the diagnosis can be challenging, particularly in older patients in whom symptoms such as steatorrhea may have resolved [186] or may not be present [188]. The most common hematologic abnormality in patients with SDS is neutropenia, which can be chronic or intermittent. Anemia and thrombocytopenia are also common manifestations. Patients with SDS are susceptible to recurrent infections [189] likely due to neutropenia. Other immune defects have also been reported. These include neutrophil chemotactic defects [190, 191], decreased proportions of circulating B cells, low immunoglobulin levels, decreased *in vitro* B-cell proliferation, lack of specific antibodies or decreased total circulating T lymphocytes, as well as decreased proliferative responses [192, 193].

Around 90 % of patients with clinical features of SDS have mutations in the Shwachman–Bodian–Diamond syndrome (*SBDS*) gene (OMIM*607444) [194], with the encoded protein being essential for normal ribosome maturation, though its precise molecular function remains unclear [195, 196]. In addition to a stem-cell defect [197, 198], patients with SDS have also a serious, generalized marrow dysfunction with an abnormal bone marrow stroma in terms of its ability to support and maintain hematopoiesis [196, 199].

Similar to other marrow failure syndromes, patients with SDS have an increased risk for MDS and AML [200], with an estimated risk of 19 % at 20 years and 36 % at 30 years [184]. There are also three reported cases of solid tumors in patients with SDS [201–203]. The reason behind this malignant predisposition is not known. However, several theories have been proposed, including chromosome instability [204, 205], accelerated apoptosis linked to increased expression of the Fas Ag and to hyperactivation of the Fas signaling pathway [206], and abnormal gene expression patterns as evident by upregulation of several oncogenes, including *LARG*, *TAL1*, and *MLL*, and downregulation of several tumor suppressor genes, including *DLEU1*, *RUNX1*, *FANCD2*, and *DKC1*, which might result in continuous stimulation favoring evolution or progression of malignant clones [207]. Accordingly, all patients with SDS should be monitored with peripheral blood counts every 3–4 months and marrow evaluation on a yearly basis, and if indicated, HSCT should be done prior to the development of overt leukemia.

18.4.3 GATA2 Deficiency

A novel inherited immunodeficiency clinically characterized by disseminated mycobacterial infections (typically Mycobacterium avium complex [MAC]), opportunistic fungal infections, disseminated HPV infections, and pulmonary alveolar proteinosis, with an increased risk of myelodysplasia, cytogenetic abnormalities, and myeloid leukemias, has been recently described [208–211]. This novel inherited immunodeficiency, termed monocytopenia and mycobacterial

infection (MonoMAC) syndrome, precedes the development of overt MDS by many years, and eventually leukemias. This form of immunodeficiency occurs either as an autosomal dominant form or sporadically [212].

Heterozygous mutations in the critical hematopoietic regulator of stem-cell integrity, *GATA2* gene (OMIM*137295), have been recently implicated as the cause of the MonoMAC syndrome, suggesting dominant interference of gene function by either dominant negative effects or haploinsufficiency [209, 213, 214]. Mutations in the same gene may result in two more different phenotypes: familial MDS/AML without other hematopoietic defects [215, 216], and Emberger syndrome, which is characterized by congenital deafness and primary lymphedema of the lower limb with a predisposition to MDS or AML [217]. The GATA family of transcription factors, which contain zinc fingers in their DNA-binding domain, have emerged as candidate regulators of gene expression in hematopoietic cells. GATA2 functions in the regulation of hematopoiesis and, in particular, is required for maintenance and survival of the hematopoietic stem-cell pool [218, 219]. GATA2 also functions in the formation of early blood and lymphatic vessels [220, 221]. The role of *GATA2* mutation in disease manifestation is incompletely understood but likely complex and thought to be linked to the generation or maintenance of progenitors required for the affected cell subsets [213].

Immunological characterization of patients with the MonoMAC syndrome revealed profoundly decreased or absent monocytes, NK cells, and B cells as well as a severe decrease in circulating and tissue dendritic cells (DCs). In most cases, GATA2 deficiency is accompanied by a severe reduction in peripheral blood NK cells, specifically the $CD56^{bright}$ subset, with marked functional impairment [209], which predispose to significant HPV and other viral infections, as well as HPV-associated SCC. Bone marrow failure resulting from loss of stem cells may underlie the multilineage cytopenias described in most patients; however, the underlying mechanisms for cytogenetic abnormalities or the leukemic transformation need to be further clarified.

18.5 Defects in Innate Immunity

18.5.1 Epidermodysplasia Verruciformis

Epidermodysplasia verruciformis (EV) is a chronic, genetically inherited skin condition characterized by increased susceptibility to cutaneous infection with certain HPV genotypes, referred to as EV-HPVs. [222, 223] EV begins during infancy or early childhood, and the more benign lesions manifest as flat, wart-like, hypopigmented, or hyperpigmented papules or pityriasis versicolor-like plaques, whereas lesions with greater potential for malignant transformation present more variably as verrucous and seborrheic keratosis-like lesions, occurring mainly on sun-exposed areas [222–224]. Approximately 30–60 % of individuals eventually develop skin malignancies, eventually in the fourth to fifth decades, with Bowen carcinoma in situ being the most frequent tumor, followed by invasive SCC and, less frequently, basal-cell carcinoma [224–227].

EV is inherited primarily in an autosomal recessive pattern [228], although both X-linked recessive and autosomal dominant modes of inheritance have been reported [229, 230]. Genome-wide linkage studies have identified two EV susceptibility loci *EV1* and *EV2*, on chromosomes 17 and 2, respectively [231]. Mutations in the *EVER1* (OMIM*605828) and *EVER2* (OMIM*605829) genes, which are part of the EV1 locus, have been identified in approximately 75 % of patients with EV [222].

The EVER proteins, localized in the endoplasmic reticulum of human keratinocytes [232], interact with ZnT-1 [233], a zinc transporter regulating cellular zinc homeostasis. Loss of EVER zinc homeostasis enhances the expression of viral genes, specifically the pro-oncogenic *E6* and *E7*, contributing to HPV-mediated carcinogenesis. Besides keratinocytes, EVER proteins are expressed in T and B lymphocytes, NK cells, endothelial cells, myeloid cells, and DCs. Zinc has been shown to contribute to TCR signaling by increasing ZAP70 phosphorylation [234]. Mutated, dysfunctional

EVER genes would disrupt zinc homeostasis and consequently produce a defect in cell-mediated immunity, which could compromise viral clearance and lead to malignant transformation [222, 233]. Although the immunological phenotype of EV might be normal, it can also manifest with decreased total T-lymphocyte counts; reduced cell-mediated immunity, as measured by reduced responsiveness to mitogens and Ags as well as cutaneous anergy to recall Ags [235, 236]; and defective cell-mediated immunity toward EV-HPVs or infected keratinocytes [237, 238].

18.5.2 Warts, Hypogammaglobulinemia, Infections, and Myelokathexis Syndrome

Warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is a rare, dominantly inherited PID characterized by warts, hypogammaglobulinemia, infections, and myelokathexis, which refers to neutropenia resulting from abnormal retention of mature neutrophils and increased neutrophils apoptosis in the bone marrow [239–241]. The incidence of WHIM syndrome has been estimated to be 0.23 cases per million births [242]. The clinical onset usually occurs during infancy or early childhood with recurrent gastrointestinal, respiratory, and cutaneous bacterial infections and increased susceptibility to HPV infection, causing numerous, recalcitrant skin and genital warts [240, 241]. Genital warts (condylomata acuminata) may undergo dysplastic changes conferring to an increased risk of malignancy [239–241]. Contrary to the long-held belief, HPV is not the only unique viral susceptibility in WHIM syndrome; more recently, EBV-associated lymphoproliferative disease [243, 244] as well as herpes zoster [245], herpes simplex virus [245, 246], and molluscum contagiosum [243] infections have been reported, indicating a generalized susceptibility to *Herpesviridae* viruses.

WHIM syndrome is primarily caused by gain-of-function mutations in the gene encoding the chemokine receptor CXCR4 (OMIM*162643) [247], a member of the G-protein-coupled receptor superfamily specific for the CXC chemokine stromal cell-derived factor 1 (SDF-1) [248], also known as CXCL12. All *CXCR4* mutations reported to date disrupt receptor downregulation leading to enhanced and prolonged chemotactic responsiveness to SDF-1 [249, 250].

Immunological and hematological abnormalities in WHIM syndrome include peripheral neutropenia, B lymphopenia with a particular reduction in the number of switched memory B cells ($CD27^+$ IgD^-), T lymphopenia with decreased number of naïve T cells, and a relative expansion of memory T cells with a restricted repertoire, deficiency of plasmacytoid DCS, and hypogammaglobulinemia [251–254]. The mechanisms by which dysregulated CXCR4 signaling affects leukocyte homeostasis and predisposes to a selective susceptibility to HPV infection and carcinogenesis are still unknown. It remains possible that defective trafficking of effector cells (T cells and NK cells) and Ag-presenting cells might contribute to defective cutaneous immunity, explaining the abnormal susceptibility to viruses affecting the skin [99].

18.6 Diseases of Immune Dysregulation

18.6.1 X-Linked Lymphoproliferative Disease

X-linked lymphoproliferative disease (XLP), formerly known as Duncan disease, is a rare and often fatal inherited immunodeficiency disorder, initially described by Purtilo et al. [255], with an estimated incidence of one to three per million male births [256]. It is characterized by severe immune dysregulation in males with a variable clinical presentation, often following EBV infection, manifesting as fulminant infectious mononucleosis and/or acquired hemophagocytic lymphohistiocytosis (HLH), dysgammaglobulinemia, and malignant lymphoma [257–260]. Other, albeit less common, clinical features of

XLP include aplastic anemia, lymphocytic vasculitis, pulmonary lymphoid granulomatosis, arthritis, colitis, and psoriasis [260–262].

Most cases of XLP are caused by germline mutations in the Src homology 2 domain-containing gene 1A (*SH2D1A*; OMIM*300490) encoding the 128 amino acid signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) [263–265]. A second XLP-like disorder caused by mutations in the X-linked inhibitor of apoptosis protein (*XIAP*; OMIM*300079) was described in 2006 [266]. Although XIAP deficiency is predominantly associated with recurrent EBV-associated HLH, no lymphoma occurrence has been reported in affected patients till now [141, 260, 266]. In humans, SAP is expressed predominantly in NK, NKT, and T cells [267–269]. It has been shown to serve as an adaptor molecule downstream of several SLAM immunomodulatory receptors family [270]. The SLAM–SAP association potentiates the development of NKT cells, T–B-cell conjugation required for the development of germinal centers and immunoglobulin production, and EBV-directed cytotoxicity by T and NK cells. In addition, it is required for normal T-cell homeostasis mediated by reactivation-induced cell death (RICD) [271, 272].

SAP-deficient patients are at increased risk of lymphoma development, as well as other lymphoproliferative diseases. Approximately 30 % of patients develop lymphoma at a mean age of 15 years at diagnosis [260, 273]. Expectedly, the majority are of B-cell origin, arising in extranodal sites, most commonly localized in the ileocecal region, with Burkitt's lymphoma comprising approximately 50–60 % of total lymphomas [260, 274, 275]. Notably, not all cases of lymphomas arise due to malignant transformation of EBV-infected B cells, as up to one-third of patients with lymphoma are EBV seronegative [260, 273, 275], indicating that the genetic defect per se can result in lymphoma. It is likely that defective antitumor immunosurveillance due to poor $CD8^+$ T- and NK-cell cytotoxic responses and lack of NKT cells contributes to lymphomagenesis [99].

18.7 Syndromes with Autoimmunity

18.7.1 Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a rare disease characterized by defective Fas-mediated apoptosis [276]. The incidence and prevalence of ALPS are unknown. Estimated cases of ALPS exceed 500 cases worldwide; however, it is not reliably confirmed. Classically, patients present with autoimmunity, lymphadenopathy, and/or splenomegaly along with elevation in TCR α/β^+ $B220^+CD4^+CD8^+$ double-negative T (DNT) cells (a constant feature of the disease with undetermined origin) and defective *in vitro* Fas-mediated lymphocyte apoptosis [277]. Furthermore, certain biomarkers may be useful to aid in diagnosis [278]. These include elevated circulating levels of sFASL, IL-10, vitamin B12, and IL-18. Patients who do not fulfill the ALPS diagnostic criteria are now classified as having ALPS-related conditions caused by germ-line mutations in *CASP8*, *NRAS*, and *SH2D1A* [277]. XLP, a genetic immunodeficiency caused by mutations or deletions in the *SH2D1A* gene, can be included in the spectrum of ALPS-like disorders, since these patients frequently display defective apoptosis in response to TCR restimulation [279, 280]. Mutations in the ALPS and ALPS-related genes often manifest with variable penetrance [281]. Thus, patients with ALPS often have family members with the same genetic mutation with no clinical phenotype or very mild symptoms. The penetrance of the mutation is not related to the type of mutation but probably depends on unknown genetic and environmental modifiers. Hence, the clinical significance of isolated detection of a heterozygous Fas mutation in a healthy relative of a patient with ALPS is not yet clear.

Autoimmunity, affecting over 70 % of patients, is mainly directed against blood cells [282]. Other autoimmune manifestations are rare and include autoimmune nephritis, hepatitis, arthritis,

uveitis, iridocyclitis, and vasculitis [283]. Autoantibodies are more common than obvious clinical disease and present in up to 92 % of patients [284]. Elevation of TCR α/β^+ DNT cells in the peripheral blood and lymphoid tissues is a hallmark of ALPS, but it is not pathognomonic as patients with other autoimmune diseases such as SLE and ITP may also have mild elevations in these cells [285].

ALPS is caused by germ-line or somatic mutations in *FAS* gene (*TNFRSF6*, or *CD95*; OMIM*134637), or germ-line mutations in the FAS ligand (*FASL*) (*TNFSF6*, or *CD95L*; OMIM*134638) or *CASP10* (OMIM*601762) genes.

Apoptosis is critical in tumor scrutiny as FAS, a putative tumor suppressor, is silenced in many tumors [286–288]. As anticipated, patients with ALPS who harbor germ-line mutations in the ALPS-related genes have an increased risk of developing malignancy [289], with the risk of NHL and HL, respectively, being 14 and 51 times greater than expected [290]. An increased risk of cancer has also been observed in unaffected family members who may inherit the same mutation but fail to develop an overt ALPS phenotype [290]. Sporadic NHL harbors somatic mutations of the *FAS* gene in 11 % [291] of cases and in the *CASP10* gene in 14.5 % of cases [292]. Furthermore, in HL, somatic *FAS* gene mutations are found in Reed–Sternberg cells in 10–20 % of cases [286, 293].

18.7.2 Autoimmune Polyendocrinopathy with Candidiasis and Ectodermal Dystrophy

Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy (APECED), also called autoimmune polyendocrine syndrome type I (APS-1), is a rare autosomal recessive disease, most commonly seen in Iranian Jews, Sardinians, and Finns. The diagnosis of APECED is reached if patients manifest at least two of the following conditions: (1) chronic mucocutaneous candidiasis (CMC), (2) hypoparathyroidism, or (3) Addison's disease. Additional autoimmune components may appear throughout life and include gonadal failure, type 1 diabetes, hypothyroidism, pernicious anemia, hepatitis, alopecia, vitiligo, and/or ectodermal dystrophies.

The disease is characterized by loss of tolerance against self-antigens [294, 295], which is caused by mutations in the autoimmune regulator (*AIRE*) gene (OMIM*607358) [296, 297]. Although the endocrine features are clearly autoimmune, the underlying immunodeficiency predisposing to CMC has been a long-standing puzzle. Recently, autoantibodies against the Th17-related cytokines IL-22, IL-17A, and IL-17F, which are implicated in protection against fungi at epithelial surfaces, were discovered in the sera of APS-1 patients [298, 299], suggesting that the underlying immunodeficiency in patients with APECED has an autoimmune basis.

Several cases of oral and esophageal SCC have been reported in APECED patients with CMC [300–303]. In a cohort of 92 Finnish patients, six had developed oral or esophageal SCC by the mean age of 37, representing 10 % of patients older than 25 years [300]. The partial T-cell defect of APECED seems to favor the growth of *Candida albicans* and predispose to chronic mucositis and the development of SCC. Besides chronic inflammation and increased cell turnover, *Candida albicans* biotypes are capable of producing the carcinogenic nitrosamine N-nitrosobenzylmethylamine [304, 305], and can also act to promote oral carcinogenesis in rats when a known carcinogen, 4-nitroquinoline-1-oxide, is repeatedly applied [306].

18.8 Other Well-Defined Immunodeficiencies

18.8.1 DNA Repair Defects

B- and T-lymphocyte development depends largely on multiplex genetic rearrangements, i.e., V(D)J recombination, class switch recombination, and somatic hypermutation, carried out by multiple DNA repair and damage response protein complexes [307]. Variations in the DNA

repair genes might compromise the delicate balance between the generation of genetic variation and replication fidelity of DNA [308, 309]. PIDs associated with defects in DNA repair, collectively termed genomic instability syndromes, are generally associated with cellular radiosensitivity, developmental defects, and predisposition to cancer [309–311]. Syndromes known to be associated with malignancies, including ataxia–telangiectasia, Nijmegen syndrome, Bloom syndrome, DNA ligase IV deficiency, Artemis deficiency, cartilage hair hypoplasia, and PMS2 deficiency, are summarized in Table 18.1. Although these defects are associated with an increased risk of lymphoid malignancies, mainly NHL, nonlymphoid tumors affecting the brain, skin, breast, and gastrointestinal tract have also been reported [311, 312, 314, 316, 319–321]. This is partly due to the fact that diverse DNA repair processes are not specific to Ag receptor diversification. DNA double-strand breaks, arising from multiple sources, including exposure to ionizing radiation, can potentially lead to replication errors, loss or rearrangements of genomic material, and eventually cell death or carcinogenesis. The DNA damage response pathway, responsible for sensing and repairing the damaged DNA, comprises the most powerful tumor surveillance mechanism [320]. The observation of an increased risk of cancer development in heterozygote carriers provides additional insight into their tumorigenic potential [321–324]. Additionally, defects in immunosurveillance mechanisms per se, similar to certain PIDs not associated with DNA repair defects, contribute to cancer development.

18.8.2 Signal Transducer and Activator of Transcription 3 Deficiency

Hyper-IgE syndrome (HIES) is a complex PID characterized by recurrent staphylococcal infections beginning early in infancy, predominantly involving the skin and lungs, chronic eczema, and markedly high serum IgE concentrations [325–327]. Skin infections due to *S. aureus* lack the usual local or systemic features of inflammation, forming so-called cold abscesses [328]. Recurrent sinopulmonary infections, resulting in bronchiectasis and pneumatocele formation frequently superimposed with bacterial and fungal infections, are the major causes of morbidity and mortality in patients with HIES [329]. Despite having extremely high serum IgE levels and eosinophilia, patients with HIES are usually free from other allergic manifestations, recognized as a marked difference from DOCK8 deficiency [325, 327]. In patients with HIES, serum IgG, IgM, and IgA levels are usually normal; however, most have impaired antigen-specific Ab response to immunization [330]. Diminished circulating memory B cells and defects in the differentiation of Th17 cells have also been demonstrated [330–332]. The multisystem nature of the disease extends beyond the immune system and accounts for the characteristics craniofacial, musculoskeletal, dental, and vascular abnormalities [333–336].

Dominant negative mutations in *STAT3* (OMIM*102582) have been identified as the major molecular etiology of autosomal dominant and sporadic cases of HIES [337, 338]. STAT3, one of the seven STAT proteins in the human, is a transcription factor and plays a critical role in responses to many cytokines and growth factors through the shared signal-transducing molecule gp130 [326, 327]. It is crucial for cell proliferation, survival, migration, apoptosis, and inflammation in various tissues [339], probably explaining the diverse clinical findings in patients with HIES.

STAT3 deficiency is associated with an increased risk of LPDs, most notably HL and NHL (relative risk: 259), with the majority of B-cell origin and aggressive histology [340–342]. Other cancers described in patients with HIES include leukemia and cancers of the vulva, liver, and lung [343]. The underlying mechanisms, however, remain unclear. The higher risk of tumor formation has been attributed to defective immunosurveillance and chronic B-cell stimulation, resulting in an increased turnover of B cells and accumulating genetic aberrations, giving rise to malignant B-cell clones [99].

Table 18.1 Clinical and Immunological Features of DNA Repair Defects Associated with Cancers

Gene	Disease	Inheritance	Clinical features	Pathogenesis	Immune defects	Associated cancers
ATM (OMIM*607585)	Ataxia-telangiectasia	AR	Cerebellar ataxia, oculocutaneous telangiectasia, chromosomal instability, radiosensitivity, thymic aplasia, recurrent sinopulmonary infections, cancer predisposition (up 40 %)	Disorder of cell cycle checkpoint and DSB repair, role in V(D)J, CSR	Often decreased IgA, IgE, and IgG subclasses, increased IgM, antibodies variably decreased, progressive T-cell lymphopenia, normal B-cell count	Lymphomas, lymphoid leukemias (mainly T cells), epithelial tumors, gastric carcinoma [311–313]
NBN (OMIM*602667)	Nijmegen breakage syndrome	AR	Severe microcephaly, bird-like face, mental and growth retardation, chromosomal instability, radiosensitivity, recurrent sinopulmonary infections, strong predisposition to lymphoid malignancy	Disorder of cell cycle checkpoint and DSB repair, role in V(D)J, CSR, SHM	Often decreased IgA, IgE, and IgG subclasses, increased IgM, antibodies variably decreased, decreased B- and T-cell counts	Hodgkin and non-Hodgkin lymphomas, leukemias (mainly B cells), brain tumors [314]
BLM (*OMIM*210900)	Bloom syndrome	AR	Short stature, bird-like face, sun-sensitive erythema, erythema, marrow failure, chromosomal instability, cancer predisposition	Role as a RecQ-like helicase	Low IgM and IgA, normal B- and T-cell counts	Leukemias, lymphomas, carcinomas [315]
LIG4 (OMIM*601837)	DNA ligase IV deficiency	AR	Microcephaly, facial dysmorphisms, radiation sensitivity, may present with RS-SCID, Omenn syndrome, or with a delayed clinical onset	Impaired NHEJ, role in V(D)J, CSR	Decreased serum Igs, decreased B- and T-cell counts	EBV-positive B-cell lymphomas, T-cell ALL [316, 317]

DCLRE1C (OMIM*602450)	Artemis deficiency	AR	Radiation sensitivity, may present with RS-SCID or Omenn syndrome	Role in V(D)J, CSR	Decreased serum Igs, markedly decreased B- and T-cell counts	EBV-positive B-cell lymphomas [318]
RMRP (OMIM*250250)	Cartilage hair hypoplasia	AR	Short-limbed dwarfism with metaphyseal dysostosis, sparse hair, bone marrow failure, autoimmunity, predisposition to cancers, impaired spermatogenesis, neuronal dysplasia of the intestine	Role in ribosomal RNA processing, mitochondrial DNA replication, and cell cycle control	Normal or reduced serum Igs, variably decreased antibodies, normal B-cell count, decreased or normal T-cell count, impaired lymphocyte proliferation	Non-Hodgkin lymphomas, basal-cell carcinoma [319]
PMS2 (OMIM*600259)	PMS2 deficiency (class switch recombination deficiency due to impaired mismatch repair)	AR	Recurrent infections, café au lait spots, cancer predisposition	Defective CSR-induced DSBs in Ig switch regions	Low IgG and IgA, elevated IgM, abnormal antibody responses, normal B-cell count, decreased, switched, and non-switched B-cell counts	Leukemias, lymphomas, colorectal carcinoma, brain tumors [320]

18.8.3 Wiskott–Aldrich Syndrome

Wiskott–Aldrich syndrome (WAS) is a rare X-linked immunodeficiency with highly variable manifestations characterized by thrombocytopenia with small platelets, eczema, and humoral and cellular immunodeficiency with increased susceptibility to pyogenic and opportunistic infections. Patients with WAS may also manifest with an increased incidence of autoimmunity and malignancies [344–349].

The disease is caused by mutations in the *WAS* gene (OMIM*300392), which is expressed exclusively in hematopoietic cells. Around 300 unique mutations spanning the *WAS* gene have been described. The effect of a given mutation on WASp expression correlates with the disease severity: mutations that cause decreased WASp levels result in the mild variant X-linked thrombocytopenia (XLT), characterized mainly by thrombocytopenia [350, 351], whereas mutations that abolish WASp expression or result in the expression of a truncated protein are associated with classic WAS. In addition, a third disorder termed X-linked neutropenia (XLN), characterized by neutropenia and variable myelodysplasia, has been attributed to activating mutations in the GTPase-binding domain of WASp [178, 352, 353].

The WAS protein (WASp) is a multifaceted protein which exists in complex with several partners involved in relaying signals from cell surface receptors to the actin cytoskeleton; lack of WASp results in cytoskeletal defects that compromise multiple aspects of normal cellular activity including proliferation, phagocytosis, immune synapse formation, adhesion, and directed migration [347]. It is therefore not surprising that lack of WASp results in a wide range of defects in cellular function involving all hematopoietic cell lineages [347].

Malignancies are relatively common in older patients (adolescent and young adults), especially in those with autoimmune manifestations, and are frequently associated with a poor prognosis [345, 348, 354]. The most frequent malignancy reported is B-cell lymphoma, which often occurs in EBV-positive patients [345, 349]. In a report of 154 patients with WAS, 21 (13 %) developed malignancies, mostly of lymphoreticular origin, with the average age at onset of 9.5 years [345]. Nonlymphoid malignancies, including glioma, acoustic neuroma, testicular carcinoma, and Kaposi sarcoma, have infrequently been reported [345, 355]. The development of hematological malignancies in WAS patients is at least partly due to NK cell and cytotoxic T-lymphocyte dysfunction [356–358], absent of invariant NKT cells [359, 360], and chronic stimulation of autoreactive cells and ineffective clearance of virally infected cells [361, 362]. It has been reported that despite normal expression levels of lytic molecules, the cytotoxic $CD8^+$ T cells from WAS patients failed to effectively kill B-cell lymphoma target cells due to inefficient polarization of cytotoxic granules toward the target tumor cells [356]. Recently, activating mutations in WASp (which give rise to XLN) have been found to lead to genetic instability through dysregulation of actin polymerization. Enhanced and delocalized actin polymerization throughout the cell was shown to inhibit myelopoiesis through defective mitosis and cytokinesis, with micronuclei formation indicative of genomic instability [363]. Despite lack of direct evidence, genomic instability might contribute to the development of malignancies in WAS patients [347].

Early HSCT is the treatment of choice for patients with classic WAS, preferably from a matched related donor [364]. Furthermore, immune reconstitution in WAS patients following HSCT leads to a decrease in cancer risk [364]. Gene therapy is an alternative to HSCT in the treatment of WAS [365]; however, the long-term outcome needs to be further monitored. This could be explained by the fact that the viruses used for therapy integrate in the host genome, with preferential insertion at transcription start sites, promoter and enhancer regions of active genes, and at conserved noncoding DNA, resulting in a high rate of transformations and the development of secondary malignancies [366, 367]. Acute T-cell leukemia due to vector insertion in the vicinity of the T-cell oncogene *LMO2* has been reported in one patient [368, 369].

18.8.4 Chromosome 22q11.2 Deletion Syndrome

Chromosome 22q11.2 deletion syndrome is relatively common (estimated in 1 in 4,000 births) [370], and about 6 % of newly diagnosed cases are familial [371]. The presenting symptoms of chromosome 22q11.2 deletion syndrome vary depending on the patients' age. While developmental delay and speech issues are the usual presenting symptoms in older children and adults, cardiac anomalies, hypocalcemia, and infection are the major manifestations in infants. Cardiac defects are seen in approximately 80 % of patients; on the other hand, tetralogy of Fallot and interrupted aortic arch type B have a strong positive predictive value for chromosome 22q11.2 deletion syndrome [372, 373]. Palatal dysfunction, feeding problems, facial dysmorphism, renal anomalies, and gastrointestinal manifestations also are seen in most of these patients [374]. Patients are also at an increased risk of atopy and autoimmune disease development [375, 376].

The immune system is affected in approximately 75 % of the patients [374, 376, 377]. The severity ranges from absent thymic tissue and no circulating T cells to completely normal T-cell counts. Many infants with low T-cell counts will demonstrate improvement in the first year of life, but after that, T-cell counts decline [378]. Patients may also suffer from variable degrees of B-cell defects [379, 380]. In a cohort of 687 patients with chromosome 22q11.2 deletion syndrome, six cases of malignancy were identified. This gives an overall frequency of 0.9 % (900 per 100,000) in this large pediatric group of patients, whereas the overall risk of malignancy in children under the age of 14 years is 3.4 per 100,000 children [381]. As reported in the literature, patients with chromosome 22q11.2 deletion syndrome have a clearly increased risk of lymphoma, particularly B-cell lymphoma [382–385]. This is a general phenomenon in patients with severe immunodeficiency. There have also been reports of myelodysplasia, acute lymphoblastic leukemia, SCC, astrocytoma, neuroblastoma, hepatoblastoma, renal cell carcinoma, and rhabdoid tumors [381, 386–390].

18.9 Concluding Remarks

The expanded life expectancy of patients with PIDs has increased the overall risk for developing cancers. However, the management of cancers in such patients remains challenging, in part due to the rarity, the increased risk for infection and other complications, as well as the rather slow pace of scientific advancement related to these conditions. Continued progress in understanding the interplay between chronic Ag stimulation, oncogenic viruses, genetic factors, and impaired host immunity during tumor formation in various PIDs will facilitate refinement of current and emerging therapeutic approaches.

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Immunosenescence, Oxidative Stress, and Cancers

19

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Contents

19.1 Introduction

The most important risk factor for cancer development is age [1]. With increasing age, numerous alterations at multiple levels including molecular, cellular, organ, and systemic levels are observed. On the one hand, cellular senescence seems to be an anticancer mechanism related to aging due to the combined effects of proliferation and environmental factors such as oxidative stress or DNA damage and telomere shortening [2]; on the other hand, there are various interactions among physiological systems which can favor the development and progression of cancers with aging where cellular senescence is also a contributor, together with hormonal changes [2]. One of the

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physiological systems involved is the immune system. After several years of debate, it is now clear that the immune system plays a major role in the control of the emergence of cancerous cells [3, 4]. With aging there are changes in the immune system collectively called immunosenescence which might adversely affect the anticancer activity [5, 6]. One of the most important characteristics of immunosenescence is the occurrence of "inflammaging" [7–9], indicating that aging is accompanied by a state of low-grade inflammation which can also contribute to the increase in cancer incidence, and, more effectively, combat the emergence of tumor cells. Experimental data implicating immune aging at various stages of cancer development are accumulating, but there remains much to discover. Here, we describe changes in innate and adaptive immune systems with age in relation to age-related increased cancer development.

19.2 Immune System and Cancer

It took some time to understand how the immune system may interact with the cancer at various stages of its development [10–12]. Currently, this synthesis of ideas developed over the decades following the original suggestion of immunosurveillance against tumors, known as "immunoediting" that describes all facets of the interaction between the immune system and cancer. Immunity plays an important role in the host defense against tumor development. Despite the fact that cancer originates from self cells and as such may be only weakly antigenic. This phase of the interaction is called the elimination stage or true immunosurveillance. At this level the immune system involves many different immune cells and is efficient at eliminating cancer cells. However, this action can result in the emergence of tumor variants and the establishment of a temporary equilibrium between the transformed cells and the efficient immune defense. At this stage, the cancer remains clinically insignificant. As the equilibrium shifts and the continuously growing genetically unstable malignant cells generate variants, the immune response can become inhibited or exhausted, and resistant cancer cells will survive and proliferate as explained by the deficit of the built-in tumor suppressor mechanisms such as cell senescence, DNA damage-induced apoptosis, etc. Eventually, the tumor escapes from immune surveillance and becomes clinically apparent. At this stage the tumor is orchestrating the behavior of the immune system by actively suppressing the immune response through the production of various inhibitory substances, such as NO, IDO, PGE2, and via other pathways. At the same time, immune suppressor cells including Tregs and MDSCs may become dominant, hence inhibiting the tumor-eliminating activity of the immune system. Thus, to eliminate the nascent tumor cells, organisms need a completely and fully functioning immune system. As we age there are several physiological alterations in the immune system ultimately contributing to the appearance of cancers with higher incidence in the elderly.

19.2.1 Immunosenescence or Immune Aging

It is currently well established that the immune response is profoundly altered with aging [13]. Most changes concern the adaptive immune system, but it is now accepted that the innate immune system is also affected [14–17]. Collectively, it is very difficult to establish whether the changes are only detrimental or are at least partly an adaptation to sustain decreasing immune responses by changing the threshold for immune activation. The presence of low-grade inflammation can be part of this adaptation process. This phenomenon can overcome the decreased immune reserve with aging. Nevertheless, as the immune response is implicated in cancer immunosurveillance, it can be hypothesized that even if the changes in the aging immune system may be adaptive in respect to the pathogenic environment, they can still contribute to the increased incidence of cancers [18–21]. The age-related changes in the innate and adaptive immune system in view of their implication in putative cancer development and progression will be discussed here.

19.2.2 Innate Immune System

The innate immune system plays an essential role in cancer immunosurveillance by directly eliminating the tumor cells and maintaining them in a quiescent state – but may also favor the development and progression of cancers in some ways. It should be stressed that interactions between the innate and adaptive immune system are recognized as essential for an efficient adaptive immune response. These functions are mediated by various innate cells including neutrophils, monocyte/macrophages, NK cells, and ILL. It is now recognized that most phenotypes and functions of the cells of the innate immune system are altered with aging, as briefly summarized in the following.

19.2.2.1 Neutrophils

Neutrophils are the most abundant innate immune cells. They are the first to arrive at the site of any aggression but are markedly altered with aging [17, 22]. It is interesting to note that not all their functions are changed with aging. Thus, the number of neutrophils and their capacity to adhere at inflammatory sites is not altered with aging [23, 24]. It is also of note that while most of the effector functions are increased with aging at the basal level, they cannot be further modulated [25–28]. The most important functions increased at quiescent state are the production of free radicals and the production of proteases [25, 26] which can be important for tumor fighting/development. Nonetheless, this can also contribute to the low-grade inflammation observed with aging, which can be detrimental. In contrast, an acute stimulation of neutrophils in the elderly reveals that they are unable to perform correctly by increasing chemotaxis, phagocytosis, and intra- and extracellular killing and to stay viable and active for a longer functional period [27]. These functions are mediated through the activation of specific receptors such as pattern recognition receptors (PRRs), Fcγ, and complement receptors. Another important function recently recognized for the elimination of foreign invaders is autophagy. Engagement of different Toll-like receptors (TLRs) such as TLR4 and TLR7 has been implicated in the activation of macroautophagy [29], which has been shown to be defective with aging [30–32] suggesting altered foreign antigen (Ag) processing. Recently, it has been shown that the inflammasome is a complex of molecules activated by specific PRRs (NLRs and AIM2) responding specifically to challenge via the activation of inflammatory caspases such as caspase-1 and caspase-5. This ultimately results in the production of a wide range of cytokines, particularly IL-1β [33], playing a role in inflammation. There are currently no data on how these inflammasomes are affected by aging. After the alterations observed in neutrophil functions, it can only be suggested that their assembly and function may be altered.

The causes of these dysregulated effector functions remain unknown, but changes in the inflammatory environment and in the signaling pathways may contribute. Neutrophils can also be stimulated via their pattern recognition receptors by Ags that may be present in higher amounts in the periphery of aged individuals, such as DNA degradation products, altered proteins, latent/chronic viral antigen, and/or tumor-derived Ags. Recently, one of the most important discoveries was of PRRs on the surface of many immune cells including neutrophils recognizing pathogen-associated molecular patterns (PAMPs) [34]. The ever-growing family of the PRRs now includes three main types: the TLRs, the retinoic acid-inducible gene 1 protein (RIG-1)-like helicases (RLRs), and the nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs) [35]. It is now recognized that they play an essential role in many cell functions, including neutrophil biology, allowing immune cells to discriminate between self and nonself and acting as danger-sensing receptors to alert the organism to the presence of microorganisms, transformed cells, or damaged cells.

There are currently 13 TLRs described with different recognition specificities and signaling pathways leading to well-characterized cellular responses [34]. Bacterial products are recognized by TLR2 and TLR4, while TLR3 and TLR7 recognize intracellular pathogens. Signaling is mediated either by the MyD88 pathway [36] or by the TRIF pathway [37, 38]. Activation of these TLRs results in the activation of NF-κB, a transcription factor furthering strong cytokine production [39].

Neutrophils from aged individuals display alterations in the signaling of these TLRs leading to their altered functionality [14, 27]. While the number of these receptors is not significantly changed with age, there is a significant alteration in the trafficking of signaling molecules in and out of lipid rafts. There is a need for further studies in order to truly appreciate the role of TLR in the altered functions of neutrophils with age [27].

Taken together, all available experimental evidence indicate that neutrophils participate in inflammaging but can no longer effectively counteract pathological challenges and as such may contribute to the inflammatory process becoming more chronic. Neutrophils also interact with other cells of the immune system, in addition to the adaptive arm such as B cells for antibody production and T cells for efficient effector functions [40, 41]. They also participate in the recruitment of monocyte/macrophages to the challenge site which take over their functions for a longer time period.

19.2.2.2 Monocyte/Macrophages

Monocyte/macrophages have been relatively poorly studied in human aging. However, currently available data indicate that there are phenotypic changes associated with altered effector functions in older individuals. Recent studies characterizing monocytes showed the existence of two distinct subpopulations: $CD14^{++}$(high) CD16- and $CD14^{+}$(low) $CD16^{+}$ [42]. These subpopulations are very distinct in their surface protein expression and their functions. The first $CD14^{++}$(high) $CD16^{-}$ subpopulation expresses CD62L, CD64, and CCR2 with low levels of CXCR1. The second $CD14^{+}$(low) $CD16^{+}$ lacks all these surface markers but expresses high levels of CX3CR1. These latter cells are considered to be mainly proinflammatory as they produce high levels of TNF-α in response to TLR2 and TLR4 ligation. By analyzing the four subpopulations of human monocytes, it was found the $CD14^{+}$ (low) $CD16^{+}$ and the $CD14^{++}$(high) $CD16^{+}$ populations were increased with aging, whereas the proportion and number of $CD14^{+}$ (low) CD16- were decreased compared to the young [43].

The few existing data suggest that monocyte/macrophages from aged individuals display age-related dysfunction [44–46]. These alterations include a decrease of cell surface TLR expression (TLR1 and TLR4), although this finding is controversial [31, 47, 48]. Other receptors also show an altered expression, such as the expression of the important T-cell CD80/CD86 co-stimulatory receptors which is decreased on monocytes upon TLR stimulation [49]. *In vitro* studies in humans demonstrated a higher proinflammatory cytokine profile, especially for IL-6 and IL-8 production by resting monocytes [9], despite the finding that cytokine production following stimulation with LPS is reduced. Consistent with this, another recent study found that the four monocyte subsets had lower IL-6 production upon TLR1/TLR2 stimulation, confirming earlier studies on TLR stimulation [50, 51], which indicates that monocytes are not a homogeneous population and react differently depending on the nature of the stimuli.

Many years ago, it was shown that several surface receptors such as Fcγ and FMLP had altered signal transduction upon appropriate stimulation, resulting in altered function [25, 26]. Recent data further suggest that in addition to the decrease in some TLR expression, the TLR signaling pathways show age-related alterations [27] linked to altered chemotaxis as evident by the reduced number of infiltrating macrophages in wounds of elderly humans. Alteration in the MAPK signaling pathways including p38 MAPK and ERK1/2 MAPKs has been reported in human monocytes with aging.

Macrophages from elderly people produce more prostaglandin E2, which suppresses T-cell activation via decreased IL-12 production [52]. Furthermore, it was demonstrated that phagocytosis, free radical production, and chemotaxis were reduced in monocytes/macrophages from healthy aged subjects [53]. No data seem to exist regarding age-related changes in the clearance of apoptotic cells, known as an important macrophage function. We can only speculate that considering the functional changes described above and the "inflammaging," the clearance of apoptotic cells may be impaired with aging. Decrease in some receptors, as well as altered signaling leading to changes in chemotaxis and phagocytosis,

supports the hypothesis that apoptotic cells are not cleared efficiently. This could lead to their persistence in becoming proinflammatory and sustaining the quiescent state stimulation of monocyte/macrophages, finally contributing to the process of "inflammaging." Furthermore, these data confirm that, like neutrophils, monocytes are to some degree activated at the basal state, but cannot be further stimulated through their surface receptors. This baseline activation state may be very important to maintain their functions for combating/constraining constant and chronic challenges but insufficient for eliminating new infections. Therefore, it seems that neutrophils and monocytes are probably both contributing to the low-grade inflammation with aging which not only impairs the immune environment but also creates a vicious circle which maintains their functioning at an adequate level whereas impairing their contribution to combating new invaders, including tumor cells. Taken together, all the experimental data available suggest that with aging, most monocyte/macrophage functions are changed with age, leading to altered tumor cell and pathogen clearing and altered regulation of the adaptive immune response and the inflammatory process resulting in chronic low-grade inflammation and ultimately to increased age-related diseases such as infections, cardiovascular disease, and cancers.

19.2.2.3 Dendritic Cells

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) that can prime specific T cells. There are several types of DCs [54]: Plasmacytoid dendritic cells (pDCs) are important in host defense as they are one of the first cells to produce type I interferon, hence initiating several other responses, including NK cell activation which amplifies host response [55–57]. The second type of DC is the conventional or myeloid-derived dendritic cell (mDC), regarded as the most important APC for T-cell activation. They express TLRs and C-type lectins for the detection of Ags and subsequently produce IL-12, IL-15, and IL-18. IL-12 is essential for induction of Th1 cell responses which will induce cytotoxic T lymphocyte responses to clear virus-infected cells [39]. They can also activate NK cells, which directly eliminate tumor cells. In addition to presenting Ag, they also provide co-stimulatory signals and cytokines for optimal T cell priming, differentiation, and proliferation [58]. Whether the numbers of DCs change during aging is still controversial.

There are several studies demonstrating alterations in pDC function in aged humans including reduced type I interferon production following TLR stimulation, e.g., via TLR7 and TLR9. It has been suggested that the increased basal oxidative stress related to aging could be the underlying cause of the decreased upregulation of the interferon regulatory factors by TLRs [59, 60]. In contrast, mDCs from aged humans showed increased expression of CD86 signaling, another sign of activation even in the "quiescent" state. However, these findings have not been corroborated by *in vitro* studies. Nonetheless, they do seem to retain the capacity to produce proinflammatory cytokines and to activate $CD8^+$ T cells [61], as well as to induce IL-17 production, which is known to recruit neutrophils [62]. DCs have also been reported to have a decreased ability in naïve $CD4^+$ T cell activation via Ag presentation [63, 64], attributed to decreased PI3K activity, a major pathway mediating cell function. Reduced PI3K was implicated in both age-related reduced DC migration and also as a negative regulator of TLR signaling. Thus, the global result of this decreased PI3K activation is a higher stimulation of the NF-kB pathway further contributing to "inflammaging" due to greater production of proinflammatory cytokines such as IL-6 and TNF-α in the basal state [63]. DCs have reduced Ag processing capacity concomitant with the altered expression and function of their co-stimulatory molecules.

Natural killer (NK) cells are one of the most important antitumor players in the innate immune system [10]. The NK cell population is now also divided into different subpopulations; those with a CD16-$CD56^+$ or $CD16^+CD56^{++}$ phenotype produce high amounts of IFN-γ and are among the most cytotoxic subtypes [65]. Subset distribution changes with aging, and the number of $CD56^{dim}$ NK cells increases, while $CD56^{bright}$ cells decrease [66, 67]. Furthermore, the expression of CD57 is

increased on $CD56^{dim}$ NK cells from elderly subjects, representing a highly differentiated subset of NK cells. These observations were recently extended by the finding that CD94 (member of the C-type lectin family) and KLRG1 expression on NK cells was significantly decreased in elderly subjects. Although the exact consequence of this decrease is not known, it was hypothesized that the decreased expression of these surface markers induces unregulated cell lysis contributing to chronic inflammatory conditions. Moreover, the same study revealed the presence of a greater proportion of IFN-γ-positive $CD3^{-}CD56^{bright}$ NK cells with aging. This may suggest a shift to a more cytotoxic, cytokine-producing and potentially immunomodulatory NK cell phenotype occurring as a mechanism to compensate for the decreased proportion of $CD56^{bright}$ NK cells. Aging also influences the dynamics of NK cells [65]. NK cells from the elderly have a significantly decreased proliferation and production rate, and there is an increased proportion of long-lived NK cells which can be related to the increased proportion of $CD56^{dim}$ NK cells. The increased expression of CD57 may also suggest that the NK cells of elderly people are late-stage or terminally differentiated, like many of their $CD8^{+}$ T cells [68]. Taken together, the data indicate that although the number of NK cells often increases with age, there is a profound redistribution of NK cell subsets with altered receptor expression, explaining the functional alterations leading either to decreased direct defense against virus-infected and tumor cells and/or decreased regulatory activity for other components of the innate immune response, ultimately resulting in decreased modulation of the adaptive immune response. Recently, it has been shown that NK cell activity is also under the control of IL15Rα/IL15, released by nonimmune cells such as muscle cells, which, by its decrease with aging can also contribute to these NK cell functional alterations [69].

Studies in very healthy elderly populations revealed that the total NK cell number tends to increase with age, while their cytotoxicity is not significantly affected [70]. However, other studies in unselected elderly populations revealed that decreased NK cell functions with aging were associated with a higher incidence of infectious diseases [71]. IL-2-induced NK cell proliferation is decreased with aging and many cytokines and chemokines produced by NK cells, such as IL-2, IL-8, are also decreased but with maintenance of IFN-γ production [72]. This decreased production of cytokines contributes to the altered activation of macrophages with aging, resulting in decreased microbicidal and tumoricidal activities. Thus, NK cells of elderly people show decreased proliferative responses to cytokines; higher total cytotoxic capacity when stimulated with certain cytokines including IL-2, IL-12, or IFN-γ; and a greater sensitivity to stimulation via CD16. The cytotoxic activity of NK cells depends on whether the whole NK cell population or activity per cell is considered. On a per cell basis it is decreased, which might be important for protection against developing cancer cells.

Furthermore, other receptors involved in the cytotoxic activity of NK cells including members of the natural cytotoxicity receptor family, namely, NKp30 and NKp46, decrease with aging [73]. NKp30 has also been shown to be important in the regulation of the cross-talk between NK cells and DCs. By this interaction the NK cells can activate the DCs to more efficiently prime T cells. DCs release Th1 cytokines which further enhance NK activation. Thus, NK cells can modulate the adaptive immune response against virus-infected or tumor cells via this interaction with DCs.

NKT cells are innate T lymphocyte population that recognize lipid Ags presented in the context of the CD1d molecule found on monocytes, macrophages, and DCs [74]. They can increase the functions of NK cells. NKT cells are rapidly recruited from the circulation during acute inflammation and interact with various APCs expressing the CD1d molecule. Recently, it has been shown that NKT cells are able to recruit neutrophils and activate them via their IFN-γ secretion [75]. Thus, NKT cells may play an important regulatory role in the acute phase of a microbial and/or tumor cell challenge by interacting with various APCs via CD1d lipid antigenic presentation and secretion of different

cytokines. There are only a few reports on NKT cell functioning in the elderly [72]. However, it can be hypothesized that the altered activation of APCs via their TLR receptors will create an unfavorable milieu for NKT activation either directly or by their cytokine secretion.

IL-17 is mainly secreted by γδ T cells, Th-17, and NKT cells [76]. This cytokine acts indirectly on neutrophil survival through stimulation of the secretion of G-CSF. IL-17 is also released by neutrophils themselves and reinforces their survival and recruitment [77]. It can also promote tumor vascularization by angiogenic factors. These immune cells as well as IL-17 itself may have pro- and antitumor activities; currently it is not known what determines this dual effect on cancer. However, their differentiation in various subtypes, expression of specific receptors, and production of various cytokines is likely to be determined by and in turn influence the tumor microenvironment [75]. How aging affects γδ T cells has not been well investigated to date.

19.2.3 Adaptive Immune System

Although there are changes in the innate immune response with aging as described above, it is still thought that the most important and relevant changes occur in the adaptive immune response. Among the cells composing the adaptive immune response, the T cells are thought to be the most affected; in addition, more and more data are emerging showing that B cells are also changed with aging. Nonetheless, it is well recognized that some of the most marked immune alterations associated with aging concern T lymphocyte subpopulations and functions [13]. The most recognized model for T-cell subpopulations identifies naïve ($CD45RA^+$ $CCR7^+$), central memory T_{CM} ($CD45RA^-$ $CCR7^+$), effector memory T_{EM} ($CD45RA^-$ $CCR7^-$), and T_{EMRA} ($CD45RA^+$ $CCR7^-$) cells. Among these subpopulations, the highly differentiated populations of EM (effector memory: *CCR7-, CD28-, CD27-, CD45RA-*) and EMRA-like CD4 and CD8 T cells (T effector memory cells re-expressing CD45RA) have been shown to accumulate in older humans [13]. Currently, the suggested reason for this accumulation is a chronic antigenic stimulation especially caused by chronic viral infections (predominantly CMV); however, other chronic inflammatory stimulations related to specific diseases may also contribute (including diabetes mellitus type 2, atherosclerosis, and possibly Alzheimer disease) [78–81]. Interestingly, there are some reports showing that these cells also accumulate in cancer, such as at the early stage of breast cancer [82] and in renal carcinoma [83]. Furthermore, they also express the characteristic inhibitory surface receptors of exhausted and/or senescent cells like KLRG1, CD57, PD-1, and CTLA-4, as well as having reduced replicative capacity and decreased survival after TCR activation [84]. The role of these cells in cancer development is still questionable. Whether they are metabolically inert as senescent cells with short telomeres and decreased telomerase activity or they are metabolically active and able to secrete various proinflammatory cytokines and contribute to cancer development is a matter which is yet to be elucidated. The cause of this exhaustion is not known with certainty, but could either be due to direct antigenic stimulation by viral Ags such as CMV or they could be innocent bystanders affected by the chronic low-grade inflammatory environment induced by such chronic antigenic stimulation caused by constant basal proinflammatory cytokines such as TNF-α produced by the innate immune system [85]. It was shown that p38 has a role in cell activation, proliferation, and cell cycle progression [86, 87]. TNF-α can further activate p38, thus contributing to immunosenescence [85]. Interestingly, p38 is constitutively phosphorylated in EM and EMRA T cells, contributing to their reduced telomerase activity. Thus, the proinflammatory environment causing hyperphosphorylation of signaling molecules, such as p38, may influence the development of T-cell subpopulations as found in aging and inflammatory diseases. Together, these changes may be well tumorigenic by altering adequate tumor-specific immune response; they may be good targets for therapeutic modulation, as recently demonstrated so encouragingly for PD-1/PDL-1 [88–90]. Considering these changes, it is reasonable to assume that an alteration in T lymphocyte activation is a central

issue in the age-related modifications of the immune response. Currently, the most important paradigm underlying these changes is the repetitive antigenic stimulation over the life span that could lead to partial unresponsiveness (immune exhaustion) and accumulation of memory cells. This has been shown for both $CD4^+$ and $CD8^+$ T cells with distinct senescent status, surface molecule expression, telomere length, and functionality. This was further supported by a longitudinal study, the OCTA/NONA study, resulting in the development of the Immune Risk Profile integrating several of these parameters [91–94]. It is of note that as appealing as the CMV paradigm may appear, it is not yet proven [95–97]. It is likely that other factors could also contribute to causing the changes in the T cell compartment of the immune system with aging including the slight but detectable amounts of the proinflammatory cytokines concomitant with increased reactive oxygen species found in this basal proinflammatory state. Moreover, the intracellular T cell redox environment influences T cell function in aging [98, 99] which will be discussed later. Concomitant with these phenotypic changes, the functions of T cells are also altered, and there is increasing evidence to implicate altered activation in the decreased T cell functions with increasing age.

Studies of elderly humans and animals have revealed that one function of T cells most noticeably altered is the production of interleukin-2 (IL-2) compared to younger counterparts [100]. It can be hypothesized that defects or alterations in the proximal events during T cell activation will strongly affect the efficiency of immune responses [100]. Thus, appropriate signal transduction cascades trigger an appropriate T-cell response, whereas alterations in the early events of T cell signaling will result in less effective, altered overall responses [101–104]. The most important changes occur in $CD4^+$ T cells resulting in decreased production of IL-2 and clonal expansion. Although there are no changes in TCR number at the cell surface, the number of CD28 co-stimulatory molecules decreases with aging, especially on $CD8^+$ T cells. One of the most important driving forces to decrease surface CD28 expression is TNF-α. This cytokine can also activate p38 which plays an essential role in fibroblast senescence [85]. Nearly all of the signaling pathways associated with TCR activation or IL-2 receptor responses are found to be altered with aging [105, 106]. There is an alteration in the early steps of T-cell activation including protein tyrosine phosphorylation, calcium mobilization, and the translocation of PKC to the plasma membrane. In addition, subsequent steps of the signaling pathways including the Raf-Ras-MAP kinase pathway are impaired. Decline in proximal and intermediate events of transmembrane signaling leads to the decreased activity of transcription factors, especially NF-kB and NF-AT. Not only activation signaling but also the negative regulatory network is altered with aging [106]. This altered signaling followed by decreased activation may be caused by a differential inflammatory state and subsequent T cell phenotypic and functional change.

There are also age-related changes in the B cell compartment [107–111]. Production of B cells is altered with aging at different levels, resulting in decreased naive B cells. In addition, an age-dependent loss of diversity of B cell receptors is also observed which has been correlated to poor health and may reflect expanded clones of memory B cells. These changes may also lead to a shift in antibody specificity and the increase of autoantibodies. These alterations in the B cell compartment may also favor the emergence of cancers related to aging.

Taken together, aging is associated with an exhaustion of the adaptive immune response, especially by rendering T cells dysfunctional and unable to appropriately respond to receptor ligation. This, together with B cell alterations, contributes to the establishment of a chronic inflammatory state, leading to higher susceptibility to diseases such as cancer and increased mortality predicted by the Immune Risk Profile [91].

19.2.4 Interaction Between Innate and Adaptive Immune Responses: Effect of Aging

It is evident that if any component of the immune response is not functioning, the outcome cannot be optimal. Thus, the first line of defense of the

organism, the innate immune response, is not only a powerful eradicator of foreign invaders but is also responsible for the activation of the adaptive immune system for long-lasting and highly specific immunity by Ag specific, clonally expanded B and T lymphocytes. The reduced functioning of both monocytes/macrophages and DCs with aging will lead to reduced Ag presentation and activation of T cell immune responses by these APCs. In addition, neutrophils secrete many molecules such as HMG-B1 and alarmins which can directly induce DC maturation or the activation of both the innate and the adaptive immune response. It is possible that the reduced neutrophil function with aging will also affect this aspect of their role in immune response.

A very efficient network exists among the different cells participating in the innate immune response aiming to eradicate invaders, restore homeostasis by resolving acute inflammation, and ultimately to efficiently activate the adaptive immune response [16]. The individual functioning of the innate immune cells was shown to be dysregulated with aging either because of receptor-driven signaling pathway alterations or because of an age-related proinflammatory milieu sustained by cytokines and oxidative stress [22]. These alterations will induce a disruption in their functioning and in their mutually supporting network resulting in inefficient eradication of the challenge, contribution in chronic antigenic stimulation, and a chronic low-grade inflammation. On the other hand, they ultimately lead to the altered and inadequate activation of the adaptive immune response.

One of the important central players of the cooperation of the innate and adaptive immune response is TNF-α. This factor is at center stage of the cytokines secreted by various cells of the innate immune system, such as monocytes stimulated by many external or internal agents leading to modulation of the T-cell response either to enhance it or dampen it via downregulation of CD28 or exhaustion of T cells [112]. TNF-α production is increased in oxidative stress, chronic antigenic stimulation, CMV infection, and visceral adiposity [113–115]. Thus, the regulation and control of this vital molecule to maintain it under a beneficial threshold may be the key to aging and age-related pathologies such as cancer.

Alterations in the T-cell compartment can also trigger changes in the innate immune system because the accumulation of memory and terminally differentiated/exhausted T cells secreting more proinflammatory cytokines and chemokines will chronically stimulate and attract the innate immune cells. The increased susceptibility to apoptosis of certain T-cell subsets like $CD4^+$ naive T cells may also chronically contribute to the stimulation of innate cells.

All these data demonstrate that with aging, alterations in both arms of the immune system, as well as in their efficient cooperation, contribute to altered protection against different challenges and participate in the development and maintenance of age-related low-grade inflammation and increased susceptibility to diseases such as cancer [9]. The same interaction between the innate and adaptive immune response may either favor the eradication or the progression of cancers depending on their state of activation, the phenotype repartition, and the microenvironment.

19.3 Inflammation Aging and Oxidative Stress

The relationship between chronic low-grade inflammation related to immunosenescence and age-associated diseases, such as cancer, remains to be elucidated. It is of note that alterations of certain proinflammatory (IL-6, TNF, IL-1) as well as anti-inflammatory cytokines (IL-10, IL-4) are observed at greater frequencies in age-associated diseases compared to healthy aging [9]. Thus, age-related immune dysregulation manifested essentially by a basic chronic low-grade inflammation and a suppression of the adaptive response may eventually lead to the development of clinically significant pathological conditions including cardiovascular disease, dementia, diabetes mellitus, osteoporosis, and cancer [8]. Age-related low-grade inflammatory process seems to accelerate the progression of chronic diseases, as well as having an immunosuppressive effect on cellular immune responses by contributing to their exhaustion. The question arises as to

whether this proinflammatory activity is the *primum movens* for disease development or just a secondary reaction following latent chronic inflammatory diseases. Moreover, this low-grade inflammation may also represent an adaptive mechanism to maintain an acceptable level of response against cells including nascent tumor cells. However, when increasing over a certain level, it could become predominantly detrimental by favoring their proliferation and the clinical appearance of cancer.

What are the molecular events underlying inflammaging? It seems that NF-kB is at the center stage of metabolic pathways, as it controls the secretion of proinflammatory molecules, such as cytokines, chemokines, MMPs, COX2, and iNOS [116, 117]. NF-kB is also activated by many of these molecules via various pathways such as the MAPK and the IP3/Akt pathway. As might be expected from knowledge of the pathways leading to their development, NF-kB activity is highest in $CD8^+$ TEMRA cells [118]. Moreover, the FOXO family of transcription factors plays a role in longevity, cell survival, and proliferation via the modulation of NF-kB by free radical production [119]. Thus, NF-kB modulating pathways are heavily implicated in the occurrence, as well as in the perpetuation of this low-grade inflammation.

Thus, what is the relation between inflammaging and free radicals which have been shown to increase with aging as a result of increased oxidative stress [120]? The degree of oxidative stress is the result of the disequilibrium between the production of ROS and endogenous antioxidant species. Free radicals are produced as by-products of aerobic respiration [121]. They are beneficial for signaling, enzyme activation, and microbial elimination, but over a certain threshold, they may become detrimental by causing mutations in DNA and oxidation of macromolecules [122]. The role of free radicals became the basis of one of the leading theories of aging and consequently has been related to many age-associated diseases including cancer [123, 124]. In this context, it has been known for many years that age-related increased ROS production due to mitochondrial dysfunction may cause DNA damage and favor cancer development [125]. Recently, it was recognized that local inflammatory processes such as in the intestine and stomach may lead to the development of cancers. However, the relationship between oxidative stress and "inflammaging" is less well established. When innate immune cells are chronically activated, they continuously release free radicals which can contribute to tumorigenesis directly as well as via the alterations they cause to the adaptive immune system, as already mentioned [126]. It is of note that free radicals can create a vicious circle by maintaining (through TLRs and inflammasome activation) the production of free radicals by other innate immune cells such as neutrophils, DCs, and monocyte/macrophages which in turn reactivate them. Thus, free radicals directly and indirectly via oxidatively modified proteins or lipids activate NF-kB leading to proinflammatory cytokine production. Similarly these free radicals and lipid peroxides also activate the Nalp3 inflammasome. These events lead to low level of activation of innate cells at the basal level and participate in its maintenance.

Oxidatively modified proteins are also continuously produced as a result of the low-grade inflammation [127, 128], accumulating in immune cells, especially in T cells, which interfere with their functioning. Many proteins including TCR, CD45, and enzymes are targeted by free radicals and become carbonylated or glycoxydated. This accumulation is further enhanced by decreased proteasome activity to eliminate these altered proteins [129, 130]. Thus, the free radicals create an altered cellular environment favoring the activation of innate cells and decreased functioning of adaptive immune cells.

Furthermore, these free radicals will affect the surrounding cells in infiltrating tissues by inducing cell proliferation, evasion of apoptosis, tissue invasion, angiogenesis, autophagy, and alterations in macromolecule functions either by gain of functions or by loss of functions. All these activities may contribute to some extent of tumorigenesis. Free radicals mediate these functions by stimulating different molecular pathways including the Ras, MAPK, PI3K, mTOR, and NF-kB pathways. Consequently, ROS also alter

Nrf2 activity which is considered to be the master regulator of the antioxidant response [126]. Nrf2 modulates a large number of genes that control several processes including immune and inflammatory responses [131]. We have shown that with T cell aging, the Nrf2 is altered [22], which is also hypothesized in innate immune cells, and further contributes to the inflammatory process and consequently to carcinogenesis. Thus, the immunosenescence associated inflammaging contributes to cancer development by many pathways, especially by t increased basal free radical production, which in turn further activates these cells by propagating inflammatory signal by free radicals.

19.4 Immunosenescence and Cancer

A causative connection between inflammation and some cancers is well established [132]. Inflammation in its uncontrolled state highly favors tumorigenesis by increasing genomic instability via the production of free radicals, persistence of proinflammatory cytokines and chemokines and the subversion of Treg, γδT cell, and MDSC functions, as well as through angiogenesis [133]. The apparent disequilibrium between the retention of a reactive innate immune response at basal state and the more severely altered adaptive immune response with aging leads to the presence of the low-grade inflammatory status commonly present in the elderly, termed as "inflammaging" as discussed above. Although the cause of this increased basal inflammatory state is certainly multifactorial, it is likely that one of the most important causes is chronic antigenic stimulation concomitant with increased free radical production related to oxidative stress. The Ag source can be exogenous, as with persistent viral infections such as CMV [95] and subclinical bacterial infections, or endogenous like the various posttranslationally modified macromolecules such as DNA or proteins which can be modified by oxidation, acylation, or glycosylation. Such altered molecules can stimulate the innate immune response, particularly macrophages via TLRs, thus contributing to a sustained proinflammatory state which is measurable in some circumstances via increased circulating levels of IL-6, IL-1β, or TNF-α. Thus, aging is accompanied by a chronic low-grade inflammatory process and by many other changes, some related to inflammaging, some independent thereof. Hence, this may be the price that has to be paid for maintaining immunosurveillance against persistent pathogens or endogenous stressors such as cancer cells. All these changes contribute to a decreasingly effective immune environment, probably unable to appropriately respond either to new Ags such as represented by the continuous risk of exposure to new pathogens, or to chronic persisting Ags such as those from CMV or tumor cells during the life span. Therefore, inflammaging related to immunosenescence is likely to be one of the most important general driving forces for cancer development. It is of note that every individual alteration at all cellular and molecular levels also contributes to increased tumorigenesis. The most important elements for immunosenescence are the decreased neutrophil, macrophage, and DC functions but maintaining uncontrolled proinflammatory cytokine production, as well as the decreased specific adaptive immune response by T cells to tumor Ags. TNF-α seems to play a particularly important role as it is secreted mainly by immune cells, in contrast to IL-6. It is the consequence of and the support for inflammaging via NF-kB and AP-1 signaling.

Furthermore, an important aspect of the inflammatory response is the production of free radicals which leads to the activation of various signaling cascades resulting in effector functions and apoptosis as well as in the further production of proinflammatory cytokines. They also increase the possibility of genomic instability and epigenetic deregulation leading to enhanced mutations [134]. These proinflammatory cytokines secreted by the cells of the innate immune system are also able to induce the production of free radicals. Thus, the deregulation of innate immune responses strongly contributes to age-related chronic inflammatory processes and associated pathologies, as well as a functionally neutral consequence of the aging

process. As a result, its modulation could be beneficial in the treatment of these diseases.

Moreover, the deregulated immune response with aging also produces directly pro-tumor molecules as well as inducing the accumulation of immunosuppressive immune cells either systemically or in the tumor microenvironment. Data suggest that pro-tumor molecules such as NO, indoleamine-2,3-dioxygenase (IDO), TGFβ, IL-10, VEGF, PD-1 are increasing with age, as well as MDSCs ($CD11b^+$, $CD33^+$, $CD34^+$, $CD14^-$ $HLADR^-$) under the high proinflammatory cytokine micro- and macroenvironment, and Tregs which suppress the antitumor activities of T cells, NK, and NKT cells [18, 21]. These changes completely alter tumor-immune interactions necessary for cancer eradication or at least for the maintenance of the equilibrium stage.

Finally, altered immune network functioning also favors tumorigenesis. The altered presentation of antigens by DC and macrophages decreases the activation of T cells, the functions of which are further altered by oxidative stress and proinflammatory cytokines produced by innate immune cells. In contrast, the altered T cell phenotype and functions are further increasing the innate cell functions. Thus, a vicious circle is created leading to the appearance of tumor cells.

19.5 Modulation

Due to our increased understanding of tumor-immune interactions now, the patient's immune system, even in nonimmunological treatments, like radiotherapy, should be taken into consideration [12, 135], in order to achieve long-term tumor control or complete tumor elimination. Thus, the patient's immune system needs to become integral to cancer therapy. It is also clear that immunotherapies are mostly used in late-stage cancers when the immune system is already subverted. Thus, immunotherapy should be initiated when the immune system is still able to react.

Dendritic cells (DCs) possess the specialized potential to present exogenously derived antigen to cytotoxic T lymphocytes in order to elicit an immune response. This process, termed cross-presentation, is crucial for the generation of immune response to viruses and tumors and in autoimmune disease. The ability of DCs to cross-present exogenous Ag to CTLs makes them an attractive target for exploitation in immunotherapy. In recent years, significant advances have been made in understanding the mechanism of cross-presentation and the DC subsets involved. The recent discovery of human cross-presenting DC has given this field a new lease of life relative to cancer immunotherapy [136]. Such an example is the injection of monoclonal antibodies (mAbs) which not only directly eliminate tumor cells but also result in the release of new tumor antigens by killing tumor cells. These can then participate in cross-presentation to T and B cells, thus amplifying the primary treatment [137].

Modern immunotherapy clearly needs to consider many aspects of tumor biology and associated immune reactions. The heterogeneity of tumors and their microenvironment combined with the diversity of immune cells/molecules will need complex approaches to immunotherapy. The new paradigm is to use autologous tumor cells for vaccine and/or in combination with personalized peptide vaccination which would lead to eradication of tumors or at least to the retardation of their development and metastasis formation [21]. In an aging/geriatric environment, certain characteristics specific to elderly subjects, such as functional status and comorbidities, should definitely be further considered.

19.6 Concluding Remarks

There is no doubt that aging is the main risk factor for the development of many diseases including cancers, type 2 diabetes, and cardiovascular and neurodegenerative diseases. Understanding the mechanisms regulating aging is the most important for the comprehension of the occurrence of these different diseases. The low-grade

inflammation seen with aging can be a common factor linking aging to these diseases; thus, it is strictly deleterious. However, from a different perspective considering inflammaging as a consequence of immunosenescence, it may be essential to survival probably ensuring that elderly can probably react to challenge much more easily and rapidly than they would be able to with an immune response similar to young people. In fact this could be an evolutionary adaptation to maintain a response without losing control if the immune system would have a youngish function in an aging milieu. There are several examples, such as the increase in the number of NK cells and CMV-specific late differentiated $CD8^+$ T cells, as well as their IFN-γ secretion [70, 95, 96]. Therefore, understanding the interaction between this low-grade inflammation and its shifting toward pathogenic pathways, either in cells or their microenvironment, can provide the key to unveiling why aging is the most important risk factor for these diseases. It is also evident that a unifying picture starts to emerge implicating genomic instability, metabolism, and immunity in the development of cancer and other inflammation-related diseases. However, the challenge is to discover why differentiation toward individual diseases occurs under the presence of the same elements. Notably, the occurrence of each disease predisposes to other conditions as well; the best example would be diabetes, recognized to be a very high risk for the development of cancer or rheumatoid arthritis, as well as cardiovascular diseases. The problem is to intervene in the aging process to maintain its reactivity toward different challenges and at the same time decrease the risk for the development of disease. Thus, understanding the real mechanism underlying aging may lead to delay in the onset of these pathologies, ultimately extending the healthiest life span possible with aging.

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Nutrition, Immunity, and Cancers

20

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20.1 Introduction

Changes in immunologic pathways have a leading role in all stages of cancer. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukins 1 and 6 (IL-1 and IL-6) are important mediators of cancer complications such as cachexia [1]. A tumor can trigger the release of cytokines such as IL-6 [2], which is associated with an increase in lipolysis and proteolysis, which in turn affect the appetite

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and host neuroendocrine axis and induce anorexia and cachexia [2, 3]. Several neuropeptides such as neuropeptide Y (NPY) and adipokines such as leptin have been implicated in the pathogenesis of cancer cachexia syndrome [3, 4]. NPY receptors appear to be resistant to NPY, and production of NPY appears to be decreased in cancer cachexia. This hypoleptinemia may play a role in increased insulin resistance seen in cancer patients [5, 6]. Nuclear factor-kappa B (NF-κB) plays an important role in cancer development and may be influenced by proinflammatory chemokines to activate inflammatory response genes and regulate cell proliferation and apoptosis [7]. NF-κB activation also promotes the cyclooxygenase-2 (COX2) cascade, leading to increased oxygen free radical synthesis and cell damage [8, 9]. Thus, an imbalance of cytokine production and neuropeptide and adipokine dysfunction may be a major cause of the nutritional consequences of cancer.

20.2 Role of Nutrition in Predisposition of Cancer from an Immunologic View

One of the known risk factors for cancer is obesity, especially with the modern lifestyle and low physical activity [1]. Dietary patterns have a significant effect on the cytokine profile; for instance, the high intake of saturated fats, especially in obese people, leads to infiltration of adipose tissue by macrophages producing IL-1b, IL-6, and macrophage inhibitory factor (MIF) [2–4]. Moreover, a decrease in the secretion of anti-inflammatory adipokines such as adiponectin may maintain proinflammatory signals and activate the production of C-reactive protein (CRP) by the liver [5, 6]. Based on previous studies, this chronic inflammatory process is related to an increased susceptibility to various types of cancer, including cancers of the gastrointestinal, respiratory, and genitourinary systems [7, 10].

Influenced by this important effect of nutrition on the immune system, characteristics of the human diet can directly stimulate gastrointestinal malignancies [11]. A diet low in fiber and vegetables may affect the regulation of carbohydrate absorption and short chain fatty acid formation, which affects the metabolism of carcinogens [12]. This process is linked to colon cancer and its progression [13]; apparently, a decrease in fiber intake may allow more time for exposure of colon cells and the immune system to the potential carcinogens, affecting intestinal transit [14]. Moreover, based on the evidence used to draw conclusions about a gluten-free diet in patients with celiac disease leading to cancer protection, it seems reasonable to consider gluten as a booster for cancer in celiac patients [15].

Meat consumption is a risk factor for some cancers, especially colon, rectum, and prostate. Red meat consumption increases the risk of colon cancer by causing increased production of heterocyclic amines [16, 17].

On the other hand, a change in the normal diet and deficiency of vitamins or minerals may affect the adequacy of either innate immunity (phagocytic activity, chemotaxis of neutrophils, or release of cytokines from monocytes) or adaptive immunity (immunoglobulin production of B cells or cell-mediated immunity) [18, 19]. Many of the consequences of malnutrition in the regulation of signal transduction and immunoregulatory gene expression were first recognized in the early 1800s as nutrigenomics [20, 21]. The majority of these changes are reversible after administration of adequate nutrition supplements [22–24].

The following list of specific dietary factors has been studied in relation to the immune aspects of cancer.

20.2.1 Protein-Calorie Balance

The formation of lymphocytes, eosinophils, and vital immune system proteins such as thymic hormones, antibody (Ab) responses to T-cell-dependent antigens (Ags), and Ab affinity are affected by protein-calorie imbalance [25]. It has long been recognized that caloric restriction with a well-balanced diet avoiding certain nutrient deficiencies can increase longevity and has cancer preventive effects in mammals [26].

20.2.2 Essential Fatty Acids

Essential fatty acids in our body can regulate the production of prostaglandins, prostacyclins, thromboxanes, and leukotrienes, causing a significant effect on the host immune system and regulation of inflammation [27].

20.2.3 Antioxidants (Selenium, Vitamin E, and Vitamin C)

These nutrients have strong antioxidative effects and may reduce the risk of cancer by neutralizing reactive oxygen species or free radicals that can damage DNA [28–30].

20.2.3.1 Vitamin A

Vitamin A plays an important role in protection against measles, white blood cell (WBC) function, resistance to carcinogens, and skin and mucous membrane defenses. Vitamin A precursor carotenoids, such as lycopene, have a potential effect on cancer prevention [31, 32].

20.2.4 Vitamin D

Vitamin D has been of interest based on ecologic studies on populations with greater exposure to ultraviolet light who had a lower risk of breast cancer [33], colon cancer [34], and prostate cancer [35]. This vitamin regulates humoral Ab response and supports a Th2-mediated anti-inflammatory profile of cytokines; therefore, its anticancer properties are strongly suggested [36].

20.2.5 Vitamin B6

Pyridoxine induces WBC responses, Th1 cytokine-mediated immune responses, and shrinkage of the thymus [36]. Epidemiologic studies and laboratory animal models have shown that vitamin B6 modulates the risk of cancer. It is not clear how vitamin B6 mediates this effect, but it has been reported that high dietary vitamin B6 attenuates and low dietary vitamin B6 increases the risk of cancer [37].

20.2.6 Folate

Folate is important for DNA methylation, repair, and synthesis [38–41]. Epidemiologic studies have shown that low folic acid intake is associated with a higher risk of various cancers, most notably colon [42], breast [43], and probably cervical cancer [43]. The fact that methylenetetrahydrofolate reductase, an enzyme predicted to reduce the risk of colon cancer, is associated with folate status supports the role of folate in cancers [42, 44].

20.2.7 Calcium

Many studies show that calcium may reduce the risk of colorectal cancer via direct and indirect effects. Calcium has a direct effect on proliferation, stimulating differentiation, and apoptosis in the colonic mucosa [45, 46]. Its indirect effect is binding to toxic secondary bile acids and ionized fatty acids to form insoluble soaps in the lumen of the colon [47, 48].

In addition to deficiency, an overdose of some micronutrients can also have an immunosuppressive effect, especially megadoses of vitamin E [49]. High doses of certain minerals such as chromium, copper, iron, manganese, and zinc also may induce cancer and immune dysfunction [50–52].

In summary, attenuated innate and adaptive immunity as a result of an inadequate diet leads to a higher risk of cancer and lower homeostasis for cancerous antigens, thus reducing nutrient intake, increasing losses, and interfering with utilization due to altering metabolic pathways. Thus, nutrition may have a significant role in prevention and treatment of cancer [40].

20.3 Aging as a Confounder of the Triangle of Nutrition, Immunity, and Cancer

Aging may be a confounder of the triangle of nutrition, immunity, and cancer; however, neither the relationships nor the mechanisms of interaction are known. Unfortunately, only a few studies have considered that nutrition and immune function simultaneously decrease in elderly individuals [53]. It is known that increased age adversely affects the function of the immune system as well as nutrient intake habits [54]. Therefore, both immunosuppression (decreased effectiveness of T and natural killer cells) and nutritional deficiencies (as defined by the 1989 recommended dietary allowances) in the elderly may have independent correlations with increased risk of infection and neoplasia development [55, 56]. One of the probable mechanisms that may affect both immunity and nutrition in old people is turnover fluctuations of cellular components in lysosomes or autophagy. Advanced age leads to a reduction in the autophagy of loading viral Ags and cross-presentation of tumor Ags into MHC class I molecules, as well as pathogen killing [57, 58]. Similarly, the capability of autophagy for energetic balance recycling of amino acids to maintain protein synthesis under starvation conditions and the capacity of intracellular lipid stores or glycogen mobilization are disturbed [59, 60]. However, only minimal information has been produced concerning human cancer initiation as a direct result of a specific dietary etiology in the elderly.

20.4 Role of Cancer in Predisposition to Malnutrition from an Immunologic View

Despite the role of nutrition in either preventing or causing cancer in humans, malnutrition is a common problem (global percentage of 56.5 %) [61], and weight loss is often predictive of shortened survival in these patients [62]. In advanced stages of cancer, up to 35 % of related deaths may be linked to improper diet [63–65]. Moreover, a proportion of patients with malignancy develop cachexia, a progressive involuntary weight loss status that is attributed to clinicopathologic factors of the tumor (origin, metastasis, and size), host immunity, and antitumor treatment (Fig. 20.2) [65, 66]. During the development of cancer-associated cachexia, several Th2-dominant condition mediators such as IL-2 and TNF-α (prognostic markers) are implicated in appetite loss and metabolic disturbances, as well as leptin, IL-1, IL-6, IFN-γ, leukemia inhibitory factor, NPY, and proteoglycan 24K [67, 68]. These immunologic and metabolic changes induce cancer cachexia syndrome, which is characterized by patient tissue wasting, anorexia, appetite loss, prolonged fatigue and lethargy, insulin resistance, microcytic anemia, hyperlipidemia, and hypoalbuminemia [69, 70]. Metabolic features of this syndrome include increases in heterogeneity of energy requirement, substrate cycling and turnover, Cori cycle activity, and hepatic protein synthesis, as well as decreases in peripheral muscle protein synthesis, serum protein lipase activity, and plasma concentration of branched chain amino acids [71, 72]. In general, the severity of malnutrition and cachexia in digestive neoplasias is in highest percentages (from 79 % in esophageal cancer to 40 % in rectum cancers) due to the involvement of all areas described in Fig. 20.2 during the development of cancer and in chemotherapy or tumor resection.

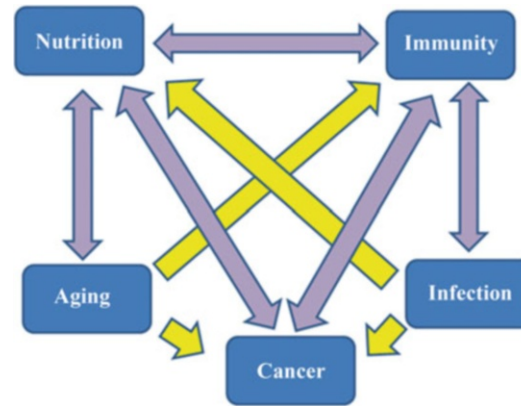

Fig. 20.1 Schematic overview of complex network of diet-immunity-cancer

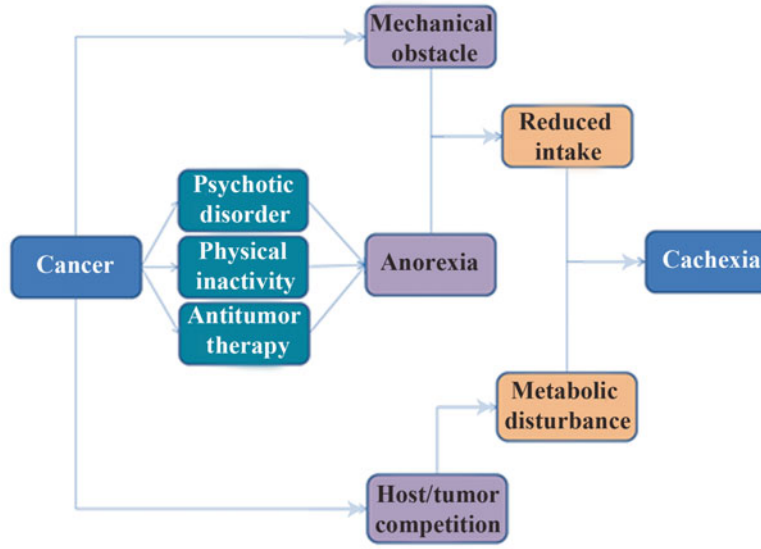

Fig. 20.2 The casual pathways of cachexia occurrence after malignancy

It should be noted that antitumor agents with their side effect on cells with high turnover may exacerbate malnutrition. This could be explained by the competition between cancerous regions and normal cells of the gastrointestinal system to use nutrients to repair the adverse effects of antitumor drugs (hypermetabolic state). Briefly, impaired caloric intake, side effects of therapy, changes in taste and mood, pain and other adverse consequences of eating, obstruction, fistula, and malabsorption all promote malnutrition in cancer patients [73–77]; therefore, well-nourished patients with intact gastrointestinal integrity have lower morbidity and mortality than others [78].

It should be noted that cachexia after cancer differs from cachexia following starvation. Increased protein and glucose turnover, high whole body synthesis and catabolism, accelerated hepatic protein production (especially acute phase agents), increased serum free fatty acid levels, and depletion of fat stores were reported only in cancer patients. However, metabolic abnormalities and, paradoxically, impaired immune response are probable consequences of cancer cachexia, as explained in the previous section [79, 80]. Increased levels of immunosuppressive mediators (e.g., TGF-β), decreased C3 and delayed hypersensitivity response, and diminished numbers and activity of (NK) cells are the most common changes in the defense system of patients with cancer cachexia, leading to more infectious complications and poor prognosis [81]. Neutrophil chemotaxis, monocyte phagocytosis and killing, number of T cells, and proliferation of lymphocytes are also defective in patients with lung cancer [82]. Phagocytic and bactericidal activities of neutrophils were low in hepatocellular carcinoma patients [83]. In addition, surgical stress in cancer patients enhances Th2 and compromises the Th1/Th2 balance and expression of HLA-DR on monocytes, which is considered to be a central marker of immune paralysis after surgical trauma [84]. Most of these immune parameters are also reduced during radiotherapy and chemotherapy because of their side effects on bone marrow. However, these factors are reversible after nutrition improvement [85].

20.5 Role of Nutritional Support in Immune Restoration of Cancer Patients

Adjuvant therapy of cancer patients by different nutritional support strategies (dietary counseling, oral nutritional supplements, enteral tube

feeding, and parenteral tube feeding) is the mainstream recommendation to increase their quality of life and to obviate the risks associated with gastrointestinal complications and reverse malnutrition [86]. However, there is no comprehensive approach based on the needs of cancer patients with cachexia or those with increased nutrient requirements [87]. Several studies have shown the effectiveness of nutritional supply in groups of patients with malignancy that resulted in weight gain, increased appetite, increased energy and protein intake, reduced gastrointestinal toxicity, and enhanced immune function [88–90]. In the clinical setting with standard treatment protocols, it turns out that the implementation of nutrition support in patients with cancer is most effective when it is limited to special, well-described circumstances. Nonetheless, the potential advantages of some specific nutrients have been described and are outlined below.

20.5.1 Arginine

Arginine is a semi-essential amino acid with immunomodulatory potential such as stimulated thymic growth and mononuclear cell response to mitogens, which enhances lymphokine-activated killer cell generation via a nitric oxide-mediated mechanism and stimulates the release of polyamines by the small intestine. In one randomized trial of malnourished patients with head and neck cancer, follow-up at 10 years indicated better survival in those who received supplemental arginine preoperatively [91, 92].

20.5.2 Glutamine

Glutamine is the most abundant amino acid in the human body and the preferential fuel of rapidly dividing cells such as lymphocytes and macrophages [93]. However, supplementing glutamine in the diets of patients with cancer may be counterproductive because glutamine (which is essential for fast growing cells in culture) may promote accelerated tumor growth [94]. A meta-analysis of studies that used parenteral glutamine postoperatively showed it was associated with a shorter hospital stay and a lower incidence of infectious complications [95].

20.5.3 Branched Chain Amino Acids

L-valine, L-leucine, and L-isoleucine can improve the immune response and maintain serum albumin level in the course of hepatocellular carcinoma recurrence [96].

20.5.4 Nucleotides, Long-Chain Omega-3 Polyunsaturated Fatty Acids, and Eicosapentaenoic Acid

These lipid agents have anti-inflammatory, anticachectic, immunomodulating, and antitumor effects [97–100].

20.5.5 Fructooligosaccharides

This group of functional fibers associated with increased lactic acid bacteria acts as an immunomodulator by stimulating IgA synthesis, promoting mucin production, modulating inflammatory cytokines, and decreasing Ag absorption [101].

20.5.6 Bioactive Compounds

Agaricaceae fungus consisting of ergosterol, oleic acid, and triterpenes may inhibit neovascularization induced by tumors and therefore attenuate cancer progression [102].

20.5.7 Vitamins C and E

Since chemotherapy may induce mucositis and bleomycin in particular induces chromosomal damage in lymphocytes, the administration of vitamins C and E may reduce the side effects of therapy [103–105].

20.5.8 Vitamin A

This fat-soluble vitamin can increase the numbers of NK cells or regulatory lymphocytes in cancer patients [66]. A recent study showed that all-*trans* retinoic acid can potentiate the chemotherapeutic effect of cisplatin by inducing differentiation of tumor initiating cells in liver cancer [106].

20.6 Concluding Remarks

In summary, due to the safety and cost-effectiveness of oral dietary therapies, nutrition counseling and the implementation of nutritional supplements should be the initial approaches to nutritional support [107]. Even though parenteral nutrition may also lead to weight gain and improvement in nitrogen balance in patients with cancer, it does not clearly improve serum albumin levels or alter whole body protein turnover even with prolonged administration. Therefore, when nutrition support is chosen as a therapy, the use of enteral nutrition is preferred if the gastrointestinal tract is functional [108, 109]. The use of parenteral nutrition should be limited to malnourished cancer patients who are receiving active anticancer treatment, whose gastrointestinal tract is not functional or who cannot tolerate enteral nutrition, and who are anticipated to be unable to meet their nutrient requirements for 14 days or more [108].

Moreover, it is proposed that preoperative and postoperative immune-nutrition intervention by total parenteral nutrition using a lipid-based regimen is the method of choice in cancer patients who have undergone major surgery to reduce immune dysfunction without enhancing tumor growth (increased augmentation of lymphocyte blastogenesis and production of helper T-lymphocyte lymphokine IL-2, increased ICAM-1 level, and decreased IL-4 and IL-10 values) [111–113]. This observed preference of parenteral nutrition is marginal, and enteral methods are always the preferable route for cancer patients with an intact digestive system. It is also reported that complement components and lymphocyte response may be better with enteral rather than parenteral nutrition [110, 113].

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Allergies and Cancers

21

Delia Rittmeyer and Axel Lorentz

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21.1 Introduction

Worldwide, especially in industrialized countries, allergies and cancer cause high morbidity, mortality, and financial burden to healthcare systems. A total of 12.7 million people were diagnosed with cancer, and 7.6 million died from cancer in 2008, whereby incidences in industrialized countries are nearly twice as high as in developing countries [1]. In developed countries, for instance, in Germany and in the USA, cancer is the second leading cause of death after cardiovascular diseases [2, 3]. Cancer rates are rising due to an increasingly aging population and changes in lifestyle [1]. Allergies are more prevalent, but mortality is much lower. In Germany, about 40 % of all adults have experienced some type of allergy during their life time, and about 300 million people are suffering from asthma worldwide [4, 5].

Interest in possible relationships between these prevalent diseases arose in the 1950s. Following studies revealed a decreased prevalence of allergies among cancer patients [6]. Since then, much research has been done, but still no generally accepted statement about the correlation has been established. As the immune system is involved in both allergic and neoplastic diseases, a connection might be obvious; nonetheless, the nature of this connection is dichotomous. On the one hand, allergies are regarded as a hyperactive state of the immune system which leads to better detection and destruction of tumor cells. On the other hand, allergic reactions go along with

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inflammatory processes which may initiate and support tumor growth [7]. Hence, there are different hypotheses on the relationship which appears to be complex and not universally applicable for every type of cancer or allergy. This chapter will give an overview about studies examining these relationships and describes possible mechanisms which could explain them.

21.2 Molecular Mechanisms of Allergy

By definition, allergy is an immunologic reaction to normally innocuous environmental antigens (Ags), so-called allergens, and it is mostly equated with type I hypersensitivity (immediate-type hypersensitivity) according to the classification by Coombs and Gell. This type is mediated by immunoglobulin (Ig) E in response to T helper cell type 2 (Th2) polarization of $CD4^+$ T cells [8]. Usually IgE is associated with defense against helminthic infections [9]. Atopy is the hereditary tendency to immediate-type reactions and increased production of IgE; however, not every allergic disease has to be atopic [10]. There are different genes associated with atopy, but environmental factors are of great importance as well. During fetal and postnatal periods, the immune system is rather Th2 polarized which shifts toward Th1 during the first years of life [9]. According to the hygiene hypothesis, infectious diseases in childhood are important for Th1 bias. This corresponds with an increasing incidence of allergic diseases in industrialized countries where excessive hygiene leads to an inadequate Th1/Th2 balance [11].

Allergic reactions are induced by low doses of allergens. Allergens are proteins, many of which are enzymes, and their allergenicity is determined by protease activity, surface features, or glycosylation patterns. Soluble allergens enter the body, orally or by inhalation, where they are taken up by antigen-presenting cells (APCs) such as dendritic cells (DCs) which present them to naïve $CD4^+$ T cells via major histocompatibility complex (MHC) class II [12]. In the presence of interleukin (IL)-4, naïve $CD4^+$ T cells differentiate into Th2 cells which are characterized by the production of mainly IL-4 and IL-5. On the contrary, Th1 cells which develop under the influence of IL-12 from the same precursor cells predominantly produce interferon (IFN-γ) and IL-2. Further factors determining polarization are the Ag's route of entry, Ag dose, and the way of Ag presentation [13, 14]. Th2 cells organize the further allergic response toward the specific allergen, as shown in Fig. 21.1. Secretion of IL-4 or IL-13 by Th2 cells causes the isotype switch to IgE in B cells. Additionally, a costimulatory signal, namely, the engagement of CD40 on the surface of B cells and CD40 ligand on the surface of Th2 cells, is required for the stimulation of the B cell [15]. As a result, sensitized B cells differentiate into plasma cells and produce allergen-specific IgE. Moreover, B cells themselves are also able to take up soluble Ags via specific B cell receptors and present them to naïve $CD4^+$ T cells inducing Th2 differentiation [9]. IL-5, IL-6, and IL-9 may enhance IgE production, whereas Th1 cytokines like IFN-γ and IL-12 act as inhibitors [14].

Most of the IgE engage to the high-affinity receptor FcεRI on the surface of mast cells even in absence of Ag. If allergens bind to specific IgE, FcεRI is cross-linked, followed by an inflammatory reaction [15]. Mast cell mediators such as histamine, lipid mediators, and cytokines are released during the effector phase of an allergic reaction and induce typical allergic symptoms. FcεRI is also expressed on basophils which are also able to release allergic mediators being stored in granules [16]. As basophils are able to produce IL-4 as well, they can amplify IgE production [17]. When specific IgE was once built, further exposition to the corresponding allergen elicits an allergic reaction without renewed sensitization [9].

Production of IL-5 by Th2 cells and mast cells activates eosinophils to secrete inflammatory mediators as well as highly toxic proteins and free radicals from their granules [8, 9]. Hours after the early phase of the reaction, the late phase may take place which is characterized by infiltration of further inflammatory leukocytes and eventually a chronic inflammation may be established [18]. The cells involved in allergic

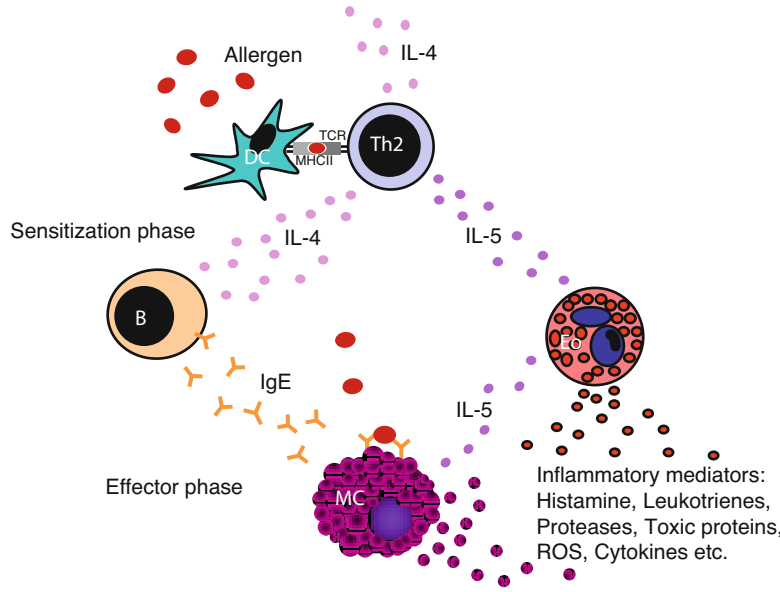

Fig. 21.1 Type I allergic reaction. *B* B cell, *DC* dendritic cell, *eo* eosinophilic granulocyte, *IgE* immune globulin E, *IL* interleukin, *MC* mast cell, *Th2* T helper cell type 2, *ROS* reactive oxygen species. For further explanation, see text

reactions reside predominantly in tissues close to the body surface as their actual function is to defend against multicellular parasites which invade primarily into skin and mucosa-associated lymphoid tissue. Therefore, these cells are specialized to evoke Th2 immune responses [8].

21.3 Types of Allergic Diseases

Allergic asthma is a chronic inflammatory disease of the airways caused by inhaled allergens. Symptoms are breathlessness, wheezing and coughing due to bronchial constriction, and increased mucus secretion. It is often accompanied by hyperreactivity of the airways to other stimuli [10, 19]. Allergic rhinitis or hay fever is an allergic inflammation of the nasal mucosa which results in sneezing, itching, and runny or blocked nose and is often combined with allergic conjunctivitis [20]. Atopic dermatitis or eczema is a manifestation of atopy which occurs predominantly among children, showing symptoms like itching, red rashes, and small vesicles on the skin [20, 21]. Food allergies mostly cause diarrhea or vomiting, but they may also affect the respiratory tract and others [8]. Anaphylaxis is a systemic reaction against an allergen with life-threatening symptoms like hypotension or airway constriction [20].

21.4 Molecular Basics of Carcinogenesis

Cancer is a genetic disease in consequence of a number of mutations in somatic cells. Unlimited growth of the mutated cells leads to formation of neoplasms. Tumor cells are capable of invading into tissues and eventually of disseminating and building metastases in distant regions of the body. The clinical phenotype is varying as well as the implications, depending on the type of cancer and the affected patient. Although the incidence of cancer increases with age, tumors occur in every age group [22].

The development of cancer, carcinogenesis, is a multistep process which requires progressive alterations in the genome of normal cells. Mutations can occur spontaneously or can be generated by so-called carcinogens [23]. A carcinogen is an environmental factor like a chemical

compound, a biological substance, a virus, or radiation that is able to interact with DNA and cause damages or alterations in the genome. Usually cells have several mechanisms to repair DNA damages. During the process of repair, the cell cycle is stalled, preventing that this mutation is multiplied. If no repair is possible, the cell is destroyed by apoptosis [24]. An abolition of these mechanisms is a precondition for oncogenesis. Therefore, mutations have to occur in genes which are responsible for the control of cell proliferation, differentiation, or apoptosis [25]. Such critical genes can be divided into two groups: oncogenes and tumor suppressor genes [26]. Products of oncogenes are, e.g., transcription factors, growth factors, or their receptors. Tumor cells are characterized by gain-of-function mutations in oncogenes, resulting in overexpression of oncogene proteins and subsequent increased growth [27]. Tumor suppressor genes, or rather their products, have a repressive effect on cell growth. Loss-of-function mutations in tumor suppressor genes result in unimpeded proliferation or evasion of apoptosis [25].

However, one single mutation is not sufficient for the formation of a cancer cell. Carcinogenesis is a multistep process involving several events that incapacitate control of the cell cycle, thereby creating a cell with growth advantages [28]. The initiation process of carcinogenesis, characterized by somatic changes, is followed by the process of promotion. Different promoters like chemical irritants, hormones, or inflammation induce proliferation of the damaged cells and further mutations, as the genome of cancer cells is very unstable [25, 29]. The next step is tumor progression. By means of alteration of cell adhesion molecules and protease activity, cancer cells are capable of leaving the primary tumor and infiltrating into tissues. Subsequently, tumor cells spread through blood or lymphoid vessels and build metastases in distant parts of the body while they are displacing healthy tissue [30].

21.5 Types of Cancers

Pancreatic cancer is one of the cancer types with the poorest prognosis, as mortality rates almost correspond to incidence rates [31]. The most common type is adenocarcinoma which affects the exocrine component of the pancreas, but other components of the pancreas may also be affected. Main causes are smoking, diabetes mellitus, and chronic pancreatitis [22]. Lung cancer is the third leading type of cancer among men and women and the leading cause of death from cancer among men. More than two thirds of the cases are caused by cigarette smoke [31]. Cancers of the colon and rectum represent the second most common type of cancer. Besides the hereditary component, dietary habits are a major risk factor [3, 31]. Skin cancer includes malignant melanoma, basal cell carcinoma, squamous cell carcinoma, and some others [22]. The first one causes more deaths; however, the others are more prevalent, yet with higher curing rates [31]. Meningioma and glioma are the two most common types of brain cancer, whereby the causes are largely unknown [32]. Lymphatic and hematopoietic cancers are, e.g., leukemia, Hodgkin lymphoma, or non-Hodgkin lymphoma. Leukemia is characterized by an abnormal proliferation of leukocytes and can be classified into acute or chronic and myelogenous or lymphocytic forms [22]. Acute lymphocytic leukemia is the most common tumor disease in childhood, whereas the etiology is still not identified [31]. Among reproductive cancer, prostate cancer in men and breast cancer in women are the leading types of cancer. Furthermore, breast cancer is the most frequent cancer-induced cause of death among women. Other common reproductive tumors are tumors of the uterus, cervix, and ovaries [31].

21.6 Antitumor Immunity

In 1970, Burnet and Thomas established the hypothesis of cancer immunosurveillance. It states that, to a certain degree, the immune system is able to detect and destroy tumor cells before they can arise to clinically detectable malignancies. Meanwhile this hypothesis has been expanded to the theory of immunoediting which is comprised of three phases: the elimination phase, the equilibrium phase, and finally the escape phase [33].

The elimination phase complies with the process of immunosurveillance. Immune cells of innate and adaptive immune response identify tumor cells by so-called tumor Ags [34]. If these are presented to an activated $CD8^+$ T cell, the tumor cell is directly destroyed by the release of cytotoxic proteins. Moreover, antigen-specific B cells produce specific antibodies which can opsonize tumor cells and lead to either antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) [35].

Besides this adaptive immune reaction, there are cells of the innate immune system involved in immunosurveillance which execute antigen-independent immune responses. Among them are natural killer (NK) cells and NK T cells which are able to recognize and directly kill tumor cells [25]. In addition, these two cell types produce IFN-γ which is probably the most important cytokine in antitumor immunity [33]. It acts indirectly by modulating the immune response, e.g., by activation of macrophages or augmentation of T cell response and NK cell activity, and it is able to increase immunogenicity of tumor cells. Moreover IFN-γ itself has anti-proliferative, apoptotic, and angiostatic capacities which directly affect tumor cells [36, 37]. However, cancer cells are capable of defending against these immune mechanisms. Either they lack certain MHC peptides, making them unrecognizable to T cells, or they do not express costimulating signals which lead to T cell tolerance [38]. Hence, if the immune system is not able to kill the entire tumor cells, the process of immunoediting reaches the equilibrium phase, characterized by dynamic dying and generation of further mutated cancer cells [34]. Following Darwin's rules, those cells, which show surviving advantages through reduced immunogenicity, resist the immune attacks. Thus tumor cells also get shaped and sculpted by immune cells, leading to cell populations that are capable of evading any immune reactions [33]. In this case, surviving tumor cells enter the escape phase. Besides the absent immunogenicity, tumor cells are also able to suppress immune reactivity so that they can proliferate continuously and eventually develop a malignant tumor [38].

Altogether the immunosurveillance hypothesis describes that the immune system is in fact able to fight tumor cells, but also promotes carcinogenesis by sculpting poorly immunogenic mutants.

21.7 Relationship Between Allergies and Cancers in General

The first studies relating to possible associations between allergies and cancer date back to more than half a century [39, 40]. Anyway until now the results have not been consistent, despite various researches in this regard [41].

Regarding cancer in general, there seems to be a balance between studies reporting positive and negative correlations with different types of allergies. While analyses of the Cancer Prevention Study II indicate a slightly decreased risk for people suffering from hay fever or asthma [42], data from the First National Health and Nutrition Examination Survey (NHANES I) show an up to 50 % increased risk of developing any type of cancer [43]. Together with several other studies [19, 21, 39, 44–55], no conclusion can be drawn which identifies the role of allergies in cancer epidemiology. As the term cancer includes diseases of diverse etiologies and a variety of affected tissues, it is necessary to distinguish between different cancer sites as well as specific types of allergy. In the following, those associations which are supported by the majority of studies are presented.

21.7.1 Cancers Positively Correlated with Allergies

Without exception, all of the evaluated studies suggest a positive association between a history of asthma and lung cancer. Without controlling for smoking, a study of 78,000 asthmatic patients found an increased risk for women as well as for men [49]. Another study observed a positive association with asthma, yet no associations with hay fever only, both asthma and hay fever, and any of these conditions [42]. A further survey

calculated a lower, but still elevated, risk for asthma when controlling for smoking. An additional analysis of the effect of respiratory drugs taken for the treatment of asthma showed no connection to cancer development [19]. In a Taiwanese study, asthma was the only type of allergy associated with risk of lung cancer [48].

The prevalence of skin cancer was predominantly examined among subjects suffering from atopic dermatitis, for other types of allergy there is only little evidence available. Atopic dermatitis was associated with a clearly increased risk of keratinocyte carcinoma which made up half of all observed excess cancers in this study. Among 6,275 hospitalized patients with atopic dermatitis, not a single case of malignant melanoma was found [50]. Another study involving patients with atopic dermatitis found an increased risk of melanoma as well as of nonmelanoma skin cancer [51].

21.7.2 Tumor-Promoting Effects of Allergies

The positive association between specific types of cancer and allergies is mainly explained by exemplary description of the relationship between asthma and lung cancer. Increased susceptibility to inhaled carcinogens due to impaired mucociliary clearance and pulmonary obstruction and, above all, inflammatory processes are regarded to be responsible for the increased prevalence of lung cancer among asthmatic patients [49, 56–58]. As described before, allergic reactions go along with chronic or subchronic inflammation. There is evidence that tumors predominantly arise at the sites of inflammation and that inflammatory cells and mediators are found in all tumors [59].

Inflammatory reactions are usually triggered by infections. Macrophages, which have detected infectious agents, release chemokines that attract other inflammatory leukocytes, such as neutrophils and further macrophages. Additionally they release cytokines which increase vascular permeability to facilitate migration of attracted cells into afflicted tissues. Leukocyte recruitment is mediated by adhesion molecules and extracellular proteases which relieve movement into the tissue [29]. Since inflammatory responses are supposed to remove the causes as well as to rebuild damaged tissues, an environment rich in growth promoting, but also rich in damage causing, factors is required. Consequently, the conditions for carcinogenesis are established.

Reactive oxygen species (ROS) released by macrophages are capable of causing DNA damages, thus promoting tumor initiation. Permanent cell regeneration raises the probability of carcinogenic mutations [29]. Cancer promotion is supported by growth factors like TGF, IL-1, IL-6, or IL-8. Furthermore, several inflammatory mediators have angiogenic properties or stimulate the production of angiogenic factors. For dissemination, cancer cells exploit the mechanisms that leukocytes utilize for extravasation into inflamed tissues. These are activation of selectin molecules, interactions between integrins and adhesion molecules of the immunoglobulin superfamily, and secretion of proteinases [29].

Apparently, an inflammatory microenvironment is essential for tumor progression, but vice versa, tumors themselves also secrete inflammatory mediators which recruit leukocytes and mediate inflammation [38, 60]. Accordingly, Dvorak described tumors as “wounds that do not heal” [61], indicating that pathogen-induced inflammation is usually self-limiting, while cancer-related inflammation is triggered permanently [29]. Oncogenic mutations that initiate carcinogenesis may also lead to the establishment of an inflammatory environment. The activation of the Ras oncogene by mutation, for instance, leads to the expression of proteins that induce the production of inflammatory mediators [38, 59]. The main mediator cells of tumor-induced inflammation are tumor-associated macrophages (TAM). They are able to release almost all of the cytokines and chemokines required for tumor progression, and their abundance has been shown to correlate with a poor prognosis [29, 62].

One of the key molecules in the connection between inflammation and carcinogenesis is the transcription factor nuclear factor (TNF)-κB. TNF-κB is an endogenous tumor promoter as it is activated immoderately by carcinogenic mutations. In addition, it is a coordinator of

inflammation by regulating expression of several proinflammatory and survival factors [59, 62].

21.7.3 Cancers Negatively Correlated with Allergies

The association between a history of allergy and pancreatic cancer seems to be quite definite. Five surveys could demonstrate an inverse association. Holly et al. reported a decreased prevalence of any self-reported allergy among pancreatic cancer patients. This correlation was available for multiple allergens like house dust, plants, molds, animals, and food. Furthermore, with increasing numbers of allergies and increasing severity of symptoms, the risk of cancer development decreased. It should be noted that even after receiving a hyposensitization therapy, allergic patients still showed a reduced risk [63]. Hay fever was correlated with a reduced risk of pancreatic cancer in Turner's prospective study [42]. Eppel et al. found a risk of pancreatic cancer in allergic patients that was scaled down by more than 50 %, but not for asthma patients. For males separately, the risk was even lower [64]. Another study that additionally investigated a possible association between variants in IL-4 and IL-4 receptor α genes and cancer prevalence found a negative correlation for any allergy, hay fever, and reaction to animals. But variants in the abovementioned genes were not correlated to cancer [65]. A more recent study detected a significantly increased survival of non-resected pancreatic cancer patients with self-reported allergies. In the cohort that has undergone a resection, results were nonsignificant [66].

Cancers of the colon and rectum are less prevalent among individuals that show a history of allergy. Several studies identified allergies to be inversely associated with colorectal cancer. The probability of developing colorectal cancer with any self-reported allergy in an Italian study was lowered, whereas the association was stronger when allergy was diagnosed at age 35 or older. Regarding colon and rectum cancer separately, the risk of rectum cancer development was lower than colon cancer, whereas the latter was not statistically significant [67]. Another case-control study obtained a protective effect of any allergy on cancer development. Self-reported allergy was inversely associated with both colon and rectum cancer [68]. The risk of colorectal cancer calculated by Turner et al. was reduced by more than 20 % among patients suffering from both asthma and hay fever, and less reduction was observed among patients suffering from hay fever only [42]. A prospective study from Iowa involving only women noted an inverse correlation for allergy in general which was the strongest in patients with skin allergies. Moreover, the risk was decreasing with an increasing number of allergies [69]. Allergic rhinitis was negatively associated with rectum cancer among Taiwanese patients, and the association was stronger for males than for females [48]. Combining the cohorts from the Cancer Prevention Study (CPS) I and II, Jacobs et al. calculated a relative risk of 0.83 for colorectal cancer mortality when having both asthma and hay fever [70].

Most studies agree about a decreased risk of tumors of the brain, specifically glioma, being associated with atopic diseases. In a hospital-based case-control study, the prevalence of glioma was reduced in combination with physician-diagnosed history of any allergy and asthma as well as with self-reported allergy to chemicals. Meningioma risk was not associated with any type of allergy. In addition, the risk of acoustic neuroma was positively associated with hay fever, allergy to food, and allergy to other substances [71]. One further case-control study found hospitalized glioma cases to be less likely to suffer from asthma, as well as hay fever, atopic dermatitis, or allergy in general. Moreover, there was a stronger risk reduction in conjunction with use of any allergic medication like nasal spray or antihistamines [72]. Wigertz et al. contrasted the prevalence of allergy among glioma and meningioma cases with noncancerous individuals. They showed a decreased risk of glioma among subjects with asthma, atopic dermatitis, and hay fever. Treatment of hay fever with nasal spray or eye drops was associated with lower risks than non-treated disease. Meningioma risk was only reduced among atopic dermatitis patients [73].

In children having asthma, a 45 % risk reduction could be observed [74]. One case-control study used IgE levels for the measurement of allergy besides a self-reported history of allergy. As IgE levels did not significantly confirm self-reported allergies, odds ratios for the risk of glioma development varied but both implicated a decreased risk [75]. A few years later the same research group reported similar risks for meningioma development [32]. A more recent study confirmed this with an odds ratio of 0.46 for allergen-specific IgE levels and glioma [76]. Besides glioma and meningioma, data from the INTERPHONE study also indicate allergies to protect from acoustic neuroma [77].

21.8 Tumor-Protecting Effects of Allergies

The majority of the presented studies attribute negative associations between allergies and cancers to an enhanced immunosurveillance among allergic patients due to a hypersensitive and hyperactive immune system. This implies that immune cells of allergic subjects are more effective in detecting and destroying cancer cells [48, 53]. The pivotal cells of immunosurveillance are NK cells by virtue of their capacity to carry out ADCC and to produce IFN-γ [37]. There is evidence for increased numbers and activity of NK cells in subjects suffering from asthma or allergic rhinitis [78–80]. Additionally, it could be proved that there is a negative correlation between cancer incidence and natural cytotoxicity which would further explain an improved potential for immunosurveillance among allergic individuals [81].

Besides the classical cells of immunosurveillance, other immune cells may be antitumor effectors as well. Below, critical cells and mediators of allergic reactions and their possible antitumor activities are given. While in nonallergic individuals their activity may be negligible due to low occurrences, their actions may be increased among allergic subjects, explaining a negative correlation between allergies and cancer incidence.

Allergic disorders are marked by increased levels of eosinophils, a condition named eosinophilia, as eosinophils are important effector cells in allergic reactions [82]. A role for eosinophils in immunosurveillance of tumors was considered since they were observed in different tumor infiltrates. Indeed, higher numbers of tissue or blood eosinophils correlated with better prognosis, e.g., improved survival rates in lung and colon cancer [83, 84]. Although eosinophils might contribute to tumor growth by release of VEGF, thereby initiating angiogenesis, *in vitro* and *in vivo* studies substantiated rather antitumor activities [6, 85].

Eosinophils are recruited by secretion of IL-5 from Th2 cells and eotaxin-1, a specific chemokine. Particularly IL-5 induces differentiation from $CD34^+$ precursor cells, stimulates synthesis of granule proteins, and activates eosinophil effector functions [86, 87]. These effector functions are mainly mediated by the release of their granule proteins which are highly toxic toward pathogens, as well as toward tumor cells. *In vitro* studies could prove the direct cytotoxicity of eosinophil cationic protein (ECP) [83, 84, 87]. ECP causes lysis of tumor cells by creating pores in the cell membrane [88]. Further granule proteins like major basic protein or eosinophil peroxidase have indirect antitumor properties in terms of triggering the release of histamine from mast cells. Besides the IL-5 dependent activation, eosinophils are also responsive to specific IgE. As they express IgE receptors on their surface, binding of IgE leads to tumor-specific antibody-dependent cellular phagocytosis (ADCP) [6].

A study which involved lung cancer patients investigated antitumor activities of eosinophils *in vitro*. For this purpose, eosinophilia was induced by IL-2 treatment in cancer patients. Eosinophils were then purified from blood samples and added to tumor cells. ADCC and direct lysis by eosinophils from IL-2 treated patients were highly increased compared to those of non-treated patients or healthy donors, which did not harm tumor cells at all [83]. This suggests that in fact there are differences in cytotoxic potentials between allergic and nonallergic individuals. The influence of IL-2 was to ascribe to secondary cytokine production because IL-2 has no direct effect on eosinophils, but stimulates lymphocytes. Thus, eosinophil activation was most likely mediated by IL-5.

Another study confirmed the involvement of eosinophils in antitumor immunity in methylcholanthrene-induced fibrosarcoma models. Among IL-5 transgenic mice, which show increased levels of eosinophils, tumor growth and incidence were reduced, whereas among eotaxin-deficient mice, incidence was increased. An even greater increase of incidence was observed in eosinophil-deficient mice. This provides evidence that, at least, chemically induced cancers may be effectively fought and inhibited in growth by eosinophils [86].

IgE is the key mediator of allergic reactions. Binding of IgE to the high-affinity receptor FcεRI on the surface of mast cells and basophils leads to ADCC, whereas binding to the low-affinity receptor CD23 on the surface of macrophages or eosinophils leads to ADCP [6]. Usually IgE is predominantly present in tissues bound to its receptors, but in allergic patients, serum IgE levels are up to ten fold higher than normal [89]. In addition to defense against helminths and hypersensitivity toward allergens, IgE antibodies may also be directed against tumor Ags, thereby mediating antitumor activities. *In vitro* studies could demonstrate IgE-mediated effector activities against human ovarian carcinoma cells [89, 90]. Furthermore, treatment of mice with IgE targeted on tumor cells resulted in decreased growth of induced cancer. The effect was significantly stronger for IgE than for treatment with IgG. Besides the curative potential of IgE, a protective long-term immunity against the specific tumor cells were observed as well [91]. The incidence of survival was monitored within a case-control study among glioma patients. Those who had elevated levels of IgE were observed to survive on average 9 months longer compared to patients with moderate or borderline IgE levels. Additionally, elevated IgE levels were more common among control subjects than in patients which might support the assumption of an antitumor capacity of IgE [92]. Among pancreatic cancer patients, IgE levels were detected to be five fold higher than in control groups, whereas levels of other Igs were similar. Tumor-specific IgE was found to mediate ADCC against tumor cells, whereas IgE isolated from healthy controls did not [93]. Recapitulating, IgE is an effective mediator of antitumor cytotoxicity as well as phagocytosis of tumor cells.

Typical Th2 cytokines are IL-4, IL-5, IL-13, and IL-10. The role of IL-5 in recruiting and activating eosinophils has already been described. IL-10 and IL-13 exhibit rather tumor-promoting than antitumor activities [85, 94]. IL-4 is known as Th2 differentiation factor and mediator of IgE isotype switch in B cells [95]. However, IL-4 also shows antitumor activities. First, IL-4 induces the infiltration of macrophages and eosinophils which mediate cytotoxicity toward tumor cells [96]. Second, IL-4 is one of the most potent inhibitors of angiogenesis by blocking migration of endothelial cells. The resulting restricted tumor growth could be proved for local as well as for systemic application of IL-4 *in vivo* [97]. Moreover IL-4 receptor has been shown to be expressed on different human tumors, and immunogenicity of melanoma cells could be increased by IL-4 by means of enhanced MHC class II expression [98].

As described, many crucial components of allergic reactions were separately shown to have antitumor activities, but only little research has been done yet to evaluate the combined effects of these cells. One study evaluated growth of inoculated tumor cells in mice that were sensitized against ovalbumin. Tumor cells in allergic mice showed the same proliferation rate like those in nonallergic mice, whereas apoptosis was increased [99]. Consequently, tumor progression was decreased in allergic mice which might support the relationship between allergy and some types of cancer in humans.

21.9 Concluding Remarks

Even despite extensive research, the relationship between allergies and cancer remains poorly understood. As there are studies which show negative as well as positive correlations, one has to take a closer look at the specific type of cancer and the location it arises. Allergies are accompanied by inflammatory reactions which constitute an optimal environment for carcinogenesis, thus promoting the development of tumors at this specific site. Additionally, systemic effects in terms

of enhanced immunosurveillance can likewise be evoked, thus preventing from cancer at other areas. The presented examples of a positive correlation between asthma and lung cancer as well as atopic dermatitis and lung cancer and a negative correlation between allergies and pancreatic cancer, colorectal cancer, and glioma fit this classification. Nonetheless, there is still a need for well-conducted epidemiological studies, as well as for investigations on the molecular level to clearly define the relationship between allergy and cancer.

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Cancer Immunology of Transmissible Cancers

22

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Contents

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22.1 Introduction

Cancer, an affliction primarily of vertebrate animals, is a disease characterised by uncontrolled cell proliferation which frequently results in the death of the host. Cancer clones are under natural selection to avoid host immune response and resist treatment, resulting in the generation of increasingly aggressive subclones. Cancers nearly always originate and spread within a single individual, ending either with the elimination of the tumor or the death of its host. Cancers may be triggered by contagious pathogens, most commonly viruses, such as human papillomavirus which can cause cervical cancer in humans or the Jaagsiekte sheep retrovirus which causes pulmonary tumors in sheep. However, cases in which cancer cells themselves form a pathogenic agent do occur, although they are extremely rare. There are only two naturally occurring cancers able to spread between individuals. These are canine transmissible venereal tumor (CTVT) found in dogs and devil facial tumor disease (DFTD) of Tasmanian devils. These cancers act as a parasite transmitting from one host to the next. While all tumor must adapt in order to avoid their host's immune response, these two tumors have evolved to avoid immune destruction not only from their original host but also from the immunologically disparate hosts that they are transmitted to. These two diseases give two different perspectives on transmissible cancers

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Table 22.1 Comparison of DFTD and CTVT

Disease	DFTD	CTVT
Species affected	Tasmanian devil	Dog, wolf, coyote, jackals, foxes
Distribution	Tasmania	Worldwide
Age of origin	About 18 years ago	At least 6,000 years ago
Likely cell of origin	Schwann cell	Macrophage
Site of primary infection	Face, mouth or neck	External genitalia
Mode of transmission	Biting	Coitus
Frequency of metastases	65 %	7 %
Mortality	100 %	Rare
Effect on host population	Decline of host population of 80 %, extinction in wild likely within 30 years	Little or no effect
Treatment	Surgical excision if treated early	Chemotherapy

and give us unique insights into the immunology of cancers. These cancers provide an ideal model for studying the battle between tumor and host. For a comparison of CTVT and DFTD, see Table 22.1.

22.2 Canine Transmissible Venereal Tumor

22.2.1 Prevalence and Transmission

Canine transmissible venereal tumor is a contagious neoplasm found in domestic dogs [1]. The disease is found worldwide, but is mostly prevalent in stray dog populations [2]. While it is most commonly found in dogs, it can be transmitted to a wide range of canine species including wolves, foxes, jackals and coyotes [3]. In some regions, such as Japan, it is the most common tumor found in dogs [4]. Transmission occurs by transplantation of viable tumor cells during coitus [3]. The tumor establishes on the external genital mucosa of the infected dog and can affect both sexes of any breed of dog [3]. Metastases are rare and are most commonly found in the lymph nodes [2]. CTVT in domestic dogs can be successfully treated with chemotherapy [3]. CTVT can also be induced in adult immunocompetent dogs by inoculation with living tumor cells [5].

22.2.2 Histology and Clonality

Histologically, CTVT is described as an undifferentiated round-cell neoplasm of histiocytic origin [6]. Cytologically, CTVT cells do not have many distinctive ultrastructural features [7]. CTVT has been proposed to be of macrophage lineage based on its expression of several macrophage characteristic proteins [8] and its ability to be parasitised by *Leishmania infantum* [9], a parasite usually infecting macrophages. The transmissibility of CTVT cells has been demonstrated by studies which found that the disease can be induced by transplanting live cells, but not killed cells or cell filtrates [3]. The chromosome number of CTVT (57–59 chromosomes) is consistent across geographically dispersed samples and is different to the normal number of dogs (76 chromosomes) [10–12]. CTVT genomes share chromosomal duplications and deletions which are not found in the dog genome [13]. Transmissibility has been further supported by the presence of a LINE insertion near the c-myc gene which is found in all CTVT tumor studied to date but is not found in the normal dog genome [14]. Recently clonal transmission of CTVT has been confirmed by molecular genetic studies. Rebbeck et al. [13] and Murgia et al. [15] found that the pattern of microsatellite polymorphism strongly suggests a monophyletic origin. The most recent common origin of CTVT may have been relatively recent, predicted to be between 47 and 470 years [13] or between 250 and 2,500 years [15]. However, the date of CTVT origin is ancient, predicted to have occurred at least 6,000 years ago in either the dog or wolf and may have predated the domestication of dogs [13]. This makes CTVT the oldest known malignant cell line.

22.2.3 Disease Progression

In experimental transplantation, the disease has a predictable growth pattern featuring three distinct stages of progressive growth, stable growth and then regression [3]. The initial progressive phase lasts several weeks and is characterised by rapid tumor growth with a doubling time of about 4–7 days [16]. During the stable growth phase, tumor expansion slows [16]. During regression, the tumor shrinks and eventually disappears [16]. Spontaneous regression is associated with an intense local lymphocytic infiltration [17]. The number and size of tumor-infiltrating lymphocyte subpopulations vary with CTVT growth phase [17]. In natural transmission, the disease will usually regress after 6–9 months of growth, unless treated earlier [2]. Experimental transplantation to immunosuppressed dogs results in tumors that do not regress [18]. Additionally, the host is immune to subsequent reinfection after remission, and offspring of infected mothers are partially protected from infection [19]. These findings demonstrate that host immune response is involved in tumor regression and protection from subsequent reinfection.

22.2.4 Immunology

CTVT is initially capable of downregulating host immune response, but in the majority of cases, it is eventually overcome by the host defences [2]. Regression of CTVT involves both humoral and cell-mediated immunity. Rejection of foreign tissue is initiated by the presence of major histocompatibility complex (MHC) antigens (Ags) on the surface of foreign cells. MHC Ags present peptides to T cells. There are two classes of MHC antigen. Class I peptides are recognised by $CD8^+$ T cells, while class II peptides are recognised by $CD4^+$ T cells. Cells without MHC, mutated or foreign MHC, or MHC presenting abnormal peptides can trigger an immune response; therefore, regulation of MHC is important for cancers to escape host immunosurveillance. CTVT has the additional distinction in that it is capable of transmitting across MHC barriers. Many tumors have selective mechanisms for downregulating MHC class I molecules to escape recognition by $CD8^+$ cells [20]. CTVT cells express none, or very few, MHC class I and II molecules during the progressive phase [21, 22]. Additionally they do not express β_2M, a component of MHC class I, on the cell surface [23].

The initial lack of cell surface MHC should result in cell destruction by natural killer (NK) cells [24]. However, migration of NK cells to the tumor is impaired due to tumor expression of TGF-β1 [25]. TGF-β1 is a potent immunosuppressive cytokine which commonly plays a role in immune avoidance in cancers [26]. TGF-β1 is expressed in high concentrations in CTVT tumors where it suppresses the killing activity of tumor-infiltrating lymphocytes (TIL) [25]. Natural killer cells, which migrate to the tumor due to the lack of cell surface MHC expression, are impaired by TGF-β1 [25]. In addition, the function of dendritic cells (DCs) is impaired with inhibited antigen uptake and presentation, impaired differentiation and apoptosis of monocytes and DCs [27].

Host expression of interleukin-6 (IL-6) appears to be critical in forcing the tumor into regression. At the onset of regression, expression of IL-6 by TILs is increased, antagonising the activity of tumor TGF-β1 [25]. By downregulating TGF-β1, the ability of interferon γ (IFN-γ) to promote MHC class I and II expressions is restored [28]. IFN-γ and IL-6 work synergistically to enhance MHC expression [28]. It has been postulated that IFN-γ induces expression of an MHC class II transactivator, resulting in an increased MHC class II expression [28]. This results in the attraction of $CD4^+$ cells which promote the generation of antibodies (Abs) against CTVT, driving tumor rejection and the subsequent immunity against it. Host IL-6 may also enhance T cell cytotoxcity when MHC molecules are expressed [25]. Additionally, during regression TILs secrete a heat-sensitive factor, enhancing MHC class I and II expressions [29]. At commencement of regression, 30–40 % of cells express both class I and II MHCs [21, 22]. DC activity is substantially recovered during regression [27]. Expression of IL-6 and the

re-establishment of DCs are believed to be the critical factors in initiating tumor regression.

There is evidence that humoral immunity is also involved in regression. Treating CTVT-infected dogs with the sera of post-regressive dogs caused regression, while dogs simultaneously given CTVT and immune serum did not develop the disease [18]. Antibodies to CTVT have been found in dogs after CTVT regression [30]. B lymphocytes and plasma cells appear in higher concentration in regressive than progressive tumors [17]. Additionally, Liao et al. [31] detected a CTVT-secreted factor which was specifically cytotoxic to B cells.

22.3 Devil Facial Tumor Disease

22.3.1 Prevalence and Appearance

Tasmanian devils are the world's largest marsupial carnivore since the extinction of the Tasmanian tiger in the early twentieth century. Devils were once widespread on mainland Australia, but today are restricted to the island of Tasmania. Although several population crashes have been reported over the last two centuries, Tasmanian devils were classified as a species of least concern prior to the outbreak of DFTD with a population of around 150,000 animals. DFTD was first witnessed in 1996 by a wildlife photographer in Mount William National Park in the far north-east of Tasmania [32]. Since then, the disease has spread rapidly across the state, with the disease found in 85 % of the devil distribution as of 2012 [33]. The disease is projected to have spread to the entirety of devil distribution by 2016 [34]. Since its emergence, DFTD has wiped out over 80 % of the devil population [34], and unless acted upon, the devil is expected to be extinct within 30 years [33]. This had led to devils being listed as endangered by the IUCN as well as national and state authorities [35].

DFTD appears as tumors mostly around the head and neck of the devil [36]. After appearance of the first lesions, death usually occurs within 6 months [36], and there have been no verified cases of devils having survived the disease. Death may occur due to starvation or complications from metastases [37]. The tumors are undifferentiated soft tissue neoplasm, believed to be derived from Schwann cell originator cells [38]. Metastases occur in around 65 % of cases [36].

22.3.2 Transmission

DFTD was discovered to be a transmissible allograft from karyotypes of the tumors and hosts [39]. Similar to CTVT, DFTD samples have a conserved karyotype which is distinct from the normal devil karyotype [39]. The DFTD karyotype is highly rearranged with the absence of both copies of chromosome 1, one copy of chromosome 5 and both sex chromosomes [39, 40]. Clonality has been confirmed by genotyping which has shown that DFTD specimens taken from different individuals are identical to each other, but usually different to their hosts, at several microsatellite markers as well as MHC genes [41]. Further support for clonality comes from next-generation sequencing [42]. The disease is spread by biting. Devils bite one another frequently when fighting over food and territory or during mating [43]. DFTD cells are transferred when a devil bites into the tumor of a diseased devil [44]. DFTD cells can then establish into a tumor around wounds in the mouth or face of the new host [39, 45].

22.3.3 Immunology

While much is known about the immunology and pathology of CTVT, very little is known about the same aspects of DFTD. Unlike CTVT, DFTD cells pass from one animal to another without provoking an immune response [46]. Both tumor and host characteristics have been hypothesised to be responsible for the ability of this cancer to spread and go undetected by the host immune system. It has been suggested that an impaired immune system and a lack of genetic diversity, in particular at MHC genes, may make the devil population susceptible to the spread of DFTD [47]. However, it is likely that the tumor itself

actively avoids immune detection. Both downregulation of cell surface MHC and the expression of immunosuppressive factors have been investigated. Each of these host and tumor characteristics will be discussed below.

22.3.4 Do Devils Have an Impaired Immune System?

Soon after the emergence of DFTD, it was hypothesised that the spread of this disease may be enabled by an impaired devil immune system [47]. Tasmanian devils are known to be highly susceptible to neoplasms [48]. However, research over the last decade has demonstrated that devils have a robust immune system functionally equivalent to other marsupial and eutherian immune systems. Their immune tissue architecture and immune cell distribution are similar to that seen in eutherians; their lymphocytes proliferate in response to mitogen stimulation, and subcutaneous injection of a cellular Ag produces a strong antibody response [46, 49]. Additionally, NK-cell responses have been demonstrated [50]. Therefore, with a robust immune response, it is unlikely that the absence of immune response to DFTD is due to a lack of functionality in the devils' immune system.

22.3.5 Devils Have Low MHC Diversity

Devils lack genetic diversity across the genome [42, 51] as well as at MHC class I and class II genes [41, 52]. Having an important role in the recognition of both cancerous and foreign cells, MHC class I presentation should be critical in the recognition of DFTD cells by the devil's immune system. However, this recognition may be impaired if MHC diversity is so low that it impairs the host immune system from recognising these cells as foreign. Siddle et al. [41] found that devils have critically low MHC diversity and suggested that this was the first link between a lack of MHC diversity and the spread of disease. However, the role of MHC diversity in DFTD spread has been questioned by recent studies. Kriess et al. [53] conducted skin grafts in devils and found that even MHC similar hosts were capable of rejection. This suggests that minor histocompatibility Ags may play a role in allograft rejection. Most recently Lane et al. [54] found no link between MHC diversity and susceptibility to DFTD, suggesting that MHC is not critical to the disease spread. However, the lack of genetic diversity in devils may still be responsible for the spread of this disease. There is likely to be low diversity at other key immune genes such as minor histocompatibility Ags and genes of the innate immune system, which may have a role in disease susceptibility.

22.3.6 Expression of Immunosuppressive Cytokines

Expression of immunosuppressive cytokines is found in many cancers and allows the cancers to avoid host detection and destruction of tumor cells by suppressing the host immune response. As discussed, the expression of TGFβ1 by CTVT cells is involved in preventing NK-cell response [25]. However, it has been recently shown that TGFβ1 as well as three other cytokines commonly expressed by cancers to downregulate immune detection are not over-expressed in DTFD tumors compared to control tissues [55]. This includes VEGF-A, IL-6 and IL-10. It therefore appears that in DFTD, unlike CTVT, suppression of the immune system by release of immunosuppressive cytokines does not play a key role in the pathology of DFTD [56].

22.3.7 Regulation of Cell Surface MHC

Like CTVT, regulation of cell surface MHC may allow DFTD to avoid rejection. Recent research has shown that DFTD cells express functional MHC class I and class IIB RNA transcripts, but little or no transcripts for genes involved in Ag processing including B2M, TAP, MHC class IIA

and DMB [56]. This study found only trace amounts of MHC I proteins at the surface of DFTD cells both *in vivo* and *in vitro* [56]. These findings may explain how DFTD cells evade recognition by T cells, though further work is needed to build a full picture of how DFTD cells avoid immune recognition.

22.4 Comparison of DFTD and CTVT

DFTD and CTVT are the only naturally occurring clonally transmissible cancers. Over the last decade, our understanding of the pathology and immunology of these diseases has greatly developed, but much is still unknown. Further research into their origin, evolution and immunology will not only provide insights into transmissible cancers, but may also have medical applications to human cancers. These two diseases differ in many aspects of their pathology and immunology. However, they also share features in common which may help reveal circumstances favoring the generation of such diseases.

While CTVT is an ancient disease having been around for at least 6000 years, DFTD is a very new disease less than 20 years old. This allows us to compare a transmissible cancer which has been through thousands of years of co-evolution with its host species to one which has recently emerged. CTVT, as a successful parasite, has likely undergone selection to become a benign tumor which does not kill its host population, resulting in the low rate of metastases and the characteristic regression. On the other hand, the newly emerged DFTD results in 100 % mortality and may drive its host population to extinction. Immunologically there are both similarities and differences in how these diseases avoid host immune rejection. CTVT initially has low or no expression of MHC classes I and II to avoid rejection by T cells, and the expression of host TGFβ1 appears to be important in preventing NK-mediated destruction of tumor cells [25]. Similarly, recent evidence has shown that DFTD has low or no expression of MHC class I on the cell surface [57]. However, TGFβ1 is not upregulated in DFTD cells [55]. Therefore, it is yet to be seen how DFTD cells avoid NK-mediated destruction.

With only two naturally occurring transmissible cancers worldwide, it is somewhat surprising that these diseases do not occur more frequently. There are several commonalities between CTVT and DFTD which appear to be significant factors making their host species susceptible to this form of disease. Firstly, for such a disease to occur, a route of transmission must be present. In both species, transmission of cancer cells appears to occur at the site of tissue damage. Devils' biting behavior [43] and the extended, rough copulation that occurs in dogs [57] provide routes of transmission for the transfer of tumor cells. The probable low genetic diversity in both of the original host populations is another commonality. Tasmanian devils have low genetic diversity, particularly at MHC genes [41, 51], while it has been hypothesised the CTVT arose in an inbred wolf population due to homozygosity at a number of loci in CTVT [15]. Additionally, a spontaneously arising sarcoma was found to be transmissible among a colony of laboratory Syrian hamsters which also had low MHC diversity [58]. A further trait which may predispose populations to disease is susceptibility to neoplasms. Devils are naturally highly susceptible to neoplasms [48]; however, this is not a trait shared by dogs or wolves. If the presence of all these factors is indeed required for the development of transmissible cancers, this may explain the rarity of such diseases. However, with wildlife species increasingly losing diversity due to anthropogenic effects, the chance of seeing similar disease occurring in wild vertebrate species may be increasing.

22.5 Evolution of Transmissible Cancers

A further intriguing aspect of DFTD is that it allows us to observe the evolution of a transmissible cancer in real time. DFTD, as a clonal cell

line, has already gone through two stages of adaptation: one in order to establish as a tumor in its initial host and a second stage of adaptation in order to be capable of transmission to other hosts. The disease is now going through further evolution as it spreads through the population. A number of strains have been identified based on karyotype [59]. Although the functional significance of these changes is unknown, when cultured, the strains display different morphology and growth rates [59]. These changes provide variation which selection can act on. The presence of devils with distinct genotypes at the disease front [60], some of which may offer partial or full resistance to the disease, may provide a strong selective force to the disease. Another possibility is that the disease may adapt to become more benign and slow growing. Devils which can survive longer with the disease have the capability of infecting more devils, possibly resulting in the evolution of a more benign form of DFTD. A similar adaptation may have occurred early in the history of CTVT. However, it is also possible that the modern characteristics of CTVT were present at its origin and have gone through little adaptation since this time.

Fassati and Mitchison [61] first suggested that epigenetics must be involved in the regulation of CTVT. Although epigenetics has yet to be investigated in CTVT, several studies are beginning to suggest that epigenetics may have a role in the regulation and evolution of DFTD. Increased expression of the DNA methyltransferase 1 gene in DFTD cells has been observed resulting in hypermethylation [62]. This results in different patterns of gene silencing in different DFTD tumors, providing variation on which selection can act. One such area that selection is likely to act is in expression of MHC. Siddle et al. [56] have shown that the regulation of Ag processing proteins, which enable DFTD cells to evade the immune system, is controlled by epigenetic mechanisms. This may mean that regulation of MHC can vary depending on circumstances. This provides a mechanism for fine tuning of the immunology of the cancer cells, allowing DFTD to adapt to immunologically disparate hosts.

22.6 Transmissible Tumors as a Cancer Model

CTVT and DFTD provide *in vivo* models for studying the battle between tumor and host immune response. CTVT has been used as a model for human cancer since at least 1980 [63, 64]. As these cancers have been propagated through many hosts over many years, they have had a long exposure to host immunosurveillance, thus providing insight into the evolutionary strategies developed by cancers to evade immune recognition. The strategy of immune evasion employed by CTVT has many features in common with many human tumors including the regulation of MHC expression and expression of immune-modulating cytokines. Thus, CTVT provides an excellent model for studying these features. Additionally, CTVT is one of the only *in vivo* models for studying tumor regression, allowing investigation into the mechanism through which host immune system overcomes tumors. Understanding how the tumor controls host immune response and how the host forces regression of the tumor could be useful in the development of cancer immunotherapy approaches in human patients. Following the discovery that host expression of IL-6 antagonises the effects of tumor-expressed TGFβ1 leading to regression, Chou et al. [65] found that a combination of IL-6 and IL-15 could induce regression in progressing CTVT tumors. Such a therapy may be useful in human cancers which also produce TGF-β1 to suppress host immune response [65]. CTVT and DFTD are similar to a number of rare cancers that can be transmitted between humans and may provide a model for these diseases. In humans, the most comparable diseases are cases of malignancies vertically transmitted during pregnancy. Mother to foetus transmissions of melanoma, lymphoma, leukemia and carcinomas have all been reported. Parallels exist between the formation of a fetus and transmissible cancers. The semi-allogenic fetus downregulates cell surface MHC class I but upregulates the nonclassical HLA-G to avoid destruction by NK cells [66], thus using a similar strategy to avoid immune

rejection as is used by both CTVT and DFTD. Fetal trophoblasts and choriocarcinomas regulate MHC in a similar way to the foetus in order to avoid immune detection, and this contributes to the very aggressive nature of choriocarcinomas [67]. Choriocarcinomas can metastasize to the mother [68] or the fetus [69]. There are even cases of choriocarcinomas forming during pregnancy and metastasizing to both the mother and the fetus [70]. Another route of tumor transmission in humans is through organ or hematopoietic stem-cell donations. About one third of recipients who receive organs from a donor who had some form of cancer at the time of donation developed the same cancer as the donor [71]. Unlike dogs and devils, donor recipients are under immunosuppressive treatment, and usually once immunosuppression is withdrawn the malignancy will regress [72]. However a parallel exists between donor-derived malignancies and transmissible tumors in MHC similarity between individuals. In organ transplantations, MHC genotypes are matched between the recipient and the donor, and this may be a feature shared with CTVT and DFTD, both of which likely originated in populations with low MHC diversity. Therefore DFTD and CTVT could be used as a naturally occurring model for transplant-transmitted cancers.

22.7 Concluding Remarks

CTVT and DFTD are two tumors which have progressed to infect thousands of individuals since their origin. These unique cancers give a fascinating perspective on cancer evolution and immunology. Little is currently understood about the pathology and immunology of these diseases. However, further investigation into these novel diseases will reveal clues as to how tumors can adapt to their host and evade the immune system. This advance in our understanding of cancer may lead to practical treatments for cancers in humans.

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Envisioning the Application of Systems Biology in Cancer Immunology

23

Julio Vera, Shailendra K. Gupta, Olaf Wolkenhauer, and Gerold Schuler

Contents

23.1 Introduction

Biomedicine has evolved extremely fast in the last decade. Many challenging new insights into the nature of biological systems and the avenue of new experimental techniques have synergized during this period to change our perception about biomedicine. Biological systems are nowadays envisioned as complex networks composed of dozens to thousands of proteins, genes, and miRNAs, which interact to control cellular- and tissue-level phenotypes. One can say that biology is the science of the ultimate complexity because in one sense every single cell

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contains as much complexity as entire solar systems or galaxies. In this context of increasing complexity, systems biology has emerged a decade ago.

Systems biology is a methodological approach that combines quantitative experimental data, mathematical modeling, and other tools from computational biology to address biological and biomedical questions from a systemic perspective. It is almost a mandatory research strategy when: (a) analyzing massive amounts of high-throughput quantitative experimental data, (b) trying to understand the function and regulation of biochemical networks enriched in regulatory motifs like feedback loops, and (c) integrating biological data from diverse sources across temporal and spatial scales. Within the methodology, the use of mathematical modeling is an essential step, necessary to integrate and analyze data, formulate and explore biological hypothesis, or perform quantitative predictions with a therapeutic aim [1]. It has a clear interdisciplinary nature because it involves expertise in biomedicine, quantitative experimental techniques, data engineering, mathematical modeling, and bioinformatics, only to mention some of the scientific profiles of researchers that can get involved in a systems biology project.

Due to this multiplicity of disciplines, over the years the concept of systems biology has become fuzzy and difficult to define precisely. At the moment, systems biology describes at least three different approaches, all of them relying on the use of quantitative experimental data and mathematical modeling. They are briefly described in the following subsections.

23.1.1 The "Omics" Paradigm and the Use of Statistical Models

In the last few years, it has become technically and economically affordable to perform quantitative, high-throughput experiments to measure the concentrations or activation state of proteins and other biomolecules like RNAs or metabolites. This has given rise to several new experimental fields (e.g., genomics, transcriptomics, proteomics, and metabolomics, collectively known as "omics" techniques). When applied to samples obtained from large cohorts of patients suffering complex multifactorial diseases, especially cancer, these techniques have already generated massive amounts of clinical and biomedical data. These data are a precious resource to discover the molecular mechanism behind the emergence of a disease. From an applied perspective, these techniques can be used to generate new protocols and tools for early diagnosis or more efficient and personalized therapeutic treatments. However, the data alone are not sufficient: human intuition and direct interpretation are not well-suited tools for the analysis of massive volumes of high-throughput data. Complex mathematical models, which rely on the intensive use of advanced statistical and computational methods, are necessary to interpret and analyze the amount and type of data generated through the "omics" paradigm.

These statistical models have been successfully exploited in the search of biomarkers for cancer progression, metastasis, or resistance [2]. In this case, patients in a clinical study are classified in groups according to the progression status of the tumor. Expression profiles of proteins, RNAs, or other biomolecules, obtained from patient samples, are analyzed using statistical models to find one or more disease-associated genetic signatures. These genetic signatures account for groups of genes having an expression pattern that, considered globally, can be used to discriminate between patient groups. The ultimate aim is to use these genetic signatures for improving diagnosis and/or prognosis. For some tumor entities, genetic signatures have been already found that could be successfully associated with progression and are currently used in prognosis tests [3, 4]. However, one has to say that the statistic elucidation of this kind of signatures should never be the end point of a research process. It has to be followed by additional *in vitro*/*in vitro* experiments and clinical studies to find a mechanistic interpretation for them [5].

23.1.2 Mathematical Modeling and Systems Theory: Dissecting the Complexity Emerging Out of the Structure of Biochemical Networks

Accumulating experimental evidences indicate that, at the molecular level, cells are organized in large and complex regulatory networks that involve genes, interacting proteins, different kinds of coding and non-coding RNAs and metabolites. When trying to find a mechanistic interpretation for the behavior behind these large networks, simple human intuition and direct data analysis fail because they involve too many interacting variables [1, 6]. Furthermore, these networks contain a plethora of cross-talking regulatory motifs, like feedback and feedforward loops that show often counterintuitive behavior. In engineering and physics, mathematical modeling has been used for a century to investigate the dynamics, regulation, and controllability of other physical or artificial systems containing similar regulatory motifs. It is therefore not a surprise that biological data-based mathematical modeling has emerged as a powerful tool, able to dissect the nature of biochemical networks, interpret the complex nonintuitive relations between their compounds, and provide support in the design of hypothesis and experiments. This strategy has been used with remarkable success in the last years in molecular oncology and cancer signaling. It has proved to be useful in: (a) the detection and analysis of the nonlinear behavior emerging from the combination of feedback, feedforward, and other regulatory motifs in biochemical networks [7, 8]; (b) the integration of diverse sources of high-throughput data accounting for the regulation and dynamics of large cross-talked biochemical networks, with hundreds of compounds [9]; (c) the derivation, analysis, and validation of hypotheses concerning the structure and regulation of cancer-related pathways [10, 11]; or (d) the design and assessment of conventional, targeted, or combined anticancer therapies [12, 13].

23.1.3 Bridging Biological Scales Through the Integration of Biological Data in Multi-scale Models

Evidences are growing in recent years pointing to the fact that, in many cases, the influence of the surrounding media in the tumor cannot be separated from the tumor biology [14]. The microenvironment interacts with the tumor and affects its progression via a number of selective forces including hypoxia, lack of nutrients, or immune-driven apoptosis, while the tumor can modify the features of its microenvironment to subvert the body's protective mechanisms [15]. This notion is the motivation behind the many efforts to develop data-driven mathematical models of cancer progression, able to account for the spatial organization of tumors and the interaction with the surrounding microenvironment [16]. The so-called cancer multi-scale models are mathematical constructs that are able to simulate global spatio-temporal features of tumors like growth, angiogenesis, as well as therapy- or hypoxia-mediated apoptosis and necrosis [17].

23.2 One Step Further: Integrating the Different Perspectives of Systems Biology into a Unified Framework

Although each one of these mathematical model-based approaches has proved to be quite successful in accelerating the discovery in tumor basic biology and clinics, they have limitations that cannot be ignored. Statistic models are extremely useful tools to analyze enormous amount of patient data and find expression patterns associated to given clinical phenotypes; however, those statistical expression patterns alone suffer with the lack of support provided by a truly mechanistic interpretation of the data, the sort of analysis that provides biological causation. Mathematical models of biochemical networks can provide insights into the biological mechanisms

underlying cancer progression, but are not able to account for the effects of the tumor-microenvironment interaction. Current multi-scale models are accurate describing biomechanical forces, cell phenotypes, and spatial interactions between tumor cells and their surroundings. However, they lack a precise description of the intracellular mechanisms driving those phenotypic features, as well as a connection to the clinical understanding of the tumor biology.

These limitations are the motivation why researches have tried to integrate the different scopes into a unified conception of systems biology in recent years [18–21, 8]. The idea is to develop a unique framework that integrates tools and methods from statistics, bioinformatics, computational biology, and mathematical modeling with the aim of integrating biomedical data across biological and spatiotemporal scales. This approach must be able to: (a) link massive clinical patient data with the function and (dis)regulation of biochemical networks; (b) provide a strategy to combine different kinds of quantitative high-throughput biological data into integrative pictures of cancer; (c) connect cancer genotypes and phenotypes from a mechanistic, causal, data-driven perspective; (d) provide tools to detect and investigate regulatory, feedback loop-like structures that extend across multiple biological organization levels like paracrine and autocrine loops; and (e) determine the consequences of this multi-level cross talk in the context of cancer and the immune response. In our vision, this ultimate version of the systems biology method involves iterative integration of data from clinical trials and *in vitro*/*in vivo* biomedical research using techniques of data analysis, bioinformatics, and mathematical modeling and simulation. The proposed workflow is sketched in the following paragraphs (Fig. 23.1).

STEP 1. In clinical cohorts of, for example, cancer patients *vs*. healthy individuals, high-throughput data of tissue and/or plasma concentrations for proteins, RNAs, or other molecules are collected together with biometric data from the patients. The data are processed, integrated, and analyzed using statistical models aiming to group them according to their gene expression *vs*. the progression status profiles. In this way, one can obtain cancer-associated genetic signatures relevant to the phenotype under investigation (e.g., chemoresistance, aggressiveness, metastatic potential). These signatures account for a group of genes, proteins, miRNAs, or other molecules, for which a robust statistical correlation is found between their combined expression pattern and the investigated cancer phenotype [5].

STEP 2. Relevant biomedical and clinical knowledge is gathered from databases, computational algorithms, and publications inspected via manual curation or text mining. This information is used to find feasible biochemical interactions (i.e., protein-protein interactions, transcriptional regulation, etc.) between compounds of the genetic signature, but also with other kinases, transcription factors, or microRNAs, all of them relevant to the investigated cancer phenotype. In this way, we can construct a network of cross-talked intracellular pathways relevant to the investigation of the aimed cancer phenotypes. Furthermore, similar networks can be constructed for the cell types in the tumor microenvironment related to the phenotype investigated. Since tumor cells and cells in the microenvironment secrete cytokines and other molecules signaling each other, the obtained network is one of cell-to-cell communication, accounting for the tumor-microenvironment interaction in the cancer phenotype under investigation. The network obtained is often called regulatory map, nothing but a visualization of the state of the art of the biochemical and biomedical knowledge about the cancer phenotype under investigation. Tools from network biology can be used to dissect the topology of the regulatory map and isolate regulatory motifs relevant for the derivation of hypothesis and experiments [22, 8].

STEP 3. The parts of the network relevant to the biomedical scenarios which are related to the investigated cancer phenotype are translated into a mathematical model. The model consists of mathematical equations, in an adequate

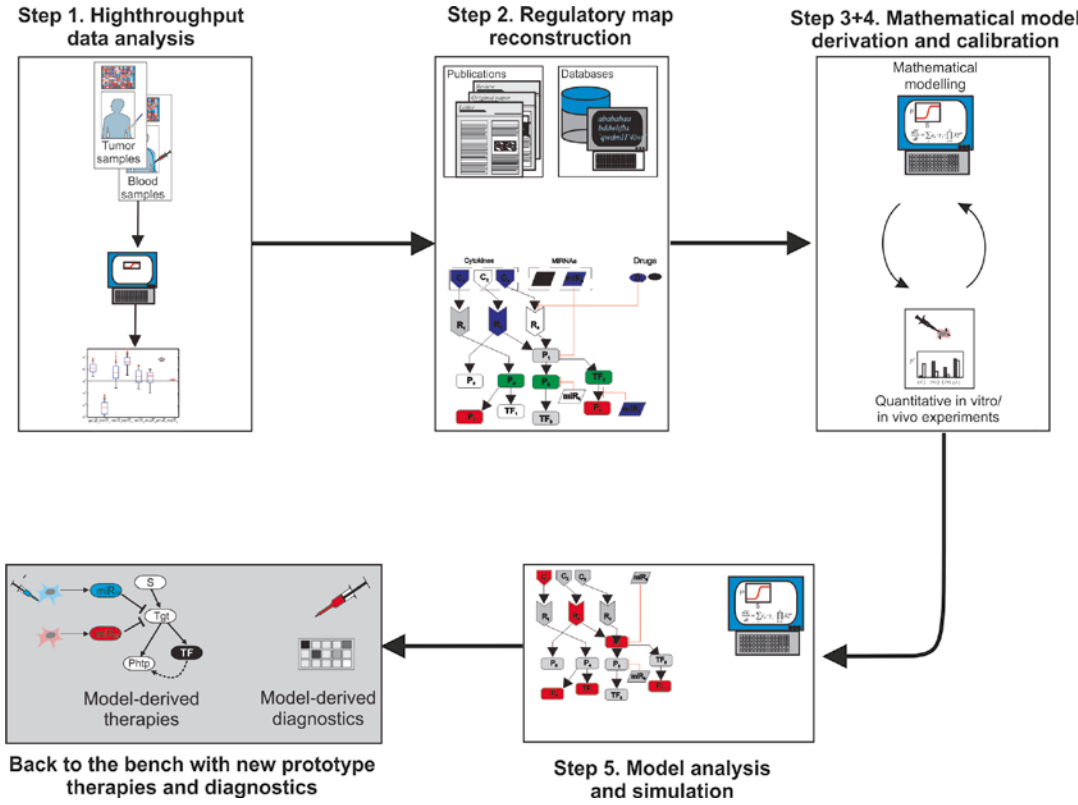

Fig. 23.1 Sketch of an advanced systems biology workflow

modeling formalism, accounting for the time evolution of the expression and/or activation status of the network compounds, as well as their connection to the phenotypes. Many modeling formalisms are available, all of which are with advantages and disadvantages [6]. To circumvent some of these disadvantages, one can combine them in hybrid models. For example, we have combined interconnected sub-modules in ordinary differential equations and Boolean logic [23]. Ordinary differential equations are excellent tools to analyze the nonlinear behavior of signaling pathways with multiple, nested feedback and feedforward loops, while logic models are an ideal representation of massive transcriptional networks. The combination of both model types allows the analysis of large-scale, nonlinear transcriptional and posttranscriptional networks and their connection to cancer cell phenotypes [23].

STEP 4. Additional quantitative *in vitro/in vivo* experimental data are used to improve the biological characterization of the model, that is, to make it more accurate in terms of prediction of the relevant biomedical scenarios. This is often called model calibration and allows assigning appropriate values to model parameters and other model features. Alternatively, this process also allows for the validation of hypothesis concerning the structure and regulation of the network in the biomedical context analyzed; in this case, iterative cycles of modeling and experimentation can be used to formulate, refine, prove, or disprove hypothesis concerning the existence and relevance of given biochemical interactions [24]. With the use of the mathematical model, one can analyze spatiotemporal regulatory features of the network that elude the elucidation via conventional experimentation, like self-sustained oscillations, or bistability.

STEP 5. In recent years, various studies have proved that a well-calibrated, data-driven mathematical model can be used with predictive purposes in the context of molecular oncology. The underlying idea is to use model simulations and other tools to assess existing therapies in a personalized manner, design new therapies, or detect sets of biomarkers for cancer prognosis. In a final step, one has to go back to the bench and design additional *in vivo*/*in vitro* experiments to confirm the model predictions. Alternatively, the model predictions can be combined with virtual screening and other techniques from computational biology and immunoinformatics and used in the process of drug discovery or vaccine development. For example, potential drug targets, identified via mathematical modeling, can be used as most promising candidates in a drug screening procedure via protein docking-based techniques [21].

23.3 Does Cancer Immunology Need a Systems Biology Approach?

In our opinion, the immune system is one of the most complex realizations of a biological system. The immune system is actually a multi-scale system (Fig. 23.2). It involves many types of cells, whose fate, proliferation, or activation status is controlled by feedback loop-regulated pathways. These pathways very often cross talk creating complex networks. Furthermore, the activation status of given immune cells depends on other immune cells by direct contact or through secretion of local or global signaling molecules, especially cytokines. In this way, the immune system is enriched in cell-to-cell communication circuits and autocrine loops. When we further consider the interaction between the immune system and a tumor, the picture becomes more systemic-like. Tumor cells and the immune cells in the surroundings communicate through chemical signals and affect each other's fate. Tumors secrete antigens (Ags) detected by immune cells like dendritic cells, while cells from the immune system secrete cytokines and antibodies (Abs) targeting the tumor cells. In addition, features of the microenvironment in which the tumor is hosted can affect the response of the immune cells. Finally, all these processes are happening at the same time and affecting each other at different biological and temporal scales. Altogether, this suggests the use of a systemic strategy to tackle the complexities of the tumor-immune system interaction. In the following section, we discuss some published results that illustrate how systems biology can be used in the context of oncology and tumor immunology.

23.4 A Quick View on Current Results

23.4.1 Computational Biology, Bioinformatics, and High-Throughput Data Analysis Used in the Design of Immune Therapies for Cancer

The availability of next-generation sequencing along with omics data shifted the paradigm for cancer treatment and opens the doors toward possible cancer immunotherapy. Like traditional vaccines that stimulate the host immune system to recognize and destroy pathogens, cancer vaccines are aimed to generate an immune response that differentiates tumor cells from the normal cells for their possible elimination. For several of the pathogenic cancers, such as cervical cancer caused by human papillomavirus; hepatocellular carcinoma caused by hepatitis B and hepatitis C virus; Hodgkin lymphoma by Epstein-Barr virus; T-cell leukemia by human T-cell leukemia virus; and Kaposi's sarcoma by Kaposi's sarcoma herpes virus, there has been considerable success in designing cancer vaccines in the past, and many of them are currently in use or in the advanced stages of clinical trials. Most of these vaccines are designed in a similar way as the traditional epitope-based vaccine-designing approaches. However, for the non-pathogenic cancer, the major challenge for the immune system is to distinguish cancer cells from the healthy cells

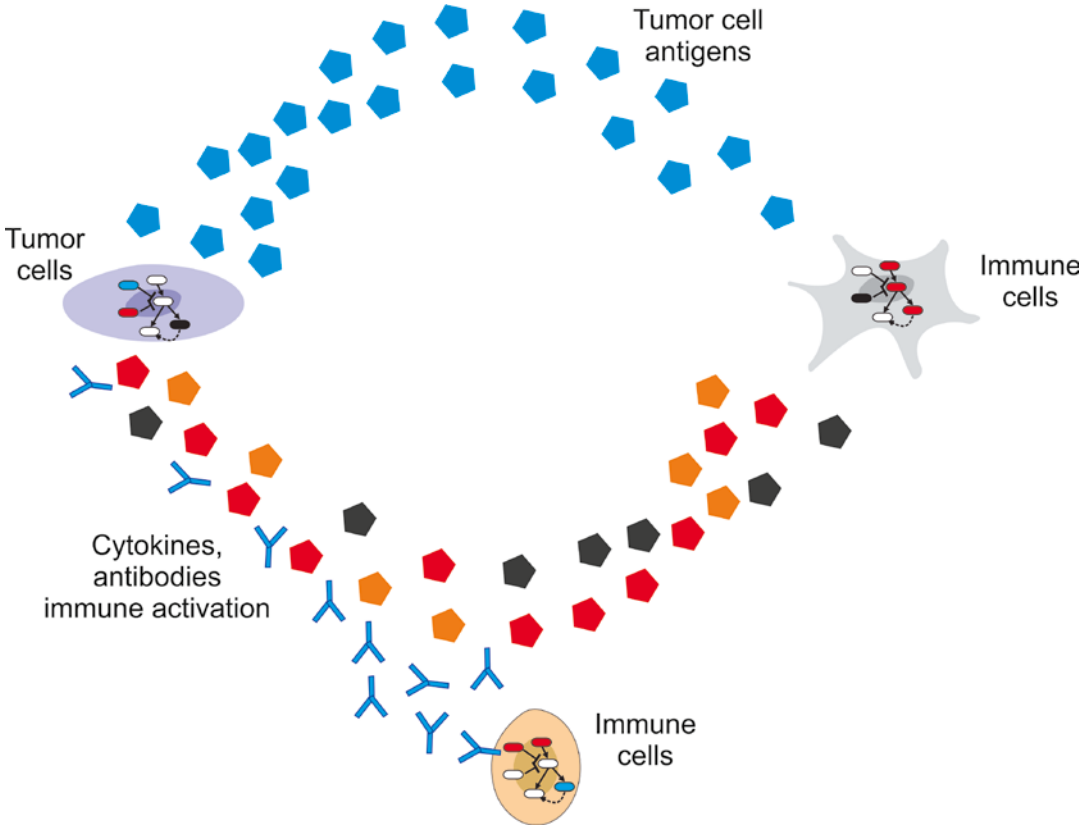

Fig. 23.2 Tumor-immune cells interaction envisioned as a multilevel system. The immune system involves many types of cells, whose fate is controlled by feedback loop-regulated pathways, but also some immune cell types affect the activation of others by direct contact or local/global signaling molecules. Furthermore, the immune system and the tumor are affected by cell-to-cell communication circuits involving tumor antigens and immune cell-secreted antibodies and cytokines

in order to activate B lymphocytes to produce Abs or T lymphocytes. In order to trigger antibody-dependent cellular cytotoxicity or phagocytosis to kill cancer cells, these Abs need to recognize specific proteins normally on the outer membrane of the cancer cells [25]. T lymphocytes have the capacity to selectively recognize peptides (antigens) derived from self/ nonself proteins attached with major histocompatibility complexes on the antigen-presenting cells (APCs). The use of cytotoxic T cells (CTLs), dendritic cells (DCs), and monoclonal antibodies is now a well-established strategy to design potential cancer immunotherapeutics [26].

The major challenge in the development of cancer vaccines is that Ags normally recognized by the immune system are expressed as the "self"-Ags to which the immune system is already tolerized. Therefore, the potential approach is to identify non-tolerogenic, tumor-associated antigens (TAAs) suitable to develop Ag-specific anticancer vaccines [27]. In spite of success in other infectious diseases, the use of small self-peptides as Ags in cancer vaccines did not attain much interest in the past because of their poor immune response and minimal therapeutic benefits. Most of these free peptides are likely to have a short half-life and poor pharmacokinetics properties and are thus rapidly cleared before they can be loaded on the dendritic cell surfaces in the complex with MHC molecules to stimulate $CD8^+$ and $CD4^+$ T cells for the initiation of adaptive immune responses [28]. However, the coadministration of suitable dendritic

cell-activating adjuvant along with short TAA peptides was shown to boost immune responses in advanced melanoma [29] and vulvar intraepithelial neoplasia patients [30]. These studies generated the hope to design effective therapeutic cancer vaccines.

In order to avoid the "self"-recognition that normally results in the weakened immune responses for cancer vaccines, researchers have validated the use of DNA vaccines in preclinical studies where the tumor-derived sequences were initially fused with the genes encoding microbial proteins [31]. This strategy helped T helper cells in the induction of Abs against tumor Ags along with epitope-specific antimicrobial $CD8^+$ T cells. Another example PROSTVAC, a DNA vaccine for prostate cancer, which includes recombinant vaccinia virus encoding prostate TAAs along with adhesion molecules and DCs stimulators, is already in the clinical trial phase III [32]. Besides, several monoclonal antibodies (mAbs) and other small molecules such as kinase inhibitors, angiogenesis inhibitors, proteasome inhibitors, and molecular receptor blockers are also combined with immunotherapy for developing targeted anticancer therapies [33]. Many Abs boost the immune response against cancer cells. Ofatumumab and ipilimumab are two such mAbs recently approved by the US FDA. While ofatumumab targets CD20 protein which inhibits early-stage B-lymphocyte activation in chronic lymphocytic leukemia [34], ipilimumab specifically targets cytotoxic T-lymphocyte-associated antigen 4 (CTLA 4) that provides inhibitory signal for activated T cells [35]. Unconventionally, mAbs are also shown to target intracellular oncoproteins; this finding opens a new possibility to predict potential targets for TAA discovery [36, 37].

Still, the detection of effective non-tolerogenic TAAs from extra-/ intracellular oncoproteins is one of the major challenges in cancer immunotherapy. To recognize TAAs, one has to carefully investigate sites for cancer-specific point mutations, chromosomal aberrations, splicing variants, alternative reading frames along with overexpressed genes, and other regulatory elements (transcription factors, miRNAs, etc.) [38–40]. For many of these data mining approaches, well-established computational pipelines already exist in the public domain. For therapeutic cancer vaccines, the idea is to either amplify or induce new immunogenic responses in cancer patients based on $CD8^+$ or $CD4^+$ T-cell responses by recognizing differentially expressing TAAs from microarray data repositories [41]. One of such database is Oncomine, which has a huge repository of gene expression profiles from microarray studies to identify differentially expressing genes in various stages of major types of cancer [42]. These data analysis pipelines facilitate the discovery of novel cancer biomarkers and drug/vaccine candidates. In the following section, we will describe the use of bioinformatics tools and computational pipelines to discover potential cancer vaccine candidates with a case study.

23.4.1.1 Case Study: Computational Approaches to Design DNA Vaccine for Cervical Cancer Caused by Human Papillomavirus

Cervical cancer is the most common and slow-growing malignant cancer present in the tissues of the cervix or cervical area in women. Persistent infection with human papillomavirus (HPV) is considered to be one of the major etiological factors for cervical cancer [43]. More than 100 different types of human papillomaviruses (HPV) have been identified [44] and categorized into high-risk and low-risk strains. A total of 16 different high-risk strains have already been identified, among them strain 16 and 18 are together responsible for approximately 70 % of all cervical cancer cases [45]. Two HPV vaccines GARDASIL and CERVARIX are currently in use as prophylactic vaccines and offer no therapeutic benefit for patients already infected with the virus or those with precancerous lesions or cervical cancer [46]; also they are not completely effective against all high-risk strains of this virus. In contrast, therapeutic vaccines generate a T-cell immune response to eliminate existing viral infection. Epitope-based vaccines provide a specific strategy for prophylactic and therapeutic

application of pathogen-specific immunity. The identification of epitopes suitable for diagnostic use and for therapeutic or prophylactic intervention is clearly a crucial prerequisite of these strategies. The selection of immunogenic, consensus, and conserved epitopes from proteins of major high-risk strains may provide an experimental basis for the design of very specific T-cell and DNA vaccines effective against all high-risk strains. Herein, we will highlight the computational pipeline adopted in one of our previously published research works which was used to design in silico DNA vaccines against (HPV) by using consensus epitopic sequences of L2 capsid protein from all high-risk HPV strains [47]. In addition, various computational parameters were optimized to increase the immunogenicity of the vaccine by considering multiepitopic sequences, codon optimization, CpG motifs optimization, and inclusion of promoter and other immune-stimulatory molecules. A generalized computational pipeline for the design of DNA vaccine is highlighted in Fig. 23.3. The work initiates with the detection of differentially expressing genes in cancer (non-pathogenic) or the identification of conserved immunogenic regions from pathogens involved as the major etiological agents. From the conserved regions, MHC class I and class II epitopes are predicted followed by the inclusion of proteosomal/lysosomal cleavage sites. Various computational approaches may follow to filter the immunogenic peptide such as 3D structure modeling to calculate the solvent accessibility of cleavage sites, post cleavage conservancy of epitopes, and then long half-life for proper immunogenicity using molecular dynamics simulations. The selected peptide can then be back-translated and optimized for codons and CpG motifs. In silico cloning experiments may also be performed for the selection of good expression systems to be used for vaccine development.

Retrieval of Sequence Data and Identification of Conserved Regions in the Protein

In case of previously designed HPV vaccines, researchers thoroughly investigated L1 and L2 capsid proteins form the virus to detect potential vaccine candidates. Some of the previous *in vitro* neutralization studies demonstrated high cross-reactivity with L2 antisera. We retrieved HPV L2 capsid protein sequences for various strains from the NCBI (http://www.ncbi.nlm.nih.gov) and the UniProt (http://www.uniprot.org) database. To identify conserved regions in the protein, we performed multiple sequence alignment using the ClustalX software. Based on the multiple alignment files, we identified conserved regions in the L2 capsid proteins using the Shannon entropy function available on the Protein Variability Server (http://imed.med.ucm.es/PVS). From the alignment file, Shannon entropy is calculated as

$$H = -\sum_{i=1}^{M} P_i \log_2 P_i$$

where P_i is the fraction of residues of amino acid type i and M is the number of amino acid types.

To identify the conserved regions in the L2 capsid proteins of all high-risk HPV strains, the cutoff score for the Shannon entropy was set to 2.0 (Fig. 23.4). The fragments with Shannon variability score $\leq$2.0 and continuous length of $\geq$9 amino acid residues were further selected for the epitope identification.

Prediction of MHC Class-I and Class-II Epitopes

Epitope mapping is always the key step in vaccine designing. Epitopes are usually thought to be derived from nonself protein Ag that interacts with Abs or T-cell receptors thereby activating an immune response. Besides nonself proteins, epitopic sequences from the host can also be recognized by MHC molecules. For an effective vaccine, it is important for the epitopes to invoke strong response from T and B cells. A large number of bioinformatics algorithms were designed for this purpose, such as Position-Specific Scoring Matrix (PSSM)-based SYFPEITHI [48], Artificial Neural Networks (ANN) [49], Stabilized Matrix Method (SMM) [50], and Average Relative Binding (ARB) [51]. In this work, we used the RANKPEP server (http://imed.med.ucm.es/Tools/rankpep.html) for the prediction of consensus binding epitopes (9 mers)

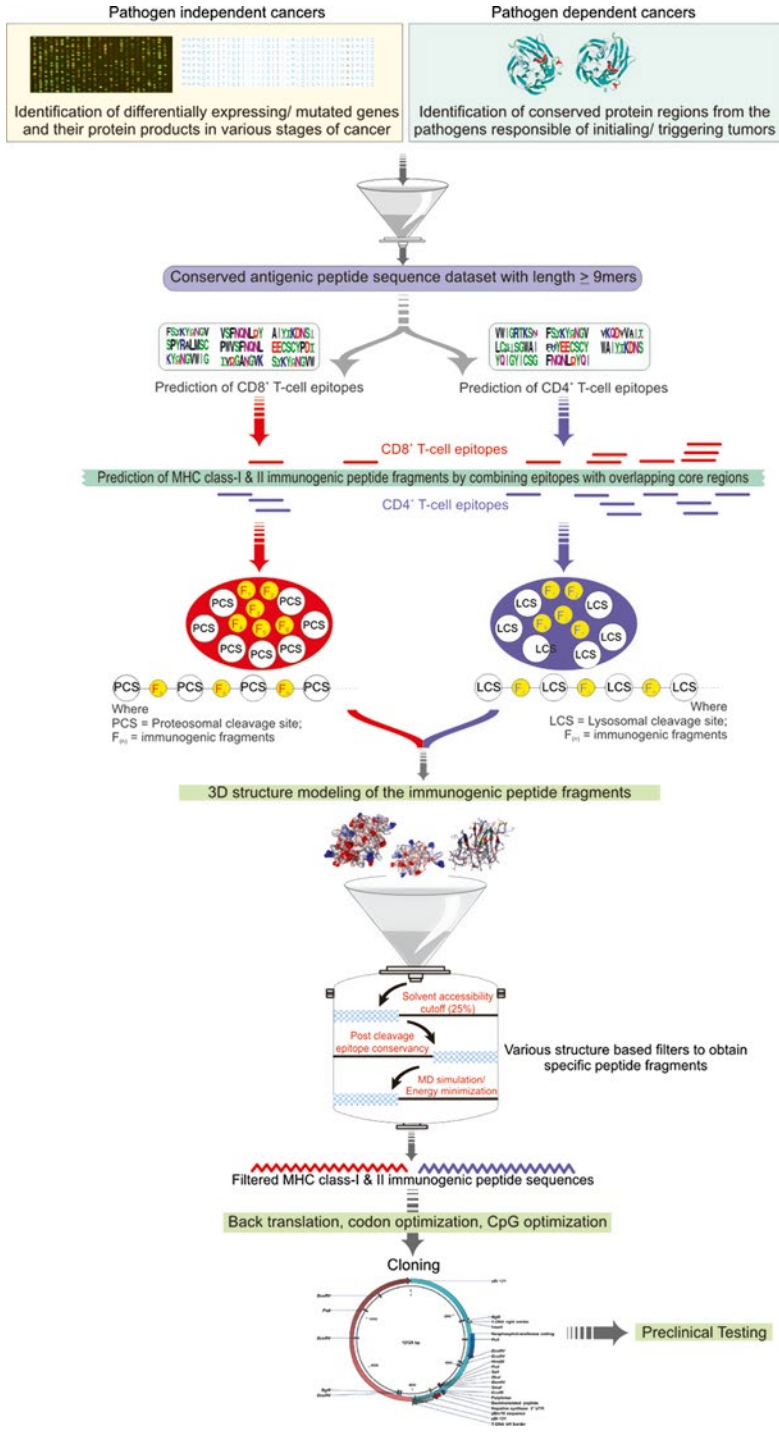

Fig. 23.3 Generalized workflow for computer-aided epitope-based DNA vaccine design

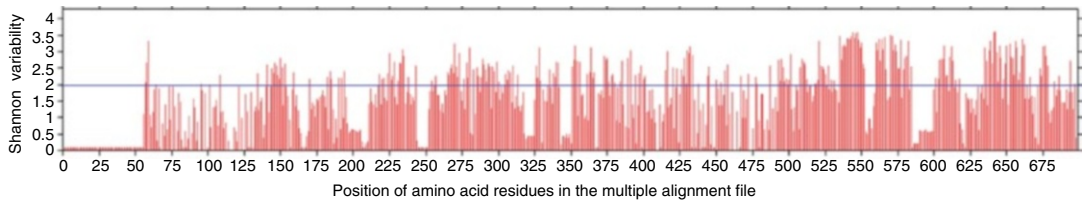

Fig. 23.4 Figure showing the Shannon variability score of individual positions in the multiple alignment files of L2 capsid protein from high-risk HPV strains. *Red bars* indicate the variability score of amino acid residue *i* at the given position in the multiple alignment file. *Blue line* represents the cutoff Shannon variability score. All the *red bars* below the *blue line* are potential conserved sites for analysis

for both MHC class-I and class-II molecules with default parameters. In total, we used 75 MHC class-I and 49 for MHC class-II matrices for the prediction of potential epitopes from all the consensus L2 capsid proteins.

Reverse Translation of Immunogenic Peptide Fragments

To back-translate a peptide sequence into the DNA sequence, a large number of bioinformatics tools are available in the public domain. Because of the degeneracy of the genetic code, the back-translation is ambiguous as most amino acid residues are encoded by multiple codons. To design an optimal DNA sequence, most of these tools use a codon frequency table specific for the organism of interest. We used Backtranseq program of mEMBOSS 6.0.1 for this purpose.

Optimization of Codons and CpG Motifs

Codon optimization is the process to enhance the efficiency of DNA expression vector to express the foreign gene in the host's cell environment. DyNAVacS server (http://miracle.igib.res.in/dynavac) was used to compute the optimal codon for each of the amino acid residue encoded by the stretch of DNA. The server optimizes codons according to the codon usage table derived from the Kazusa Codon Usage Database (http://kazusa.or.jp/codon). We used a codon frequency table for *Homo sapiens* that ranks codons by analyzing their frequency of occurrence in 93,487 coding sequences [52]. Immunogenicity of Ag-specific DNA vaccine was previously shown to significantly increase by the optimization of CpG motifs [53]. We again used the DyNAVacs server for CpG optimization [54]. In this process, the consensus motif XCGY (where X is any base but C and Y is any base but G) was incorporated in the sequence as triplet (XCG or CGY) by substituting the less frequent codons that codes the same amino acid residues.

Insertion of Cleavage Motifs and Finalization of DNA Sequence

For the purpose of generating specific epitopes, proteasomal and lysosomal cleavage motifs were also included before and after each MHC class-I and class-II epitope, respectively. These cleavage motifs are targeted by the proteasomal and lysosomal cleavage machineries to generate immune responses in the host. The corresponding nucleotide sequence of the 12-residue long peptide HEYGAEALERAG was added as proteosomal cleavage motif before and after the optimized DNA sequence of each MHC class-I epitope. The HEYGAEALERAG motif contains all five cleavage sites Y3-G4, A5-E6, A7-L8, L8-E9, and R10-A11 defined for eukaryotic proteasomes in which A5-E6 is the major cleavage site [55]. Similarly, the nucleotide sequence of the 5-residue long peptide KFERQ was added as lysosomal cleave motif before and after the DNA sequence of each MHC class-II epitope. KFERQ specifically acts as a recognition motif toward heat shock proteins and facilitates further steps for the degradation of proteins by lysosomes [56] to generate MHC class-II epitopes. At the end, start and stop codons were added to finalize the DNA vaccine. Arrangement of the epitopes is very crucial and one of the deterministic factors for the efficacy of the DNA vaccine. The folding

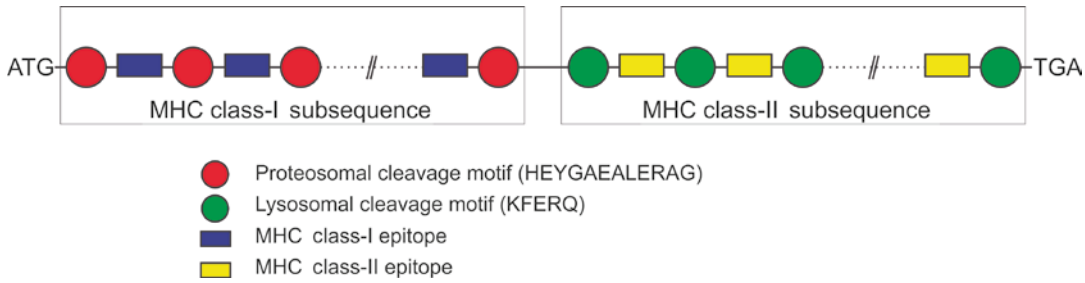

Fig. 23.5 Arrangement of various segments of DNA vaccine constructs. The arrangement of epitopes in the sequence is very crucial to increase the efficacy of DNA vaccine

of the protein product in the host will largely depend on the arrangement of these epitopes and also determine the solvent accessibility of the cleavage motifs. Various computational tools can be used for this purpose including molecular dynamics simulation approaches. The overall arrangement of the DNA vaccine construct is shown in Fig. 23.5.

In Silico Cloning Experiments of DNA Vaccine Construct

Several expression systems have been successfully designed in the past, for the cloning of the number of genes encoding surface antigens from pathogens to facilitate vaccine development. A good DNA vaccine vector should be designed with minimal functions so that the only gene expressed in mammalian cells is the *antigen-encoding* gene. We performed the cloning experiments using clc-DNA Workbench 5.0.1. For our purpose, the pVAX1 vector was selected as an expression system. pVAX1 is a nonfusion vector specifically designed to stimulate cellular as well as humoral immune responses [57] and requires that the inserted gene of interest contains the Kozak translation initiation sequence, an initiation codon (ATG), and a termination codon (TAA, TGA, or TAG). When this designed DNA vaccine is injected into the host, the antigenic protein gets translated and alerts the body's immune system to generate immunization memory cells.

The methodology described above highlights how various bioinformatics algorithms and computational tools can be combined to design novel and effective vaccine candidates before being subjected to *in vitro* confirmatory studies.

23.4.2 Mathematical Models Used in Basic Oncology Research

23.4.2.1 Pathways and Networks

The successful use of systems biology to elucidate the regulation and function of cancer-related pathways is well proved by a large body of literature published in the last decade. In this context, mathematical modeling has been used to investigate the time-dependent behavior of biochemical systems, to integrate multiple data sources, or to validate the existence of new regulatory or transcriptional interactions in given regulatory pathways. A question in biochemical networks for which data-driven mathematical modeling is necessary is the elucidation of the nonlinear properties emerging from the combination of regulatory motifs containing positive/negative feedback and coherent/incoherent feed-forward loops. When biochemical pathways or networks hold these regulatory structures, they often display behavior that evades direct reasoning. Many papers, which use a data-driven modeling approach, succeeded proving how signal amplification [11], sustained oscillations [58], or bistability [59] emerged as hallmarks of signaling and transcriptional networks.

To mention an example on immune-related pathways, Das and colleagues [60] integrated different modeling approaches with *in vitro* experiments to elucidate the interplay between Ras

activation and SOS proteins in the activation of T and B lymphocytes. What makes their work interesting is that both proteins, Ras and SOS, are integrated in a positive feedback loop that participates in the Ag receptor stimulation of lymphocytes. In this feedback loop, Ras gets strongly activated upon membrane receptor stimulation, a process which is mediated by members of the SOS family. In turn, SOS activity at the plasma membrane is allosterically upregulated by active RasGTP. To validate the existence of this positive feedback loop and its functional consequences, the authors combined model simulations and time-dependent *in vitro* experiments with human and chicken lymphocytic cell lines. They found that under some stimulatory conditions, the biochemical system displays bistability. That is, for high doses of stimulus, the pathway works like an all-or-nothing system: transient but intense stimulus can trigger a sustained activation of the system and the downstream pathway. When we consider a population of lymphocytes, this property may induce the emergence of a bimodal response, with a subpopulation of lymphocytes getting full and sustained activation, while others remain inactive. From an immunological perspective, the authors hypothesize that this system induces the emergence of a short-term mechanism of molecular memory. This mechanism can improve the activation of T lymphocytes which were stimulated in previous serial encounters with rare antigen-bearing cells.

In the study by Das et al. [60] the focus was to elucidate the dynamics of a small signaling system containing regulatory loops. In other cases one tries to address how several pathways cross talk to each other and integrate their signals to achieve the regulation of given phenotypic responses. This has also been explored using mathematical models of large regulatory networks in the context of cancer [61] and immunology [62]. For example, Carbo and collaborators [63] used a systems biology approach to investigate the regulation of the pathways underlying $CD4^+$ T-cell differentiation. By collecting and organizing the state of the art of biomedical knowledge, they constructed a comprehensive regulatory map of the critical pathways regulating the differentiation of naïve $CD4^+$ T lymphocytes into Th1, Th2, Th17, or iTreg. The regulatory map was translated into a mathematical model in ordinary differential equations and characterized using perturbation experiments, in which different concentrations of relevant cytokines were used to stimulate the shift between different signaling and transcriptional pathways and therefore the distinctive differentiation of the naïve T cells. Once the model was calibrated and validated, model simulations and sensitivity analysis were combined to determine the model parameters controlling the activation of different pathways. They found that the pathway regulating the nuclear receptor PPARc function plays a major role controlling the shift between the Th17 and iTreg transcriptional and phenotypic programs. Based on these findings, they foresee a therapeutic potential to the regulation of PPARc signaling in the context of chronic inflammatory and infectious diseases. In this way, the authors show how a full systems biology strategy can be extremely useful to dissect the signaling and transcriptional networks controlling differentiation and plasticity of immune cells.

23.4.2.2 Genotype-Phenotype Mapping

Mathematical models can be used to bridge the gap between intracellular pathways and the cellular phenotypes they regulate. In this case, the idea is to develop mathematical models that consider how genetic or epigenetic changes in critical cancer-related pathways can affect the fate of tumor cells and trigger (or disrupt) phenotypic responses at the cellular level. Some authors call this the genotype-phenotype mapping [64].

We have recently applied this idea to investigate the deregulation of critical cancer signaling-transcriptional networks during the emergence of a phenotype of chemoresistance ([8], see Fig. 23.6). To this end, we constructed a data-driven mathematical model in ordinary differential equations (ODEs) accounting for an intracellular network around E2F1, a transcription factor involved in abnormal cell proliferation, apoptosis, and chemoresistance. The network included the interaction of E2F1 with

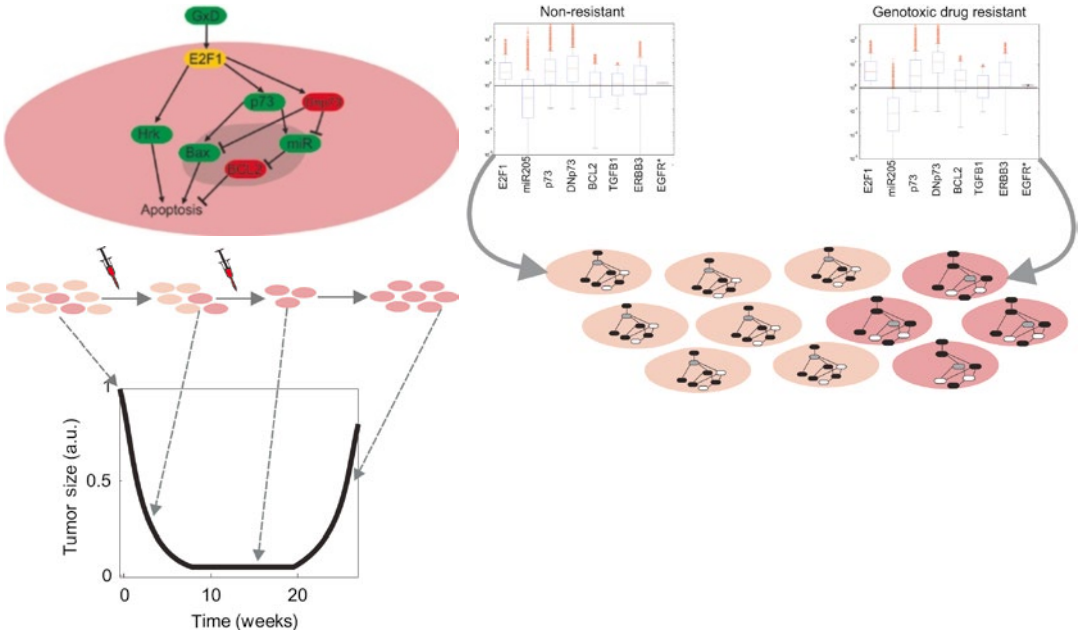

Fig. 23.6 Model-based genotype-phenotype mapping: modeling genetic signatures promoting chemoresistance. In Vera et al. (2013) [8], we derived a data-driven model in ODEs accounting for an intracellular network around E2F1, involved in cancer resistance to genotoxic and cytotoxic drugs. We connected the network model with an additional equation describing the size of a population of tumor cells whose response upon anticancer drugs administration was controlled by the E2F1 network. Model simulations of heterogeneous tumors predicted that genotoxic drugs can favor the selection of subpopulations of chemoresistant tumor cells

two isoforms of *p73* and the microRNA miR-205, as well as a number of transcriptional targets whose regulation after anticancer drug administration is controlled by these E2F1/p73/miR-205 networks. To make the genotype-phenotype mapping possible, we connected our model with an additional equation that describes the size of a population of tumor cells whose response upon stimulation with anticancer drugs was controlled by the E2F1-centered network. In this equation, basic phenotypic traits of the modeled cells like proliferation or death rate were connected and therefore controlled by the E2F1-regulated transcriptional targets. These transcriptional targets represent the triggering of proliferative, apoptotic, or antiapoptotic programs in the model.

This equation computationally connects the genome of cancer cells with their phenotypic response by linking the expression of intracellular network components to the dynamics of the tumor cell population. We could simulate tumor heterogeneity by considering several subpopulations of tumor cells, each one of them represented with a set of model equations. Using model simulations, we detected genetic signatures for the network that conferred resistance to either genotoxic or cytostatic drugs and even double drug resistance. Furthermore, our model predicted that genotoxic drugs, when applied to heterogeneous tumors, can favor the selection of subpopulations of chemoresistant tumor cells.

23.4.2.3 Multi-scale Modeling

In a more refined version of the previous strategy, systems biology and data-driven modeling can be used to account for spatial features of tumor organization and the interaction of the tumor with the surrounding microenvironment. This is the rationale for the so-called cancer multi-scale models, which has been successfully used in the last years to investigate the detailed dynamics of tumor growth or angiogenesis [17]. In the recent literature, there are several excellent reviews about the topic [65], as well as a number of

examples of cancer multi-scale models [16, 66], many of which referred to angiogenesis.

To mention an example with a cancer immunology focus, Pak and coauthors [67] derived a mathematical model to investigate features of the delivery of recombinant immunotoxins, a family of new molecules with anticancer activity. They are composed of an Ab fragment targeting specific tumor cell Ags and a protein toxin fragment, which is released and triggers cytotoxic effects upon recognition, internalization, and processing of the molecule. The authors derived a mathematical model that links recombinant immunotoxin dosing and changes in tumor volume. In the model, a tumor is divided into a series of spherical subunits that contain a blood vessel and a number of tumor cells surrounding it, which can be present as normal, intoxicated, or dead tumor cells. For each one of these structures, the model contains a set of differential equations accounting for the dynamics of immunotoxin, from its release from the blood vessel until its internalization in a tumor cell, which becomes intoxicated. In this way, the model accounts for the amount of immunotoxin released, present, and degraded in each tumor subunit. The other part of the model describes the dynamics of tumor cell populations existing in the subunit. This part of the model considers processes like cell growth, immunotoxin-related death, and cell migration to occupy the space cleared after the death of highly intoxicated cells. Using model simulations, Pak and colleagues found that Ag shedding, a key mechanism in the dynamics of tumor-specific surface Ags, is critical for the success of the therapy. Using model simulations, they found that Ag shedding homogenizes the distribution of the immunotoxin in solid tumors, therefore increasing the efficiency of the therapy.

23.4.2.4 Mathematical Models Used to Assess and Design Therapies

Previous results illustrate the potential of systems biology and data-driven modeling to explore the structure, function, and regulation of biochemical networks, as well as their interplay with cancer-related cell and tissue phenotypes. In addition, systems biology can play a major role in translational medicine, providing tools for clinical data integration, as well as for design, assessment, and personalization of anticancer therapies [68, 69]. In the following, we illustrate these possibilities with several recent examples.

Assessment of Conventional Therapies

A very promising use for systems biology is the personalized assessment of anticancer therapies. The literature contains many recent works illustrating how data-driven modeling can be used to maximize the efficiency of current therapies but also to detect patient subpopulations for which they are not suitable. For example, mathematical models can be used to determine under which conditions a conventional therapy: (a) is toxicologically safe [70, 71], (b) does not induce further resistance [8, 72], and (c) can be combined with other therapies [8]. Furthermore data-driven models can be used to establish the drug dosage and timing that optimizes the anticancer effect and/or reduce toxicity [73].

For example, Engel and collaborators [70] made use of data-driven mathematical modeling to look for the optimal administration dose and timing of several conventional anticancer drugs minimizing the risk of acute neutropenia, a side effect of anticancer therapy in malignant lymphoma and other cancers. What makes therapy-associated acute neutropenia important for cancer patients is that they get a drastic reduction of neutrophil blood levels, which makes them more vulnerable to bacterial infections and increases the risk of life-threatening sepsis. Engel and coworkers derived, characterized, and tested a quantitative data-based ODE model that describes the generation, proliferation, and differentiation of neutrophils and other human granulocytes. The model was extended to account for the changes in the granulocyte dynamics suffered by patients with lymphoma and treated with cytostatic drugs and recombinant GSCF, an adjuvant therapy that stimulates granulocyte production and accelerates the recover from neutropenia. The model was characterized with patient data obtained from several large randomized clinical trials, in which

efficacy and safety of multidrug chemotherapies were assessed. The obtained model describes precisely the time response of white blood cell levels for ten different therapeutic regimes. Furthermore, the authors suggest that the model can be used as a predictive tool, able to assess the safety of other non-explored conventional anticancer drugs regimes. Although the model was characterized with data from patients suffering malignant lymphoma, they claim the model can be adapted to assess the risk of therapy-associated neutropenia in other tumor entities.

This idea can be extended to other conventional anticancer therapies. For example, Ribba and colleagues [71] developed a multi-scale model to investigate the effect of some tumor features in the efficiency of radiotherapy. The authors constructed a model for colorectal cancer progression that links cell cycle progression, DNA damage level, and other signaling pathways to the sensitivity of individual cells to the irradiation doses. Their model integrated four modules, implemented using different modeling frameworks. Some of the key features of the model are: (a) it includes regulatory pathways controlling cell cycle, cell division, and apoptosis; (b) these pathways are connected with the fate of individual tumor cells and actually control tumor cell death and proliferation; (c) the model also considers the spatial structure of the tumor, that is, how cells get distributed and interact with the tumor microenvironment through gradients of growth and antigrowth factors and hypoxia; (d) additional model equations describe how different irradiation dosing (time and dose) triggers DNA damage in proliferative tumor cells. When they simulated radiotherapy administration with their model, they found that the efficacy of conventional irradiation protocols can be improved if the cell cycle-regulated dynamics of tumor growth is considered when planning the schedule of irradiation sessions. This result is in line with others suggesting similar optimal schedules of chemotherapy sessions, something known as cancer chronotherapy [74].

Design of New Chemo- and Immune Therapies

Systems biology has become a valuable approach to boost the procedure of drug discovery and the design of combined therapies that integrate conventional and targeted chemotherapy. The underlying idea is to combine predictive model simulations, sensitivity analysis, and other advanced model-based computational methods to help detect single or combined potential drug targets. These model-obtained potential drug targets can later direct the search from new drugs [21, 75, 76]. In a quite remarkable example of this strategy, Schoeberl and colleagues combined high-throughput and time series data with mathematical modeling of the receptor tyrosine kinase signaling family to detect new anticancer drug targets [12, 77]. They derived, calibrated, and validated an ODE-based mathematical model describing the known features of the ErbB/PI3K signaling network in the context of cancer progression. Predictive model simulations were combined with computational sensitivity analysis to identify which members of the ErbB family have a major effect in the activation of AKT signaling in cancer cell lines. They later synthesized a human monoclonal antibody that inhibits the phosphorylation and subsequent activation of their top one model-detected drug target, the ErbB3 receptor. The model predictions were validated by showing that this antibody stops the growth of human tumor xenografts in mice models. Interestingly, the team is entirely composed of researchers from a biotech company devoted to the use of systems biology in drug discovery (Merrimack Pharmaceuticals, Cambridge, USA).

This strategy has also delivered some interesting results in the context of immune anticancer therapies. Kim and Lee [78] used data-driven modeling of the lymph node-tumor interaction to analyze whether preventive vaccination with cytotoxic T lymphocytes (CTLs) can be employed to promote the clearance of microtumors before clinical detection (Fig. 23.7). Toward this end, they derived a hybrid mathematical model composed of two

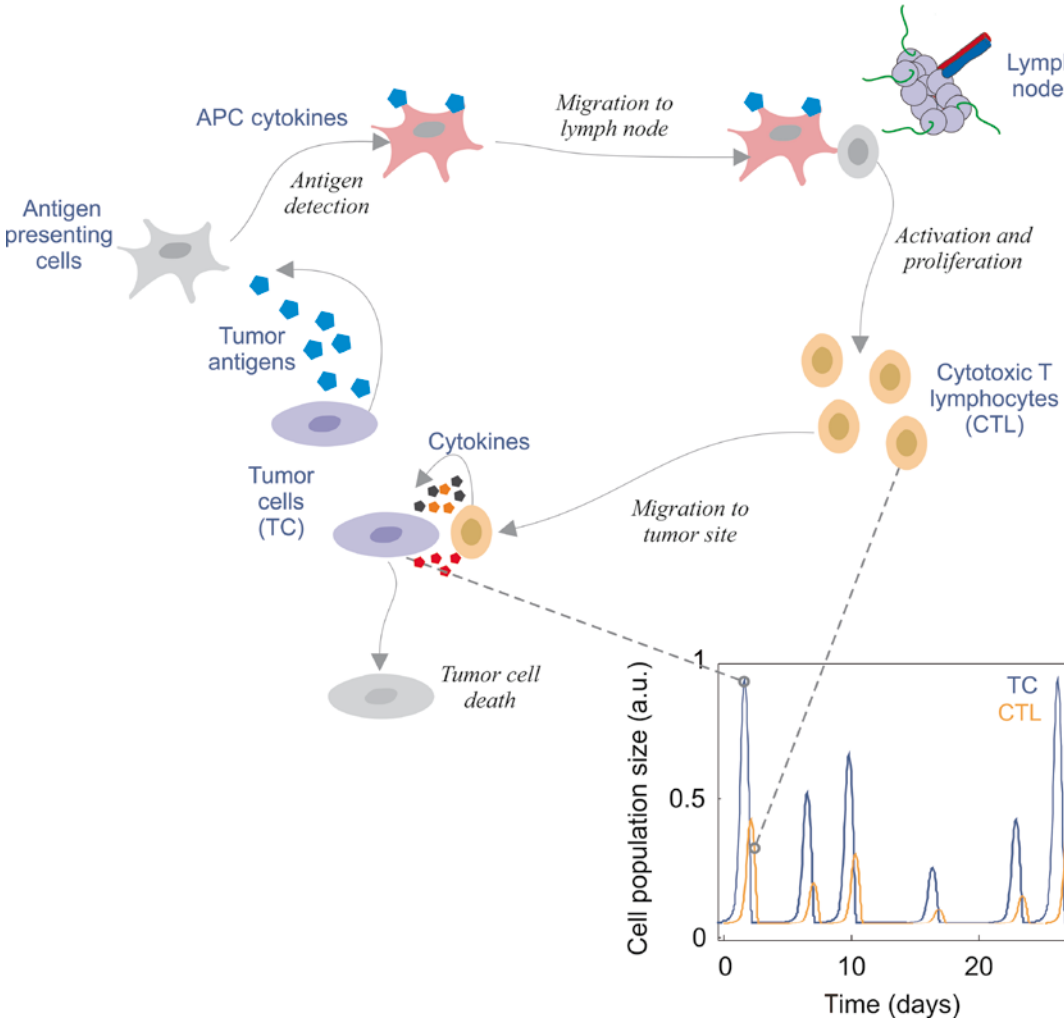

Fig. 23.7 Data-driven modeling of the lymph node-tumor interaction and the clearance of microtumors with cytotoxic T-lymphocyte (*CTL*) vaccination. The model describes the dynamics of CTL activation, including tumor Ag production, its detection by antigen-presenting cells, and the activation of cytotoxic T lymphocytes by the matured antigen-presenting cells. In addition, the model describes tumor cell detection by CTLs and CTL-mediated tumor cell death. The model can simulate variations over time for the populations of the different immune cells and the tumor cells

interconnected modules. The first module describes the dynamics of CTL activation, including the tumor antigen production at the tumor site, its detection by antigen-presenting cells, and the subsequent maturation and their migration to the lymph node. Furthermore, the module includes the activation of CTL by the matured APCs and its subsequent proliferation, maturation and migration, as well as the emergence of memory T cells. The second module describes the interplay between active cytotoxic T lymphocytes and tumor cells, including tumor cell detection, recruitment of additional CTLS, and CTL-mediated tumor cell death. The model was characterized using data from breast

cancer. The authors used the mathematical model to determine a threshold in the size of the anticancer memory CTL pool able to promote an effective clearance of microtumors. Furthermore, the model predictions attribute an important role in the success of the immune response to the rapidity in which CTLs detect the tumor site. Paradoxically, the model simulations suggested that tumors with fast growth rate are more prone for CTL destruction due to the faster production of tumor antigens and, hence, faster detection by CTLs.

Unconventional Therapies

A fascinating option with data-driven mathematical modeling is to explore therapies inspired in not yet experimentally proven concepts and ideas. In this sense, modeling is used to formulate new hypothesis on the origin and progress of cancer, as well as to foresee how one could derive new therapies based on this. In the recent literature, there are some examples of this procedure [79, 80]. In a series of recent papers, Gatenby and coworkers hypothesized that adaption to chemotherapeutic agents has an energetic cost for cancer cells, and this can be exploited to design anticancer therapies [80, 81]. In fact, the starting point of their hypothesis is that chemoresistant cells need additional energetic resources to keep working the resistance mechanisms against drugs. Their adaptive therapy relies on considering the existence of several coexisting subpopulations of cancer cells in the tumor, with different genetic and phenotypic backgrounds regarding chemoresistance. In their hypothesis, one can favor the proliferation of chemosensitive cells by manipulating the timing and dose of conventional chemotherapy, in a manner which these cells can effectively compete with chemoresistant ones for space and resources and delay the development of a fully resistant tumor. To substantiate their hypothesis, they have derived a series of *in vitro* data-driven mathematical models, which describe the growth of tumors composed by chemosensitive and chemoresistant cancer cell subpopulations. For the most updated version of the model, they performed *in vitro* experiments under conditions of normal growth and genotoxic drug administration using either normal MCF-7 cell lines or mutant cell lines overexpressing proteins involved in the efflux of anticancer drugs. Using data from these experiments, they characterized the rates of growth and drug sensitivity of both tumor cell subpopulations in the model. Later, model simulations were performed to analyze the tumor growth rate when different versions of their adaptive therapy were used; they compared the results with the tumor growth rate under conventional genotoxic chemotherapy. They found that the combination of their adaptive therapy (which tunes the timing and dose of conventional chemotherapy) with the administration of non-chemotherapeutic membrane pump substrates (a kind of "competitive" inhibitors of drug efflux) and 2-deoxyglucose (an inhibitor of glucose transporters and glycolysis) provokes a fourfold increase in the progression-free survival in their computational models.

23.5 Concluding Remarks

Systems biology emerged a decade ago as a methodological approach that combines quantitative experimental data, mathematical modeling, and other tools from computational biology, aiming to understand the regulation of these complex biochemical systems. The interaction between tumors and the immune system is not an exception to this scenario. The immune system is by definition a multi-scale system not only because it involves biochemical networks that regulate the fate of immune cells but also because immune cells communicate with each other by direct contact or through secretion of local or global signals. Furthermore, tumor and immune cells communicate, and this interaction is affected by the features of the microenvironment in which the tumor is hosted. Altogether, we are envisioning a complex multi-scale biological system, whose analysis requires a systemic view to succeed integrating massive amounts of quantitative experimental data coming from different temporal and spatial scales.

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Principles of Immunological Diagnostic Tests for Cancers

24

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Contents

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24.1 Introduction

Through the use of deliberate mutation of immunoglobulin genes, the immune system has evolved the ability to produce antibodies (Abs) able to bind targets with exquisite specificity (i.e., recognition of ONLY the target) and impressive affinity (i.e., strong binding to the target). These abilities explain why Abs remain an invaluable tool for the detection and measurement of biological phenomena and already represent some of the treatment modalities of the present and near future. While most of the work with Abs is currently *ex vivo*, their use *in vivo* has already shown significant progress and benefits. Antibodies are currently used for biosensing of specific targets in the body, in order to deliver radioactive isotopes or cytotoxic drugs (reviewed in Ricart and Tolcher [1]). Antibodies have also been used for visualizing specific biological processes such as tumor shrinking and tumor growth [2–5] or to aid in the imaging of tumors. These types of applications for antibodies will likely become more common as immunoglobulin engineering becomes more sophisticated, increasing the potential of using Abs *in vivo* for the targeting of specific lesions or tumors or even for the neutralization of specific biological processes. In the meantime, Abs are widely used in multiple formats and platforms to aid in the detection of a wide range of cancers. This chapter will introduce the structure of the immunoglobulin protein, including the most commonly used altered

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and engineered variants created by researchers, and provide detail on how these various Abs can be labeled to allow their detection. A number of different applications then become possible. The principles of these applications and the ways in which they can be combined to create diagnostic tests will be outlined, including how diagnostic assays are increasingly being designed to include the detection of large numbers of targets simultaneously, a technique known as multiplexing.

24.2 Overview of Antibodies

Antibodies, or soluble forms of immunoglobulin (Ig), possess a vast array of possible specificities and a structure that is one of the more stable among mammalian proteins. Researchers have capitalized on the large pool of specificities provided by naïve B lymphocytes as well as on the refinement of specificities for the recognized motif, or epitope, provided by the process of somatic hypermutation during clonal expansion of activated B cells. However, the *ex vivo* generation of Abs is becoming the standard for the purposes of research, diagnostics, and therapy. This allows for an increased amount of versatility through a large number of sources and formats. Clinicians and researchers have the choice of intact Ab molecules or fragments, as well as polyclonal or monoclonal antibodies (mAbs) from a number of different species. Each of these various Ab molecules can also be chemically linked to a multitude of reporter molecules, allowing the use of Abs in a wide range of assay platforms. The most common of these platform variants are described below.

24.2.1 Monoclonal vs. Polyclonal Antibodies

A polyclonal Ab preparation consists of a mixture of immunoglobulin molecules with multiple specificities, all of which are directed against the target. Most polyclonal Ab mixtures are created by the injection of a purified full-length recombinant protein into an animal, which can lead to the generation of Abs that recognize many portions of the protein. In other instances, a short peptide comprising a more specific region of interest is used, creating a number of different Abs that recognize a very specific region or "epitope." In most cases the rabbit is used to generate polyclonal Ab mixtures. Many other species can also be used to create these Abs, contributing to the multiplexing flexibility of Abs. The injected peptide or protein, known as an immunogen, is selected to include a very specific, and preferably unique, region of interest in a target molecule. When the injected animal's immune system recognizes the peptide or recombinant protein as foreign, the resulting immune response will generate multiple immunogen-specific Abs, which can then be isolated from the animal to yield a polyclonal antiserum. In some cases, this antiserum is purified further using affinity chromatography [6].

Because of a higher degree of confidence in their affinity and specificity, mAbs are often chosen over polyclonal preparations when possible. Kohler and Milstein developed the first mAbs in the mid-1970s by expanding on the techniques used to generate polyclonal Ab preparations. As with polyclonal Ab stimulation, an immune response is elicited to an injected immunogen. In this case, however, multiple antibody-producing daughter B cells are isolated from the spleen of the injected animal after several days. Myeloma cells are then fused with the harvested antibody-producing B lymphocytes to generate hybridomas. These hybridomas can produce large amounts of the Abs expressed by the original activated daughter B cells and are capable of proliferating in culture indefinitely. Single hybridomas are separated and expanded in culture to create monoclonal populations. The Abs produced by the monoclonal populations are then screened for affinity and specificity [7, 8].

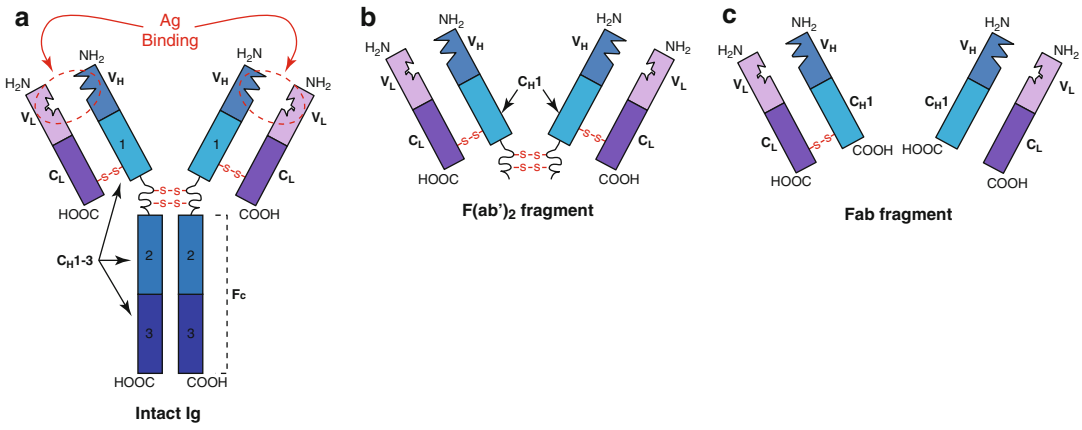

Fig. 24.1 Intact immunoglobulin and common antibody fragments. (**a**) Schematic representation of an intact immunoglobulin molecule. Each heavy chain (*blue*) consists of three constant domains (C_H1-3) and the variable domain (V_H). C_H1 and C_H2 are linked by the flexible hinge region, which forms two disulfide bonds with the hinge region of the complementary heavy chain. Each light chain (*purple*) consists of one constant domain (C_L) and one variable domain (V_L) and is associated with the heavy chain through a disulfide bond proximal to the carboxy-termini of the two chains (COOH). The antigen-binding regions of the molecule (Ag Binding) are found at the amino-termini of the V_H/V_L pairs (NH_2) and are circled in red. The Fc portion of the molecule, consisting of C_H2–3, is indicated. Domain labels are constant throughout the figure. (**b**) The F(ab')$_2$ antibody fragment. Enzymatic digestion of intact immunoglobulin with pepsin results in the cleavage of the molecule at the hinge region, maintaining the disulfide bonds and yielding the F(ab')$_2$ fragment. (**c**) Papain cleaves the hinge region of intact immunoglobulin just above the disulfide bonds, generating two Fab fragments. Fab fragments can also be created through genetic manipulation. The heavy and light chains can associate non-covalently (*right*) or may maintain a disulfide bond near the carboxy-termini (*left*)

Several technologies for more cost-effective, rapid, and simpler generation of mAbs have since been developed. Chimeric or "humanized" Abs have been made possible by recombinant techniques, combining human Ab DNA with the sequence encoding the binding site of a mouse mAb [9]. Recent years have also seen the emergence of bacterial expression of antibodies, which allows for the selection of advantageous Ab specificities via phage display. The displayed Ab fragments are generated from the plasma cells of human donors or from the spleen of an immunized animal. Increasingly, however, these phage libraries and other screening tools are generated by genetic engineering (discussed in greater detail in Donzeau and Knappik [9]). The highly specific high-affinity mAbs required for therapies, diagnosis, and basic research are created using these methods.

24.2.2 Antibody Fragments

Depending on the requirements of the assay platform, Abs can be used in a number of different formats, including the intact immunoglobulin molecule as well as multiple types of smaller fragments (Fig. 24.1). The Fab fragment includes the entire light chain, as well as the variable and first constant region of the heavy chain, and can form stable H/L heterodimers without being covalently linked. In some cases Fab fragments can remain joined through a C-terminal disulfide bond (Fig. 24.1c) [9]. Fab fragments can be created by papain digestion of intact immunoglobulin molecules, or more recently, through genetic manipulation. The F(ab')$_2$ fragment is similar, in that it also retains the disulfide bond which covalently links the two chains of the Fab fragment (Fig. 24.1b). In the case of the F(ab')$_2$ fragment,

however, a portion of the flexible hinge region remains intact following its creation by digestion of intact Abs with pepsin. Additional small fragments and multivalent engineered Abs can also be created through genetic engineering and may enjoy increasing use in diagnostic assays and possibly cancer therapy in the coming years.

24.2.3 Reporter Labeling

There are a number of reporter molecules available for use in visualizing and even quantifying the binding of an Ab to its target [10]. One such class of reporters is the group of laser-activated fluorescent molecules called fluorophores or fluorochromes, commonly used in flow cytometry (see Sect. 24.8). Other reporters can be enzymatic and therefore depend on chemical reactions to be detected. For these reporters, the Abs are chemically linked, or conjugated, to an enzyme such as alkaline phosphatase (ALP) or horseradish peroxidase (HRP). An intense color is generated by the product created when these enzymes are incubated with chromogenic substrates, allowing measurement with a spectrophotometer. It is also possible to incubate these enzyme-linked Abs with a chemiluminescent substrate, the product of which gives off light, which can then be quantified by a number of different instruments and even captured on film.

A common third approach, often used to allow greater flexibility for the multiplexing of targets, includes biotin-conjugated Abs [11]. Biotin recognizes streptavidin with a high level of specificity and affinity, forming one of the strongest known non-covalent bonds. Streptavidin can be linked either to fluorophores or to enzymes like HRP and ALP, providing the flexibility to use a particular biotinylated Ab across multiple assay platforms. Similarly, within a single platform, the same biotinylated Ab can be used in multiple wells or tubes and, if necessary, be identified by different colors by using varied streptavidin-conjugated reporters, as with the multiple fluorophores used in flow cytometry [12].

24.2.4 Primary and Secondary Antibodies

Some diagnostic assay formats require the use of Ab pairs for detection (see Fig. 24.6b for a schematic representation). The first, or primary, Ab is specific for the target. A secondary reporter-conjugated Ab can be used in cases where the primary Ab does not include a reporter. Anti-species Abs, which are directed against immunoglobulin molecules produced by a different species, are commonly used as secondary Abs. For example, mouse immunoglobulin is injected into a goat to produce an immune response, resulting in a polyclonal goat anti-mouse Ab preparation that can be labeled with a reporter molecule. The goat anti-mouse Ab preparation is used to detect the presence of the primary mouse mAb wherever it may be bound to the target. However, in order to avoid possible cross-reactivity and to minimize the complexity of the assay, simpler assays in which the primary Ab is directly conjugated to a reporter are preferred when the assay system permits.

24.3 Immunoprecipitation

For many years, specific Abs have been used as a means to bind and concentrate targets in solution [13]. This process, known as immunoprecipitation (IP), involves the mixing and incubation of the specific Ab with a solution containing the molecule of interest (Fig. 24.2). After sufficient time to allow the Ab to bind the target, the Ab itself can be captured through binding to beads coated with bacterial protein A, protein G, or a mixture of both. The solution can then be centrifuged to pellet the beads at the bottom of the tube, allowing the supernatant to be transferred or discarded. Through this process, the target has been isolated and greatly concentrated and is now more readily detected.

When searching for comparatively rare proteins, which are present at much lower concentrations, a larger number of cells or volume of bodily

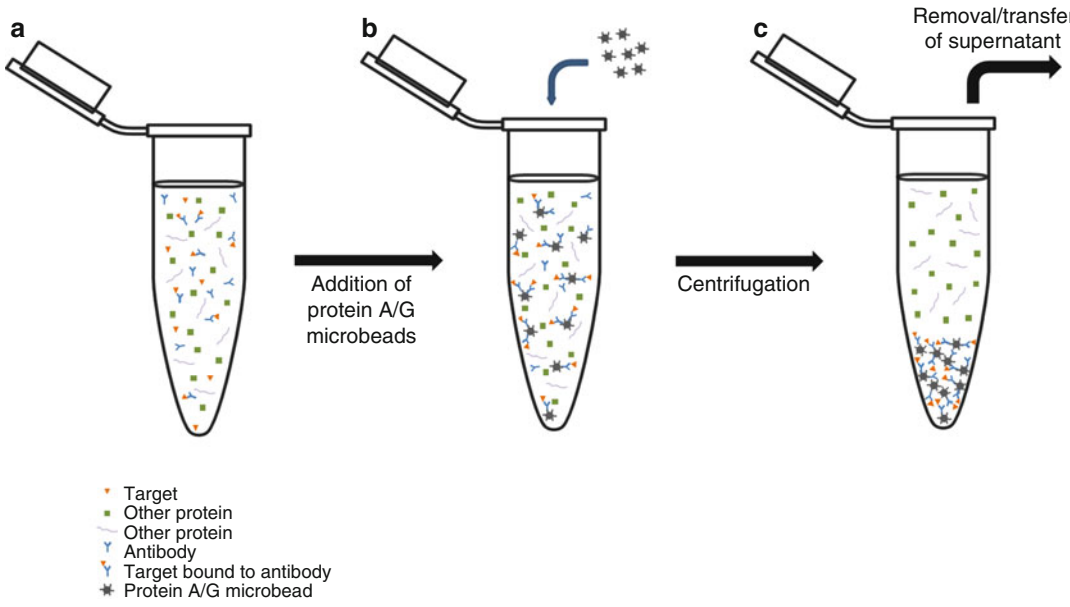

Fig. 24.2 Immunoprecipitation. (**a**) Cell lysate or other biological sample is incubated with specific antibody (*Ab*), which binds to the target in solution. (**b**) Microbeads coated with bacterial protein A, protein G, or a combination of both are added to the solution. The Abs, whether bound to target protein or free, will be bound by the bacterial proteins coating the bead. (**c**) Following centrifugation, the beads and their cargo of Ab and target protein will form a pellet at the bottom of the tube. The supernatant, now depleted of the target protein, can be transferred to another tube or discarded. These schematic representations of Abs and their targets will be used for all subsequent figures

fluids like plasma are required. This larger amount of material often presents problems for the detection system, which can be solved through the capture and concentration of the target by IP. In other cases, IP is used to diminish the amount of background detected by the assay system. The background can be minimized either by pulling the target out of the sample mixture for detection or by specifically depleting the mixture of an unwanted protein(s) that has been found to conflict with the detection of the target. IP is often used as a first step before detection by immunoblotting.

24.4 Immunoblotting

Also known as Western blotting, immunoblotting (IB) makes use of specific Abs for the detection of proteins of interest [14]. Sodium dodecyl sulfate (SDS) and heat are used to denature the proteins in a sample, which can range from a bodily fluid such as plasma, to a solution of cellular proteins released from cells by treatment with a lysis buffer. These proteins are separated according to mass via polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a membrane for detection (Fig. 24.3). The specific primary Ab is washed over the surface of the membrane for a prolonged incubation period, allowing it to bind the target protein, followed by incubation with a secondary enzyme-conjugated anti-species Ab. After the addition of a chemiluminescent substrate, a band of light will be generated at the position where the primary and secondary Abs are bound to the membrane. The amount of protein present dictates the amount of primary and secondary Ab bound to the membrane, which in turn dictates the intensity of the light generated. This light signal is traditionally detected by exposure to autoradiography film, but advances in low-light camera-based systems have led to increasing use of these documentation

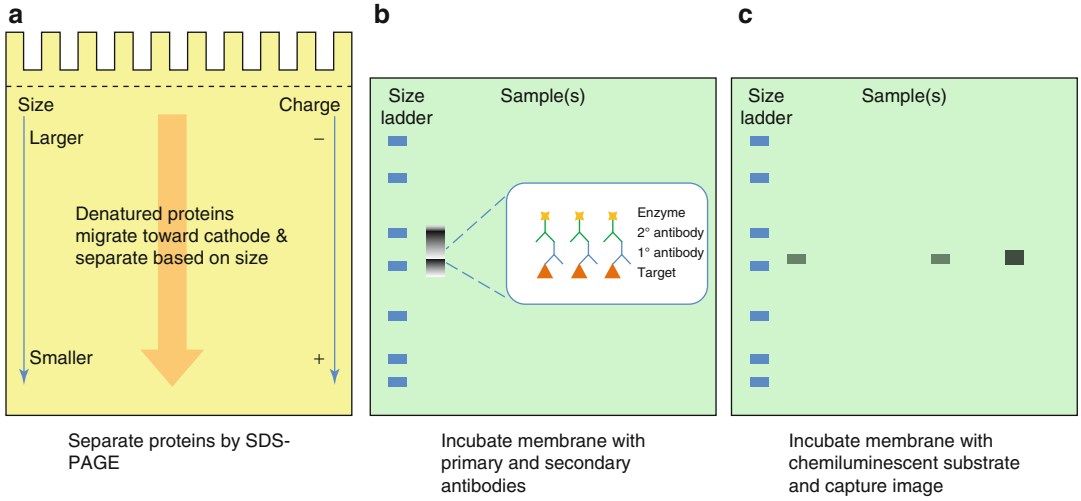

Fig. 24.3 Immunoblotting. (**a**) Samples are denatured in lysis buffer, loaded onto a polyacrylamide gel, and separated by electrophoresis (*PAGE*). The presence of sodium dodecyl sulfate (*SDS*) in the buffer masks the native charges of the proteins and lends an overall negative charge, allowing the proteins to migrate toward the cathode according to size, with smaller proteins traveling farther through the matrix than large proteins (SDS-PAGE). Proteins can also be analyzed by their native conformations under non-denaturing conditions in the absence of SDS (not shown). (**b**) Separated proteins are transferred to a nitrocellulose or polyvinylidene fluoride (*PVDF*) membrane via the application of electrical current. The membrane is then probed with primary Ab specific for the target protein or residue, followed by an enzyme-conjugated secondary anti-species Ab (more detail on secondary antibodies and reporters is given in Fig. 24.5). A molecular weight standard containing multiple proteins of known molecular weights is usually included in each experiment (size ladder), to provide an estimation of the distribution of the sample proteins. The proteins in these ladders are often dyed, sometimes with multiple colors, to allow visualization on the membrane. (**c**) The target is visualized by incubating the membrane with the chemiluminescent substrate of the reporter enzyme, which emits light. The signal is captured by exposure to autoradiography film or by a camera-based gel-documentation system. The quantity of target can then be extrapolated from signal intensity and/or band size, with larger bands corresponding to more bound target, although this measure is not truly quantitative, but relative to the other samples in that experiment only

methods. On a traditional immunoblot exposed to film, lower-intensity signals correspond to fainter, thinner bands, while larger amounts of signal create fatter, darker bands (Fig. 24.3).

Due to the fact that it provides an opportunity to physically view the interactions of an Ab with the proteins present in a sample matrix, immunoblotting is still widely used in a research setting despite being an older technique. This characteristic can help researchers determine the specificity of an Ab during the development of a cancer test, even if another technique will ultimately be used for detection. However, despite the fact that the method is comparatively time-consuming and labor intensive, there are still some cancer-related diagnostic tests which make use of Western blotting. Examples include confirmatory tests for Ri, Hu, or Yo, which are found in paraneoplastic syndromes associated with a number of cancers. The proteins of interest in these Western-based tests are actually Abs themselves. The Ri immunoblot detects the anti-Ri Ab present in patients with paraneoplastic myoclonus/opsoclonus syndrome, which is most often associated with gynecological cancers, breast cancer, and small cell lung cancer. The Yo, or Purkinje cell, Ab is also found in patients with breast, ovarian, and other gynecological cancers, in this case suffering from paraneoplastic cerebellar degeneration. Hu antineuronal nuclear Abs are detected by Western blot in a small percentage of patients with small cell lung cancer and are associated with paraneoplastic sensory neuropathy and encephalomyelitis. The highly specific Abs used in these Western blots provide confirmation of the identity of the Hu, Yo, and Ri Abs initially detected by first-line screening tests.

24.5 Radioimmunoassays

One of the first highly sensitive methods for measuring the levels of proteins such as hormones in the blood was the radioimmunoassay (RIA) [15]. In a classic RIA, a known quantity of purified target protein is radiolabeled, most often with a gamma radioisotope of iodine. This "hot" protein is mixed with a specific Ab that has been immobilized on a surface, and then the biological sample containing unlabeled or "cold" protein is added to the mixture (Fig. 24.4). In a standard competition assay, the cold protein will then compete with the radiolabeled protein for binding to the Ab, leading to the displacement of a fraction of the radiolabeled protein. The amount of target protein present in the sample can then be extrapolated by measuring the amount of displaced radioactivity.

RIA technology allowed some of the first specific and sensitive tracking of important hormones like insulin in human blood [16] and is still used in some cancer-related diagnostics today, including thyroid hormone testing. Some thyroid hormone tests, including reverse T3, free T4, and especially thyroid-stimulating hormone (TSH), are still offered via RIA. These thyroid hormone tests are included as diagnostic tests in the preliminary characterization of thyroid nodules as malignant or benign and in the diagnosis of TSH-secreting pituitary adenomas. In the interest of laboratory safety, however, technology has moved away from techniques requiring the handling of radioactivity, and the RIA method has largely been replaced by enzymatic immunoassays.

24.6 Enzymatic Immunoassays

Enzymatic immunoassays, or EIAs, are the archetypal antibody-based detection format and a foundation of basic cellular biology research. The best known EIA format is the enzyme-linked immunosorbent assay (ELISA) [17], which has been used for the detection of targets in both cell lysates and in nearly every bodily fluid, ranging from whole blood to sputum to cerebrospinal fluid. Most commonly, ELISA assays are performed in microtiter plates containing 96 or more wells, providing the opportunity to test a large number of samples in a single run. Further, as the treatment of each well is often identical, the format of the ELISA assay lends itself to a high degree of automation using liquid handling robots and plate washers. Since the ELISA often contains multiple lengthy incubation steps, the ease with which it can be automated provides valuable time and labor savings in a high-throughput cancer diagnostics laboratory.

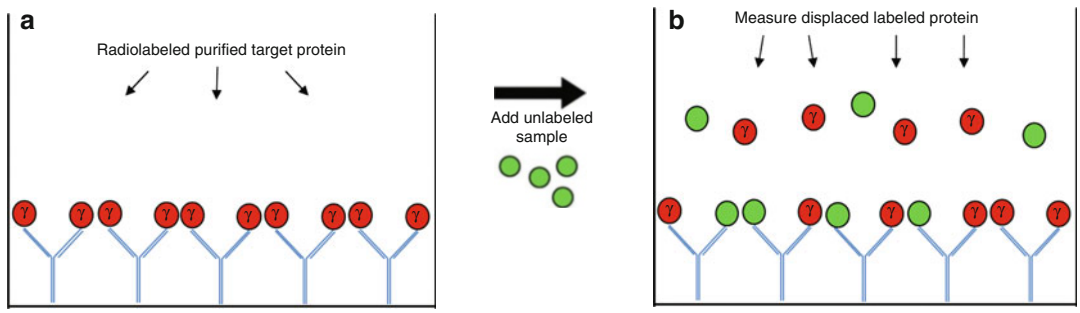

Fig. 24.4 Radioimmunoassay. (**a**) Purified target protein is radiolabeled, often with the gamma isotope of iodine (γ) and incubated with immobilized specific antibody (*Ab*). Sample containing unlabeled target protein is then added to the well. (**b**) The unlabeled target protein competes with the purified radiolabeled protein for binding to the Abs, displacing some of the radiolabeled protein when present at high enough concentrations. The unbound protein is removed from the well, and the radioactivity of the displaced radiolabeled protein is measured to give an indirect measure of the amount of unlabeled target protein present in the sample

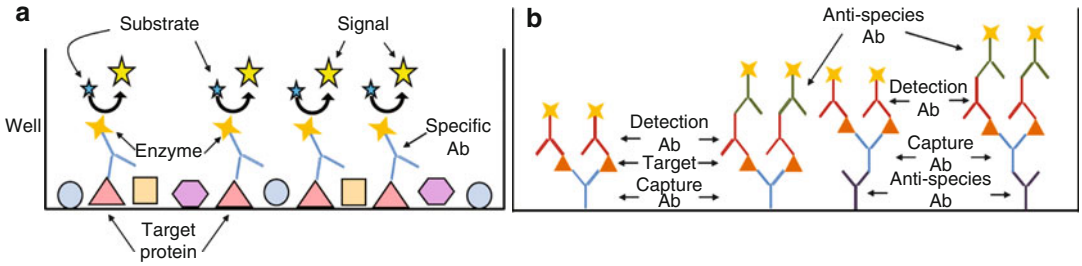

Fig. 24.5 ELISA. (**a**) The simplest ELISA consists of proteins adsorbed to the surface of a well and incubated with specific enzyme-conjugated Abs. After binding of the Abs to the target protein, the well is washed, and the colorimetric or chemiluminescent substrate is added. The reporter enzyme acts on the substrate, generating signal in the form of color or light, respectively. (**b**) The sandwich ELISA and its possible variations. The specific capture Ab can be directly coated onto the surface of the well or be bound itself by an anti-species Ab. After capture of the target protein, the target is bound by the detection Ab, which can be conjugated to a reporter itself or bound by a reporter-conjugated secondary anti-species Ab. Each of these permutations is represented

ELISA formats can range from simple to complex, incorporating from one to four Abs (Fig. 24.5) [17]. At the most basic end of the spectrum is the "direct" ELISA, which uses a single reporter-labeled primary Ab to detect the target that has been adsorbed to the surface of the well or plate (Fig. 24.5a). More commonly used, however, is the "sandwich" ELISA, which can use from two to four Abs as shown in Fig. 24.5b. In many cases the sandwich format is preferred due to the greater level of specificity conferred by requiring two different specific antibodies to bind the target before detection is achieved. The first Ab which binds the target is referred to as the "capture" Ab and is bound to the plate/well either through direct adsorption or through interaction with a corresponding anti-species Ab that is bound to the plate instead. The capture Ab will bind the target during incubation with the lysate or bodily fluid, after which the irrelevant proteins are washed away, leaving the enriched and purified target. The second, or "detection," Ab is now incubated in the well and allowed to bind to the target wherever it has been captured in the well. The detection Ab can be directly labeled with a reporter or can be detected itself by a secondary reporter-conjugated anti-species Ab. The important consideration to remember when designing a sandwich ELISA is that if a secondary anti-species Ab will be used for detection, the capture and detection Abs must have been generated in different species, to prevent the binding of the secondary detection Ab to both.

The flexibility made possible by the sandwich ELISA allows the detection of specialized protein motifs. Examples include the differentiation between isoforms created by alternative splicing [18] or detection of posttranslational modifications such as phosphorylation, acetylation, glycosylation, methylation, ubiquitination, and even protein cleavage [18–23]. The turnover rate of important proteins, the activation status of specific pathways, and other important cellular activities can be inferred from the posttranslational modifications of important cell signaling proteins. For detection of these modifications, the target protein can be bound by the capture Ab, the unbound background protein is washed away, and then a detection antibody specific for the modification of interest can be used to determine whether the protein contains that posttranslational change. The opposite approach can also be taken, in which a detection Ab specific for the target protein can be used to probe the proteins pulled out of solution by a capture Ab specific for phosphotyrosine, for example. In some cases, the posttranslational modification at a specific amino acid residue is even included in the immunogen, in order to generate an Ab specific ONLY for the version of the protein containing a phosphorylated residue at a given position rather than the non-phosphorylated version.

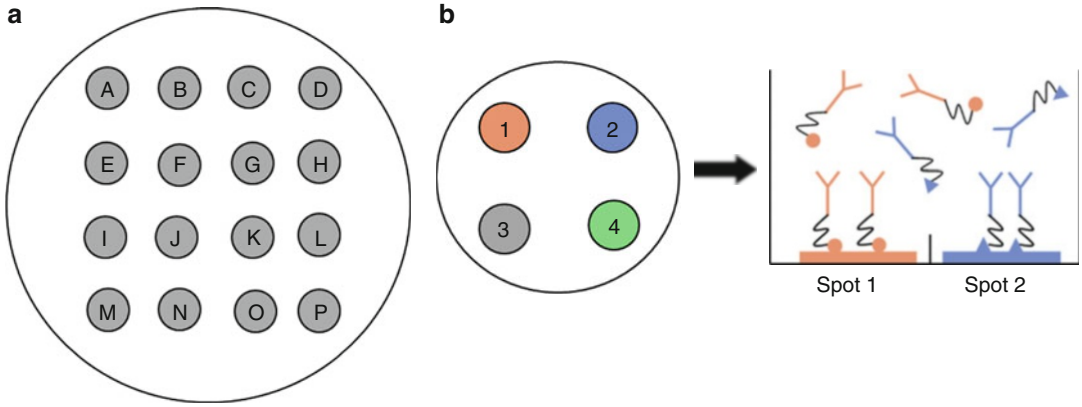

Fig. 24.6 Multi-spot ELISAs. (**a**) A schematic representation of a 16-spot multi-spot ELISA well. Each spot, or letter, corresponds to a different capture Ab that is carefully applied to the plate in one discrete area, usually by robot. A single sample can then be incubated in the well and 16 different sandwich ELISAs performed simultaneously on one small volume of analyte. (**b**) Chemical linkers can create multi-spot assays without robotic spotting of the capture antibodies, allowing mixing and matching of desired analytes. Each capture Ab is conjugated to one of several chemical linkers and incubated simultaneously in the well. Each linker binds only to its corresponding spot, isolating each capture Ab in one specific region of the plate. Multiple sandwich ELISAs can then be performed as in (**a**)

It is also theoretically possible, though generally technically difficult, to use a sandwich ELISA to detect the protein product of a gene fusion, such often happens in cancer. One such example is the BCR-ABL fusion protein which is the result of the so-called Philadelphia chromosome, or the reciprocal translocation t(9;22);(q34;q11), that occurs most often in chronic myeloid leukemia (CML). In this example, a capture Ab specific for the BCR protein would immobilize both wild-type (WT) and fused BCR, while only the fusion protein would be bound by the anti-Abl detection Ab.

The ability to detect multiple targets side by side in a single aliquot of sample can provide a great deal of important information, as well as maximize the information derived from the often inadequate and precious samples received in cancer diagnostic laboratories. Newer ELISA technologies have emerged in the last decade that make multiplexing possible through the use of multi-spot wells. In this assay layout, a number of different capture Abs are bound to the bottom of each well in discrete spots, ranging from 2 to 4 up to 100 (Fig. 24.6a). Flexibility has been further increased by breakthroughs in chemical linkers, which allow assay designers to mix and match the capture Abs in a given well and do it in-house (Fig. 24.6b). These linker-conjugated capture Abs are used with specialized plates, in which the binding partner of each chemical linker has already been spotted in a specific position on the bottom of the well. Each capture Ab will therefore only bind to one particular spot within the well, and the sample can then be added to the well and interrogated for the presence of many target proteins at once.

These sorts of multiplexed ELISA platforms generally require camera-based detection systems that include sophisticated software capable of discriminating and parsing the signal generated by multiple spots in a single small well. Adding an ever greater level of control over the process, some more advanced ELISA platforms now include computer-controlled initiation of the chemiluminescent reaction. In this system the reporter is a true electrochemiluminescent (ECL) reagent, requiring an electrical current to undergo the chemical reaction, and the assay is performed in a specialized plate containing a small electrode in each well. The computer controls the application of current, usually breaking the plate down into sections read in sequence. These sorts of adaptations to the ELISA platform represent

some of the advances made in the last decade and will likely see increasing uptake in the design of cancer tests.

This versatility in the sandwich ELISA platform, as well as the flexibility provided by the large number of available reporter/detection formats, suggests that similarly ingenious ELISAs will continue to be developed. Most commonly in cancer diagnostics, however, more straightforward sandwich ELISAs are used for the purposes of quantitative detection and monitoring of relevant proteins. An example is the HER2 ELISA, which measures the level of HER2/neu present in the serum of breast cancer patients. With the inclusion of a standard curve on the ELISA plate, the amount of HER2/neu protein present in the well can be quantified, and the concentration of the protein circulating in the body can be extrapolated. These data can be used by the clinician to assess the patient's prognosis and to determine the likely response of the patient to a given therapy. Further, if a baseline concentration of the circulating protein is established prior to administering therapy, subsequent longitudinal measurements can be compared to that baseline and used to monitor the efficacy of therapy.

24.7 Immunocytochemical and Immunohistochemical Assays

Immunohistochemistry (IHC) and immunocytochemistry (ICC) are similar techniques used by researchers and pathologists to recognize particular cell types or to determine the location of important proteins within the cell. These proteins can include indicators of apoptosis or proliferation, as well as tumor markers. IHC and ICC assays can provide a wealth of information to the trained observer (Fig. 24.7) [24, 25]. The cells being studied can be found in an intact tissue section as is the case in IHC or taken from suspension or from a smear as in ICC. As with an ELISA, these cells are incubated with the primary Ab specific for the protein of interest and can be detected either through direct conjugation of that primary Ab or by the binding of a

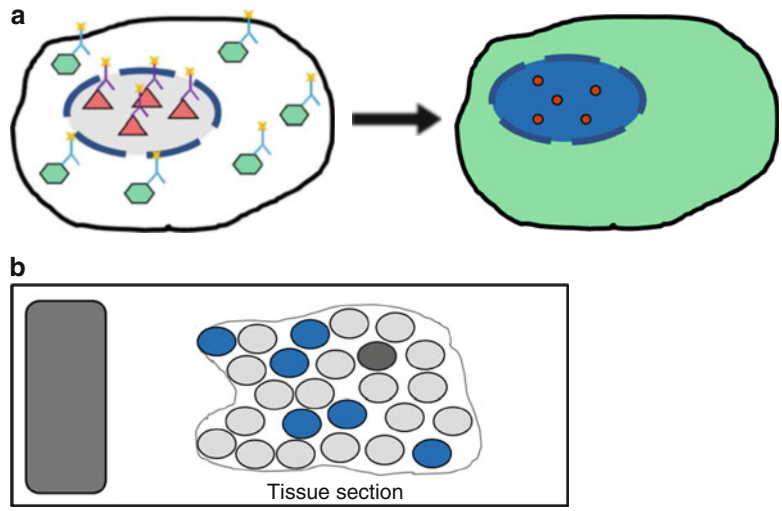

Fig. 24.7 Immunocytochemistry and immunohistochemistry. (**a**) Simplified schematic of ICC, depicting a single cell probed for two specific proteins. One protein is found to be localized to the cytoplasm (*green*), while the other protein is localized to the nucleus (*red*). This nuclear localization is confirmed by a co-stain which identifies the nucleus (*blue*). (**b**) Simplified schematic of IHC, depicting a slide-mounted tissue section. Only a few cells in the tissue section express the protein for which the sample has been stained (*dark blue*). IHC and ICC can make use of both colored stains and fluorescent markers and often require microscopes with multiple excitation and/or emission filters (not shown)

secondary reporter-conjugated anti-species Ab. ICC and IHC can use both enzymatic and fluorescent reporters; the use of fluorescent reporters is also sometimes referred to as immunofluorescence, differentiating the technique slightly due to the requirement for a fluorescent or confocal microscope, as opposed to the light microscope that can be used to visualize enzymatic reporters. Additional common antibodies or dyes are often used to identify structures within the cell, such as the nucleus. The prepared samples are viewed using advanced microscopy techniques and often computer-based image analysis systems as well.

In recent years, advances in automation have generated higher-throughput solutions for IHC and ICC. One such advance, tissue arrays, allows the placement of multiple patients' samples on a single slide, which leads to a significant increase in the uniformity and speed of slide preparation. Further, increasingly sophisticated software and new automation systems reduce the amount of time that is required to screen slides, thereby greatly increasing throughput. An example is the InScape system, which includes the scanning of the slide to create a high-resolution digital image, and automated determination of results using marker-based algorithms after the region of interest is chosen by a pathologist. The result is then verified by the pathologist, saving a great deal of time in the analysis of IHC stains.

ICC and IHC continue to be valuable tools for pathologists due to the ability of the technique to map the location of the target protein to a specific position within the cell. Some types of proteins, such as transcription factors, are regulated wholly or in part by localization. For example, many transcription factors are found in the cytoplasm when inactive and shuttled to the nucleus following activation. Mutations in some proteins that lead to improper localization within the cell have been demonstrated to contribute to malignancy. ICC/IHC assays for the visualization of the localization of these proteins, as well as assays that detect the presence or absence of posttranslational modifications, different isoforms, and even mutant proteins, are all valuable diagnostic and prognostic tools for pathologists.

One of the best known and most commonly used IHC tests in cancer diagnostics is the staining of breast cancer sections for the presence of the estrogen receptor protein (ER). As a predictive marker, ER is currently the most useful test for establishing patient prognosis. In addition, it continues at this time to be the best predictor of patient response to hormone therapies. ER is often ordered in tandem with IHC staining for the progesterone receptor (PR) as well, which provides similar, if less statistically significant predictive information.

24.8 Flow Cytometry

One of the most powerful techniques to make use of the versatility of Abs is flow cytometry [26]. An ever-increasing number of fluorophores are available as reporters, allowing high orders of multiplexing with newer instruments; in some cases, up to 11 different parameters can be recorded simultaneously. These reporter fluorophores absorb the energy provided by laser light at a specific "excitation" wavelength and then emit energy at a different "emission" wavelength. This emitted light is captured by the cytometer using an elegant and elaborate series of optical filters and photomultipliers (Fig. 24.8). In newer cytometers, multiple lasers are used to increase the available excitation spectrum and thus take advantage of the range of available fluorophores; these cytometers therefore require computer-controlled timing of the lasers and optical filters. The combination of these numerous reporters with the adaptability provided by streptavidin conjugation of the fluorophores and pairing with biotin-conjugated Abs provides an impressive number of possible analyte combinations that can be studied for a particular cell type or biological fluid.

Initially, and perhaps still predominantly, flow cytometry was used as a platform for the study of intact cells, intended to measure the levels of proteins present on the surface of the cell. The multiplexing ability provided by the range of fluorophores and number of possible parameters allows the analysis of several surface markers simultaneously and

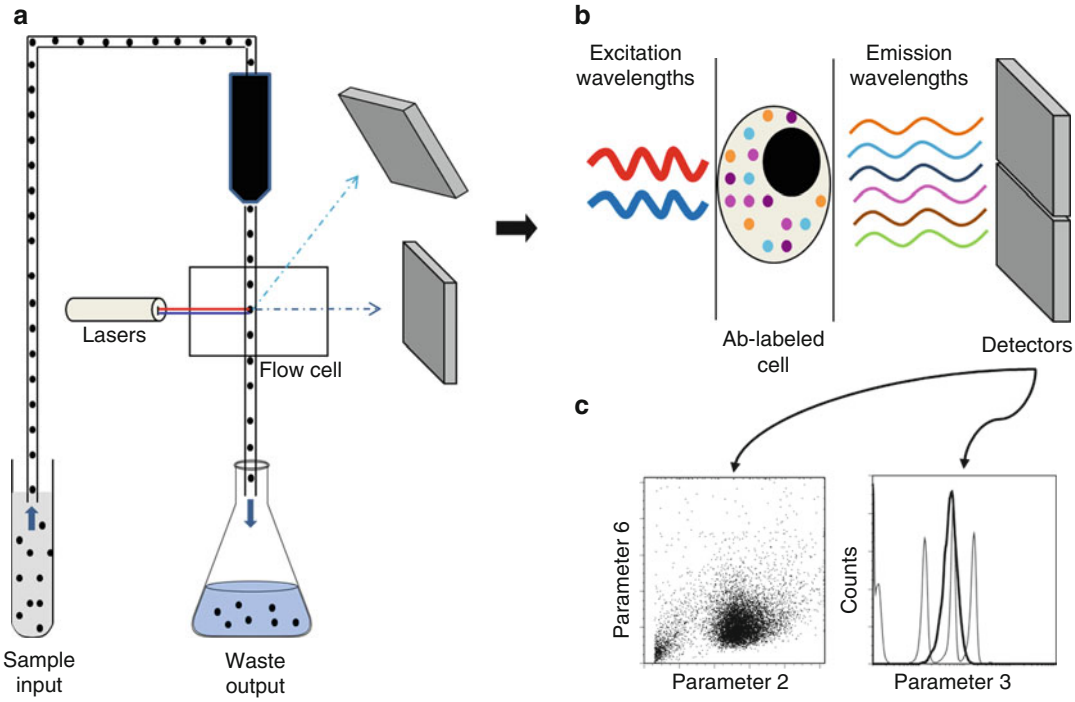

Fig. 24.8 Basic principles of flow cytometry. (**a**) Cells, which have been incubated with fluorophore-conjugated Abs, are drawn from the sample tube into the machine, where they pass the beam(s) of laser light in single file and continue on to a waste receptacle. (**b**) As the cells pass the interrogation point, any bound fluorophores are excited by the laser light. The excited fluorophores then emit light at slightly different wavelengths, which are captured by detectors after passing through a complex system of optics (not shown). (**c**) Software manipulation of the recorded light signals results in data that can be analyzed in many ways and combinations. Each target assayed, or parameter, can be analyzed in tandem with any other in a dot plot (*left*; see Fig. 24.9 for more details) or analyzed singly in the form of histograms and then compared to the histograms of other samples (*right*)

has made possible the characterization of the numerous subsets of cell types present in the human body. However, advances in the technology in the last few decades have also allowed the detection and quantitation of both intracellular and soluble proteins using flow cytometry, as well as cellular DNA content, greatly expanding the possibilities afforded by this platform.

The events occurring inside a given cell can provide valuable insights, including whether the cell is activated, in the process of proliferating or in the process of dying under particular conditions. In more traditional cell biology research, these questions would generally be answered using Western blotting or perhaps even ELISAs. Despite being powerful methods which characterize the response of a population of cells to a given condition, both techniques actually offer the average response of the entire population tested. Even the most carefully purified cell preparations generally contain a mixture of different cell types, and this heterogeneous population may very well express the protein of interest at different levels or even exhibit a differential reaction to the conditions being studied. This heterogeneity can make it difficult to interpret results and represents a major roadblock for the study of rare cell types, which are in short supply and often difficult to adequately purify. For these reasons, the ability of flow cytometry to discriminate between lineages by surface marker expression, and combine this with intracellular cytokine staining in preparations of fixed and permeabilized cells, is an important advance in studying intracellular events in mixed populations of

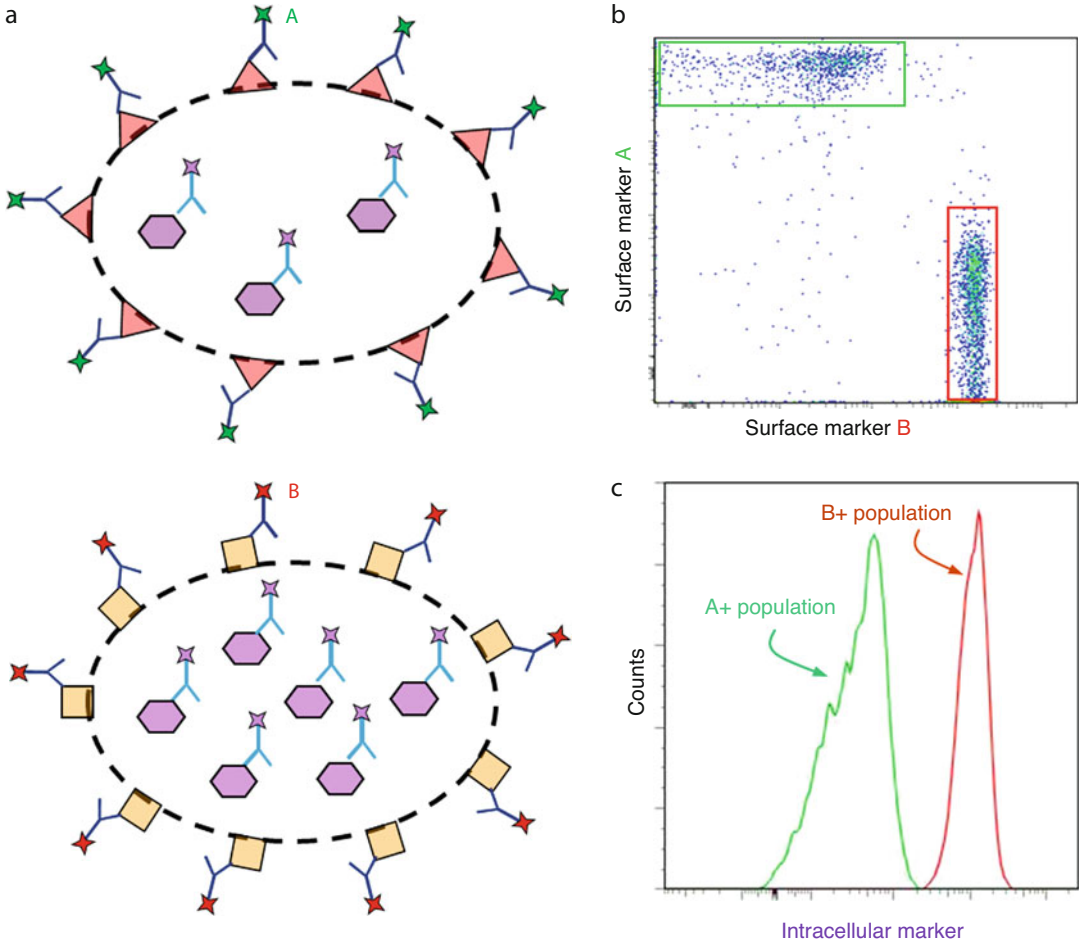

Fig. 24.9 Surface and intracellular cytokine staining of permeabilized cells. (**a**) Mixed cell populations are labeled with Abs specific for surface markers that identify subsets such as different lineages, different activation states, and others. Two different cell subsets are indicated here by binding to two different surface marker Abs, represented here by green ("A," *upper*) and red ("B," *lower*) reporters which will be seen by the cytometer as different parameters. The cells are then permeabilized to allow passage of Abs across the membrane, represented by the dashed line surrounding the cell. Permeabilized cells are incubated with Abs specific for the intracellular target (*purple* reporter), which will be seen by the cytometer as a third parameter that is the same for all cells. (**b**) After sample acquisition by the flow cytometer, the different cell subsets are differentiated by their expression of the surface markers for which they were stained. Comparison of two parameters is generally done with a dot plot, in which each dot represents a single cell; the dot plot shown here is colored like a heat map to indicate areas of greater and lesser cell density. Surface marker "A" (*green* reporter; y-axis) is present at high levels on the upper cell, while surface marker "B" (*red* reporter; x-axis) is absent, indicating that these cells will fall in the top left corner of the dot plot. Conversely, the lower cell shows high levels of marker "B" and low levels of marker "A," placing them in the lower right corner of the dot plot. These expression patterns create two distinct populations in the dot plot. "Gates" can then be drawn around the populations (*rectangles*), telling the software to consider only those cells falling within the gate in downstream analyses. (**c**) The cells within each gate are analyzed for levels of the intracellular protein (*purple* reporter). Levels are suggested by the intensity of the staining for the third parameter ("Intracellular Marker," x-axis). The diagram in (**a**) depicts the upper cell as having a lower level of the target intracellular protein, and this is reflected by the green histogram falling farther to the left on the scale than the red histogram, indicating a higher intensity of staining in the surface marker B-positive cells than in the marker A-positive cells

cells (Fig. 24.9) [27–32]. These sorts of intracellular cytokine staining protocols have allowed the study of cell signaling cascades in intact normal cells [33], as well as characterization of aberrant signaling in mutation-bearing cancer cells and in cancer cells exposed to emerging therapies.

Further advances in flow cytometry have even made it possible to mix samples from two different sources, including from two discrete patients or from a single patient pre- and posttreatment, using a "barcoding" method [34]. Each sample is mixed with a different fluorescent dye that emits at a distinct "signature" wavelength, which, when the samples are mixed, allows discrimination of each through sorting based on the detection of the signature. Although a boon for researchers, this technique has yet to become standard practice in clinical oncology diagnostics laboratories. Flow cytometry itself, however, is firmly entrenched, primarily as a valuable tool for hematopathologists, who use flow cytometry to examine the populations of circulating cells in the blood in order to discover subsets of abnormal cells, such as those present in hematological malignancies like leukemias and lymphomas. Flow cytometry panels for differential diagnosis of leukemia/lymphoma can contain upwards of 20 cell surface markers, and algorithms characterizing the patterns of these markers on the surface of cell populations in the blood help pathologists identify the particular type of leukemia or lymphoma present.

24.9 Bead-Based Assays

As with the detection of intracellular proteins, the study of soluble proteins present in bodily fluids and in cell culture supernatants was traditionally performed by immunoblots or ELISA. But again, as with intracellular proteins, flow cytometry now represents an additional platform for the detection of soluble proteins through the use of bead-based assays. In a design that combines the best features of IP and sandwich ELISAs, Abs are coated onto microbeads rather than plates, and these beads can then be incubated with the sample fluid putatively containing the protein of interest. Following capture by the beads, the protein can then be bound by a specific detection Ab. As with sandwich ELISAs, the bead-based assay can use up to four Abs, but again, fewer Abs are generally preferred (Fig. 24.10). One successful application of this technology is the detection of soluble proteins released into the bloodstream by dying leukemia cells [35–37]. Despite the similarities of the technique to the sandwich ELISA, the bead-based assay benefits from greater multiplexing possibilities, including the Luminex and cytometric bead array technologies.

As stated above, the most advanced cytometers can measure upwards of 11 or more parameters. This often presents calibration issues due to the slight spectral overlap of the fluorophores available. One approach to avoiding this problem is to use a single fluorophore to measure different analytes, rather than a large number of different "colors." The cytometric bead array (CBA) makes use of beads of different sizes, one size for each of the different capture antibodies to be used. All detection antibodies can then be conjugated to the same reporter fluorophore, because the discrimination between the different proteins detected will be provided by the size of the bead, which is one of the parameters measured as the particle flows past the cytometer's detector. These different bead sizes will result in easily distinguishable populations and thus analytes, as shown in Fig. 24.11a, while the level of protein captured and detected by a given antibody pair will be quantified by the intensity of the reporter's fluorescence (not shown). In this way, the CBA assay allows the measurement of multiple analytes side by side in the same sample.

Beyond just determining the relative amounts of protein captured by the CBA assay, however, researchers have applied a standard curve to the assay, allowing the quantitation of detection Ab molecules bound to a bead. Each experiment includes a tube containing four groups of beads, each with a different known level of bound reporter fluorophore. The data derived from this sample is used to generate a standard curve, plotting the known number of reporter molecules against the mean fluorescence intensity (MFI)

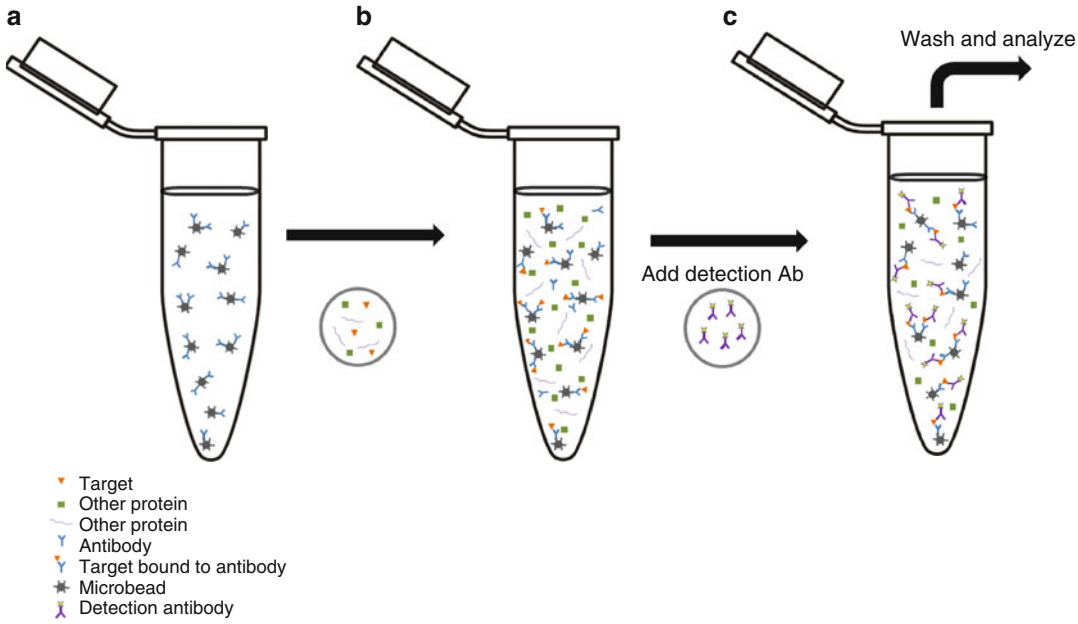

Fig. 24.10 Bead-based flow cytometry assays. (**a**) Capture Abs are coated on microspheres. (**b**) The beads are incubated with proteins in solution (e.g., lysate, cell culture supernatant, or plasma) and bind only the target protein. (**c**) The target protein is bound by fluorophore-conjugated detection antibody, the sample is washed to remove unbound detection antibody, and the beads are analyzed by flow cytometry

measured by the cytometer. Using this curve and the MFI value recorded for a given sample, the number of bound reporter-conjugated detection Abs can be calculated. This technique provides an even more accurate quantitation of the level of the target protein present in the matrix and can even be applied to the more traditional non-bead-based flow cytometry methods of intracellular and surface protein detection.

The Luminex technology makes use of a combination of the advantages of both microbead assays and flow cytometry, creating a method ostensibly able to analyze up to 100 targets in one well (see Luminex Corporation for examples). Luminex makes use of polystyrene microspheres impregnated with carefully controlled levels of both red and infrared dyes. These different titrations create different color signatures for each population of beads, much like the barcoding technique described above (Fig. 24.11b). These different beads can then be coated with discrete capture Abs, mixed together, and incubated with the biological matrix. Following capture of the target proteins, detection Abs are added, all conjugated to the same reporter fluorophore as in the case of the CBA assay. The data are then collected using the basic principles of flow cytometry, in that the dyes inside the beads are excited with a red laser to reveal the "signature" identifying which target should be captured by that particular bead, and a green laser is used to excite the reporter fluorophore to allow the measurement of the levels of protein actually captured [38]. The multiplexing capabilities of this platform provide the potential for Luminex to provide as much information about a sample as some types of antibody microarrays or multi-spot ELISAs (see below) and is therefore currently more often used in a cancer research or clinical trial setting.

24.10 Antibody Arrays

The antibody microarray makes possible the detection of a very large number of analytes in a complex sample, similar to its predecessor, the DNA microarray [39, 40]. Most antibody

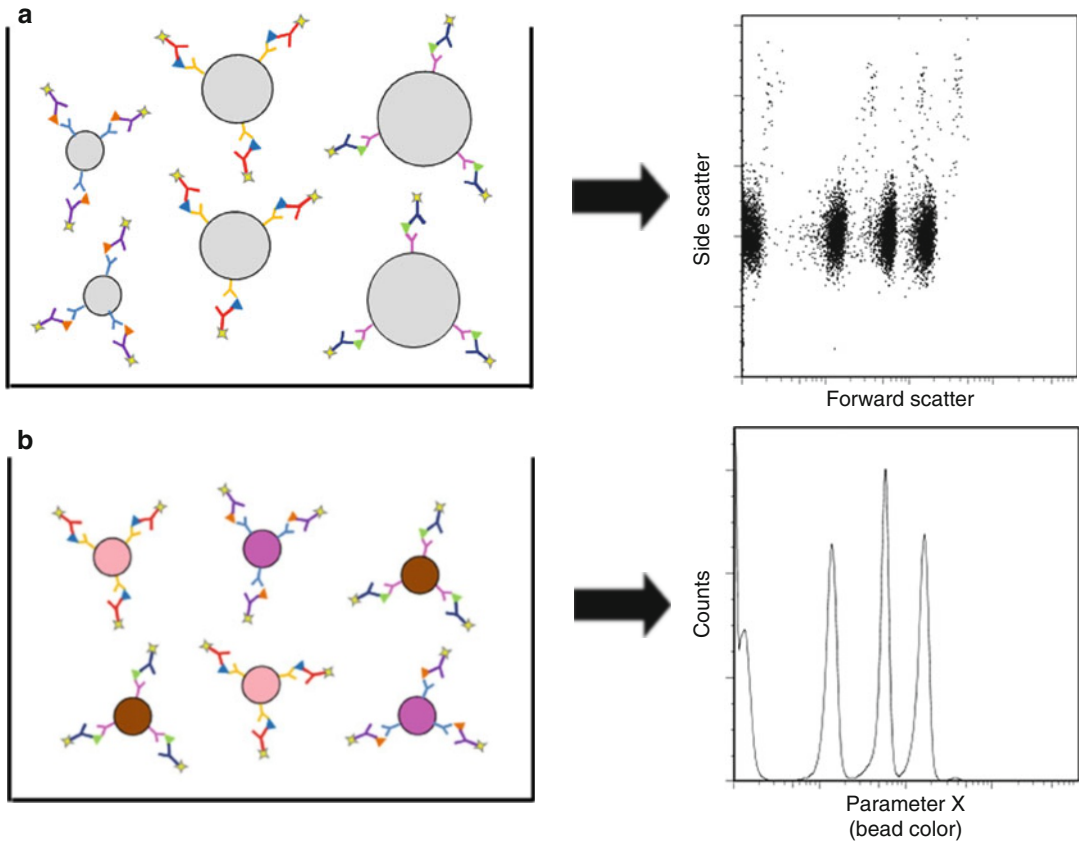

Fig. 24.11 Cytometric bead array and Luminex technologies. (**a**) The CBA platform consists of the Abs specific for each target being conjugated to beads of a different size. The beads are incubated with the sample at the same time, allowing capture of the target proteins. The beads are then incubated with detection Abs for each target, all conjugated to the same fluorophore (*left*). When analyzed, the different bead sizes are recognized by the cytometer via the forward and side scatter parameters and are identifiable as discrete populations that can be analyzed separately via gating (*right*). (**b**) Luminex technology makes use of beads of the same size which have been impregnated with dyes of slightly different wavelengths. Each set of beads is coated with a different capture Ab, incubated with sample to capture target protein, and detected with a fluorophore-conjugated detection Ab (*left*). The cytometer-based analysis instrument detects the slight variations in the color of the bead (Parameter X), creating discrete populations based on bead color which can be gated (*right*). The reporter fluorophore intensities within each population can then be analyzed, yielding information about the concentration of each target analyte

microarray formats are essentially ELISAs on a necessarily grand scale, as shown in Fig. 24.12. These arrays are valuable both for basic research and in the search for diagnostic and prognostic markers of cancer. A small volume of biological material can yield a substantial amount of information using this technique, and often of greater importance, relationships and patterns within the data can be recognized and characterized in a single snapshot experiment. Antibody microarrays can be designed in a number of different formats, including the variable of whether it is protein or antibody bound to the array itself.

In its infancy, antibody array technology most closely paralleled that of DNA microarrays by spotting the surface of the array with probes consisting of mAbs. Universally labeled proteins are then incubated with the array, and the captured protein is identified by its binding position on the array (Fig. 24.12a) [39]. The protein-labeling process includes either direct labeling with reporters or indirect detection using biotin or digoxigenin.

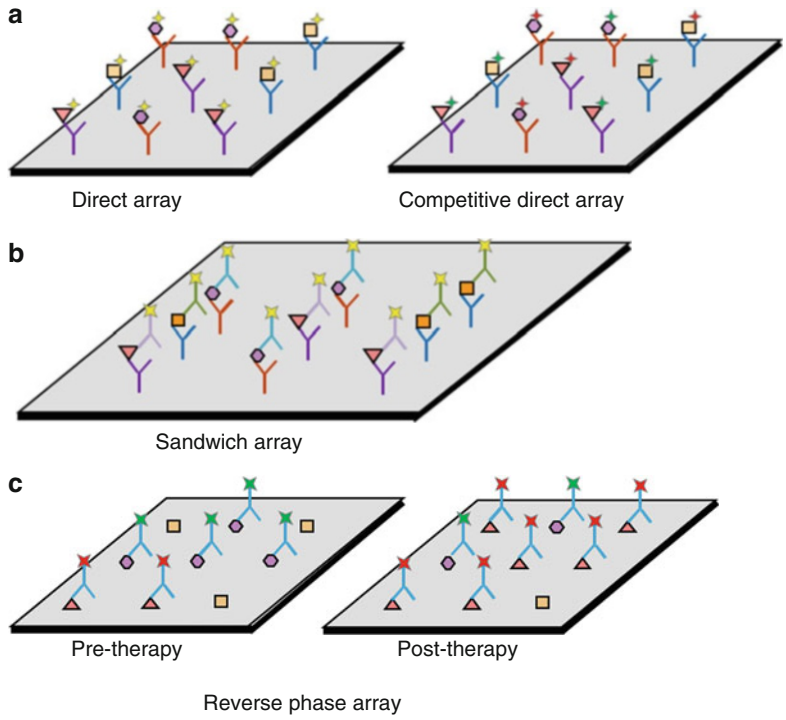

Fig. 24.12 Antibody array formats. (**a**) Direct antibody arrays involve the spotting of specific Abs onto a surface. The array is then incubated with reporter-labeled proteins (*left*). The identity of a target protein that binds to the array is determined by matching the location of the signal to the known layout of the Abs. In a competitive direct array, the proteins in two separate samples are labeled with distinct reporters (*red* and *green*) and incubated with the array simultaneously (*right*). The target proteins will compete for binding to the Abs on the array, and the relative signal intensities will indicate which sample contained greater quantities of each protein assayed. (**b**) The sandwich antibody array is highly similar to the sandwich ELISA depicted in Fig. 24.5b, simply with a large number of capture Ab specificities combined into a single assay and requiring one small volume of analyte. (**c**) The reverse-phase array consists of the proteins in a sample being adsorbed to the array surface, followed by detection with reporter-conjugated Abs as in Fig. 24.6a. Although the number of targets that can be analyzed simultaneously is limited here, the value of the reverse-phase array is that it allows multiple samples to be analyzed side by side. The example represented here is pre- and post-therapy, and the changes in protein expression resulting from the treatment are clear

Through the use of multiple reporters, it is also possible to compare two samples by incubating them together in a classic competition assay (Fig. 24.12a). This antibody array format is generally referred to as a direct array and is the best option for assaying truly large numbers of analytes in a single array, as the only major limitations are space and the availability of specific antibodies for the desired targets. To date, most arrays offered commercially contain analytes numbered in the hundreds. The primary technical hurdles encountered when using direct Ab arrays include limited specificity and sensitivity and filtering out background signal. In addition, there is always the concern that the direct labeling of the proteins may interfere with recognition of the protein by the Ab due to the physical masking or alteration of the epitope.

With these limitations in mind, additional antibody microarray formats were developed to include both capture and detection antibodies (Fig. 24.12b) [41]. Specificity is greatly enhanced when relying on the recognition of the target protein by two different Abs for detection, as one source of background is minimized. In addition, the problem of possible epitope masking is also solved by removing the necessity of labeling the proteins. One limitation of this sandwich

approach, in both basic ELISAs and the antibody array, is the occasional lack of good matched antibody pairs. Another concern is the problem of cross-reactivity among the detection antibodies, which generally serves to limit the number of possible targets when using a sandwich microarray in contrast to a direct array. However, as the targets of greatest interest or benefit for a given model or cancer type are determined, highly customized arrays are being developed for diagnostic, prognostic, and research uses. For example, some arrays are designed to study groups of putative or known breast cancer markers, while others are used to screen the effects of drug candidates on their target cells.

There is also, as might be expected, an antibody microarray design in which it is the protein mixture that is immobilized on the surface of the array (Fig. 24.12c) [41]. These protein spots can then be probed with reporter-conjugated specific Abs. This reverse-phase array allows the immobilization of multiple samples' proteins on a single array, providing side-by-side analysis, and simplifies the analysis of insoluble proteins. This assay format is also plagued by nonspecific interactions, however, and restricted to a smaller number of detection Abs by the limited reporter multiplexing options. In spite of these technological restrictions, the reverse-phase Ab array is also a valuable tool for clinicians and researchers alike.

24.11 Concluding Remarks

Many of the most spectacular breakthroughs in the field of cancer diagnostics in recent years have been on the molecular side of the coin, with the advent of next-generation or advanced sequencing leading the charge. In the shadow of such advances, many of the techniques described in this chapter tend to look outdated and simplistic. Despite this (likely unfair) comparison, many of the diagnostic assays based on the platforms discussed herein continue to be the foundation of cancer patient workups and represent many of the gold standards in diagnosis, prognosis, and treatment decision-making. One chief reason for the importance of these assays is that molecular assays don't tell the whole story. For example, it has been amply demonstrated that the level of mRNA, though often useful as a marker in and of itself, does not always directly correlate to the level of the protein that will be translated. Similarly, molecular assays reveal nothing about the posttranslational modifications that can dictate subcellular localization or activation of a protein, which can be a more telling measure of aberrant function than the sequence of the gene. The ability to study the actual protein of interest itself is an important aspect of learning as much as possible about the malignancy, to better fight and defeat it. To this end, researchers have harnessed the power of the immune system to create clever tools for the study of proteins via the exquisite sensitivity of Abs, and these tools continue to be absolutely invaluable in the diagnostic workup of cancer patients.

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Flow Cytometry in Cancer Immunotherapy: Applications, Quality Assurance, and Future

25

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Contents

25.1 Introduction

Cancer immunotherapy seeks to elicit or augment the antitumor immune response in a patient with detectable tumor or remaining tumor cells in the adjuvant setting in order to enlist the help of the patient's own immune system for tumor control. In this context, active cancer immunotherapy refers to the use of cytokines (e.g., IL-2 in melanoma

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and renal cell carcinoma), immunomodulatory monoclonal antibodies (e.g., antibodies (Abs) against CTLA-4, PD-L1, and PD-1), cell-based products (e.g., sipuleucel-T for metastatic hormone-refractory prostate cancer), or experimental vaccines based on various antigen (Ag) formats. When evaluating immunotherapies, particularly in experimental settings, it is essential to monitor the immune response elicited by the treatment. Immunomonitoring delivers evidence of immunogenicity, guides the choice and dosage of antigens, assesses the effects of immune modulators and therapy combinations, and has the potential to reveal early biomarkers of clinical efficacy. In this respect, immunomonitoring is helpful for rational clinical development and supplements clinical efficacy parameters such as disease-free period or survival, which are often only available at later clinical trial stages.

In view of their role in the anticancer immune response, the quantity and quality of tumor antigen-specific effector $CD4^+$ and $CD8^+$ T cells are of particular interest. In addition, the role of immune regulatory cells, e.g., regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs) that can suppress the effector immune response to a tumor, is increasingly recognized. Informative analysis requires multiple markers for identification of phenotypic and functional properties and the accurate quantification of cell subsets that are typically found at relatively low frequencies in the peripheral blood. These characteristics call for an assay that is multiparametric, robust, and sensitive enough to characterize rare individual cells.

The canonical multiparameter assay for the characterization of single cells in solution is polychromatic flow cytometry, and hence, it is ubiquitously used for immune monitoring in preclinical tumor immunology and in cancer immunotherapy trials. While the first fluorescence-based flow cytometer dates back to 1968, the past several years have brought major advances in cytometer technology, reagents, range of applications, automated analysis techniques, and minimal information standards. Much has also been learnt from large-scale proficiency testing programs about the challenges facing th use of increasingly complex flow cytometry assays, and what needs to be done to harmonize the assays across multiple laboratories. This chapter describes the main flow cytometry methods being applied in cancer immunotherapy, with an emphasis on recent progress in the field, challenges associated with quality control, its promise to reveal biomarkers of clinical efficacy, and further developments that are likely to be rapidly implemented in routine cancer immunology.

25.2 Main Flow Cytometry Assays in Cancer Immunotherapy

Together with immunohistochemistry, immunophenotyping by flow cytometry is probably the most commonly used assay to investigate immune and other cell subsets of interest in cancer immunology. Flow cytometry distinguishes human immune cells via a combination of physical properties and fluorescent markers such as labeled monoclonal antibodies (mAbs) targeted against cell-specific molecules. Physical properties measured by the cytometer are forward scatter light (FSC) which is roughly proportional to the cell size and side-scattered light (SSC) which reflects the granularity of cells. Markers targeted by fluorescent mAb are mostly categorized in Clusters of Differentiation (CD) nomenclature [1]. To date, the Human Cell Differentiation Molecules Association (http://www.hcdm.org) has indexed more than 360 CD markers. Commonly used "basic" CD markers are CD3, CD4, and CD8 for T-cell subsets, CD19 for B cells, CD14 for monocytes, CD11c for subsets of dendritic cells, CD56 for natural killer (NK) cells, and CD15 for granulocytes. In addition to whole blood and PBMC samples, enumeration of the number and frequencies of immune cell types can also be performed on single-cell suspensions obtained from tissues (for instance malignant tumors) [2, 3]. When analyzing tumors, further markers can be added to identify endothelial cells (CD31), fibroblasts (ER-TR7), epithelial cells (EpCAM, i.e., CD326), and particular tumor cells (e.g., CAIX for renal cell carcinoma).

Many cell populations can currently only be identified by the use of multiple mAb

simultaneously; this is the case for natural regulatory T cells (nTregs) [$CD4^+$/CD25high/$Foxp3^+$/CD127low or various subsets of MDSCs [4]. Polychromatic flow cytometry is also necessary to characterize the activation status, maturity, clonality, and differentiation status of T lymphocytes. Commonly used markers for this purpose include CD25, CD27, CD28, CD45RA/RO, CD69, CD137, and CD154, as well as antibodies to different TCR Vβ family members [5–9]. A combination of mAbs against activation markers and chemokine receptor (i.e., CCR7) can be used to identify naïve, effector memory, central memory, terminally differentiated effector memory (TEMRA), and memory T cells with stem cell-like features [10–12]. These differentiation stages are associated with changes in functional and proliferative properties [13], are altered in the elderly [14], and hence are relevant for adoptive transfer therapy and for possibly predicting response to vaccination in aging cancer patients. However, up to now, there is no gold standard for markers that are necessary and sufficient to identify most immune cell subsets; this is not surprising as our appreciation of the complexity and plasticity of human immune cell subsets is constantly evolving.

A major interest in immunotherapy clinical trials is to characterize the specificity of tumor antigen-specific T cells, most notably in settings of active immunotherapy with defined Ags. The most direct characterization of antigen specificity is via the use of HLA-peptide multimers, which bind directly to the peptide-specific T-cell receptors (TCR). First described more than 15 years ago [15], the HLA-class I multimer assay currently serves as a versatile tool for enumerating, characterizing, and following $CD8^+$ T cell immune responses, and staining protocols are broadly available [16–18]. Hence, HLA-multimers are widely used to monitor T-cell responses, especially in the context of peptide-based vaccination approaches [19–22]. They can easily be combined with mAb panels to determine the phenotype and differentiation status of antigen-specific $CD8^+$ T cells [23–25]. Limitations of HLA-multimers are that both the precise T-cell epitope (i.e., the exact amino-acid sequence of the peptide recognized by the TCR) and its HLA-restriction (i.e., the HLA-molecule which binds and presents the peptide to the TCR) must be known in advance. To date, there also remains a lack of general availability of class II multimers for $CD4^+$ T-cell detection [26].

Intracellular cytokine staining (ICS) is another common assay used for antigen-specific T-cell immune monitoring. It is the flow cytometric method of choice when HLA-multimers are not available, if the exact T-cell epitope is unknown, and for routine monitoring of $CD4^+$ T-cell responses. ICS enables monitoring of multiple effector functions of both $CD4^+$ and $CD8^+$ T-cell subsets [27–29], including polyfunctional T cells that have been associated with pathogen protection [30, 31]. A few groups have described polyfunctional T cells after cancer vaccination in patients, but whether these cells are associated with beneficial and long-lasting antitumor T-cell responses remains an open question [32, 33]. Optimized Ab combinations, protocols, and standardization approaches have been published [34–36], and ICS assays are widely used in clinical studies.

Cytotoxicity or proliferation assays, which have traditionally relied on the detection of radioactivity (i.e., ^{51}Cr release or ^{3}H thymidine incorporation) can also be conducted by flow cytometry. For assessment of killing activity, target cells (control and antigen-loaded cells or tumor cells expressing the antigen endogenously) are differentially labeled using fluorescent dyes (e.g., Paul Karl Horan (PKH) or 6-carboxyfluorescein diacetate succinimidyl ester (CFSE)) and incubated with the effector T cells to be tested. Apart from the obvious safety aspects over radioactivity-based assays, advantages of flow cytometry methods are that (1) several targets can be tested in the same tube; (2) as compared to a classical ^{51}Cr release assay, effector-target incubation time can be significantly prolonged (up to 24 h); and (3) the assay has been reported as being sensitive and effective even when low numbers of effectors are available [37, 38, 39]. Another approach to indirectly determine the cytotoxic capacities of T cells is the use of a mAb directed against CD107a (LAMP-1)

which becomes extracellularly detectable after cytotoxic granules have fused with the cellular membrane (degranulation) [40]. For measuring proliferation by flow cytometry, effector cells are first labeled with fluorescent dyes (CFSE or other tracking dyes such as CellTrace™ reagents) and cultured for several days in the presence of relevant stimuli. Since the dyes are diluted from the mother to the daughter cells, the number of cell divisions is visible in the number of fluorescent peaks detected [41]. The frequency of proliferating cells can also be assessed directly *ex vivo* by staining of the proliferation-associated nucleus Ag Ki67, expressed at all phases of the cell cycle except the resting G_0 stage [4, 42]. These measurements of target killing or cell division by fluorescent dyes have rarely been used in large-scale vaccine studies so far [38, 43], probably because they are time-consuming and require careful optimization and technical expertise to achieve reproducible results.

Finally, cell-free cytokine analysis can also be performed by flow cytometry with the use of multiplex beads, a method that has been recently adapted to meet GCLP standards [44–46]. The method uses mixes of beads of different size and fluorescence that are each coated with Abs specific for the different cytokines of interest. The soluble cytokines present in the sample (i.e., culture supernatant, serum, or plasma) bind to these Ab-coated beads, and a second Ab coupled to another fluorescent label is used to visualize the amount of bound cytokine. Simultaneous quantification of several soluble factors in one sample can be done by comparison to standard curves provided by the manufacturer, for example, to evaluate Th1/Th2 profiles [28]. The assay is as sensitive as ELISA, with detection limits in the range of 20 pg/mL for most cytokines, and can be even more sensitive when an enhanced sensitivity system is used.

The examples above clearly show that flow cytometry is a versatile tool for investigations of the phenotype, frequency, and functional properties of immune cell subsets. Furthermore, assays can often be combined for multiparametric probing of cell properties which is beneficial as precious patient samples are spared. However, the need for both robustness and sensitivity to detect tumor antigen-specific T cells and/or rare cell subsets poses specific challenges for the use of this complex tool in clinical research applications. This is addressed in the following sections.

25.3 Panel Development and Quality Assurance

Current state-of-the-art polychromatic flow cytometry in cancer immunotherapy involves multistep, multi-reagent assays followed by sample acquisition on sophisticated instruments that are able to capture up to 20 parameters per cell at a rate of tens of thousands of cells per second. Analysis of these data can be a challenge, as standard tools require multistep gating strategies and preselection of the parameter combinations to be investigated. Obtaining reproducible results from such a complex assay requires well-trained staff, stringent quality management, and detailed standard operating procedures (SOPs) for panel development, cytometer calibration, reagent qualification, sample preparation, use of appropriate technical and biological controls, and careful data analysis.

We start by considering the factors important to understand when developing a mAb staining panel. Target molecules in flow cytometry for cancer immunotherapy can have vastly different expression levels. While lineage markers such as CD45, CD3, or CD8 can be expressed at very high copy numbers per cell, some important markers such as transcription factors (e.g., FOXP3 for CD4 Tregs) or chemokine receptors (e.g., CCR5 on CD4 Th1 cells) are expressed at much lower levels. In addition, the available probes (such as mAb or HLA-peptide multimers) can have variable affinities for their respective targets. Probes are labeled with different chemical classes of fluorescent dyes that must be matched to the instrument, considering factors such as the availability of a high-power laser line with a wavelength close to the maximum absorption of the fluorescent dye and with a detector (photomultiplier plus filters/mirrors) that has a high sensitivity in the spectral emission range of the

given dye. Complicating matters further, cellular autofluorescence (i.e., fluorescence due to cellular molecules such as NADPH even in the absence of all dyes) limits the sensitivity that can be achieved with a given fluorescent probe, laser, and detector. In practical terms, autofluorescence of lymphocytes is usually limited to a distinct range of emission and absorption wavelengths [47, 48]. In general, the degree of autofluorescence determines the limit of detection, which in earlier reports was of 3,000 molecules for a standard flow cytometer [49]. Consideration of all these factors leads to the following recommendations for detecting cellular markers expressed at very low levels: use a high affinity Ab conjugated to a fluorescent dye with high quantum yield with emission spectral range far away from cellular autofluorescence, for which the cytometer has an appropriately matched high-power laser line and detector.

For polychromatic flow cytometry, additional constraints are set by the phenomena of optical spillover and spreading. In flow cytometry, cells are analyzed in a near-physiological aqueous solution to preserve the structural properties of biomolecules. Due to the spectral absorption of water and air, the useful spectral space is limited to the range from Near-UV (ca. 200 nm) to Near-IR (ca. 1,000 nm). Also, in aqueous solutions, both the absorption and emission of fluorochromes show relatively broad spectral lines. Together, this means that the number of fluorochromes that can be analyzed at the same time is ultimately limited: the combination of 15–20 different fluorochromes appears to be the upper feasibility limit [50].

As a further consequence, spectra of fluorescent dyes routinely overlap ("spillover") [51], requiring software deconvolution of true and observed signals ("compensation"). However, compensation cannot correct other errors caused by measurement, binning, and photon noise, and these errors accumulate to give an irreversible effect termed as "spreading error" [52] or "spillover spreading" [53]. Spreading error will cause the presence of one bright fluorochrome to reduce sensitivity for spectrally close fluorochromes present on the same cell. Use of a high-power laser close to the absorption maximum can reduce errors in photon counting, and narrow bandpass filters can reduce spillover; both these measures will reduce spreading error. Finally, probe combinations should be designed so that overlapping fluorochromes are chosen for labeling markers which are expected to be expressed on different cells.

In practice, panel development usually starts with the definition of a "wish list" of cellular targets, followed by the prioritization of these cellular targets, characterization of their expression levels, and checking for the availability of probes and conjugated dyes appropriate for the cytometer to be used. Guidance documents [54] and helpful software (CytoGenie: www.woodsidelogic.com, Fluorish: www.fluorish.com, Chromocyte: www.chromocyte.com) are available. A practical limitation can be the lack of commercially available fluorochrome conjugates for individual antibody clones. Indirect staining with secondary reagents (such as the biotin-streptavidin system) is possible but often not practical for multicolor applications. A better alternative is the use of new methods now available for the self-conjugation of small amounts of Ab to fluorescent dyes [55, 56]. Based on the discussion above, the cornerstones of panel development guidance are the assignment of "bright" probes for "dim" targets and strategies to avoid spreading error and autofluorescence in channels relevant for "dim" targets. It is also possible to change the optical pathway of the flow cytometer to optimize the instrument (e.g., choice of filters) according to the requirements of the panel. As the amount of potential interference between dyes rapidly increases with the number of colors in the panel, and as a large number of critical parameters should be optimized, development of large ($\geq$8 colors) panels and especially those involving separate staining steps for intracellular and extracellular targets can be an expensive iterative process requiring several man-months of dedicated work. Hence, the flow community is encouraged to share rigorously calibrated and optimized polychromatic panels via the "Optimized Multicolor Immunofluorescence Panels" (OMIPs) project [57].

Quality assurance of a flow cytometry assay starts with the flow cytometer itself, consisting of optimization, calibration, and standardization of the machine, and we refer the reader to the technical report by the Roederer group for details [58]. These optimization steps must not be neglected, as they may identify faulty parts that need replacement, such as a photomultiplier tube (PMT) with reduced sensitivity or suboptimal filters, and are important to optimize general instrument parameters. Conveniently, some (but not all) of these steps have been incorporated in vendor software packages, such as the Cytometer Setup and Tracking (CS&T) application within BD FACSDiva 6 that uses a proprietary mixture of calibration beads. For long-term immunomonitoring, it is essential to maintain accurate records of daily monitoring checks to track reproducibility and stability.

For cell staining, reagent quality can be an issue, especially if the assay is performed repeatedly over time. Often, reagents used are classified as "research use only" (RUO) and can show considerable batch-to-batch variation in important properties, such as concentration of antibody-dye conjugate, concentration of free dye, and even in the spectral properties of the dye (as in the case of tandem dyes). In addition, the shelf life designated by vendors is not always based on quantitative specifications. As a result, individual reagent batches have to be pretested and pre-titrated, and tests repeated even during the designated shelf life of a reagent. As batch sizes available from vendors are often limited, this can result in the requirement of reagent bridging (demonstration of the comparability of reagent batches) during the course of a study, leading to complex logistic and tracking processes. Reagent quality assurance may be facilitated by the preparation of mixtures of lyophilized reagents ("lyoplates") [59] that can reduce pipetting error and lead to increased reagent stability.

Appropriate use of technical and biological controls is also vital for assay interpretation. In addition to instrument calibration beads, unstained and single-stained beads are used to determine the spillover matrix for compensation. Isotype and "fluorescence minus one" (FMO) controls can help with setting gate boundaries at the analysis stage by defining the "negative" region. Pretested, aliquoted, cryopreserved samples with prescreened, predictable properties (such as being positive or negative for individual markers in the mAb panel) can serve as valuable biological controls which can be used in each assay run to track the variations in assay performance between operators and over time.

As flow cytometry-based methods become incorporated into clinical trials, the need for a stable and unlimited source of cell specimens that contains defined numbers of functional antigen-specific T cells as batch controls becomes paramount. Moreover, cell samples containing a known number of T cells specific for a defined Ag would allow easy assessment of the quality and accuracy of assays and provide standard controls for comparison of results across laboratories or time. Currently available sources for reference samples are either (i) based on leukapheresis or buffy-coat material from healthy donors – which are restricted to reactivity against immunogenic viral Ags, expensive and available in limited amount, or (ii) dependent on the ability to generate and propagate T-cell lines/clones on a repetitive basis which is a burdensome task. The Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) group has recently established a process for the generation of reference samples (RS) that can be used in T-cell assays. In a first proof-of-principle study, we showed that retrovirally TCR-transduced T cells spiked at defined numbers in autologous PBMC can be used as standard samples. The T cells could be accurately detected at all dilutions in a linear fashion, down to frequencies of at least 0.02 %, and the feasibility of RS was confirmed in a small-scale proficiency panel [60]. Subsequently, we established, optimized, and standardized the production of RS obtained by transfection of modified and stabilized RNA. Such a platform offers a simple, virus-free, and scalable process for the manufacturing of reference samples. In proof-of-concept studies for HLA-multimer experiments, the feasibility of using such RNA-engineered RS was shown. RS offered favorable properties across a variety of $CD8^+$ and $CD4^-$ T-cell-derived

TCRs against multiple Ags, including clear clustered populations, reproducible results, high stability over time, and the potential for linear dilution. Moreover, the analysis of the RS is similar to that of the tested cell samples in that the same gating strategy (and even the same gates) can be used. This suggests that RS are a useful tool to control T-cell assay performance. The suitability of these RS samples was subsequently tested in a proficiency panel organized recently (manuscript submitted).

A final, critical aspect of quality management is the careful documentation of each procedure performed, as well as provision of detailed standard operating procedures (SOPs) for each stage including data analysis. Technical staff needs to be well trained and perform the analyses on a regular basis to keep up performance. Participating in proficiency panels will also help improve laboratory standards.

25.4 Proficiency Programs Addressing Flow Cytometry Assays

While HLA-multimers and ICS are commonly used for monitoring experimental vaccines or other anticancer immunotherapies such as adoptive transfer of *in vitro* expanded T cells, there are still notable obstacles to the advancement of these T-cell monitoring assays as robust biomarkers for clinical trials [61, 62]. First, there is no gold standard protocol for any of these assays. Second, correlations between *in vitro* immunomonitoring results and patient clinical benefits have rarely been reported [4, 28, 63–67]. The reality is that assays performed at different institutions are not equal; this results in difficulties in comparing the efficacy of the various immunotherapy approaches tested for recruiting a meaningful anticancer T-cell response, in turn hampering progress in the field.

One approach for addressing these problems is by assay validation and standardization and/or centralization of the immunomonitoring at a dedicated core facility. An attractive alternative to these strategies is assay harmonization. The pros and cons of assay harmonization *vs*. standardization have been discussed in detail elsewhere [62, 68].

Assay harmonization is based on the participation of single laboratories in iterative testing exercises called proficiency panels. Pretested PBMC samples, synthetic peptides, and/or HLA-peptide multimers are shipped from a central lab to all panel participants who then use their own reagents, protocols, and analysis strategies for detecting antigen-specific T cells. Participants then report their data, which are centrally analyzed, allowing comparison of individual assay variables and performance to detect T cells. Thus, parameters involved in assay performance may be successively identified, corrected, and confirmed to exert an impact in subsequent panels (i.e., multistep approach). Finally, benchmarks and guidelines are formulated and disseminated to the community. Participating laboratories benefit by being able to measure their own performance in reference to peer laboratories, and regularly taking part in proficiency panels over time can also be seen as a quality control of assay performance for individual labs. Additionally, the working group can guide laboratories to improve performance if needed, while providing an exchange platform for assays and their application.

Proficiency panels can in principle be applied for any T-cell assay, including those based on flow cytometry [69–71]. In 2005, two consortia, the European Cancer Immunotherapy (CIMT) Immunoguiding Program (CIP) and the Cancer Immunotherapy Consortium of the Cancer Research Institute in the USA (CIC/CRI) launched a large program of proficiency panels and synergistically pioneered the concept of assay harmonization [62, 68]. From 2005 to 2012, the CIP (www.CIMT.eu/workgroups/CIP) has organized 15 small- to large-scale proficiency panels, dedicated to the measurement of antigen-specific $CD8^+$ T cells by HLA-multimers, ELISPOT, and intracellular cytokine staining.

Proficiency panels have taught us that there are large variations in the performance of T-cell assays among the flow community. While the majority of labs do detect antigen-specific T cells present at quite high frequencies in PBMC samples

(approx. >0.2 % of $CD8^+$ cells), the detection rate drastically decreases for low-frequency effectors (<0.05 % of $CD8^+$ cells). This is very relevant for cancer immunotherapy, as tumor-specific T cells are expected to be present at low frequencies in the blood, even after patient vaccination. Another lesson is that comparable performance is achievable with different laboratory-specific protocols and reagents, and full interlaboratory standardization is not necessary for good results. Surprisingly, we also found that operator experience in a method does not necessarily predict performance, underlining the utility of regular quality control of established methods. Finally, adoption of simple measures can lead to significant improvements in assay performance. For example, staining and acquiring larger numbers of $CD8^+$ cells increase the ability to detect low-frequency HLA-multimer-positive cells, and inclusion of a cell-resting phase improved sensitivity in the IFN-γELISPOT. In contrast, a high background production of the cytokine (IFN-γ) both in ICS and ELISPOT is clearly associated with decreased performance [72, 73].

Over several proficiency panel iterations, it also became clear that all steps of the assays, starting from cell handling (freezing/thawing/resting), assay conditions (reagents and protocols for mAbs and HLA-multimer staining, conditions of antigenic stimulation in ICS), result acquisition including instrument settings, down to the data analysis, can benefit from harmonization for achieving comparable results between laboratories. In flow cytometry specifically, instrumentation performance may be an issue, as we recently observed in a panel dedicated to the simultaneous detection of four Ag T-cell specificities by HLA-multimers (manuscript in preparation). Both CIC and CIP have also observed in independent panels conducted for ICS [73, 74], as well as for HLA-multimer staining [75, 76], that suboptimal gating strongly influenced the ultimate results – i.e., the detection and deduced frequencies of antigen-specific T cells. We also showed that analysis (gating) performed by a unique user substantially decreased the variation in the frequencies of specific cells as compared to those reported by single labs analyzing their own data (unpublished data). This is not a surprise, since manual gating is subjective and highly dependent on the experience of the experimenter and tradition in the lab. Further work is therefore needed with a focus on both data acquisition and analysis, including the potential for automated analysis strategies to reduce the subjectivity inherent in gating as described in Sect. 25.7.

25.5 Structured Reporting of Immune Assay Experiments

An increasing number of minimal information projects have emerged in the last years to provide guidance for structured reporting of biological assays. The first minimal information project that set the scene was the Minimal Information About Microarray Experiments (MIAME) published in 2001 [77]. It is now an established and mandatory standard for publishing microarray data for a growing list of highly recognized journals (http://www.mged.org/Workgroups/MIAME/journals.html). More than 30 such guidelines have emerged, asking for minimal information on reported results, including minimal information for cellular assays (MIACA) (http://miaca.Sourceforge.net/), specification for in situ hybridization and immunohistochemistry experiments (MISFISHIE) [78], and flow cytometry experiments (MIFloCyt) [79]. Information on the majority of available MI projects can be found in a central portal for minimal information on biological and biomedical investigations (MIBBI) (http://mibbi.Sourceforge.net/). These guidelines aim at achieving two major goals: first, to annotate data to such extent that they give transparent evidence on the quality, reliability, and possible error sources of reported results and, second, to use the reporting standard to systematically feed public databases [80].

More recently, structured reporting guidelines have also been provided for the specific context of immune assay experiments. As outlined before, the continuous conduct of proficiency panels over several years led to the identification of steps in the assay that critically impact the results, namely, (i) the sample, (ii) the assay, (iii) the data

acquisition, (iv) the data analysis, and (v) certain characteristics of the lab environment. In concordance with these findings, a flow chart of decisions that can affect the quality of data produced in clinical trials in which immunological parameters are monitored by flow cytometry was listed in a landmark publication [81]. Although the variables critically affecting the quality of results are – for most of them – well known, only very few scientific publications provide sufficient information on these aspects in their material and method descriptions. This lack of transparency is one of the major reasons preventing meaningful comparison of published results generated across institutions. In contrast, study results reported with transparent information on the essential variables of assay conduct, explicitly indicate awareness of the investigator to control critical variables, thus can be much better interpreted and reproduced.

To reduce the discrepancy between available knowledge on immune assay conduct and lack of critical information in scientific publications, a group of T-cell immunologists from the cancer immunology, infectious diseases, autoimmunity, and transplantation fields initiated the Minimal Information About T-cell Assays (MIATA) project [82]. The group conducted an intensive vetting process with two public consultation periods, two open consensus workshops, and several webinars [83]. The process towards reaching a broadly acceptable guideline on the minimum information that should be provided for T-cell assays [84] can be found at the project's webpage www.miataproject.org. With the MIATA consensus guidelines becoming available, the implementation of more structured reporting for T-cell immune monitoring can begin and should be considered by all investigators, especially for conducting T-cell assays in clinical trials [85]. So far, three peer-reviewed journals endorse the MIATA guidelines and assign the "MIATA label." The label indicates that authors of accepted manuscripts take great care about reporting on and control of variables that matter for T-cell assays. All MIATA compliant manuscripts will be listed on the MIATA homepage leading to greater exposure of the published work, which may increase interest and citations over time. The authors therefore recommend considering structured reporting of results from T-cell assays whenever possible.

25.6 Organization of Immune Monitoring in Multicenter Trials

Clinical trials will often require the recruitment of patients at multiple sites in order to reduce the overall duration and costs of the trial. The laboratory data generated from all patients and at different sites should be comparable, but as the regulatory framework for the conduct of clinical trials (ICH-GCP) is not very detailed with respect to standards of laboratory analyses, further details are specified by the more recent concept of good clinical laboratory practice (GCLP) [86–88].

Two general strategies emerge on how analytical assays can be performed among different sites [89]: in the distributed analysis paradigm, each site analyzes its locally derived samples. In contrast, in the central lab paradigm, all samples are transported to a central lab for analysis. In either case, flow cytometry poses additional challenges due to the fragility of the sample and the complexity of the assay.

For distributed analysis, the assay and instrumentation at different sites must be comparable. This can be achieved via full interlaboratory standardization, as is already routinely performed in clinical flow cytometry with *in vitro* diagnostic (IVD)-certified reagents and instruments [90]. Due to the high development costs, the number of clinical flow cytometry products for IVD on the market is limited and focuses on the clinically most relevant tasks as, e.g., the quantification of $CD4^+$ T cells in blood. In many cases, these applications lack the technical capabilities of modern polychromatic flow cytometry. Full-scale interlaboratory standardization (with demonstrated low interlaboratory variation) of research assays with RUO-grade reagents and customized flow cytometric instrumentation has been demonstrated by some groups but requires great efforts [91]. An alternative to full interlaboratory

standardization discussed in Sect. 25.4 is harmonization which can be achieved via regular participation in proficiency panels.

For highly complex flow cytometric assays within clinical trials, having all samples analyzed by the same central laboratory eliminates the need for full-scale interlaboratory standardization of participating institutes and may be less demanding. However, maintaining sample quality becomes a critical issue with this strategy. The initial sample material for flow cytometry contains living cells (in most cases derived from blood with the addition of anticoagulants). From this sample material, cells have to be isolated before the start of the flow cytometric assay. Cells are usually more fragile compared to biomolecules or small molecules. Several studies have been performed to determine how long blood can be stored or transported before peripheral blood mononuclear cell (PBMC) isolation (mostly using density gradient centrifugation) and how stable isolated cells are before the assay is started [34, 92, 93]. For simple phenotyping (e.g., CD4 counting), a 48 h delay before centralized analysis is acceptable, while the most demanding applications (such as some functional T-cell assays) require isolation of the cells within 8 h of venipuncture, followed by immediate analysis or cryopreservation of the cells [94]. Shipment to a central lab followed by processing of blood samples within 8 h is however not feasible in international multicenter trials. Therefore, a mixed model may be chosen [4], whereby cells are isolated and cryopreserved from peripheral blood at individual labs close to the patient and then shipped in the frozen state to the central lab where they are stored frozen before analysis. All stages of isolation, cryopreservation, and transport conditions should be fully standardized in this model. Standardized labeling of samples that allow the unambiguous assignment of a sample to a trial, site, patient, and visit is also critical. GCP regulation also requires special care to protect the privacy of patients, and this may be achieved by pseudonymization. These procedures have to be clearly defined in the clinical trial protocol and are usually further detailed in the clinical trial laboratory manual.

As an example demonstrating feasibility of this approach, an international, multicentric immunotherapy trial was conducted recently including T-cell immunomonitoring in which more than 40 clinical sites were trained in blood sampling, labeling, and shipping, with labels and collection tubes provided by a central laboratory. Local PBMC isolation laboratories were centrally supplied with pretested kits containing all critical reagents required for isolation and cryopreservation of PBMCs. All laboratory technicians were trained and qualified on central SOPs describing in detail the PBMC isolation and cryoconservation processes. Where required, the fresh blood was transported from the clinical sites to the PBMC isolating labs using temperature controlled shipments. The isolated frozen PBMCs were shipped to the central lab in validated dry ice containers. Patient visits involving a PBMC sampling were carefully coordinated in advance among the clinical sites, the PBMC isolating laboratories, and the logistic service providers to ensure that the blood could be processed within 8 h after venipuncture of a patient. This process led to a successful logistic chain for 361/362 (99.7 %) PBMC samples and an overall evaluability rate of 64/68 (94 %) patients for T-cell immunomonitoring [4].

25.7 Towards Automated Analysis

As discussed in Sects. 25.4 and 25.5, the standard approach for analyzing flow cytometry data is by the visual identification of cell subsets of interest on histograms or two-dimensional scatter plots. With multiparameter data, gating consists of first choosing a gating strategy – a sequence of dot plots that is designed to allow identification of the cells of interest. For example, a possible gating strategy for identifying HLA-multimer-positive $CD8^+$ T cells might be FSC-A/FSC-H (singlets), FSC-A/SSC-A (lymphocytes), CD3/viability dye (viable T lymphocytes), CD4/CD8 (basic T lymphocyte subsets), and CD8/multimer. In each dot plot, cells of interest are included and other events excluded by the use of elliptical or polygonal gates or sometimes by splitting the

dot plot into quadrants. The exact location and shape of these gates may be based on experience or by comparison with negative (e.g., isotype, FMO, or unstimulated control in ICS) and positive (reference sample or T-cell clone or superantigen stimulation) controls. After a gating strategy has been set, it is typically applied in common to all flow cytometry samples in the batch being analyzed. Some researchers will also adjust gates for individual samples to take individual variability into account. In general, there is no consensus or accepted standard gating strategy, and individual laboratories may apply different gating strategies to identify the same target cell subset. Notably, proficiency panels have made it very clear that the subjectivity of gating forms a significant source of assay variability between laboratories in the absence of a harmonization program [72, 95].

To increase the objectivity of flow cytometry analysis, automated methods in which cell subsets are directly quantified by machine algorithms have been proposed [96–98]. In broad terms, these algorithms have to first partition all the events in a data sample into disjoint subsets, based on properties of each individual event and its relationship to other events, and then to assign these subsets to biologically meaningful categories (e.g., HLA-multimer-binding $CD8^+$ lymphocytes). In the context of cancer immunology, a specific challenge for automated approaches is the high sensitivity required, since antigen-specific responses (e.g., HLA-multimer positivity or polyfunctional cells) may be relevant at relative frequencies of 0.01–0.1 %. Data from multiple laboratories significantly increases the challenges for automated analysis, since the algorithms have to also account for the variability across laboratories and issues with harmonization of sample annotation.

A typical automated analysis preprocessing pipeline starts with the extraction of the essential matrix of information stored in a flow cytometer FCS file, where each row represents an event and each column represents a detector channel, either scatter or fluorescent intensity. Preprocessing algorithms may apply compensation or specific transformations to regularize the data distribution (e.g., bi-exponential transformation). Specific channels may be explicitly excluded from analysis at this stage if they are not likely to be informative for the cell subset targets of interest. Often, a quality control filter is also applied at this stage, and data sets with inconsistent annotation, too few events, and anomalous event distributions or signatures may be flagged for manual evaluation [99].

The core of most automated analysis is the unsupervised partitioning of events into cell subsets. There are a variety of approaches that can be taken to partition or cluster events, as summarized in a recent publication [98]. One popular approach is the use of statistical mixture models, either identifying cell subsets with individual mixture components (which are typically multivariate Gaussian, student T, or skewed versions of these distributions) or using features of the estimated density to assign events to cell subsets [100–102]. Such probabilistic approaches provide a declarative framework to model domain knowledge and support formal statistical inferences for structure learning, classification, and prediction. The underlying statistical model for the domain knowledge can also be naturally extended in different contexts – for example, to incorporate specific assay details for combinatorial multimer encoding [103] or to incorporate multilevel effects via hierarchical modeling [104]. The power of probabilistic models comes at a price, in that these models tend to be much more computationally demanding than non-probabilistic approaches [105–108], and the runtime for analysis of high-volume, high-dimensional data sets may be prohibitive. However, recent developments in the use of highly parallel graphical processing units (GPU) [109] have accelerated run-times by orders of magnitude, making the probabilistic approaches a viable approach for many applications in cancer immunology.

The essential step in postprocessing is the alignment of cell subset clusters across multiple data samples, since comparative analysis of equivalent cell subsets is a necessary requirement of flow cytometry analysis in clinical research. Perhaps the most straightforward approach is to align each data sample with respect to either a reference or consensus clustering via an optimization

routine that minimizes some distance between pairs of clusters (e.g., Euclidean distance between cluster centroids). Other possible approaches skirt the problem entirely by enforcing a common clustering across all data samples or partition the clusters from fitting all data samples into "super-clusters" – all clusters in the super-cluster are then assigned to the same cell subset. The final step of assigning meaningful cell subset labels to the aligned clusters is typically done manually, although there have been recent efforts to develop heuristics that can automatically label clusters by establishing a concordance between cluster features and cell phenotype characteristics in the Cell Ontology. Innovations in the visualization of high-dimensional cytometry data have also greatly increased our ability to interpret the results of automated analysis [110–112].

The detection of antigen-specific cells poses a specific challenge for automated algorithms because of the extremely low frequency of these cell subsets in many patient samples – for example, as few as 0.01–0.1 % of the $CD8^+$ T lymphocyte population may be specific for a particular tumor Ag multimer. Two nonexclusive approaches for improving the ability of automated algorithms to improve the limit of detection are biased subsampling to enrich the sample for rare events [111, 113] or to increase the complexity of the statistical model [104]. The development of algorithms that can accurately and robustly identify rare cell populations is a driving motivator for much current research in automated flow analysis, and we expect rapid advances in this area. Illustrative examples comparing manual and automated analysis of antigen-specific cells for HLA-multimer and ICS assays are shown in Fig. 25.1.

Finally, we note that most of these automated analysis tools are developed under open source licenses and so free to use without restriction. Some packages require a modicum of programming ability to use effectively (e.g., R or Python scripting skills) and others are available online, but in general, these algorithms are probably not easily used by the average flow operator in a clinical research laboratory. In the coming years, we expect that these automated analysis tools will become increasingly accessible to the average flow operator with the following developments – developers of these tools will continue to improve their ease of use; the most successful algorithms will be incorporated into commercial software analysis packages; and more workshops will be organized to train people in the use and potential pitfalls of these exciting new technologies.

25.8 New Methods and Technologies

Flow cytometry has played an instrumental role in our comprehension of the immune system and its interplay with human tumors. The technique has recently experienced dramatic advances and the methods and technologies are evolving continuously. Due to space limitations, we focus here on the recent innovations that in our opinion have the potential to transform the field of general cytometry and are directly relevant for cancer immunotherapy.

Since the first description of a tumor Ag targeted by human T cells [114], many tumor-associated proteins and HLA-class I- and class II-restricted epitopes have been identified. However, the antitumor T-cell immune response as a whole, i.e., the repertoire of Ag specificities recognized by T cells of individual patients, has only rarely been dissected [115, 116]. This is indeed a difficult task, due to the inherent complexity of such projects (many Ags and HLA-allele restrictions have to be taken into account), along with the limited amount of patient material generally available, and high requirements in terms of cost and time. Two groups simultaneously described a combinatorial encoding method which is a very elegant way to circumvent most of these hurdles [117, 118]. The technique is based on the combination of many HLA-peptide multimers, whereby a single multimer is coupled to several (two or three) fluorochromes, generating a color code for each tested TCR specificity. Currently, up to 27 HLA-multimers labeled with eight fluorochromes can be combined in routine analysis [117]. Coupled to the production of HLA-monomers by the UV exchange technology, this high-throughput method represents an important technical

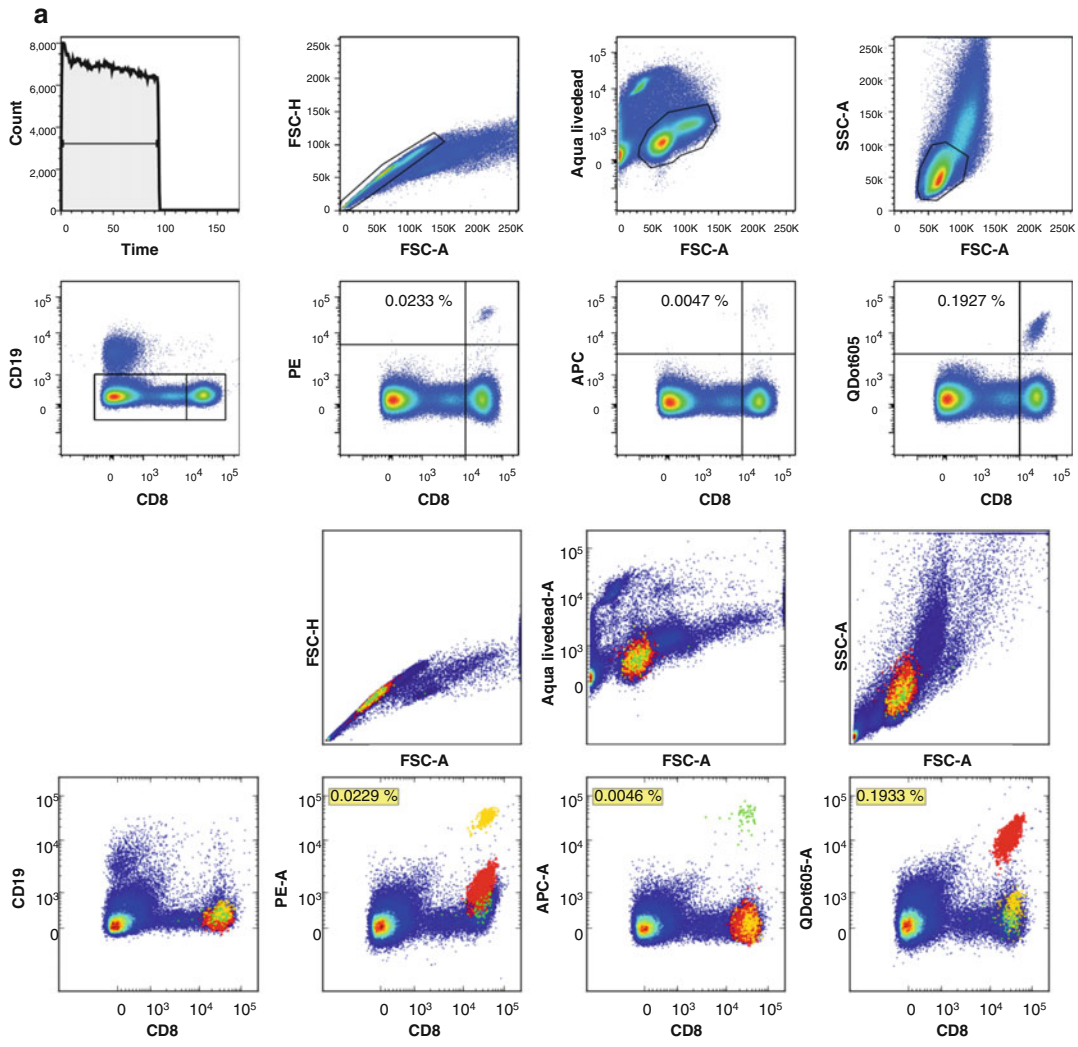

Fig. 25.1 (a) Manual and automated identification of antigen-specific MHC class I multimer-positive CD8⁺ T lymphocytes among PBMC of a HLA-A2⁺ healthy donor. *Top panel* shows a manual gating strategy to identify CD8⁺ T cells specific for three HLA-A*0201-restricted epitopes derived from a EBV, influenza, and CMV viruses. From *left* to *right*, the plots show gates to exclude artifacts due to flow stream bubbles or clumps (count/time), find singlets (FSC-A/FSC-H), exclude nonviable cells (FSC-A/Aqua LiveDead), identify lymphocytes (FSC-A/SSC-A), exclude B lymphocytes (CD8/CD19), and quantify CD8⁺ T cells binding to EBV BRFL1 peptide-MHC multimers (CD8/PE), influenza matrix peptide-MHC multimers (CD8/APC), and CMV pp65 peptide-MHC multimers (QDot605). *Bottom panel* shows the corresponding peptide-MHC binding CD8⁺ T cells identified using an automated analysis approach that fitted a Dirichlet Process Gaussian Mixture Model with 256 components to the data [103]. Essentially identical frequencies of peptide-MHC multimer positive cells are found with manual and automated analyses. (b) Manual and automated analysis of antigen-specific T cells among PBMC of a second HLA-A2⁺ healthy donor tested in an intracellular cytokine staining (*ICS*) assay after incubation with a synthetic peptide corresponding to an HLA-A*0201-restricted epitope of pp65 CMV. Manual analysis finds cells positive for IFN and TNF, and a few events positive for IL-2. Without further gating, it is not possible to tell if the IFN- and TNF-positive events come from two separate or a single bifunctional population. Automated analysis reveals that there is indeed a single-cell population positive for IFN and TNF, with no evidence for an IL-2-positive population. Again, the frequencies of antigen-specific events identified by expert gating and automated analysis are almost equivalent

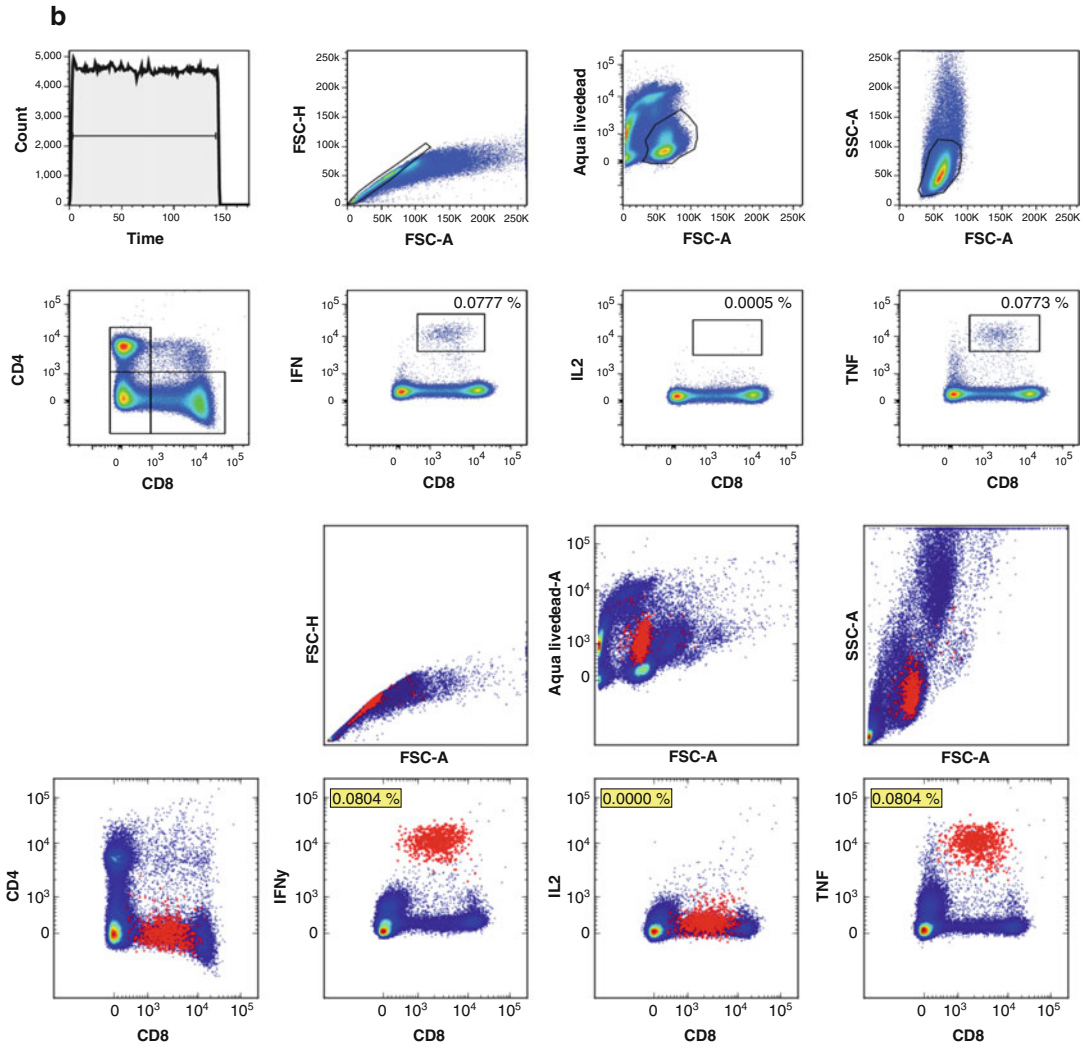

Fig. 25.1 (continued)

achievement for the T-cell immunology field and has started to deliver precious information by dissecting the anti-melanoma TIL repertoire in melanoma patients [119, 120]. Combinatorial staining could easily be implemented for monitoring vaccination trials, for example, when applying cocktails of antigenic peptides for which many specificities need to be tested in a single PBMC sample.

The combination of extracellular phenotyping with determination of intracellular changes in phosphorylation patterns upon stimulation is starting to provide new insights into signaling pathways in healthy and disease conditions [121, 122]. The binding of cytokines to their specific cell surface receptors generally results in the activation (i.e., phosphorylation) of the downstream signal transducers and activators of transcription (STATs), which in turn regulate the expression of many genes involved in cell growth, survival, differentiation, and polarization. Next to cytokines, the effect of unspecific mitogenic stimuli such as phorbol myristate acetate (PMA), phytohemagglutinin (PHA), or MHC-peptide complexes binding to the T-cell receptor (TCR) can be studied by measuring the level of other key signaling molecules such as phosphorylated (p)-Erk, p-S6, and p-NF-κB in T and B cells, whereas Toll-like receptor (TLR)

ligand-induced activation can be followed with p-Akt, p-Erk, and p-NF-κB in B cells and monocytes. The proof of principal for a "single-cell network profiling (SCNP) method" was obtained on healthy donors PBMCs [123]. In this initial study, age as well as race differences were observed, whereas intra-donor variability needs to be established by testing blood samples taken at different time points over time. As T-cell signaling defects have been described in cancer patients [124, 125], insights in the intracellular phosphorylation patterns of T cells, including during immunotherapy, may soon deliver precious information.

A fundamental advance in flow cytometry in recent years is an increase in the number of parameters that can be simultaneously evaluated on single cells. Access to an increasing number of reagents and fluorochromes including tandem conjugates, semiconductor nanocrystals (quantum dots or eFluors), and organic polymers (brilliant violet family) [126–128], together with the wide availability of sophisticated flow cytometers, is making polychromatic analysis mainstream.

However, spectral overlap ultimately limits the number of fluorochromes in a single panel to an upper bound of approximately 20, as described in Sect. 25.3. An exciting new technology that has the potential to greatly increase the number of measurable parameters is mass cytometry (CyTOF), which uses stable heavy metal ions tagged to Abs (or, e.g., MHC multimers) in place of fluorochromes. These isotope labels are detected by time-of-flight mass spectrometry after vaporization of the cell. Although isotope labels generally produce a signal of low intensity, they have a lower background and virtually no spillover, making the measurement of a much larger number of markers feasible.

Mass spectrometry has been reported to be qualitatively and quantitatively equivalent to flow cytometry, with the simultaneous analysis of more than 30 parameters being already possible [129]. However, this promising new technology has the current following limitations as compared to traditional flow cytometry: lower label sensitivity, substantial cell loss, low acquisition rate, and the impossibility to sort living cells. Nevertheless, this method has started to reveal the complexity of healthy hematopoietic cells and of $CD8^+$ T lymphocytes subsets and will certainly mature to become an indispensable technique in cancer immunology and immunotherapy [129, 130].

25.9 Concluding Remarks

Flow cytometry is the prototypical multiparameter single-cell assay, with applications in cancer immunotherapy ranging from epitope screening to immune monitoring of clinical studies. Due to its ability to characterize complex immune phenotypes and flexibility in measuring multiple immune functions such as Ag binding, expression of activation and inhibitory markers, cytokine production, cytotoxicity, and proliferation, flow cytometry is indispensable in cancer immunology research. However, because of the complexity of the assay and the fragility of the sample, it is challenging to apply and maintain robustness, sensitivity, and reproducibility, especially across multiple laboratories. Factors to consider when using flow cytometry in clinical research include understanding the range of flow-based assays available, as well as best practices for instrument, reagent, sample, and data analysis.

In order to harmonize laboratory protocols, practices, and analysis strategies, flow cytometry proficiency testing programs have been organized to learn and raise awareness of best practices. We believe that participation in proficiency testing programs, along with other initiatives delivering protocols, assay guidelines and reporting frames, is critical for raising the standard of flow cytometry analysis and strongly recommend that all clinical research laboratories that perform immune monitoring for clinical trials join such programs.

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Immunohistochemistry of Cancers

26

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Contents

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26.1 Introduction

Immunohistochemistry (IHC) is the art of using antibodies (Abs) to detect specific antigens (Ags) in tissues. Histopathologic evaluation of diseases has been altered and enhanced by the advent of IHC, and some sophisticated techniques have been replaced by IHC due to its easy and versatile immunohistochemical techniques. Of course, disorganized application of IHC could be misleading.

Immunohistochemistry is based on specific Ab-Ag interactions. The Abs which are used to detect Ag(s) are called primary Abs. Primary Abs are linked to enzymes (main part of chromogenic system) via another Ab called link Ab. This linkage to enzymes is mediated by polymers or some molecules such as streptavidin-biotin complexes. Peroxidase is the enzyme mostly used in immunohistochemistry. Alkaline phosphatase is also used (but less frequently). Some mechanisms are shown in Fig. 26.1.

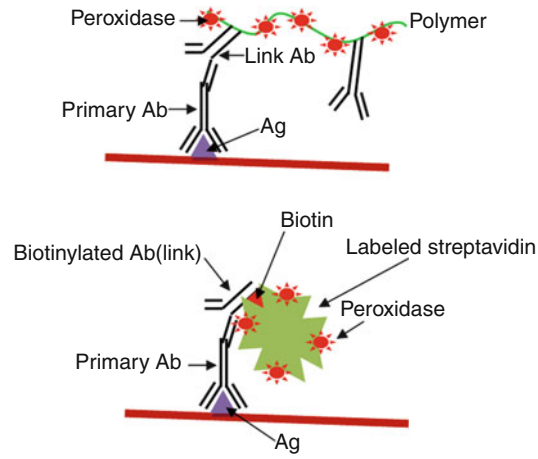

Fig. 26.1 Schematic mechanism of two immunohistochemistry methods. *Top*: secondary antibodies and enzymes link to polymer molecule. *Bottom*: biotinylated secondary antibody and labeled streptavidine

Immunohistochemistry has wide application including research uses, diagnostic purposes, and prognostic and therapeutic aims. IHC is a nice technique for tracking of proteins and haptens, so it is used to define expression of specific genes at the level of proteins. It is also very useful in diagnostic pathology including definition of cellular lineage (epithelial, vascular, lymphoid, etc.) or subtyping of some specific lesions and malignancies such as malignant lymphomas. Prognostic and therapeutic applications have gradually become widely popular such as the definition of hormone receptor status of breast cancer (ER, PR, and AR) and oncogene products (e.g., Her2, EGFR, c-kit, etc.) which could be a part of guidelines for targeted therapy of the tumors.

26.2 Immunohistochemistry of Skin Tumors

26.2.1 Markers of Normal Skin

Skin tissue is composed of epidermal and adnexal components as well as mesenchymal dermal components. All epithelial cells in the epidermis, folliculosebaceous unit, and sweat glands reveal pan-keratin markers such as AE1/AE3 (Fig. 26.2a). Keratinized squamous cells

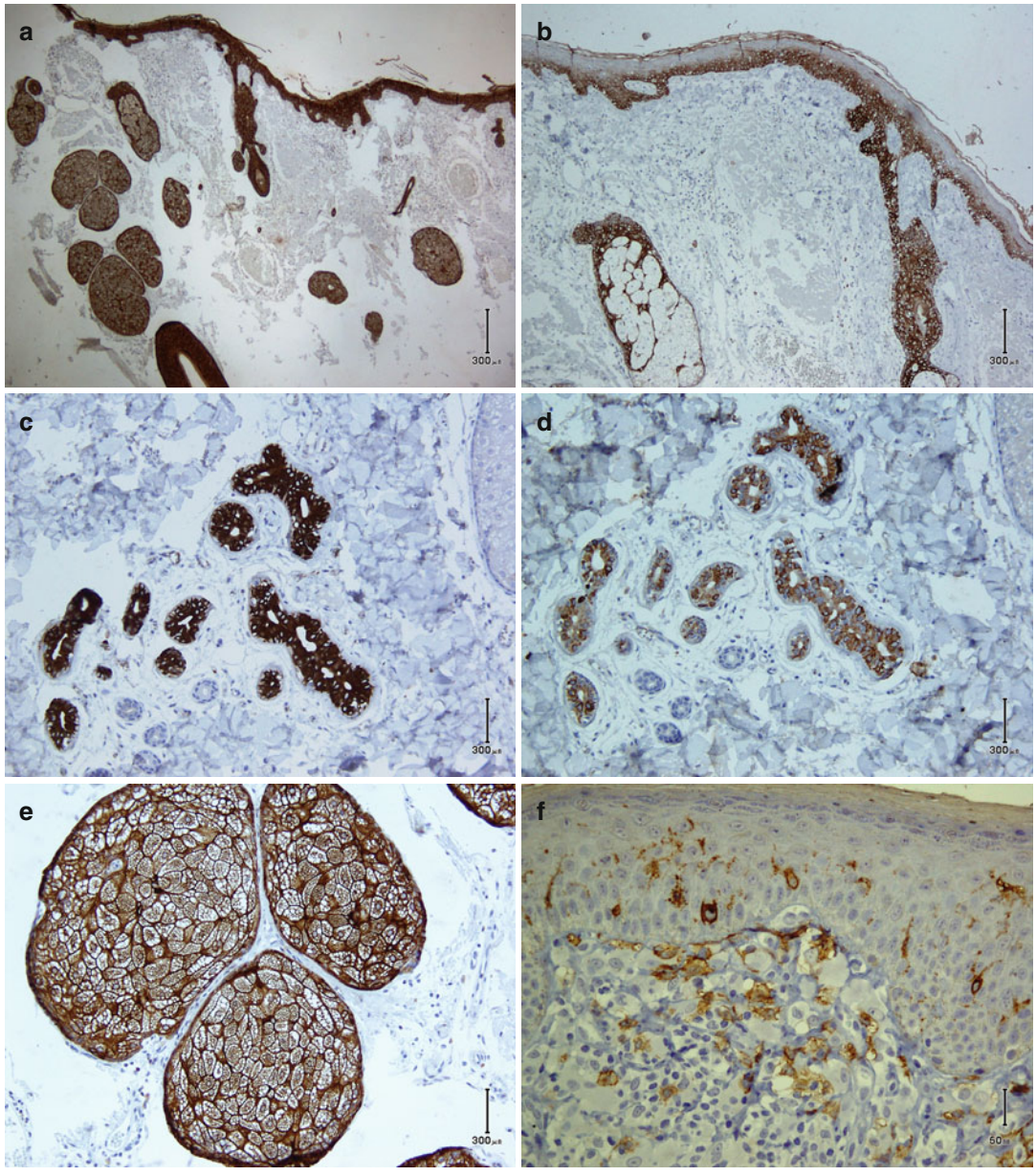

Fig. 26.2 Normal skin. (**a**) Pan-keratin of AE1/AE3 stains the epidermis, folliculosebaceous unit epithelium, and sweat glands. Basal keratinocytes are highlighted by CK5 (**b**). Sweat glands are immunostained by CK7 (**c**) and CK20 (**d**). EMA (**e**) reacts with sebaceous glands rimming cytoplasmic vacuoles, and CD1a highlights dendritic Langerhans cells in the epidermis (**f**)

and proliferative keratinocytes express cytokeratin (CK) 6/16, nonkeratinized squamous cells reacts with CK4/13, and basal keratinocytes exhibit reactivity for CK5/14/15 (Fig. 26.2b). Squamous cells in palm and sole are reactive for CK1/9/10 [1, 2]. Eccrine and apocrine glands comprise sweat structures of the skin. Normal eccrine glands show reactivity with CD7, CD20 (Fig. 26.2c, d), CEA, and S100, while apocrine glands exhibit immunostaining for CEA and GCDFP15 [3, 4]. Sebaceous glands exhibit reactivity for CK10 as well as EMA rimming

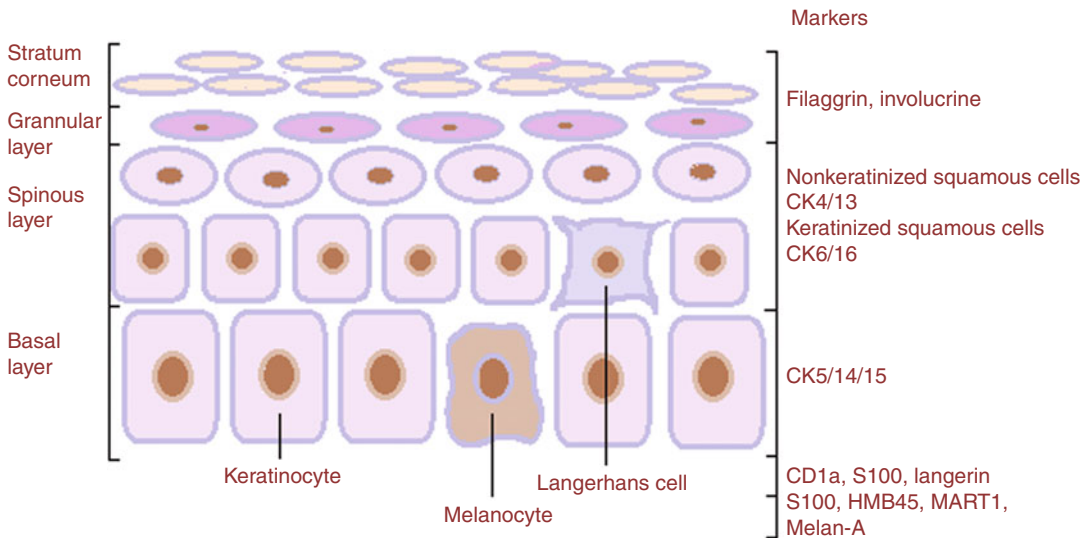

Fig. 26.3 Immunohistochemistry antibodies in schematic normal epidermal components

Table 26.1 Immunoprofile of normal epidermis, folliculosebaceous, and sweat gland structures in comparison with respective tumors

Cell	Antibodies	Tumor	Markers
Keratinocyte	CK6/16	Squamous cell carcinoma	EMA, p63
Basal keratinocyte	CK5/14/15	Basal cell carcinoma	BerEp4
Eccrine cell	CK7, CK20, CK5/14, CK1/10, CEA, S100	Eccrine carcinoma	EMA, CEA, CD15, p63, S100
Apocrine cell	CEA, GCDFP15	Apocrine carcinoma	EMA, CEA, CD15, p63, CA72.4, GCDFP15
Trichogenic cell	CK14/15/19	Trichilemmal carcinoma	CEA, S100
		Proliferating trichilemmal carcinoma	EMA, CD34
Sebaceocyte	CK5/14/15, CK8/18	Sebaceous carcinoma	EMA

cytoplasmic lipid vesicles (Fig. 26.2e) [5]. Normal melanocytes express S100, HMB45, and MART-1/melan-A but do not react with tyrosinase [6]. Langerhans cells are stained with CD1a (Fig. 26.2f), S100, langerin, and CD31 [7]. Displaying neurotactile differentiation, Merkel cells of normal skin are reactive for CK20, MOC-31, neurofilament, and CD56 [8–10]. Markers of the normal epidermal components are depicted in Fig. 26.3. The immunoprofile of normal skin components and respective cancers is summarized in Table 26.1.

26.2.2 Epithelial Tumors

Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are derived from the spinous layer and basal layer of the epidermis, respectively. Well-differentiated SCC expresses high molecular cytokeratin, while those with poor differentiation express low molecular cytokeratin. Cytokeratin, p63, and vimentin are present in the sarcomatoid variant of SCC [11]. EMA, one of the human milk fat globule proteins not expressed in normal keratinocytes, is expressed on malignant

squamous cells. Basal cell carcinoma expresses BerEp4 (Fig. 26.4) but does not demonstrate reactivity with EMA and p63, distinguishing it from SCC [12].

26.2.3 Sweat Gland Tumors

Malignant eccrine tumors are distinct from benign eccrine tumors by displaying reactivity with EMA. Eccrine tumors display CEA, CD15, and p63 which are also common with apocrine tumors. Differentiating markers of apocrine tumors are TAG-72 (CA72.4) and GCDFP15 (Fig. 26.5) which are not expressed on eccrine tumors [4]. S100 is demonstrated in 50 % of eccrine tumors, but not in apocrine tumors. A remaining challenge is distinguishing primary eccrine carcinoma from metastatic carcinoma by immunoprofile of CK5/6 and p63 which are positive in eccrine carcinoma, but not in metastatic carcinoma [13]. Paget disease is an intraepidermal extension of neoplastic cells into the epidermis which shares similar histopathologic features with malignant melanoma and Bowen disease. Immunohistochemistry study can be a helpful method in differentiating these tumors as denoted in Table 26.2 [14]. CK20 and GCDFP-15 are useful markers in distinguishing primary and secondary perianal Paget diseases, respectively [15].

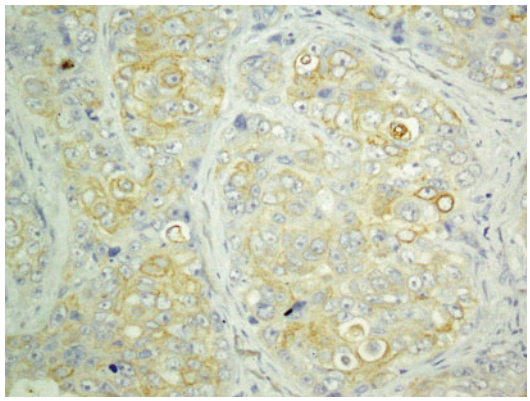

Fig. 26.4 Immunoreaction of basal cell carcinoma with BerEP4

26.2.4 Trichogenic Tumors

Tumors with trichilemmal differentiation display reaction with CK14/15/19, BerEP4, and p63 but do not react with EMA (except proliferating trichilemmal tumor), CEA, S100, CD15, CA72.4, HMB45, and GCDFP15 [3]. Trichilemmal carcinoma displays reactivity with CEA and S100, and proliferating trichilemmal carcinoma (malignant proliferating tumor) shows reactivity with EMA and CD34 [17]. Desmoplastic trichoepithelioma shares histopathologic similarities with infiltrating BCC and microcystic adnexal carcinoma. The immunoprofile of these tumors are demonstrated in Table 26.3.

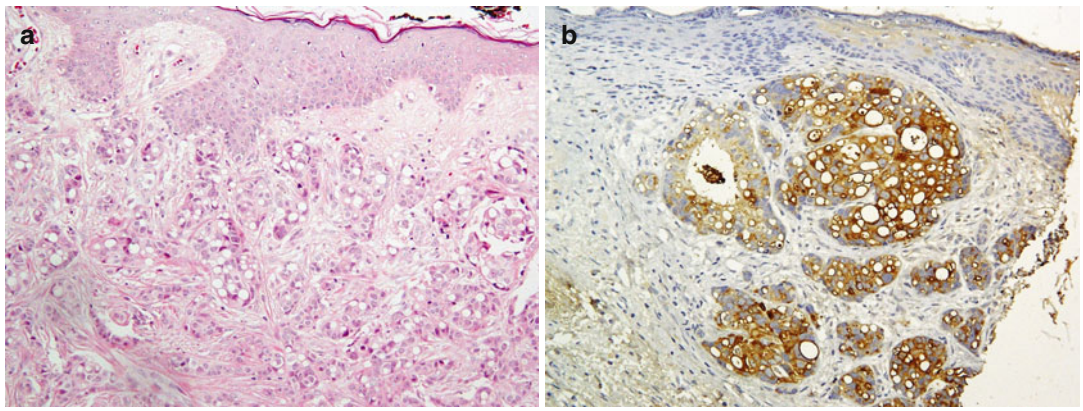

Fig. 26.5 Primary skin apocrine carcinoma (**a**) immunostained by GCDFP15 (**b**)

Table 26.2 Immunophenotype of mammary and extramammary Paget disease (PD), Bowen disease, and malignant melanoma

Makers	Mammary PD	Extramammary PD (apocrine carcinoma in situ)	Bowen disease (SCC in situ)	Melanoma (in situ)
CK7	+	+	–	–
CEA	+	+	–	–
CAM5.2	+	+	–	–
GCDFP15	+	+	–	–
MUC1	+	+	–	–
MUC5AC	–	+	–	–
CA15-3	+	–	–	–
CA72.4	–	+	–	–
KA-93	–	+	–	–
CK5/6	–	–	+	–
S100/HMB45/MART	–	–	–	+

Refs. [14–16]

Table 26.3 Immunoprofile of desmoplastic trichoepithelioma (DTE), infiltrating basal cell carcinoma (IBCC), and microcystic adnexal carcinoma (MAC)

Tumor	DTE	IBCC	MAC
Panel antibodies	EMA, CK5/6, CD10 (stroma), CK15, CK20, p63, Bcl-2, BerEP4	CK5/6, CD10 (epithelial), p63, Bcl-2, BerEP4, stromelysin-3, p53	EMA, CK7, Ck5/6, CK15, p63, SMA

Refs. [18–20]

26.2.5 Sebaceous Tumors

Sebaceous tumors exhibit reactivity with CK5/14/15, CK8/18, EMA, CD15, anti-adipophilin (ADP) and androgen receptor. CK15 is positive in sebaceoma but does not exhibit reactivity with sebaceous carcinoma [21]. Sebaceous tumors do not express CEA, S100, CA72.4, and GCDFP-15 in comparison with sweat gland tumors, which are positive for these markers [4, 22]. Sebaceous carcinoma is differentiated from BCC by showing reactivity for EMA (Fig. 26.6) and negative reaction to BerEP4, vice versa of BCC [23]. Proliferating markers are good markers to differentiate sebaceous adenoma from sebaceous carcinoma (Table 26.4).

26.2.6 Melanocytic Tumors

Being a sensitive but a nonspecific marker of melanoma, S100 is a calcium-binding protein given its name because of solubility in 100 % saturated ammonium sulfate solution. Other S100-positive tumors include undifferentiated carcinoma, nerve sheath and glial tumors, adipose tumors, and histiocytic and Langerhans cell proliferations [26, 27]. Considering as highly specific marker of melanocytes, the gp100 group includes HMB-45 and MART-1/melan-A with 60 and 80 % sensitivity, respectively. Melanoma antigen recognized by T-cells-1 (MART-1) is a protein which serves as a potential target for cytotoxic T lymphocytes recognized by two monoclonal antibodies (mAbs), A103 and melan-A [28]. Desmoplastic/spindle cell variant of melanomas does not show reactivity with HMB45 and MART/melan-A. Instead, these melanomas are more reactive with S100, p75-NGF-R, and tyrosinase [29]. Small cell melanoma is another variant of the melanoma which could be distinguished from other small cell undifferentiated tumors of the skin and subcutaneous tissue by Abs panel (Fig. 26.7). The immunoprofiles of these tumors are summarized in Table 26.5.

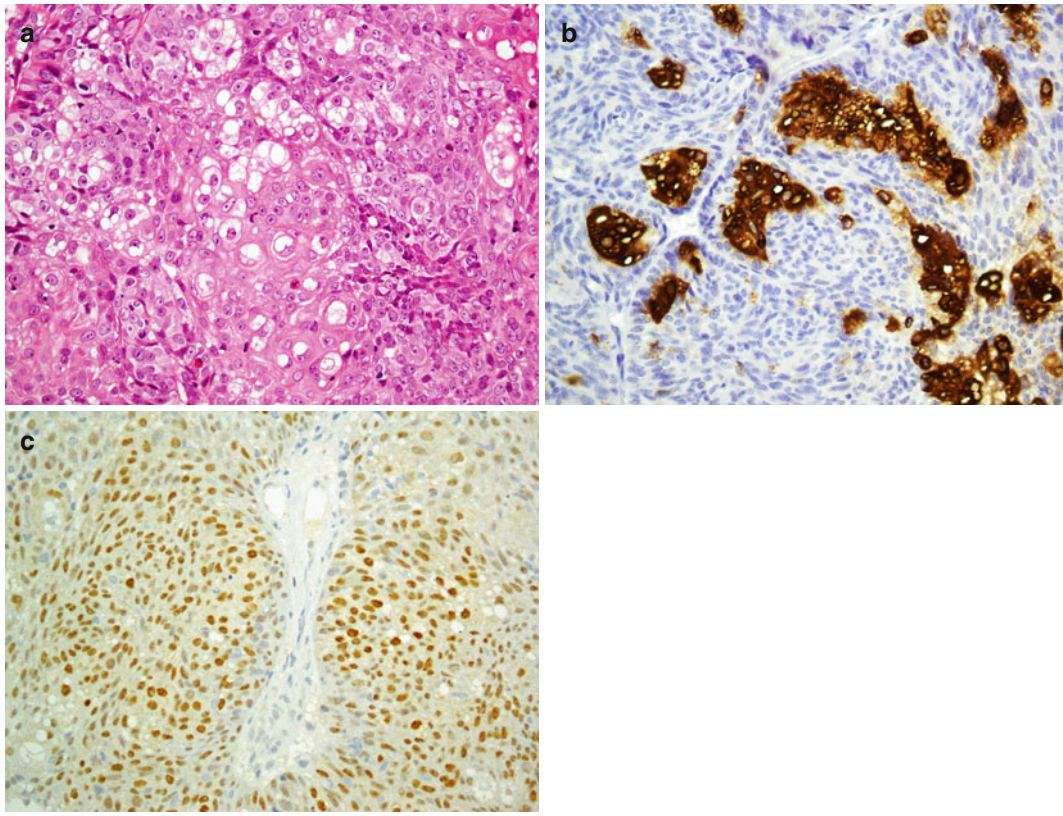

Fig. 26.6 Sebaceous carcinoma (**a**). Sebocytes are stained with EMA (**b**). Nuclear reactivity of tumor cells for androgen receptor (**c**)

Table 26.4 Immunoprofile of sebaceous adenoma (SA) and sebaceous carcinoma (SC)

Tumor	Ki-67 (%)	p53 (%)	Bcl2 (%)	p21 (%)
Sebaceous adenoma	10	11	56	34
Sebaceous carcinoma	30	50	7	16

Refs. [24, 25]

26.2.7 Prognostic Markers of Melanoma

Detection of *BRAF p.V600E* mutation by immunohistochemistry in melanomas could be used as a first step to identify patients with melanoma as candidates for BRAF inhibitors. Displaying by immunohistochemistry, melanoma progression is correlated with MERTK expression: highest in metastatic melanomas, followed by primary melanomas and nevi [32, 33]. Other prognostic markers correlated with melanoma progression and prognosis include MIB-1 (Ki-67), Bcl2, p53, p16, cyclin-D1, cyclin-D3, osteopontin, NM23, E-cadherin, beta-catenin, Wnt5a/frizzled, Cdc42, and CXCR4 [34–40].

26.2.8 Specific Mesenchymal Tumors of the Skin

Mesenchymal tumors are discussed in soft tissue tumors, but some tumors which are more seen in skin are discussed here. Kaposi sarcoma which originates from endothelial cells is an intermediate malignant potential vascular tumor of the skin positive for a highly sensitive and specific Ab

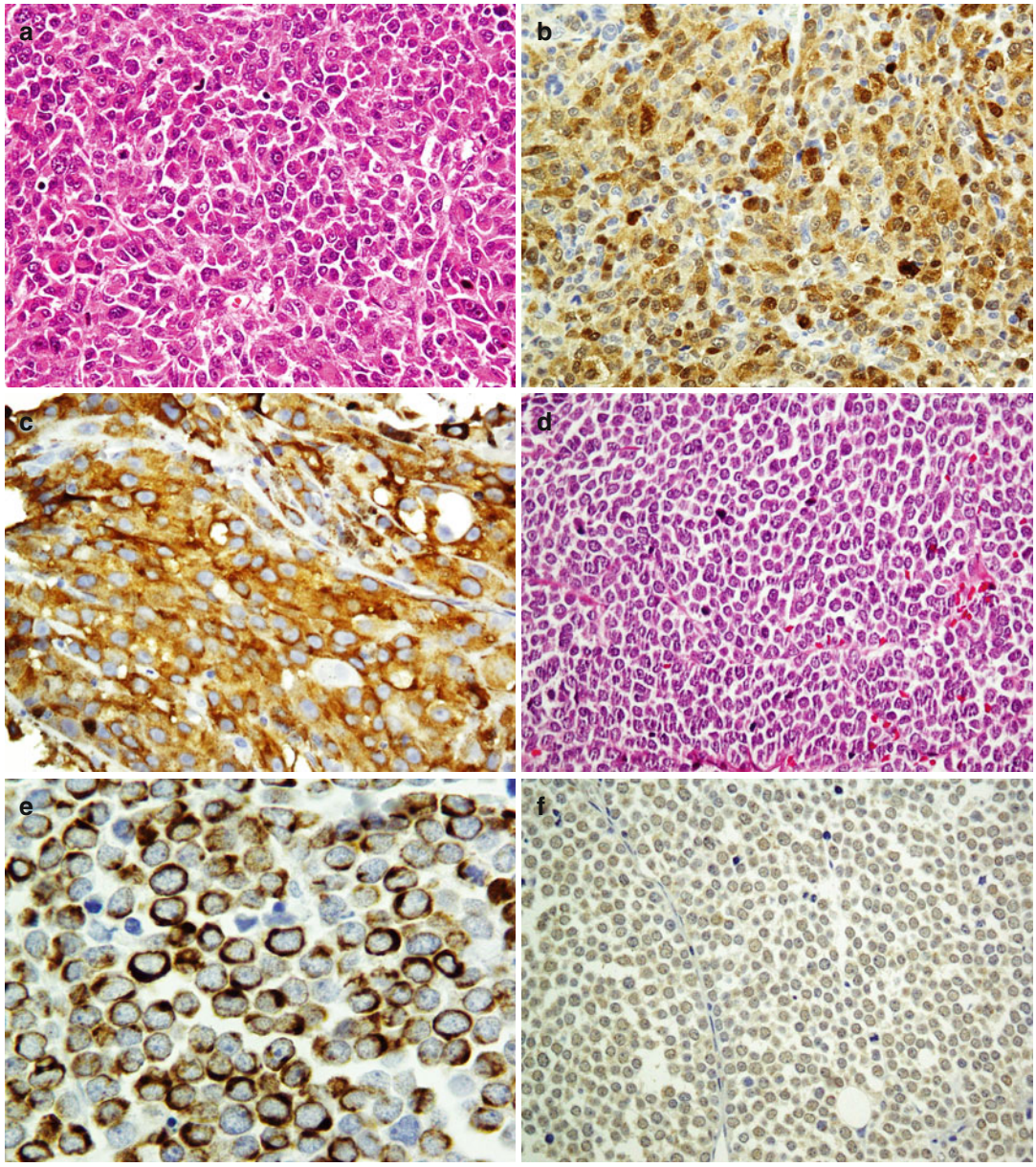

Fig. 26.7 Small round cell tumor in the skin. Malignant melanoma (**a**) reacts with S100 (**b**) and melan-A (**c**) antibodies. Merkel cell carcinoma (**d**) immunostained by CK20 as paranuclear dots (**e**) and shows weak reaction with CD99 (**f**)

called HHV8 latent nuclear antigen-1 [41]. Dermatofibrosarcoma protuberance is an intermediate tumor of fibrohistiocytic cell origin which is diffusely positive for CD34 (Fig. 26.8) and negative for factor XIIIa separate from dermatofibroma which is in reverse of DFSP (*CD34*−, factor XIIIa+) [42]. Considering it as a superficial variant of malignant fibrous histiocytoma, atypical fibroxanthoma is a fibrohistiocytic tumor exhibiting reactivity with vimentin, CD10, and CD99 (Fig. 26.9) [43]. Among tumors with smooth muscle differentiation, leiomyoma and leiomyosarcoma are reactive for SMA, desmin, and caldesmon similar to extracutaneous equivalents [44, 45]. Neurothekeoma (NTKs) is a distinctive neoplasm of the skin showing schwannian and

Table 26.5 Immunopanel of small cell melanoma (SCM), Merkel cell carcinoma (MCC), small cell squamous carcinoma (SSCC), small cell eccrine carcinoma (SEC), peripheral neuroectodermal tumor/extraskeletal Ewing sarcoma (PNET/ES), lymphoma, rhabdomyosarcoma (RMS), and metastatic pulmonary small cell carcinoma (MPSC)

Panel antibodies	SCM	MCC	SSCC	SEC	PNET/ES	Lymphoma	RMS	MPSC
S100/HMB45/MART	+	–	–	–	–	–	–	–
CK20/CD56/SYN/CGN	–	+	–	–	–	–	–	–
CK/EMA	–	–	+	+	–	–	–	+
CD15/MOC31/TAG-72	–	–	–	+	–	–	–	–
CD99/CD56/SYN/CGN	–	+	–	–	+	–	–	–
LCA/CD3/CD20	–	–	–	–	–	+	–	–
DES/MSA/MYG	–	–	–	–	–	–	+	–
CEA/TTF-1	–	–	–	–	–	–	–	+

Refs. [26–31]
Note: *CGN* chromogranin A, *DES* desmin, *MYG* myogenin, *MSA* muscle-specific antigen. *LCA* leukocyte common antigen, *SYN* synaptophysin

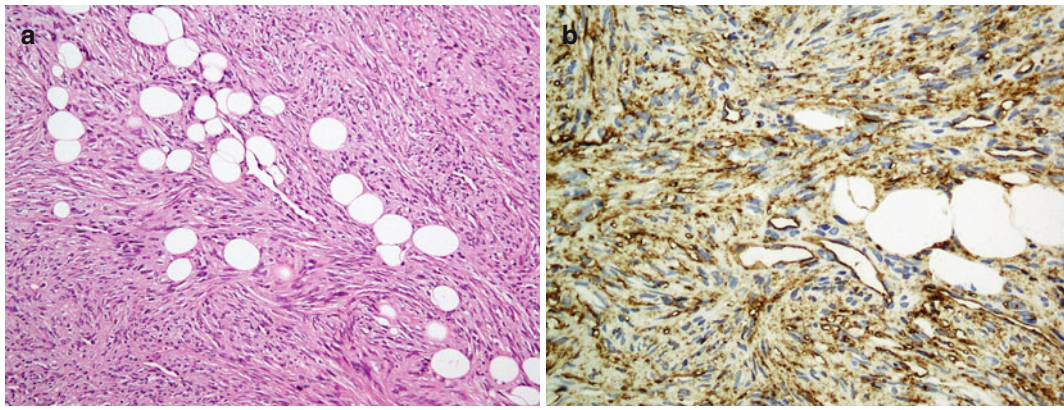

Fig. 26.8 Dermatofibrosarcoma protuberans. Spindle fibrohistiocytic cells, entrapping subcutaneous fat tissue (**a**) highlighted by CD34 (**b**)

neuroectodermal differentiation which typically labels with S100 (conventional variant), CD99, and NKI-C3 (cellular variant) [46].

26.3 Immunohistochemistry of Head and Neck Tumors

26.3.1 Tumors of the Nasal Cavity and Paranasal Sinuses

Tumors of the nose and paranasal sinuses can be categorized in two groups of small cell carcinomas and undifferentiated carcinomas. Small cell carcinomas of the nasal cavity and paranasal sinuses include olfactory neuroblastoma (ONB), melanoma, lymphoma, rhabdomyosarcoma, small cell neuroendocrine carcinoma, and ES/PNET (Table 26.6). Undifferentiated carcinomas include sinonasal undifferentiated carcinoma, undifferentiated nasopharyngeal carcinoma (Fig. 26.10), and undifferentiated neuroendocrine carcinoma (Fig. 26.11) [47, 48]. All poorly differentiated and undifferentiated carcinomas express cytokeratin [49]. Undifferentiated nasopharyngeal carcinoma reacts with EBV, and undifferentiated neuroendocrine carcinoma is positive for neuroendocrine markers and S100 [50]. NUT midline carcinoma (NMC) is an aggressive tumor with translocation of the *NUT* (*nuclear protein in testis*) gene resulting in the formation of *BRD4-NUT* fusion gene. Recently, new mAbs against the NUT Ag have been designed which will improve the diagnosis of

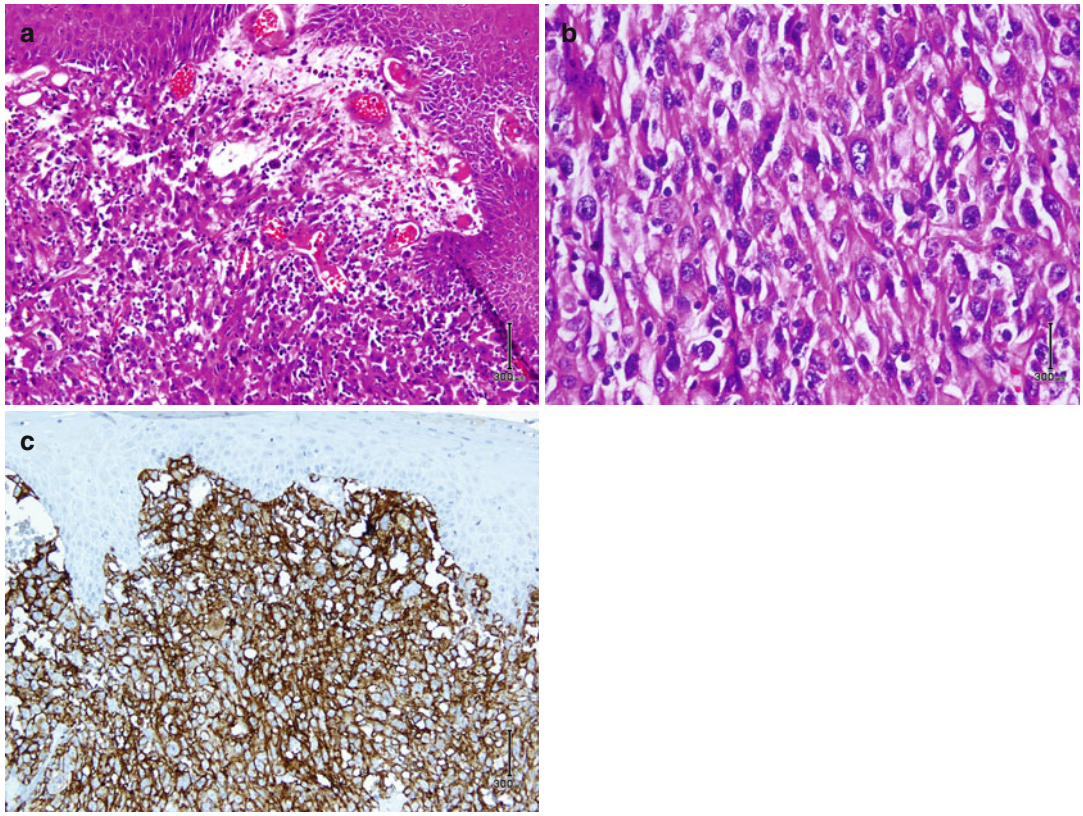

Fig. 26.9 Atypical fibroxanthoma. Atypical pleomorphic cells with vesicular nuclei in the dermis (**a**, **b**) are immunostained by CD10 (**c**)

Table 26.6 Immunohistochemistry of small cell carcinomas of nasal cavity: olfactory neuroblastoma (ONB), rhabdomyosarcoma (RMS), Ewing sarcoma/peripheral neuroectodemal tumor (ES/PNET), and small cell neuroendocrine carcinoma (SNEC)

Tumor	ONB	Melanoma	Lymphoma	RMS	SCC	ES/PNET	SNEC
Immunoreactive markers	SYN	HMB45, S100, vimentin	LCA, vimentin	Desmin, Myogenin, vimentin	AE1/AE3, EMA, SYN	CD99, SYN	Cytokeratin, neuroendocrine markers

Refs. [49, 53–57]

NMC [51]. Immunohistochemistry of poorly differentiated and undifferentiated carcinomas are denoted in the Table 26.7.

26.3.1.1 Theranostic Application

In olfactory neuroblastoma, immunoreactivity with bcl-2 may predict response to neoadjuvant chemotherapy and seems to be associated with worse survival [52].

26.3.2 Tumors of the Larynx, Nasopharynx, and Oropharynx

Squamous cell carcinoma (SCC) is the most common malignancy in the head and neck. Typically, head and neck SCCs are positive for cytokeratin cocktails, AE1/AE3, and pan-cytokeratin. Human papilloma virus (HPV) is detected in some SCCs

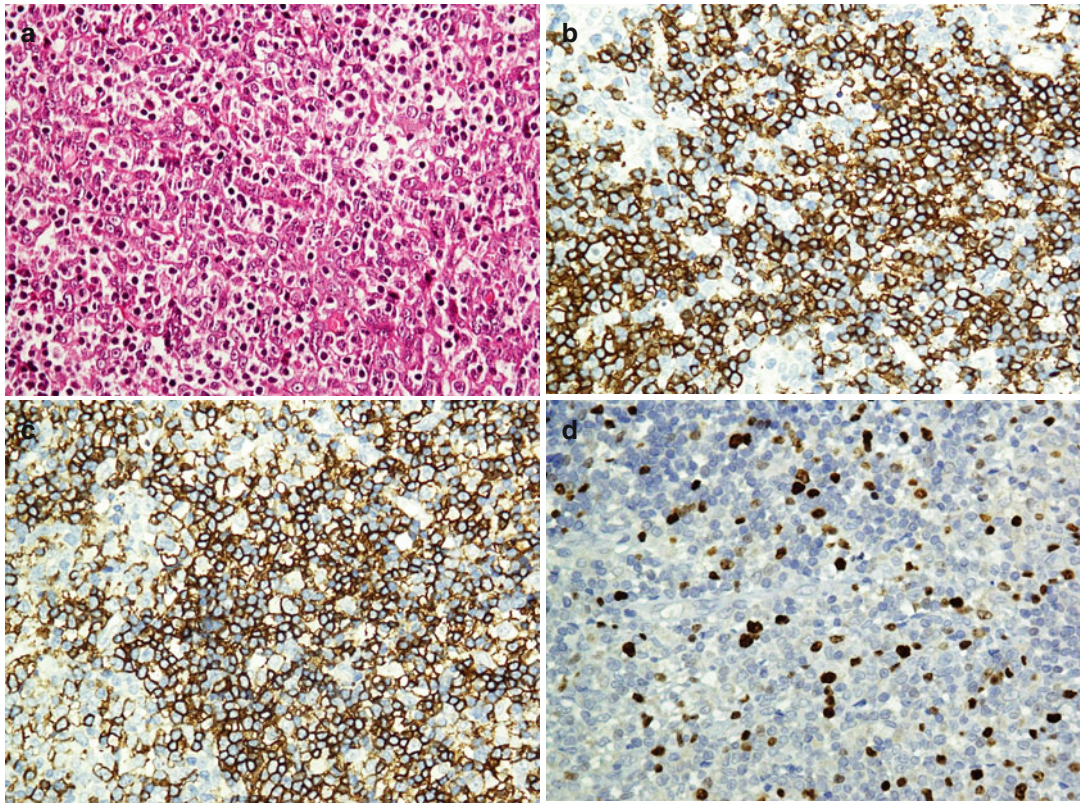

Fig. 26.10 Undifferentiated nasopharyngeal carcinoma shows infiltration of large undifferentiated cells with intermixed small lymphocytes (**a**). Cytokeratin antibody highlights malignant cells (**b**), and intermixed lymphocytes react with LCA (**c**). Ki-67 antibody reacts with about 20 % of malignant cells (**d**)

of the oropharynx and known as a risk factor of head and neck SCCs [60, 61]. Being as a variant of SCC, basaloid squamous cell carcinoma (BSCC) is another tumor with predominance of basaloid components. Basaloid squamous cell carcinomas express p63 which is relatively specific but also found in other squamous tumors (Fig. 26.12). Neuroendocrine markers are negative in BSCC [62]. Spindle squamous cell carcinoma (SSCC) is a cytokeratin-negative SCC in which spindle cell component is uniformly and strongly positive for vimentin [63]. Undifferentiated nasopharyngeal carcinoma shows reactivity to EBV immunostaining as well as some SCCs and BSCCs [64, 65].

26.3.2.1 Prognostic Marker

As a transcription repressor of E-cadherin, Snail-1 is expressed in more than half of the cases of SSCC but not in SCC. In addition, it can be a novel marker for the prediction of metastasis [66].

26.3.3 Tumors of the Salivary Glands

Salivary glands are tubuloacinar exocrine glands having two-layered epithelium which comprise of luminal (acinar and ductal cells) and abluminal (myoepithelial and basal cells). Luminal cells are positive for low molecular cytokeratin, whereas myoepithelial and basal cells react with high molecular cytokeratin and myoepithelial markers. The majority of salivary gland carcinomas can be diagnosed by routine hematoxylin and eosin (H&E)-stained slides, and immunohistochemical (IHC) staining has only a limited role in the diagnosis of salivary gland tumors [47, 67]. Figure 26.13 summarized the various components

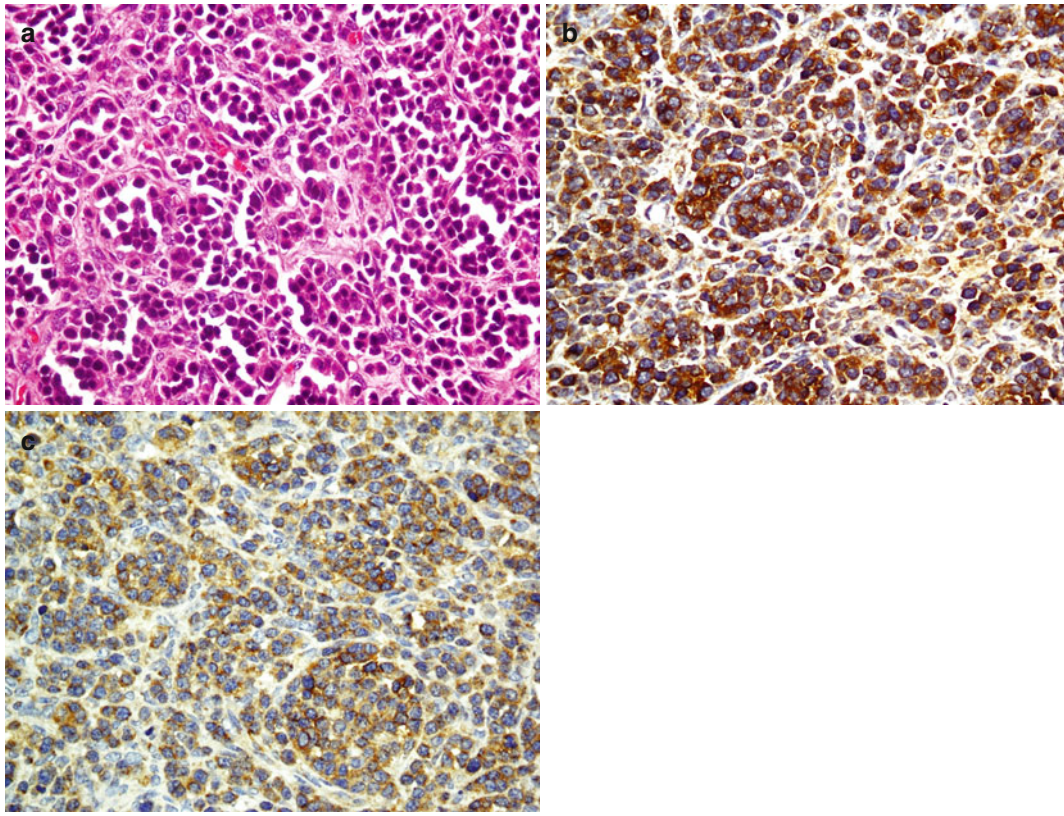

Fig. 26.11 Neuroendocrine carcinoma (**a**). Tumor cells are immunostained with synaptophysin (**b**) and NSE (**c**)

Table 26.7 Immunohistochemistry of poorly differentiated and undifferentiated carcinomas of nasal cavity: sinonasal undifferentiated carcinoma (SNUC), undifferentiated neuroendocrine carcinoma (UNEC), and undifferentiated nasopharyngeal carcinoma (UNPC)

Markers	SNUC	UNPC	UNEC (Fig. 26.11)
Cytokeratin	+	+	+
EBV	–	+	–
Neuroendocrine	–	–	+
CD99	–	–	+/–
S100	–	–	+

Refs. [49, 58, 59]

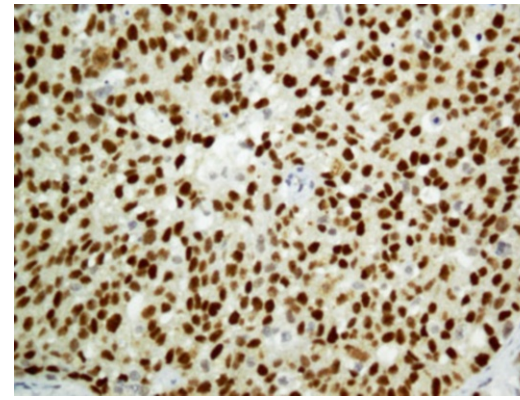

Fig. 26.12 P63 immunoreaction in basaloid squamous cell carcinoma

of the normal salivary glands with an emphasis on the immunohistochemistry Abs.

26.3.4 Immunohistochemistry of Salivary Gland Tumors

The most common malignant tumors of salivary glands consist of acinic cell carcinoma, adenoid cystic carcinoma (Fig. 26.14), basal cell adenocarcinoma, epithelial-myoepithelial carcinoma, mucoepidermoid carcinoma (Fig. 26.15), myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, and salivary duct carcinoma. All tumors are cytokeratin positive; however,

different immunoprofile patterns exist [68]. C-kit (CD117) is positive in acinic cell carcinoma and adenoid cystic carcinoma [69, 70]. Acinic cell tumor and mucoepidermoid carcinoma demonstrate reactivity with membrane-bound mucin (MUC) [71, 72]. Myoepithelial carcinomas are positive for both epithelial and myoepithelial markers but do not exhibit reaction with EMA and CEA [73]. Malignant monophasic salivary gland tumors include acinic cell carcinoma, myoepithelial carcinoma, mucoepidermoid carcinoma, and polymorphous low-grade adenocarcinoma. Immunophenotype profiles of monophasic and biphasic tumors are denoted in Tables 26.8 and 26.9. Application of CK7 and CK20 is a useful panel in distinguishing primary salivary gland carcinoma ($CK7^+$, $CK20^-$) from metastatic carcinoma ($CK7^-$, $CK20^+$) [74].

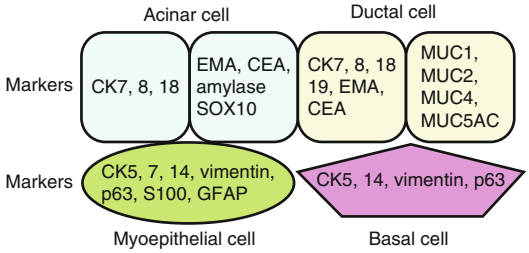

Fig. 26.13 Normal salivary gland components with immunohistochemistric antibodies

26.3.4.1 Prognostic Marker

In mucoepidermoid carcinoma, MUC1 expression is correlated with tumor progression and worsened prognosis, whereas MUC4 expression is related to a better prognosis [72].

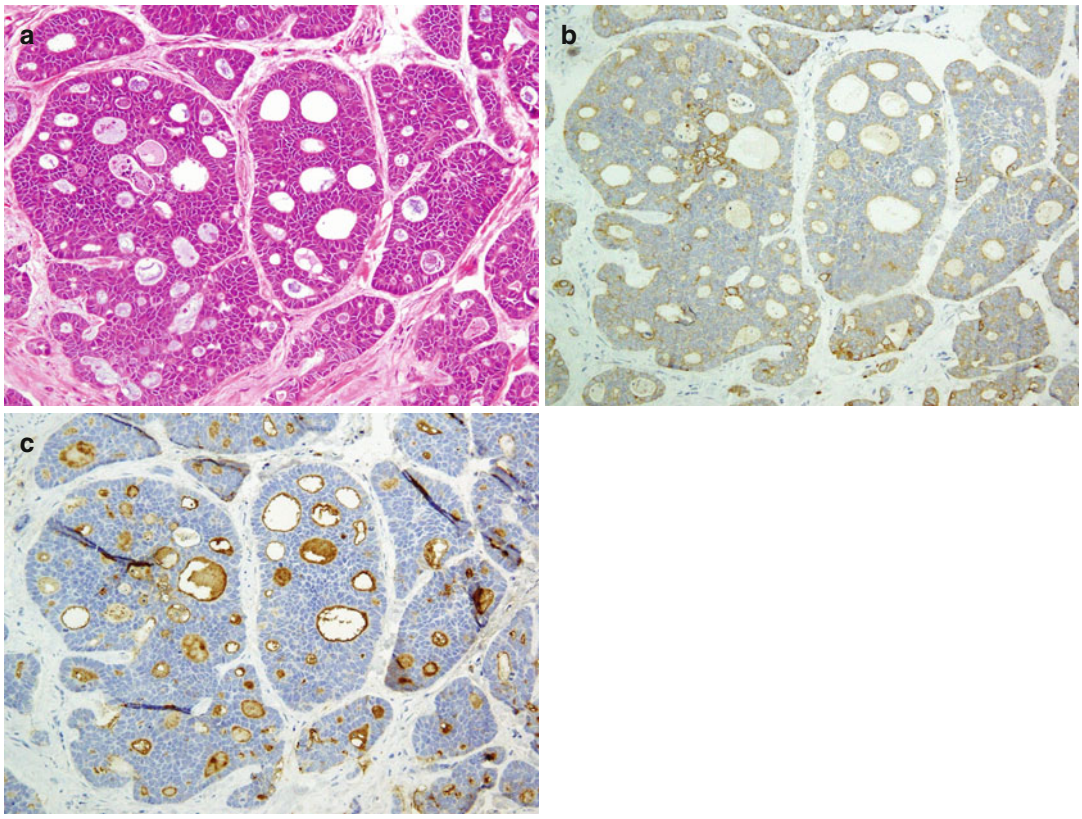

Fig. 26.14 Adenoid cystic carcinoma with typical cribriform pattern (**a**) shows immunoreaction with EMA (**b**) and CEA (**c**)

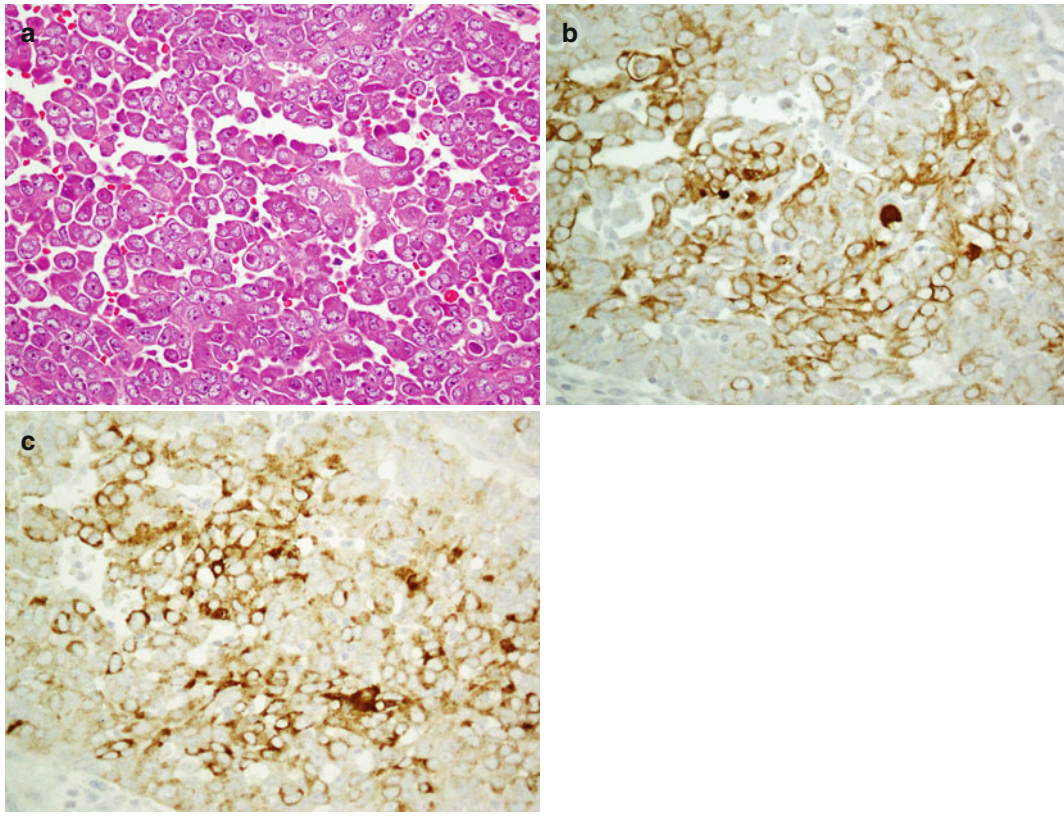

Fig. 26.15 Poorly differentiated mucoepidermoid carcinoma with polygonal atypical epidermoid cells (**a**) exhibits immunostaining with CK7 (**b**) and EMA (**c**)

Table 26.8 Immunophenotype of monophasic malignant salivary gland tumors: acinic cell carcinoma (ACC), myoepithelial carcinoma (MC), mucoepidermoid carcinoma (MEC), and polymorphous low-grade adenocarcinoma (PLGC)

Tumor	AC	MC	MEC	PLGC
Epithelial Markers	CAM5.2, CK7/8/18, EMA, CEA, MUC3	AE1/AE3, CAM5.2, CK14, 34βE12	CAM5.2, CK7/8/14/18/19, EMA, CEA, MUC1/4/5 AC, 5B	CAM5.2, CK7, 14, EMA
Myoepithelial/ basal markers	N	p63, calponion, SMA, myosin	p63 (epidermoid component)	p63
Other markers	C-kit, S100	Vimentin, S100, GFAP	–	S100

Refs. [68, 69, 71–73, 75, 76]

Table 26.9 Immunophenotype of biphasic malignant salivary gland tumors: adenoid cystic carcinoma (ACC), basal cell adenocarcinoma (BCA), epithelial-myoepithelial carcinoma (EMC), and salivary duct carcinoma (SDC)

Tumor	ACC	BCA	EMC	SDC
Epithelial markers	CAM5.2, CK7/14/19, EMA, CEA	AE1/AE3, CAM5.2, CK7, EMA, CEA	AE1/AE3, CAM5.2, CK14	AE1/AE3, EMA, CEA
Myoepithelial/ basal markers	p63, calponin	p63, calponin, SMA	p63, calponin, SMA	p63
Other markers	C-kit, S100	C-kit, S100	S100	AR, GATA3, HER2/neu

Refs. [68, 69, 71, 77–82]

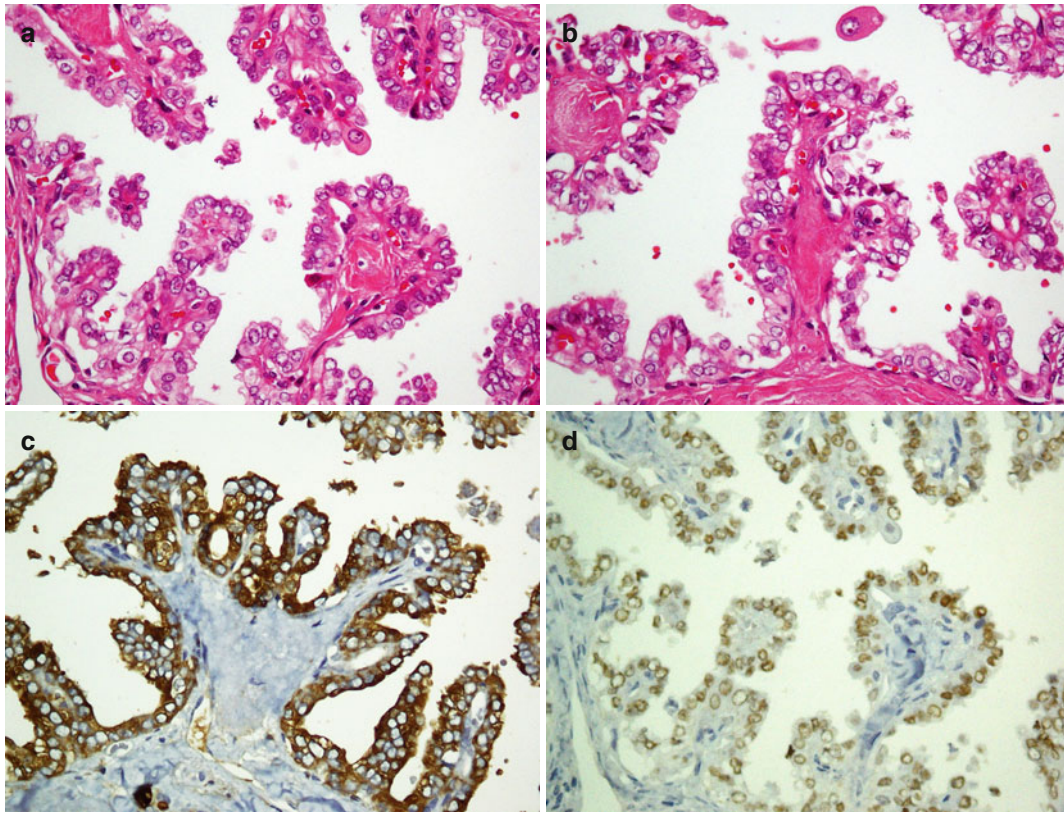

Fig. 26.16 Thyroid papillary carcinoma. Papillary projections with intranuclear inclusions (**a**) and Orphan Annie nuclei (**b**) are highlighted by thyroglobulin in the cytoplasm (**c**) and TTF1 in the nuclei (**d**)

26.3.5 Tumors of Thyroid and Parathyroid Glands

The functional unit of thyroid is the follicle which is composed of follicular cells and C cells. Follicular cells exhibit reactivity with thyroglobulin, TTF1, PAX8, AE1/AE3, EMA, and CK7 and CK8/18/19, whereas C cells are positive for calcitonin, TTF1, CK7, synaptophysin, and chromogranin. Being as a nuclear transcription factor, TTF1 is expressed on follicular and C cells. A follicular cell-specific marker is thyroglobulin which does not react with C cells (Fig. 26.16). As a member of the *paired box (PAX)* gene family, PAX8 is a sensitive marker of thyroid tumors similar to TTF1. Among intermediate filaments, CK19 is more expressed in papillary carcinoma than other tumors. Parathyroid hormone (PTH) and parafibromin are markers of parathyroid tumors. Parafibromin is uniformly expressed in parathyroid adenomas, whereas its expression is often reduced in parathyroid carcinomas. Table 26.10 shows an immunopanel of thyroid and parathyroid tumors. Figure 26.17 depicts thyroid medullary carcinoma.

26.4 Immunohistochemistry of Lung Tumors

26.4.1 Adenocarcinoma

The most frequent IHC pattern observed in lung tumors is positivity for CK7, TTF1, and Napsin A, along with negative staining for CK20, CDX2, and MUC2. It is highly advocated to consider the fact that there are recently increasing reports of primary pulmonary adenocarcinomas with intestinal differentiation which are CK7 and TTF1 negative but CK20 positive which can be highly

Table 26.10 Immunopanel of thyroid and parathyroid tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
CK+, TTF1+, TGB+	PAX8+, CK19+	Papillary carcinoma (Fig. 26.16)
	PAX8±, VIM+	Follicular carcinoma
CK+, TTF1+, TGB−	Calcitonin+, SYN+, CGN+	Medullary carcinoma (Fig. 26.17)
CK±, TTF1+, TGB−	p53+, VIM+, PAX8±	Anaplastic carcinoma
CK+, TTF1−, TGB−	PTH+, CGN+, parafibromin±	Parathyroid tumor

Refs. [83–102]
Note: *CGN* chromogranin, *SYN* synaptophysin, *TGB* thyroglobulin, *VIM* vimentin

misinterpreted as metastatic colorectal adenocarcinomas. Therefore, the importance of physical examination and imaging studies is highlighted. It should be noted that neuroendocrine markers including chromogranin, synaptophysin, NSE, and Leu7 (CD57) can be positive in lung non-neuroendocrine carcinomas such as adenocarcinomas and SCC. Recent studies have shown *EGFR, Her2*, and *BRAF* mutations in lung cancers which can increase the chance for targeted therapies in these cancers [103–106].

26.4.2 Mesothelioma

Neoplasms of the pleura are very rare, and most tumors in this area are usually metastatic lesions.

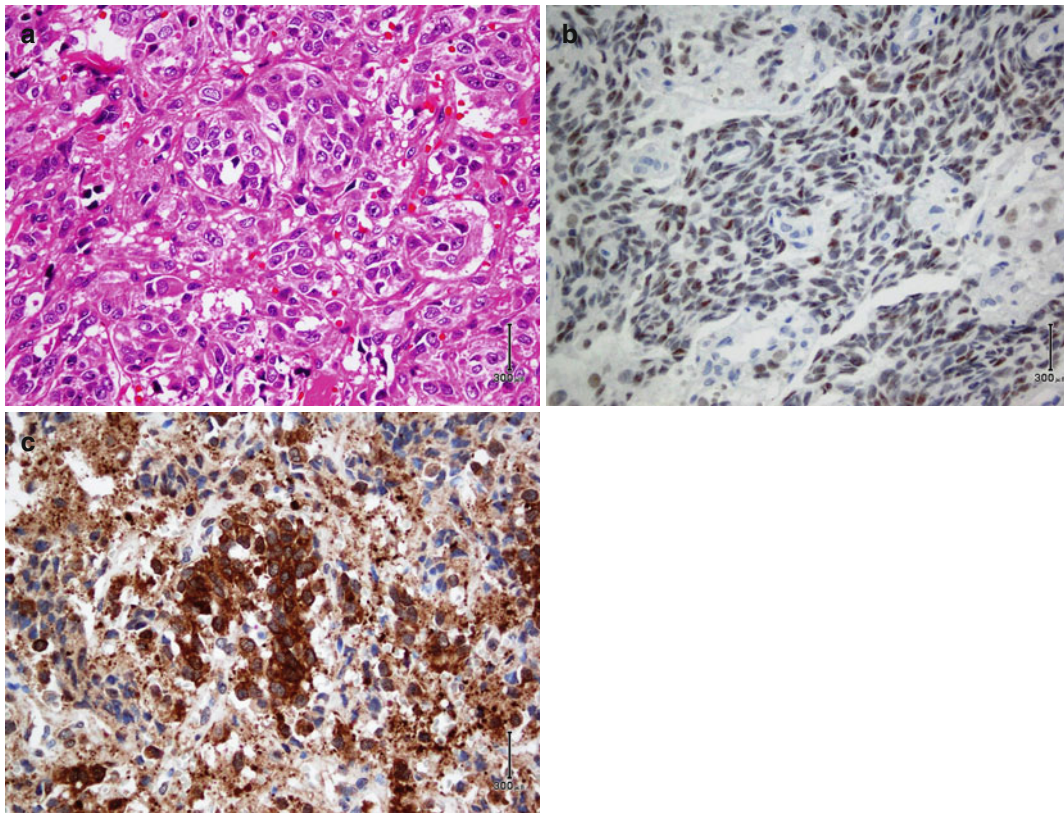

Fig. 26.17 Thyroid medullary carcinoma. Solid nests with medium-sized atypical cells (**a**) exhibit immunoreaction with calcitonin (**b**) and chromogranin (**c**)

Table 26.11 Immunohistochemistric differentiation of pulmonary adenocarcinoma (*PAC*) and malignant mesothelioma

Marker	Pulmonary AC	Mesothelioma	Comment
Calretinin	R	Usually +	The most specific and reproducible positive marker in mesothelioma
CDX2	R	–	About 13 % positive, in pulmonary mucinous carcinomas
Cytokeratin	AE1/AE3, CK5/6 (R), CK7,	CK5/6 (S), CK7 (used to differentiate mesotheliomas from sarcomas)	CK7: Most common CK in primary lung cancer (About 100 % in AC, 40 % in small cell carcinoma, about 20 % in carcinoid tumor, and none of SCC arising from lung) CK5+ specially in lung SCC
D2-40	–	+	Usually positive specially in sarcomatoid variants of mesothelioma
EMA	S (cytoplasmic)	S (membranous)	
TTF1	+	–	
Mesothelin	–	+	
p63	–	–	Positive in pulmonary SCC
pCEA	+	–	
S100	+	–	
SMA	–	50–60 %	
SP-A (surfactant protein A)	50 %	–	
Thrombomodulin	–	+	
Vimentin	+	–	
WT1	–	60 %	

Note: *pCEA* polyclonal CEA, *SMA* specific muscle antigen

One of the most important applications of IHC is to assist pathologists in differentiating mesotheliomas from lung adenocarcinomas [107–109]. Table 26.11 shows the most frequent markers stained by IHC staining in mesothelioma compared with pulmonary adenocarcinoma (Fig. 26.18).

26.5 Immunohistochemistry of Gastrointestinal Tumors

Immunohistochemistry is used in gastrointestinal and colon cancers to particularly determine the tumor subtype and origin, especially for poorly or undifferentiated cancers for which morphology alone cannot determine the origin. Generally, it should be noted that definite tissue diagnosis in clinical practice needs combination of IHC results and clinical information, including biopsy site and the patients' clinical history [110]. Previous studies show that blinded use of an IHC panel for differential diagnosis can primarily identify about 83 % of tumor origins *vs*. 65.6 % of metastasis. Several publications on IHC studies are available, and each recommends its own IHC panel for differential diagnosis. This makes it clear that there is no single IHC panel, or standard of care, for tissue determination, and pathologists have long known that tissue of origin identification is inherently a multiplex problem [111–113].

Here, the authors have briefly tried to introduce the major and common IHC markers used to differentiate frequent gastrointestinal tumors. It should be noted that the average positivity of a marker in a specific tumor differs from one study to another, as well as in different textbooks. In this chapter the most prevalent and reliable data are provided.

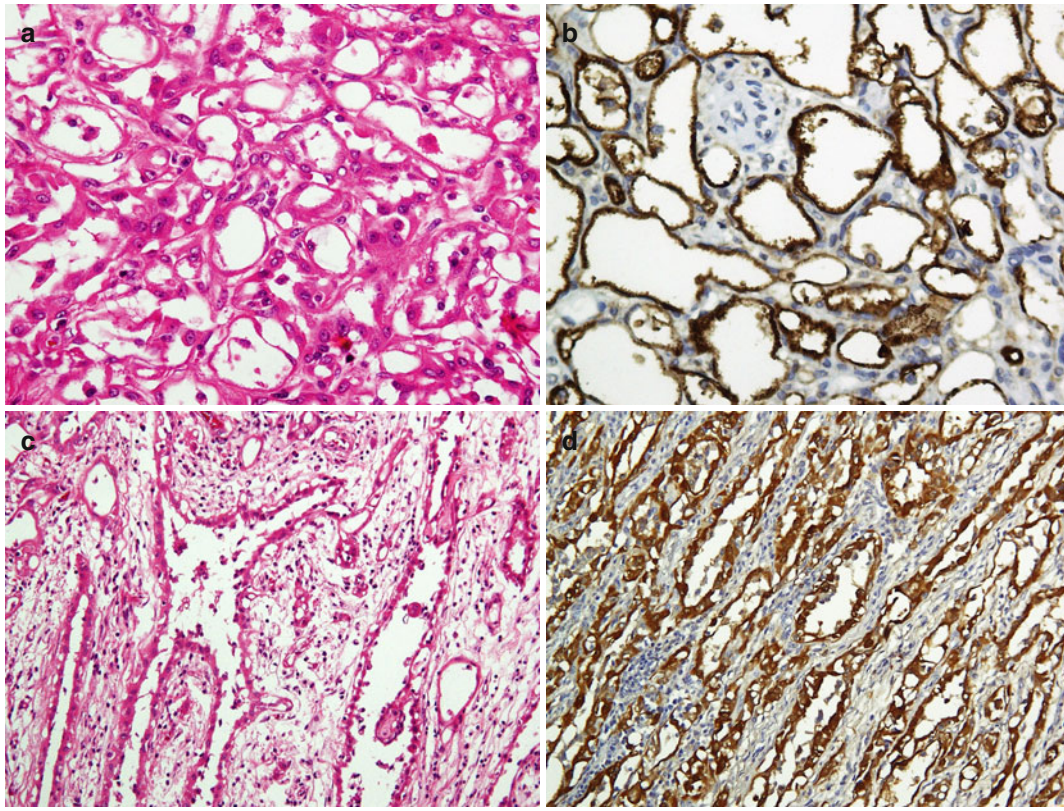

Fig. 26.18 Mesothelioma. Adenomatoid type (**a**) shows immunostaining for mesothelin (**b**), and tubular type (**c**) shows immunoreaction for calretinin (**d**)

Table 26.12 Immunohistochemistry of normal liver

	Markers				
Normal tissue	Hepatocellular	Adenocarcinoma	Carcinoma	Canalicular	Others
Hepatocytes	HepPar1, TTF1 (cytoplasmic)	MOC31	CAM5.2	CD10, pCEA	B-catenin
Bile duct cells	–	CK7, CK19 (+/–), MUC6	CAM5.2, CKAE1/AE3, EMA, BerEp4	–	B-catenin

26.5.1 Liver

The most common primary hepatic cancer is hepatocellular carcinoma which is well known to have a wide spectrum of histologic differentiation and a great diversity of appearances. It necessitates the application of IHC as an ancillary aid for better diagnosis of the lesion. It is important to reiterate that IHC is after all an ancillary aid. A significant clinicopathologic correlation seems mandatory for the final diagnosis. If a definitive diagnosis cannot be clinched, at the least, certain differential diagnoses can be excluded [114–118]. Immunophenotype of normal liver is summarized in Table 26.12 (Figs. 26.19 and 26.20).

Cholangiocarcinoma is a malignant tumor with characteristics mostly similar to other types of adenocarcinomas. The tumor is usually positive for CK7, CK19, CAM5.2, CK AE1/AE3, pCEA, mCEA (noncanalicular pattern), and MOC31. MUC4, MUC5AC, and MUC6 can also be useful not in diagnosis but in classification and predicting the prognosis.

Additionally, CD56 which is positive in benign bile ductular proliferations and negative in cholangiocarcinomas can be useful in differentiating malignant lesions from benign proliferation. The exception for this rule is clear cell cholangiocarcinoma which is positive for CD56. Staining for CK7 and CK19 in cholangiocarcinoma can help to differentiate this tumor from HCC, which is negative for the mentioned markers [119, 120]. Table 26.13 indicates the immunophenotypes of hepatocellular carcinoma and cholangiocarcinoma.

26.5.2 Esophagus

The most common esophageal cancers are adenocarcinomas and SCC. Adenocarcinoma of the esophagus is immunophenotypically similar to gastric adenocarcinomas, and there is no IHC panel to distinguish these two. Esophageal SCC is usually positive for most CK markers including CK AE1/AE3, CK 34bE12, CK5/6, CK19 (positivity increases with tumor grade whereas benign squamous lesions are negative for this marker), and p63. Additionally, most SCCs are negative for CK7 and CK20 which can be useful in distinguishing poorly differentiated SCCs from poorly differentiated adenocarcinomas positive for these two CK markers [121–123].

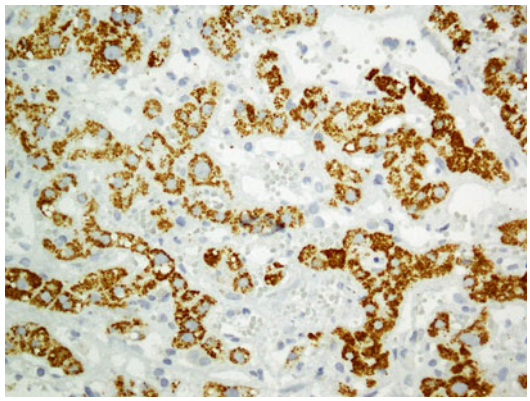

Fig. 26.19 Normal liver stains with HepPar1 showing typical cytoplasmic coarse granules of hepatocytes

26.5.3 Stomach

Stomach glandular epithelium expresses CK20 and less commonly CK7 (*CK7+, CK20+*) and MUC5AC, distinguishing it from small intestine and colorectal epithelium. Immunoprofile of normal gastrointestinal mucosa is denoted in Table 26.14. Gastric adenocarcinoma has many histologic variants, but they have almost similar immunophenotyping. It should be mentioned that synaptophysin and chromogranin as neuroendocrine markers can be positive in gastric adenocarcinomas; therefore, positive staining with these markers is not sufficient for the diagnosis of neuroendocrine carcinoma [124–126]. Immunoprofile of gastric adenocarcinoma is demonstrated in Table 26.15 (Fig. 26.21).

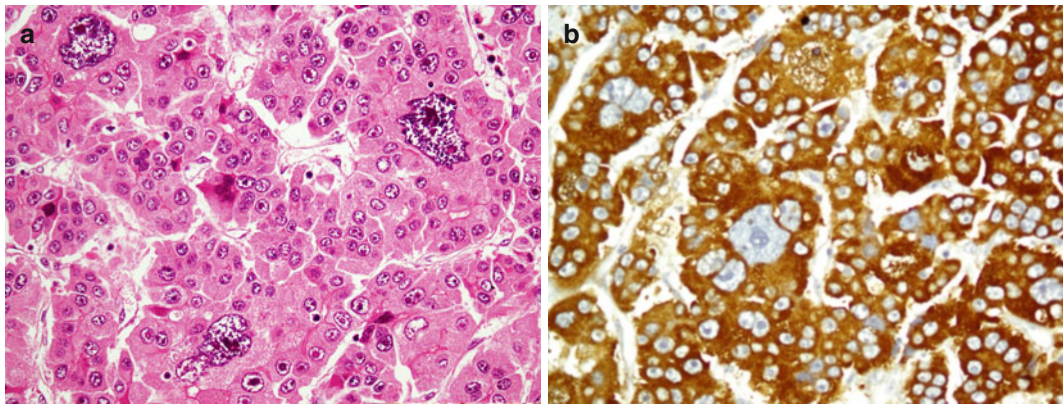

Fig. 26.20 Hepatocellular carcinoma with huge bizzare giant nuclei making diagnosis simple as malignant (**a**) exhibits reactivity with HepPar1 (**b**)

Table 26.13 Immunohistochemistry of hepatocellular carcinoma and cholangiocarcinoma

Tumor	Markers				
	Hepatocellular	Adenocarcinoma	Carcinoma	Canalicular	Sinusoidal
Hepatocellular carcinoma	HepPar1, TTF1 (cytoplasmic)	–	CAM5.2, EMA (–/+)	CD10, pCEA	CD34, FVIII
Cholangiocarcinoma	–	MOC31, CK7, CK19, MUC4, MUC5AC, MUC6	CAM5.2, CKAE1/AE3	pCEA, mCEA (noncanalicular)	–

Table 26.14 Immunoprofile of normal gastrointestinal mucosa

Normal tissue	Simple epithelial marker					MUC		CDX2 (intestinal marker)	CD15
	CK7	CK20	AE1/AE3	CAM5.2	CEA	Gastric (MUC5AC)	Intestinal (MUC2, MUC4)		
Stomach	+/–	+	+	+	+	+	–	–	+
Small intestine	–	+	+	–	–/+	–	+	+	+
Large intestine/ appendix	–	+	+	+	+	–	+	+	+

Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)

Table 26.15 Immunoprofile of gastric, small intestine, and colorectal adenocarcinoma (AC)

Tumor type	Tumor associated marker						MUC		CDX2 (intestinal marker)	CD15
	CK 18/19	CK7	CK20	AE1/AE3	CAM5.2	CEA	Gastric (MUC5AC)	Intestinal (MUC2, MUC4)		
Gastric AC	+	+/–	–/+	+	+	+	–/+	–/+	–/+	–
Small intestine AC	+	+/–	+/–	+	–/+	–/+	–/+	+/–	+/–	–
Large intestine/ appendix AC	+	–	+	+	+	+	–/+	+/–	+	–

Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)

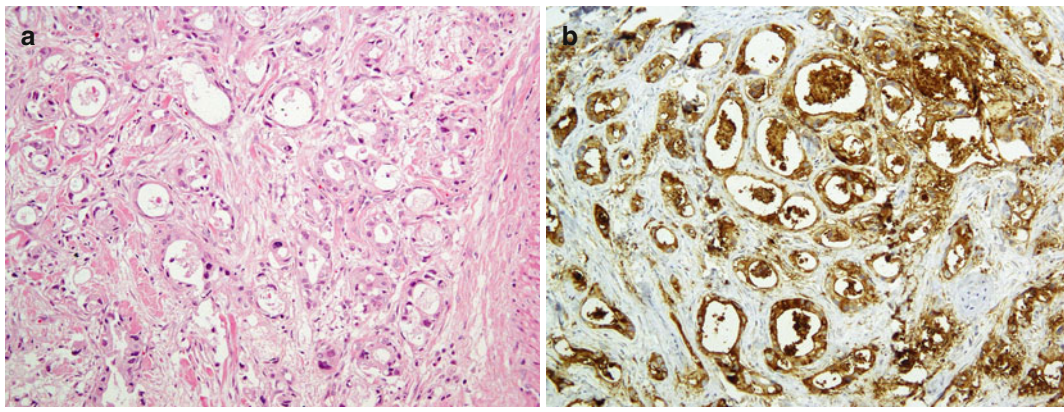

Fig. 26.21 Adenocarinoma of the stomach with atypical glands and nuclear pleomorphism (**a**) immunostained with CEA (**b**)

Table 26.16 Immunoprofile of colon adenocarcinoma based on chromosomal instability and MSI pathways

Chromosomal instability pathway (80–85 %)		MSI pathway (15–20 %)	
CK20	100 %	CK20	Can be negative in about 30 %
MUC2	Usually positive	MLH1	Complete absence of staining with a sufficient internal control is needed for a positive result
MUC5AC	Usually negative (about 30 % positive, especially in mucinous carcinomas)	MSH2	
CAM5.2	Usually positive	MSH6	
MOC31	Usually positive	PMS2	
CDX2	About 90 %	CDX2	Can be negative in about 20 %
CK7	5–10 %		
CEA	Usually positive specially monoclonal type		
CK8	Usually positive		
CK18	Usually positive		
CK19	Usually positive		
CKAE1/AE3	Usually positive		
MSI-related markers	These markers are usually positive in this subtype of colon carcinomas		

26.5.4 Small Intestine

Immunophenotyping of adenocarcinoma is also valuable in neuroendocrine tumors (NET) [127–129]. Tables 26.14 and 26.15 summarize the immunoprofile of normal small intestine, its adenocarcinoma, as well as their comparison with stomach and colon adenocarcinoma.

26.5.5 Colon

In contrast to older studies which have discussed colon cancers generally, recent studies reveal that colon cancers arise from two different pathways (chromosomal instability of APC gene vs. microsatellite instability (MSI) pathway) with different immunophenotypic features [116, 130–136]. Immunoprofile of normal and colon adenocarcinoma is denoted in Tables 26.14, 26.15, and 26.16.

26.5.6 Anal

The most frequent anal cancers are SCC and adenocarcinoma. Anal SCC is almost similar to SCC of other origins; nonetheless, the role of HPV is highlighted. Adenocarcinomas of the anus are usually positive for CK7 and negative for CK20, CDX2, and CK5/6 which helps to differentiate them from adenocarcinomas of colon origin [135, 137, 138].

26.5.7 Appendix

Mucinous adenocarcinomas of appendix origin can be distinguished from mucinous colorectal carcinomas with immunostaining for CK7 and MUC markers [139–141].

26.5.8 Pancreas

Pancreas is composed of glandular/ductal, acinal epithelium, and endocrine cells. Pancreatic neoplasms can be roughly divided into two categories of exocrine and endocrine system neoplasms. This part mostly discusses the exocrine system and mostly adenocarcinomas of this area. Additionally, tumor suppressor genes including DPC4 and SMAD4 are inactivated in about 50–60 % of the adenocarcinomas of this site [116, 142, 143]. Immunoprofile of normal pancreas and some pancreatic tumors are summarized in Tables 26.17 and 26.18. Figure 26.22 depicts solid pseudopapillary neoplasm.

Table 26.17 Immunoprofile of normal pancreas

Marker			Normal tissue
Exocrine	Glandular/ductal	Epithelial	CAM5.2, AE1/AE3, CK7, CK8/1/8/19
		MUC	MUC1, MUC6
		ONP	–
	Acinar		Trypsin, chymotrypsin, lipase, amylase, elastase
Endocrine			CGN, SYN, NSE

Table 26.18 Immunoprofile of some pancreatic tumors: pancreatic ductal adenocarcinoma (PDAC), acinar cell carcinoma (ACC), neuroendocrine carcinoma (NEC), and solid pseudopapillary neoplasm (SPN)

Marker			PDAC	ACC	NEC	SPN (Fig. 26.22)
Exocrine	Glandular/ductal	Epithelial	CAM5.2, AE1/AE3, CK7, CK8/18/19, pCEA, PSCA	CAM5.2, AE1/AE3, CK8/18, EMA	CAM5.2, AE1/AE3, CK19	– (Positive for β-catenin, vimentin, PR, CD10)
		MUC	MUC1, MUC3, MUC4, MUC5AC, MUC6 (+/–)	–	–	–
		ONP	CA19.9, CA125, B72.3, DUPAN-2, CECAM1	–	–	–
	Acinar		–	Trypsin, chymotrypsin, lipase, amylase, elastase	–	α1-antitrypsin
Endocrine		–	CGN, SYN	CGN, SYN, NSE, CD56, CD57	CGN, SYN, NSE, CD56	–

Note: *CGN* chromogranin, *NSE* neuron-specific enolase, *ONP* oncoprotein, *PR* progesterone receptor, *SYN* synaptophysin

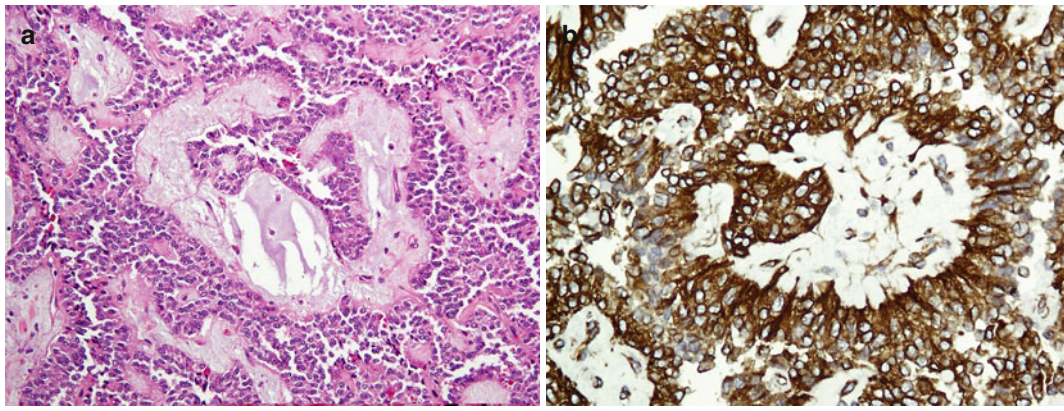

Fig. 26.22 Solid pseudopapillary neoplasm. Papillary projection covered by relatively bland-looking cells supported by a hyalinized stroma (**a**) highlighted with vimentin (**b**)

26.5.9 Gastrointestinal Stromal Tumor

Gastrointestinal stromal tumor (GIST) is a soft tissue tumor of the GI wall which is in the differential diagnosis of leiomyoma and fibromatosis. Most GISTs express c-kit (>95 %), CD34, and CD99 (Fig. 26.23). Sometimes weak positivity for S100, SMA, desmin, and synaptophysin (but not chromogranin) can also be found [135, 144, 145].

26.5.10 Neuroendocrine Carcinomas

Neuroendocrine tumors arise from different organs. Most have similar morphology and tumor marker expression, and the most important diagnostic clues are histologic features, as well as immunostaining for synaptophysin, chromogranin, and NSE (Fig. 26.24). In addition to the mentioned markers, most of neuroendocrine tumors can express the tissue markers in which they originated which help to diagnose the origin of metastatic neuroendocrine tumors [143, 146–148].

26.6 Immunohistochemistry of the Urinary Tract

26.6.1 Kidney

Renal cell carcinoma (RCC) is the most common tumor of the kidney with variants of clear renal cell carcinoma (CRCC), papillary

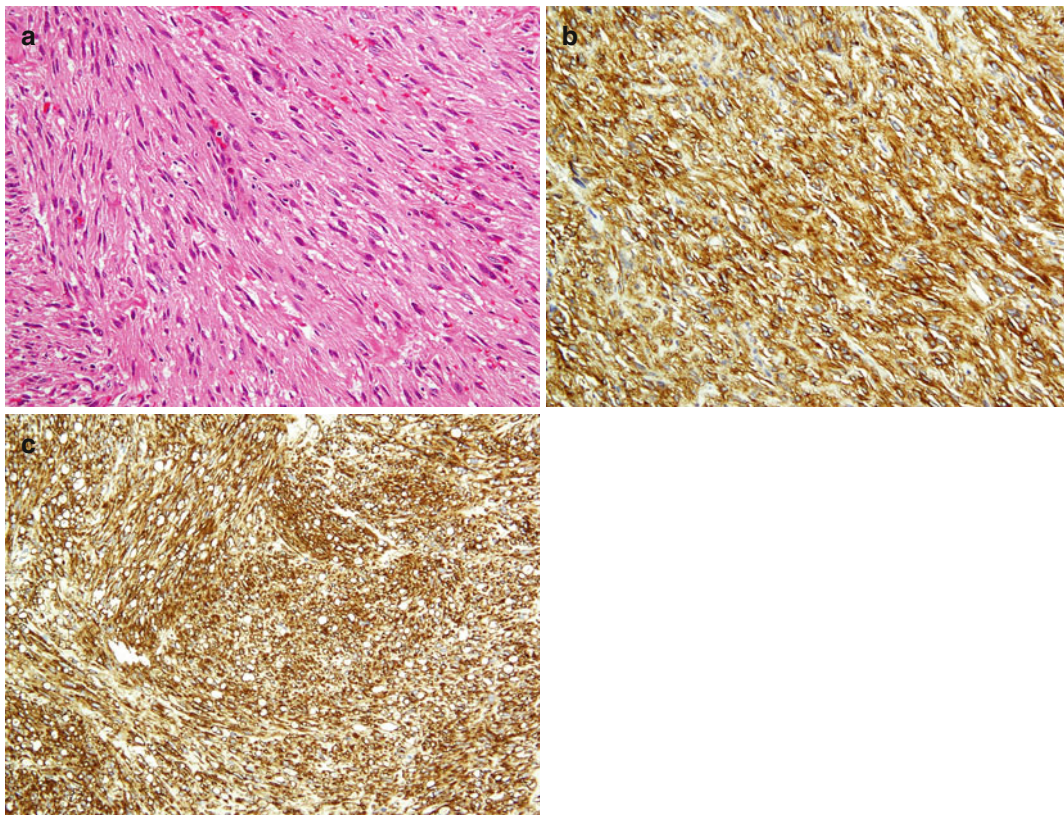

Fig. 26.23 Gastrointestinal stromal tumor. A low-grade intestinal wall tumor shows uniform spindle cells with elongated nuclei (**a**), with immunoreaction to c-kit (**b**) and CD34 (**c**)

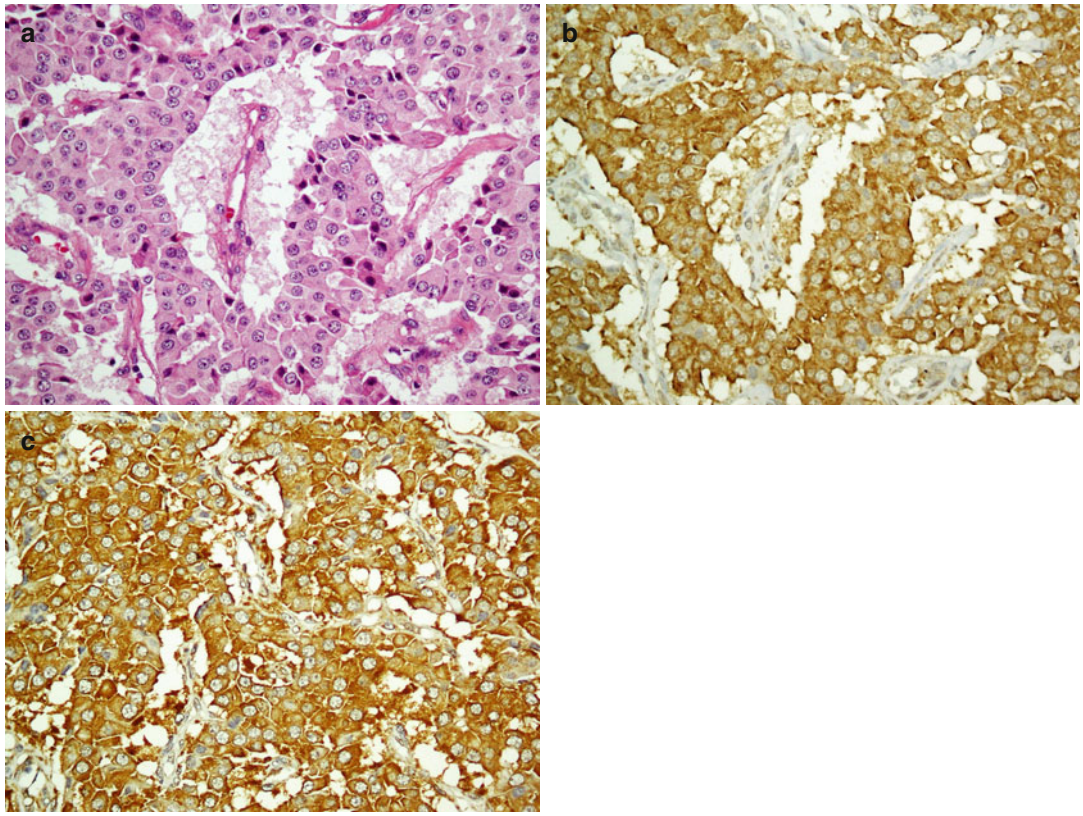

Fig. 26.24 Neuroendocrine carcinoma composed of atypical cells with round nuclei and dusty chromatin (**a**). Tumor cells are immunostained with chromogranin (**b**) and synaptophysin (**c**)

renal cell carcinoma (PRCC), and chromophobe carcinoma (CC). Commonly used immunohistochemical Abs in the urinary system are summarized in Table 26.19. Immunohistochemistry is an ancillary test used to distinguish variants of RCC as well as tumors with histopathologic similarities including collecting duct carcinoma and urothelial carcinoma of the renal pelvis. Carcinomas with clear cell feature include CRCC (Fig. 26.25), papillary renal cell carcinoma, and transitional (urothelial) cell carcinoma of the renal pelvis. Differential diagnoses of carcinoma with oncocytic appearance are chromophobe carcinoma, oncocytoma, and oncocytic papillary RCC (Fig. 26.26) [149–154]. The immunophenotype of collecting duct carcinoma is 34βE12$^+$, CD10$^-$, and AMACR$^-$, in contrast to PRCC which is 34βE12$^-$, CD10$^+$, and AMACR$^+$ [150, 155]. Considering the histopathologic pattern, the following immunopanels (Tables 26.20 and 26.21) compare the immunohistochemical Abs in these tumors.

26.6.2 Bladder

Normal urothelium exhibits a unique pattern of cytokeratin expression characterized by coexpression of simple epithelium cytokeratin (CK7, CK20, and CAM5.2) and HMWCK (CK5/6 and 34βE12). While CK20 is expressed in umbrella cells of the normal urothelium, in

Table 26.19 Immunohistochemical markers in urinary system tumors

Marker	Function	Immunoreaction in tumor
AE1/AE3	Pan-CK epithelial marker	RCC
CAIX	Carbonic anhydrase IX: maintenance of intracellular and extracellular pH, regulatory role in cell proliferation	PRCC
CAM5.2	Intermediate cytoskeleton filament	RCC, PRCC, CC, CDC
CD10 (CALLA)	A zinc-dependent cell membrane metalloprotein	RCC, PRCC
CD117 (c-kit)	Transmembrane glycoprotein receptor tyrosine kinase	CC, CDC, OC
CK7	LMWCK (simple epithelia)	PRCC, CC, UC, PAC (+/–)
CK20	LMWCH (simple epithelia)	UC (+/–), PAC (+/–)
34βE12	HMWCK (CK1, 5, 10, 14)	CDC, UC
EGFR	Receptor with tyrosine kinase activity	UC (+/–)
Ep-Cam	Glycosylated transmembrane cell surface epithelial protein in distal nephron	PRCC (+/–), CC, CDC
HMWCK	Intermediate cytokeratin filaments of prostate basal cell	"Negative" marker in PAC
Ki-67 (MIB1)	Nuclear protein expressed in all phases of the active cell cycle (G1, S, G2, M)	Proliferative marker
Ksp-cadherin (kidney-specific)	Calcium-dependent cell adhesion molecule plays an important role in the maintenance of tissue integrity	CC, OC
p53	Tumor suppressor protein	UC
p63	A member of p53 family transcription factor, marker of basal cells	"Negative" marker in PAC
P501S (Prostein)	A 553-amino acid protein localized to the Golgi complex	PAC
P504S (AMACR)	Enzyme mainly localized to peroxisomal structures	PRCC, PAC
PAX2/PAX8	Members of the paired box (PAX) gene family expressed in the development of the urogenital tract	RCC, PRCC, CC, CDC, OC (+/–)
PSA	330-kD glycoprotein, prostate-specific antigen	PAC
PSAP	100-kD glycoprotein, prostate-specific antigen	PAC
PSMA	100-kD glycoprotein, prostate-specific antigen	PAC
RCC	200-kD glycoprotein expressed in epithelial cells lining the normal renal proximal tubule	RCC, PRCC
Thrombomodulin	75-kD glycoprotein, to convert thrombin from a coagulant protein to an anticoagulant	UC
Uroplakin III	A transmembrane protein unique to urothelium	UC
Vimentin	Intermediate cytoskeleton filament	RCC, PRCC, CDC

Refs. [150–184]
Note: *CC* chromophobe carcinoma, *CDC* collecting duct carcinoma, *OC* oncocytoma, *PAC* prostatic adenocarcinoma, *PRCC* papillary renal cell carcinoma, *UC* urothelial carcinoma

dysplastic urothelium and carcinoma in situ, it is expressed in all layers of the urothelium [150–154, 168, 169]. CD44 is expressed in the basal layer of normal urothelium and shows focal staining of basal layers of the dysplastic urothelium [170]. Urothelial carcinomas are divided into (1) noninvasive papillary carcinoma and (2) invasive carcinoma which can appear as papillary or non-papillary itself (Fig. 26.27). Immunohistochemistry can be helpful to differentiate urothelial carcinoma from direct extension of an adjacent primary carcinoma (prostate, colorectal, cervix, and uterine) as well as metastasis and also to distinguish variants of urothelial carcinoma. Common immunohistochemistry Abs in normal urothelium, urothelial hyperplasia, urothelial dysplasia, and urothelial carcinoma are summarized in Table 26.22.

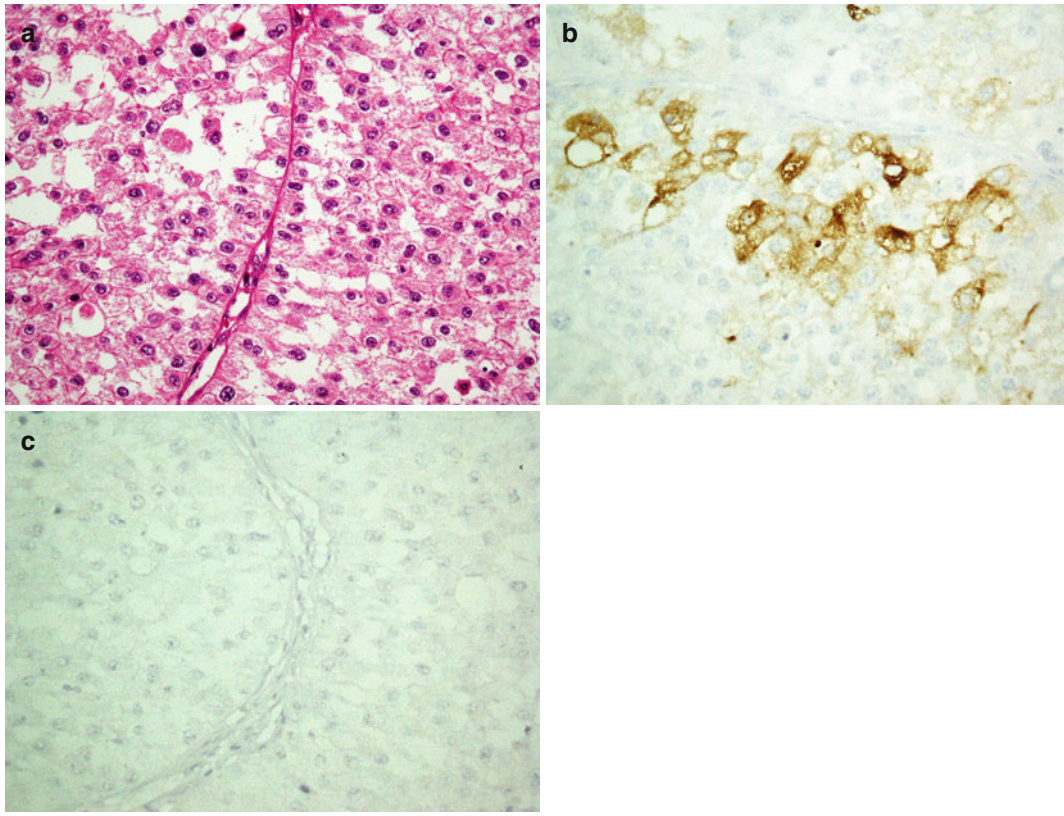

Fig. 26.25 Renal cell carcinoma, eosinophilic to clear cells (**a**) is immunostained with CD10 (**b**) but not with CK20 (**c**)

26.7 Immunohistochemistry of Female and Male Genital Tumors

26.7.1 Uterine Cervix

The most important and also frequent cervix cancers are cervix SCCs and adenocarcinomas. Cervix SCC markers are similar to those seen in SCCs of other origins. p16 is a unique marker expressed in tumors of the cervix which can help in differentiating this lesion from the same counterparts from uterine or other origins. Adenocarcinomas of the cervix also express most adenocarcinoma markers. One of the advantages of IHC is to differentiate adenocarcinomas of the cervix from the endometrium. Cervix adenocarcinomas usually express p16 and CEA, and are negative for vimentin and ER, whereas endometrium adenocarcinomas have a reverse expression pattern [176–181].

26.7.2 Vulva and Vagina

As other organs, various malignancies can occur in these two organs, but similar to cervix, the most common cancer of these two sites is SCC, with IHC marker expression similar to cervix counterparts [182, 183].

26.7.3 Uterine Corpus

Uterine tumors are of myometrium or endometrium origin. The myometrial tumors are usually

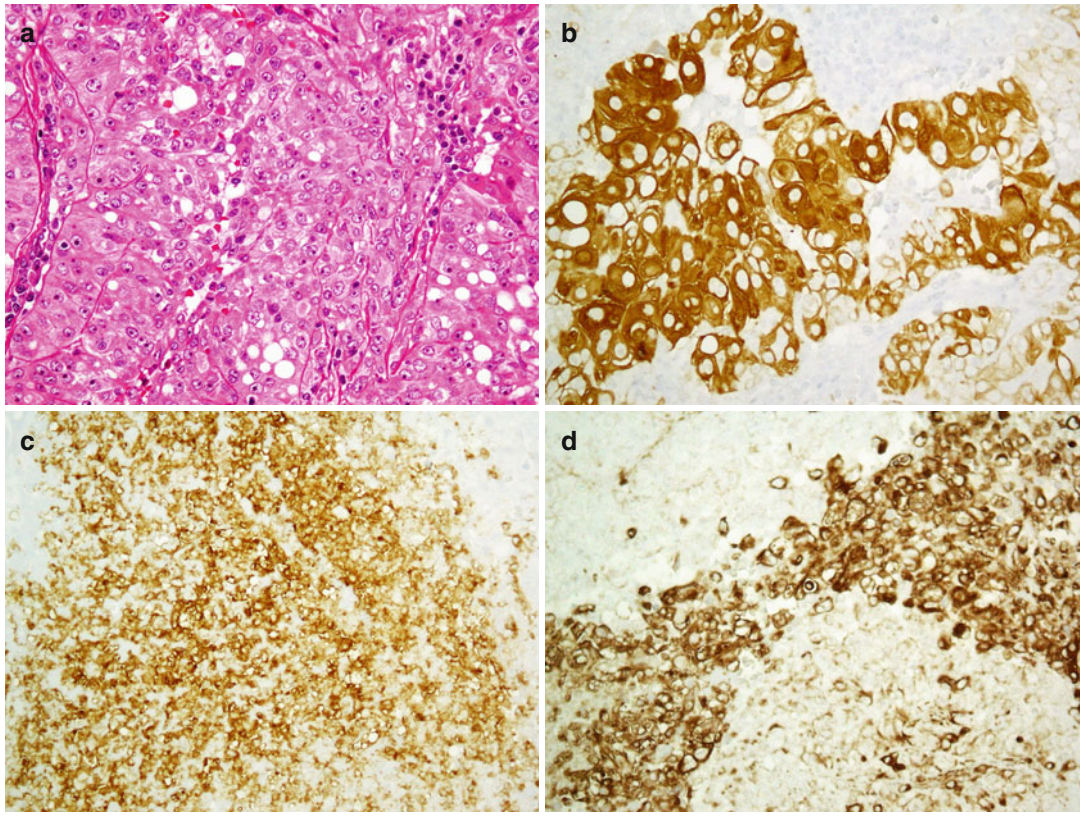

Fig. 26.26 Papillary renal cell carcinoma with oncocytic feature (**a**). Tumor cells are positive for CK7 (**b**), CD10 (**c**), and vimentin (**d**)

sarcomas and were discussed in the sarcoma section. The endometrium may develop various cancers, but the most frequent one is endometrial adenocarcinoma. Endometrial adenocarcinoma has some variants in which endometrioid adenocarcinoma is the most frequent one. Endometrioid adenocarcinoma usually expresses CK7, CA125, ER, PR, and vimentin but is negative for CEA, CK20, and p16. Some endometrial carcinomas express Her2/neu marker, which along with ER and PR markers can be used in targeted therapies [176–181, 184–187].

26.7.4 Ovary

Except the intestinal type of mucinous adenocarcinoma, all primary ovarian carcinomas are CK7 positive and CK20 negative (Fig. 26.28). This can be used in differentiating primary ovarian carcinoma from metastatic tumors [139–141, 180, 188–191]. The immunophenotype of primary ovarian tumors is described in Table 26.23.

26.7.5 Breast

Breast cancer is one of the most common malignancies with various histopathological types; however, adenocarcinomas and its two subtypes including invasive ductal (IDC) and lobular carcinomas (ILC) comprise the majority. Most breast cancers including IDC and ILC are positive for mammaglobin, GCDFP15, ER, and PR, and some are positive for Her2/

Table 26.20 Immunoprofile of kidney carcinoma with clear cell appearance: clear RCC (CRCC), papillary RCC (PRCC), and urothelial carcinoma (UC)

Tumor	CK7	CK20	Vimentin	RCC	CD10	PAX2/8	AMARC	Uroplakin	p63
CRCC	–	–	+	+	+	+	–	–	–
PRCC	+	–	+	+	+	+	+	–	–
UC	+	+	–	–	–	–	–	+	+

Refs. [150–160]

Table 26.21 Immunoprofile of kidney carcinoma with oncocytic cell appearance: oncocytic papillary RCC (OPRCC), chromophobe carcinoma (CC), and oncocytoma (OC)

Tumor	CK7	CK20	CAM5.2, EMA, AE1/AE3	Vimentin	RCC	CAIX	CD10	CD117	Ep-Cam	Ksp-cadherin
OPRCC	+	–	+	+	+	+	+	–	+	–
CC	+	–	+	–	–	–	–	–	+	+
OC	–	–	–	–	–	–	–	+	–	+

Refs. [163–167]

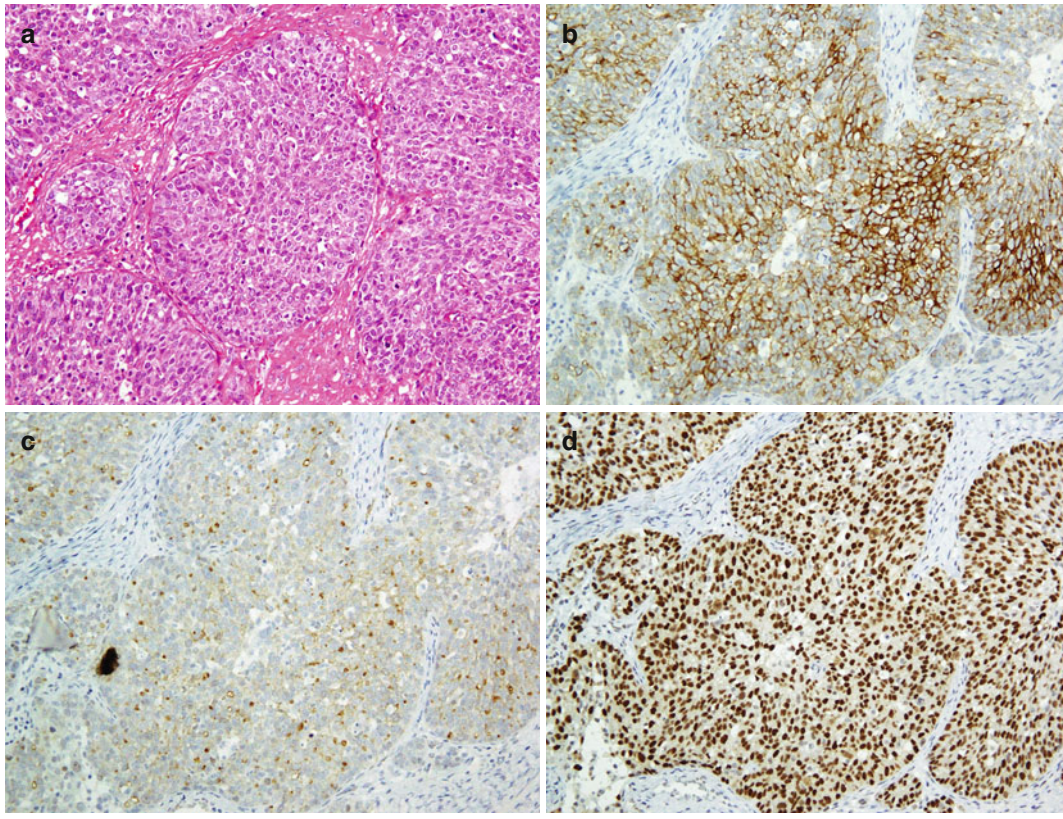

Fig. 26.27 Transitional cell carcinoma, invasive, non-papillary type (**a**). Tumor cells exhibit immunoreaction with CK7 (**b**), CK20 (**c**), and p63 (**d**)

Table 26.22 Antibody immunoprofile in normal urothelium, urothelial hyperplasia, dysplasia, and carcinoma

Marker	Normal urothelium	Urothelial hyperplasia	Urothelial dysplasia	Urothelial carcinoma
CK7	+	+	ND	+
CK20	+ U	+	+	+
34βE12	+B	ND	ND	+
CD44	+B	ND	−/+	ND
EGFR	−/+	+	+/−	+/−
p63	ND	ND	ND	+[a]
UPIII	+ U	ND	ND	+[a]
TM	+ U	ND	ND	+[a]
p53	−	−	+	+[a]

Refs. [168–175]
Note: + (>90 %), +/− (>50 %), −/+ (<50 %), – (<10 %). *B* basal layer, *TM* thrombomodulin, *U* umbrella cell, *UPIII* uroplakin III
[a]Noninvasive carcinoma > invasive carcinoma

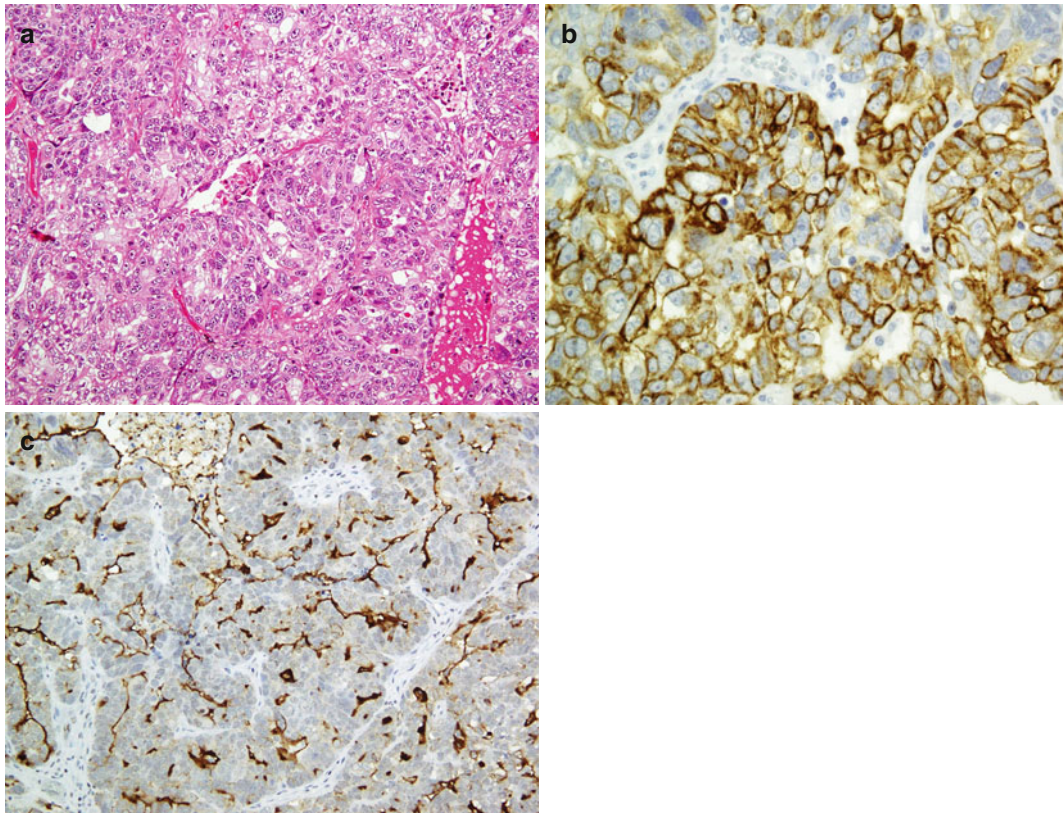

Fig. 26.28 Ovarian serous carcinoma poorly differentiated (**a**) shows immunoreaction with CK7 (**b**). CA125 is highlighted in the luminal surface (**c**)

Table 26.23 Immunophenotype of ovarian cancers

Epithelial tumors		Germ cell tumors				Stromal tumors (almost always negative for EMA)	
Serous (Fig. 26.28)	Mucinous	Dysgerminoma	Yolk sac	Embryonal carcinoma	Choriocarcinoma	Granulosa cell tumor	Sertoli-Leydig cell tumor
EMA	EMA	PLAP	PLAP	PLAP	HCG	Inhibin	CK
CK7	CK7	CD117 (c-kit)	AFP	Oct-4	Inhibin	CD99	CD99
CA125 DPC4 ER PR WT1	CK20 mCEA CDX2 MUC5A	Oct-4 D2-40	CK AE1/AE3 Glypican-3	CK AE1/AE3 CD30	CK	WT1 Calretinin CD56	WT1

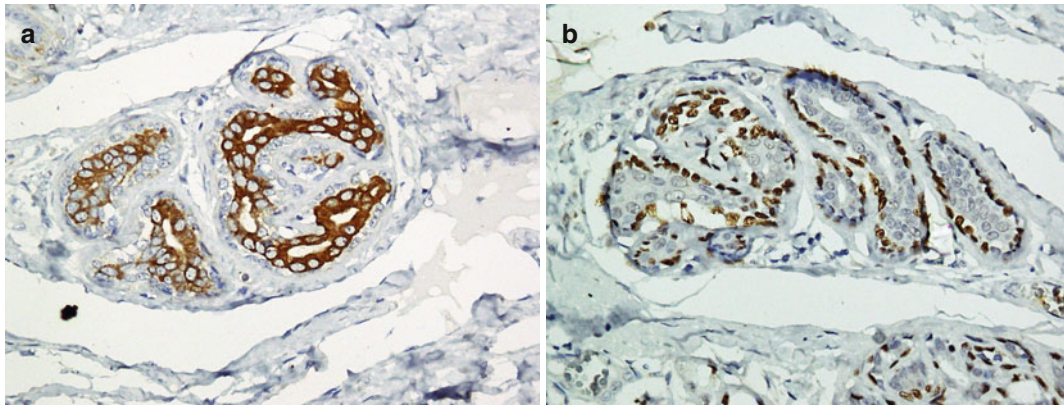

Fig. 26.29 Cytokeratin (**a**) stains epithelial cells and p63 (**b**) stains myoepithelial cells of normal breast glands

neu markers. Additionally, epithelial tumor markers, CK (especially CK7) and EMA, are also positive in these tumors [192–197]. The lack of reaction with myoepithelial markers is in favor of an invasive carcinoma. Both normal (Fig. 26.29) and proliferative glands (Fig. 26.30) and ductal carcinoma in situ (Fig. 26.31) exhibit reactivity with myoepithelial markers. Application of p63 and calponin or p63 and SMA is a good way to evaluate the presence of myoepithelial cells [192, 198]. Immunoprofile of normal breast glands and breast cancers are summarized in Tables 26.24 and 26.25 (Figs. 26.32 and 26.33).

26.7.6 Prostate

Prostate gland is composed of two layers, epithelium and basal cell layer. Normal prostate epithelium exhibits immunoreactivity with prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostate-specific acid phosphatase (PSAP), prostein (P501S), and α-methylacyl-coenzyme-A racemase (AMACR) enzyme, whereas prostate basal cells display immunostaining with HMWCK (34βE12), p63, and S100A6 (Fig. 26.34) [149–154]. Immunolabeling for basal cell markers is usually used in a mode of "negative" diagnostic marker in order to show

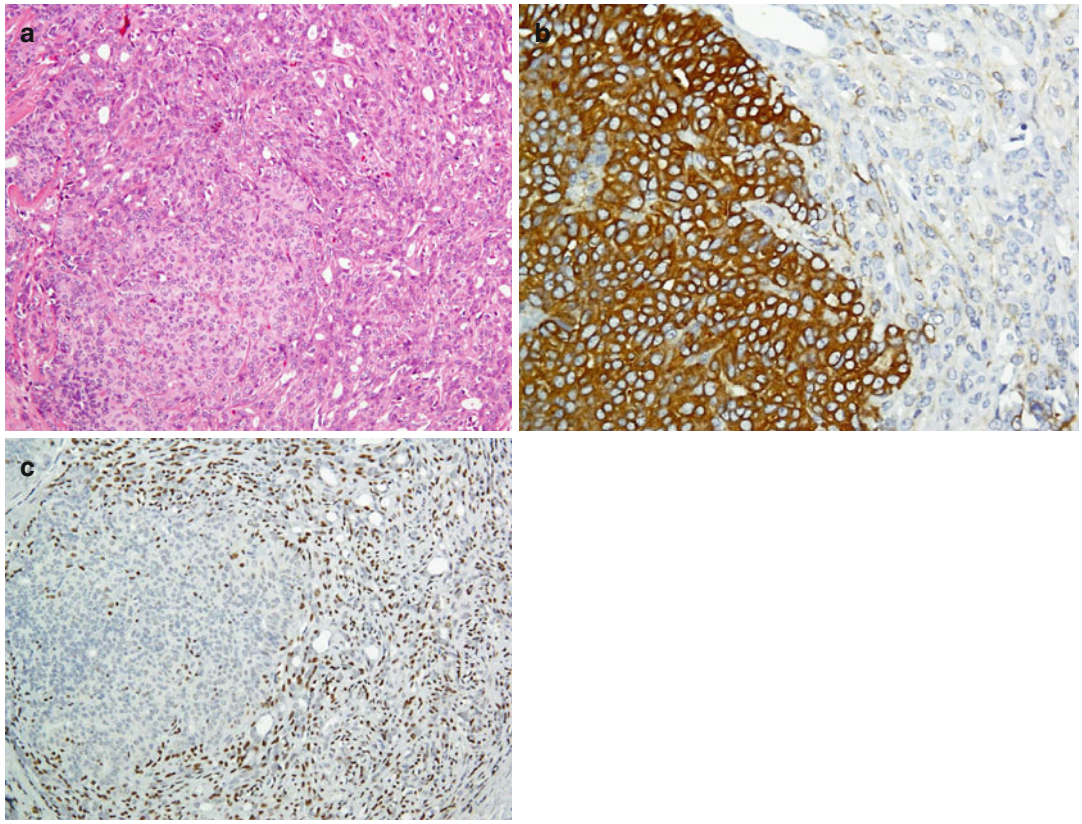

Fig. 26.30 Breast proliferative lesion (**a**). Presence of myoepithelial cells confirmed by immunoreaction to HMWCK (**b**) and p63 (**c**) which is indicative of a benign process

the absence of basal cells in prostate carcinoma (Fig. 26.35). Basal cell cocktail is a mixture of basal cell markers (HMWCH and p63 or CK5/6 and p63) used to highlight the presence of basal cells in normal glands which differentiates benign lesions from prostate intraepithelial neoplasia (PIN) and prostate adenocarcinoma [201]. Metastatic carcinoma of prostate origin exhibits reactivity to CK 7 and CK20 as well as PSA (Fig. 26.36). Table 26.26 summarized the immunoprofile of normal prostate glands as compared with PIN and adenocarcinoma.

26.7.7 Testis

Testicular tumors are classified into germ cell tumors and sex cord stromal tumors. Germ cell tumors are the most common type with classic seminoma subtype comprising the majority. The definite diagnosis of these tumors is dependent on proper application of the immunohistochemistric markers and histopathologic evaluation of the biopsy (Figs. 26.37, 26.38, and 26.39). Table 26.27 summarized the immunophenotype of testicular tumors.

26.8 Immunohistochemistry of Lymphoma

Immunohistochemistry is an integrated part of diagnostic surgical pathology of Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). Various Ags, mostly CD markers, are the

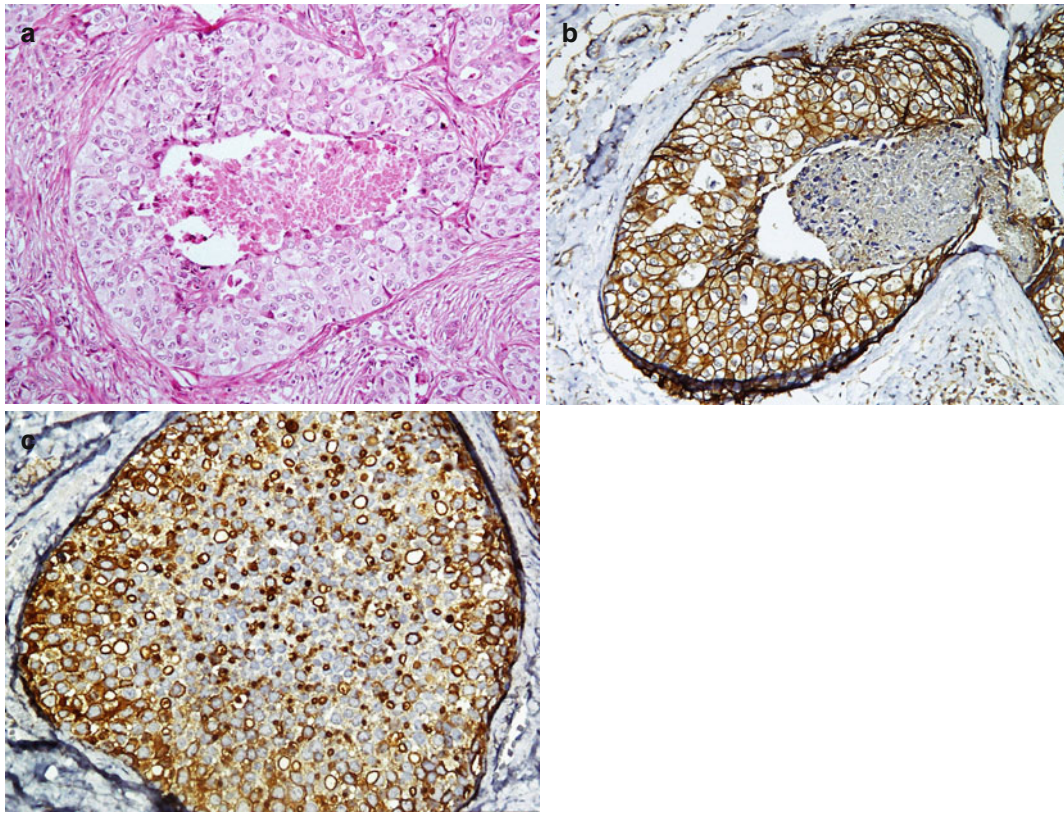

Fig. 26.31 Ductal carcinoma in situ (**a**) is immunostained with Her2neu (**b**) and CA15.3 (**c**)

targets of IHC. Neoplastic lymphoid cells express the same CD Ags with some aberrancy in type and amount. Several oncogene products are also expressed in some lymphomas (i.e., follicular lymphoma). These Ags have diagnostic and probably prognostic value. Proliferative Ags like Ki-67 are also of great value.

Morphology is the main stem of lymphoma diagnosis; nonetheless, IHC seems mandatory for the diagnosis and typing of malignant lymphoma. As a general rule, panels should be used for immunophenotypic evaluation, and there is no single marker absolutely specific for one definite lymphoproliferative disorder. Some routinely used markers are shown in Tables 26.28, 26.29, 26.30, 26.31, and 26.32 and Figs. 26.40, 26.41, 26.42, and 26.43.

26.9 Immunohistochemistry of Soft Tissue and Bone Tumors

Soft tissue sarcomas are a diverse family with different histologic origins and common histopathologic features. Given similar histopathologic features, immunohistochemistry is an ancillary method in distinguishing soft tissue tumors in order to attain a final diagnosis. As soft tissue tumor classification is based on specific line tissue origin, immunohistochemistry study by using specific Abs can be valuable in distinguishing them. Soft tissue tumors are vimentin-positive and keratin-negative tumors of a divergence family with heterogeneous tissue origins. Vimentin, a nonspecific marker, appears to react with all soft tissue tumors

Table 26.24 Immunoprofile of normal breast gland tissue

Normal epithelium	Immunoreactive antibodies
Luminal cells (LC)	CK8/18, CK19
Myoepithelial cells	CK5/6, CK14, CK17, p63, SMA, calponin, CD10
Both LC and MC	Pan-CK, AE1/AE3, CK7, S100

Table 26.25 Immunoprofile of invasive ductal carcinoma (*IDC*) and invasive lobular carcinoma (*ILC*) (Figs. 26.32 and 26.33)

Marker	IDC	ILC
Mammaglobin	–/+	+/–
ER	+/–	+
GCDFP15	–/+	–/+
E-cadherin	+	–
p120	+	+
34βE12	–	+

Refs. [192, 197–200]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)

and is considered as a control marker preserved in the tissue [246–252]. Immunohistochemistry of normal mesenchymal tissues with related tumors are summarized in Table 26.33

26.9.1 Epithelial Markers

Recognized as an intermediate filament protein, keratin is a sensitive and specific marker in the diagnosis of carcinomas among malignant tumors. Epithelial membrane antigen (EMA), derived from the mammary epithelium, is another epithelial marker expressed in most epithelial cells except squamous cells. Keratin and EMA are expressed exceptionally in some soft tissue tumors including synovial sarcoma, epithelioid sarcoma, chordoma, and myoepithelioma/myoepithelial carcinoma (previously known as parachordoma) [253].

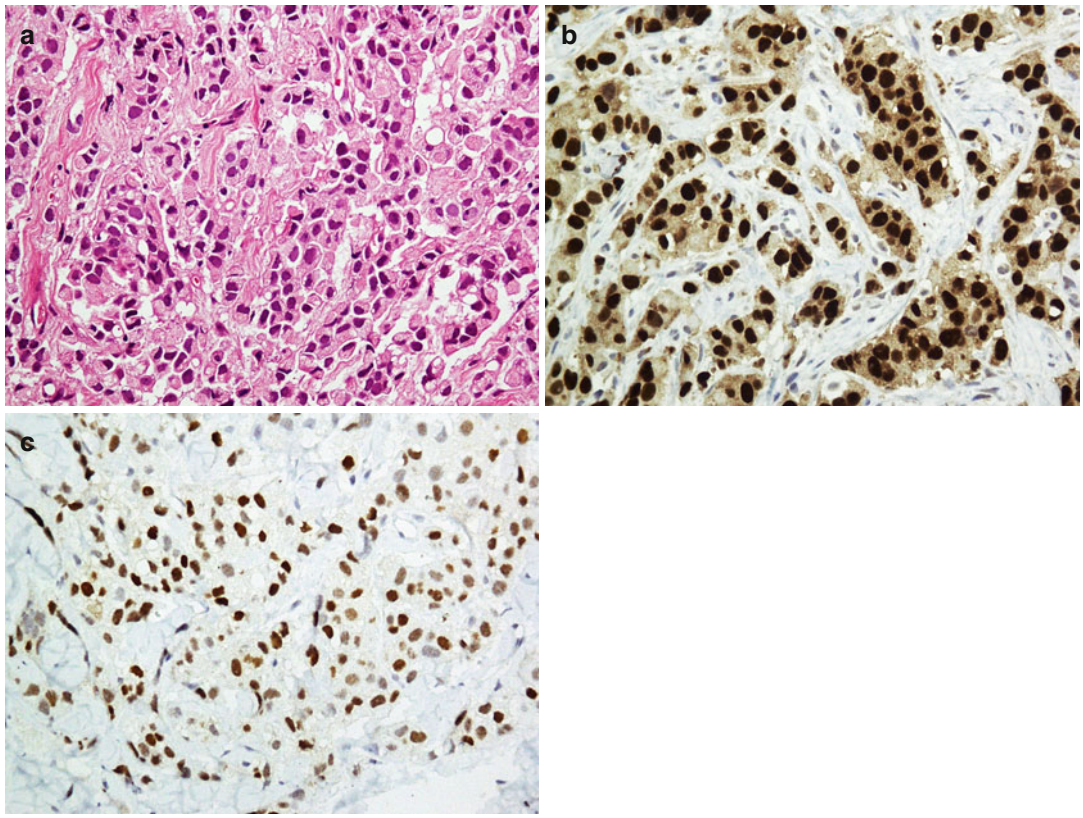

Fig. 26.32 Invasive ductal carcinoma (**a**) with ER (**b**) and PR (**c**) immunoreaction

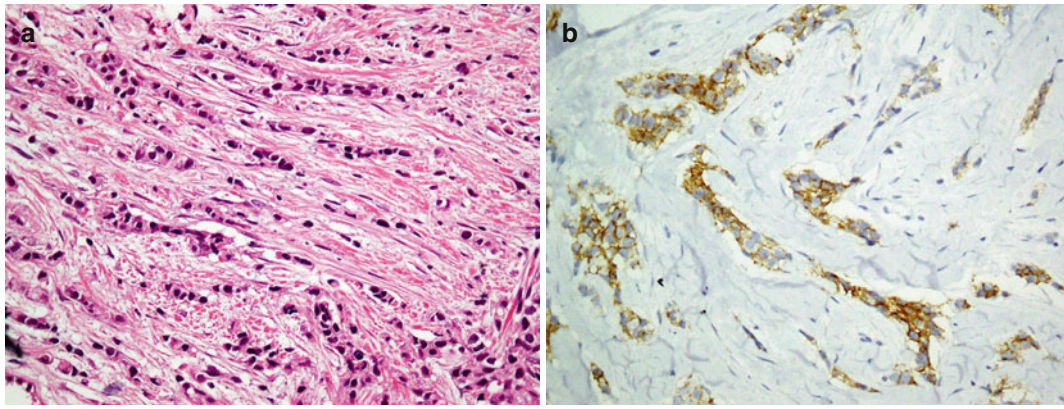

Fig. 26.33 Infiltrating carcinoma with Indian file pattern simulating lobular carcinoma (**a**), revealing immunoreaction with E-cadherin which is in favor of invasive ductal carcinoma (**b**)

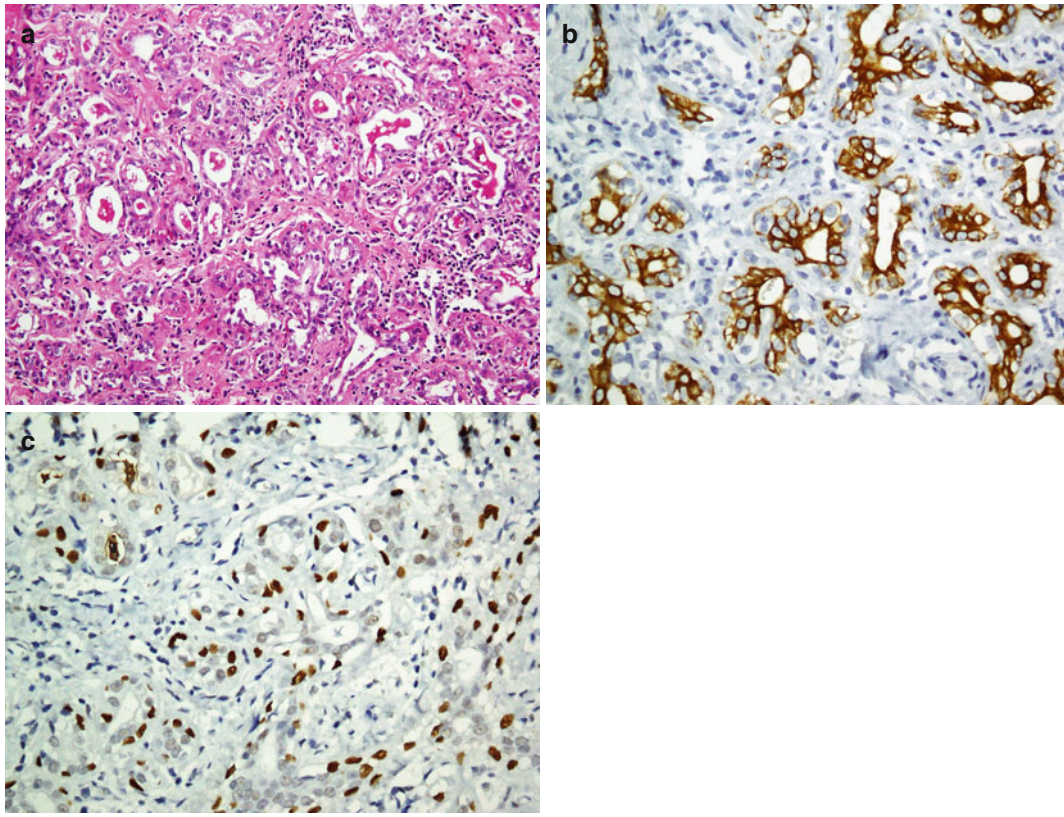

Fig. 26.34 Normal prostate tissue (**a**). The epithelium is immunostained with PSA (**b**), and basal cells are immunoreacted with p63 (**c**)

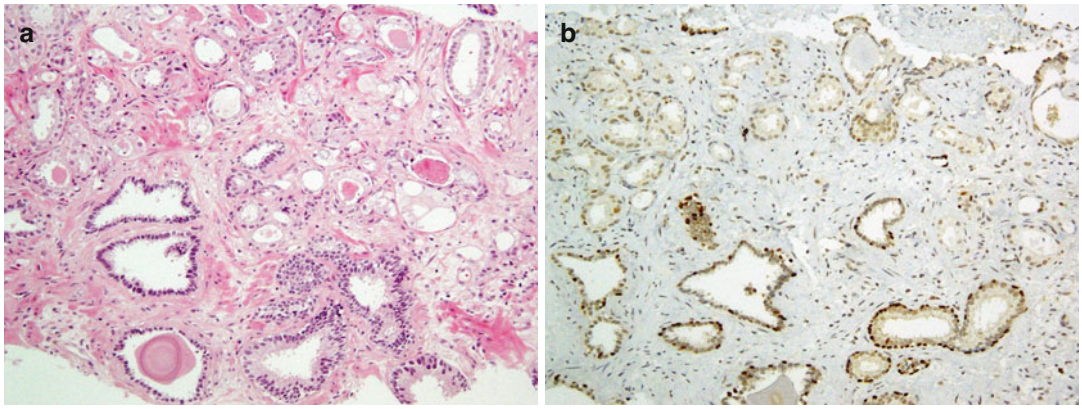

Fig. 26.35 Atypical prostate glands in the *top* of the picture which are highly suspicious of adenocarcinoma (**a**) show negative reaction to p63 (**b**). Some normal glands at the *bottom* of the picture exhibit reaction with p63

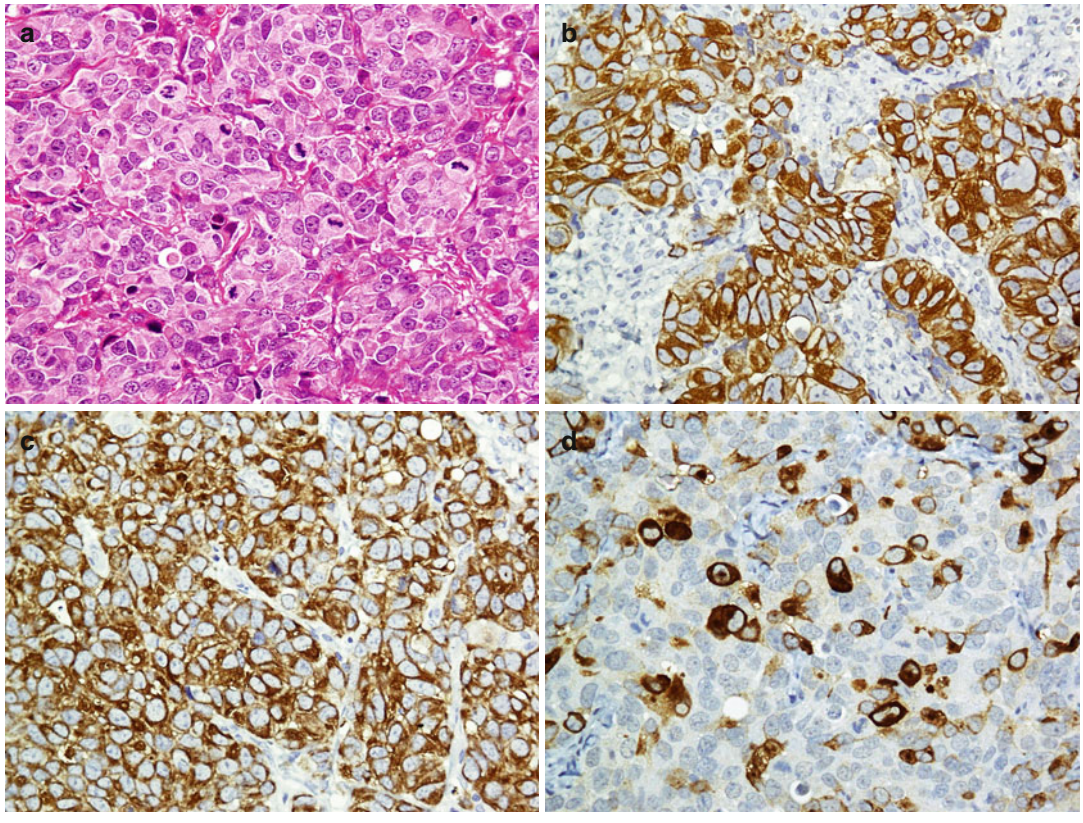

Fig. 26.36 An undifferentiated carcinoma from the pelvis with high mitotic rate (**a**) demonstrates cytoplasmic reaction with CK7 (**b**), CK20 (**c**), and PSA (**d**) which support the origin of this tumor as prostate

Table 26.26 Immunoprofile of normal prostate (NP), high-grade prostate intraepithelial neoplasia (HGPIN), and prostate adenocarcinoma (PAC)

Marker	NP	HGPIN	PAC	Application
PSA	+E	+	+	Weak reaction in HGPAC or metastatic carcinoma, to differentiate HGPAD from other undifferentiated carcinoma (colon, urothelium)
PSAP	+E	+	+	Similar to PSA
PSMA	+E	+	++	Correlated with grade and stage, more intense in HGPAC
P501S	+E	+	+	To differentiate high-grade PAC from other high-grade adenocarcinomas (colon, urothelium)
P504S (AMACR)	–	++	++	Combine with basal cell markers to differentiate HGPIN and PAC from normal prostate
HMWCK (34βE12)	+B	Partial loss	–	Complete loss in PAC ("negative" marker)
p63	+B	Partial loss	–	More sensitive than HMWCK ("negative" marker)
CK5/6	+B	Partial loss	–	More sensitive than HMWCK ("negative" marker)

Refs. [201–209]
Note: *B* basal cell, *E* epithelium

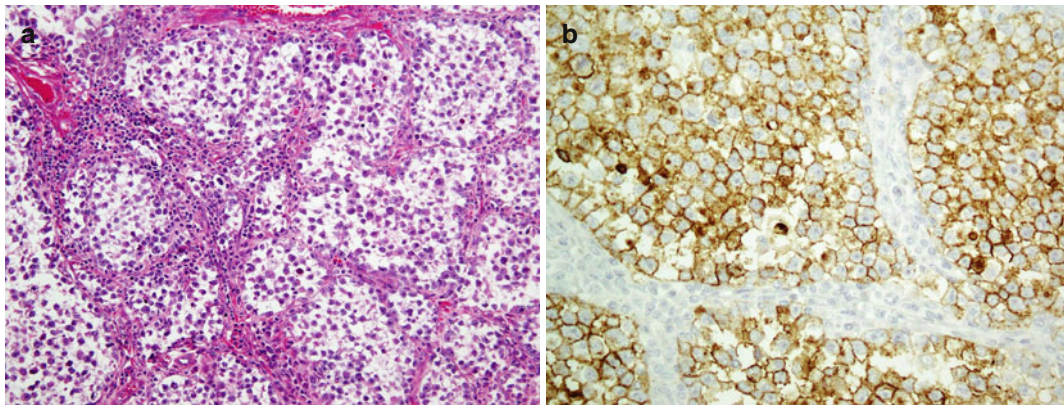

Fig. 26.37 Classic seminoma with polygonal cells and abundant watery cytoplasm (**a**) shows immunostaining with PLAP (**b**)

26.9.2 Myogenic Markers

There are some Abs which react with myogenic cells including desmin, actin, myoglobin, myo-D1, myogenin, caldesmon, and calponin. Desmin is an intermediate filament protein present in the cytoplasm of smooth and skeletal muscles. The Ab against this protein reacts with myogenic tumors such as rhabdomyoma, leiomyoma, rhabdomyosarcoma, and leiomyosarcoma (Fig. 26.44) [254]. Similar to desmin, actin is another myogenic protein detected in smooth and skeletal muscles. In addition, smooth muscle actin may react with some other cells like myofibroblasts and myoepithelial cells [255–257]. Myoglobin is exclusively seen in skeletal muscle cytoplasm, whereas myo-D1 and myogenin are nuclear transcription factors which are specifically expressed in skeletal muscle nuclei [258–260]. Myogenin has technical advantages over those of MyoD1, as the latter may cross-react with an unknown cytoplasmic Ag in non-muscle cells and tumors [261, 262]. However, Abs against these Ags are useful in determining rhabdomyosarcoma (Fig. 26.45). Calponin, a smooth muscle protein, is also expressed in

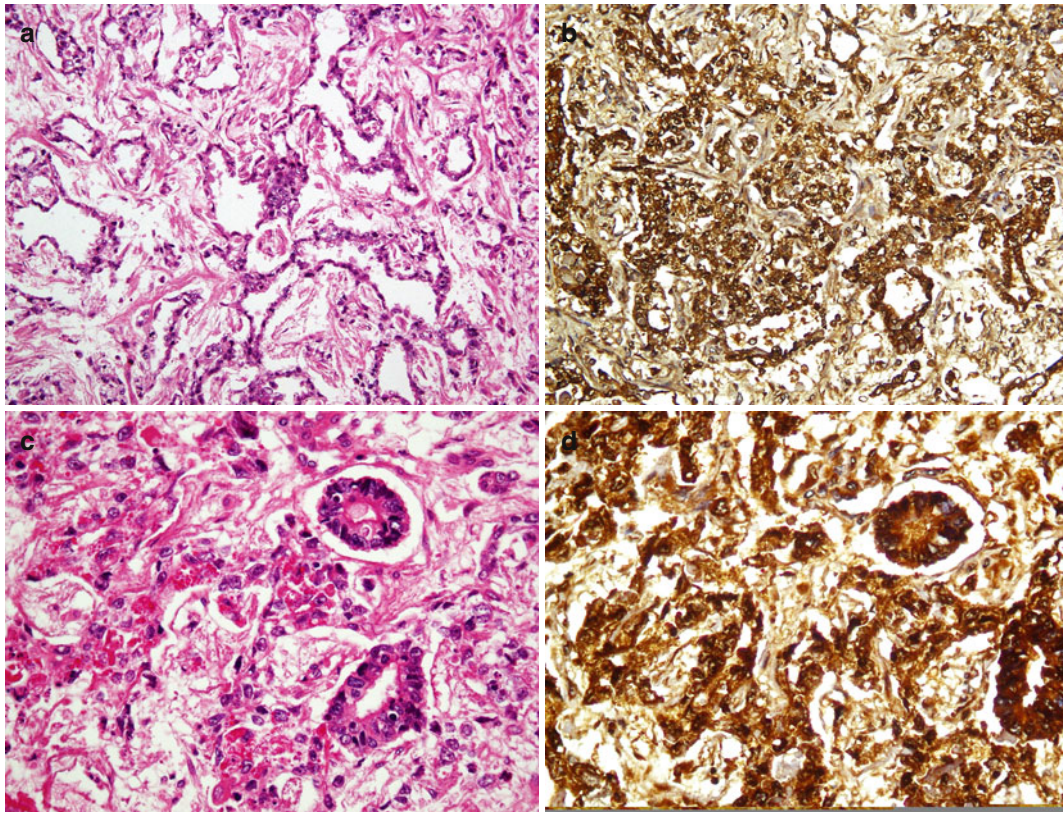

Fig. 26.38 Yolk sac tumor with tubuloglandular structures exhibits immunostaining with AFP (**a**, **b**) and glandular structures with numerous hyaline globules which are positive for AFP (**c**, **d**)

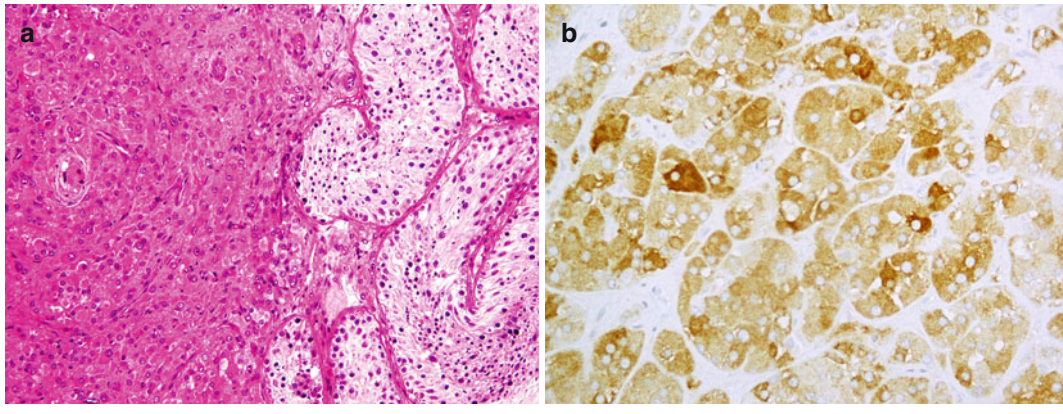

Fig. 26.39 Leydig cell tumor. Eosinophilic polygonal cell growth in the adjacent of seminiferous tubules (**a**) show immunoreaction with inhibin A (**b**)

Table 26.27 Immunophenotype of testicular tumors: classic seminoma (CS), spermatocytic seminoma (SS), embryonal carcinoma (EC), yolk sac tumor (YST), choriocarcinoma (CC), Sertoli cell tumor (SCT), and Leydig cell tumor (LCT)

Germ cell tumors (PLAP+, inhibin–)					Sex cord stromal tumors (PLAP–, inhibin+)	
CS (Fig. 26.37)	SS	EC	YST (Fig. 26.38)	CC	SCT	LCT (Fig. 26.39)
C-kit+	C-kit+/–	C-kit+/–	C-kit+/–	Inhibin+	AE1/AE-/+CAM5.2+	AE1/AE-/+GAL-3+
OCT3/4+		OCT3/4+	AE1/AE+	AE1/AE3+	Vimentin+	Vimentin+
CD117+		AE1/AE3+	AFP+	Glypican-3+	SMA+	CD99+/–
D2-40+		AFP+/–	Glypican-3+	HCG+	SYN+	
		CD117+	HepPar-1+		NSE+	
		CD30+				

Refs. [154, 210–223]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %),–(<10 %)

Table 26.28 Immunoprofile of precursor lymphoid neoplasms (Fig. 26.40)

Lymphoma	CD2	CD5	CD20	CD79a	PAX5	CD45	CD34	CD10	CD99	Tdt	CD43	CD56
B ALL/LBL	–	–	+/–	+	+	–/+	+	+	–	+	+	–
T ALL/LBL	+	+	–	–	–	–/+	+	+/–	+	+	+	+

Refs. [224–230]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)

Table 26.29 Immunoprofile of small B-cell lymphomas: B-cell small lymphocytic lymphoma/chronic lymphocytic lymphoma (B SLL/CLL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), mucosa-associated lymphoid tissue (MALT), follicular lymphoma (FL), lymphoplasmacytic lymphoma (LPL), and hairy cell leukemia (HCL)

Lymphoma	CD20	CD23	CD10	CD5	BCL6	MUM1	CD43	CyclinD1	AnnexinA1	BCL2
B SLL/CLL	+ (weak)	+	–	+	–	+/–	+	–/+	–	+
MCL	+	–/+	–	+	–	–	+	+	–	+
MZL (nodal)	+	–	–	–	–/+	+	+/–	–	–	+
MZL (MALT)	+	–	–	–	–	+/–	+/–	–	–	+
MZL (splenic)	+	–	–	–	–	+/–	–	–	–	+
FL	+	–/+	+	–	+	–	–[a]	–	–	+
LPL	+	–/+	–/+	–	–	+[b]	–/+	–	–	+
HCL	+	–/+	–/+	–	–	NT	NT	+	+	+

Refs. [224–227, 231–239]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)
[a]Maybe positive in grade 3
[b]More intense in plasmacytoid cells

Table 26.30 Immunoprofile of some aggressive mature B-cell lymphomas: diffuse large B-cell lymphoma (DLBL), T-cell/histiocyte-rich B-cell lymphoma (TC/HRBCL), and anaplastic large cell lymphoma kinase (ALK)

Lymphoma	CD20	CD10	MUM1	Bcl-2	Bcl-6	CD30	Ki-67	EMA	CD45	CD138
DLBCL (NOS) (Fig. 26.41)	+	+[a]	–[b]	+/–	+[a]	–/+[a]	<90 %	–	+	–
TC/HRBCL	+	–/+	–/+	+/–	+	–	<90 %	+	+	–
DLBCL plasmablastic	–[a]	–	+	–	–	+/–	>90 %	+	–[a]	+
DLBCL-ALK+ (Fig. 26.42)	–	–	+/–	–	–	–	<90 %	+	+ weak	+
Burkitt lymphoma	+	+	–	–[a]	+	–	>95 %	–	+	–

Refs. [224–227, 236, 239–242]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)
[a]Some cells may be weakly positive
[b]Positive in non germinal centers (35–65%)

Table 26.31 Immunoprofile of some mature T-cell/NK-cell lymphomas: mycosis fungoides (MF), adult T-cell lymphoma/leukemia (ATLL), angioimmunoblastic T-cell lymphoma (AILT), anaplastic large cell lymphoma (ATCL), and T-cell lymphoma (TCL)

Lymphoma	CD3	CD5	CD4	CD8	CD30	ALK	TIA1	CD56
MF	+	+	+	–	+[b]	–	+[b]	–
ATLL	+	+	+[a]	–[a]	+/–	–	–	–
AILT	+	+	+	–	+[b]	–	–	–
ALCL	–/+	+	+	–	+	+ (60–80 %)	+/–[c]	–
Subcutaneous panniculitis-like TCL	+	–	–	+	–	–	+	–
Cutaneous TCL	+	–	–	–/+	–	–	+	+
Hepatosplenic TCL	+	–/+	–	–/+	–	–	+	+
Nasal or nasal-type NK/TCL	+ (Cytoplasmic)	–	–	+/–	–	–	+	+
Enteropathy-type TCL	+	–	–	+	+/–	–	+	+[d]

Refs. [224–227, 243–245]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)
[a]Most cases
[b]Some large cells
[c]More often ALK-positive cases
[d]Subset with monomorphic small cell morphology

Table 26.32 Immunophenotypic features of classic Hodgkin lymphoma (CHL) and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) (Fig. 26.43)

Lymphoma	CD20	Pax-5	CD15	CD30	Facsin	EMA	ALK-1
CHL	+/–	+(weak)	+	+	+	–/+	–
NLPHL	+	+	–	–/+	–	+/–	–

Refs. [224–227]
Note: + (>90 %), +/– (>50 %), –/+ (<50 %), – (<10 %)

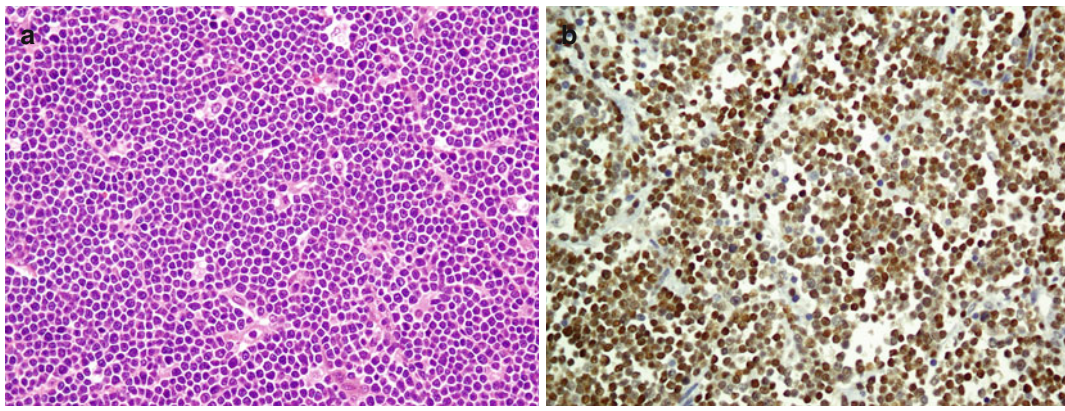

Fig. 26.40 Lymphoma with starry sky feature declares a highly proliferative phase (**a**) in which antibodies to terminal deoxynucleotidyl transfer (TdT) marks it as a precursor lymphoid neoplasm

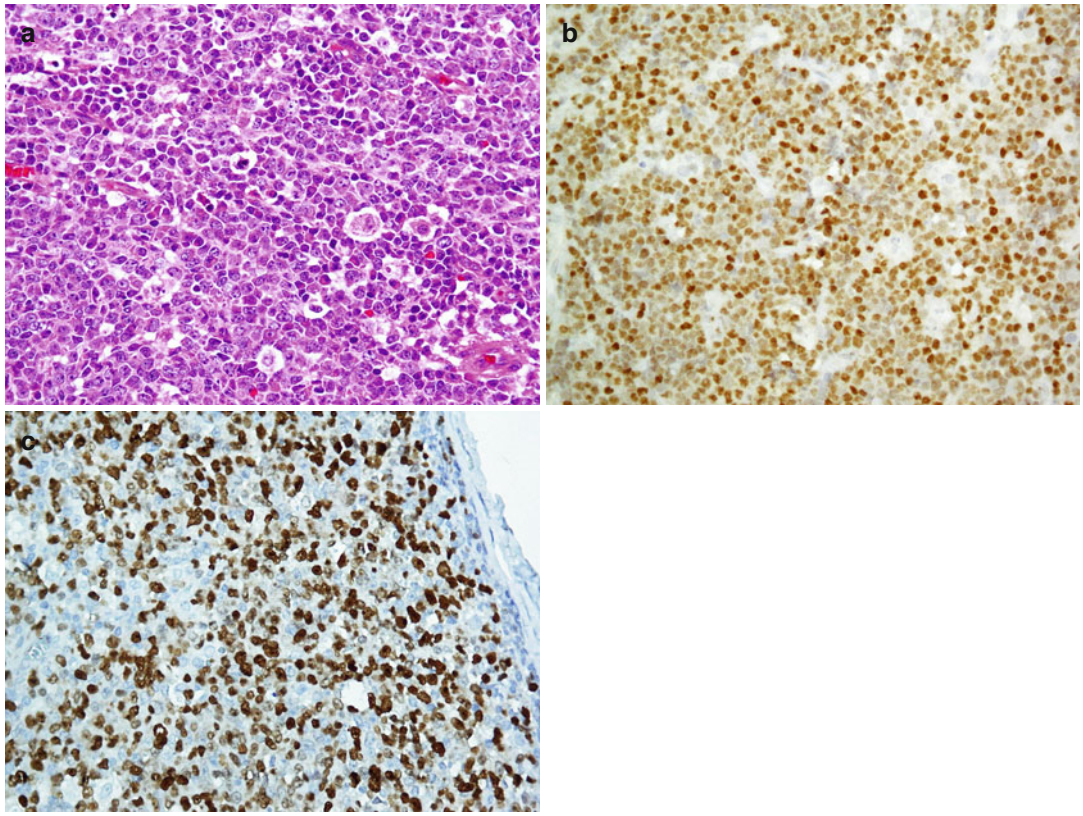

Fig. 26.41 Diffuse large B-cell lymphoma (NOS) (**a**) weakly reacts with Bcl-6 and (**b**) indicates a high proliferative index by Ki-67 (**c**)

myofibroblasts and myoepithelial cells and limits the usefulness of diagnostic pathology [45]. A relatively smooth muscle-specific marker being expressed in cytoplasm, caldesmon is a useful Ab in distinguishing smooth muscle tumors from myofibroblastic tumors [263]. A novel Ag of smooth muscle differentiation, transgelin is a calponin-related protein found in smooth muscle showing higher sensitivity and specificity than other markers [264].

26.9.3 Nerve and Schwann Cell Markers

First isolated from the central nervous system (CNS), S100 protein is known as a marker of nerve sheath tumors as well as melanocytic and chondrocytic tumors. S100 is expressed by a wide range of cell types including glial cells, neurons, Schwann cells, melanocytes, chondrocytes, lipocytes, myoepithelial cells, sustentacular cells, Langerhans histiocytes, interdigitating reticulum cells, and various epithelia [26]. CD56 (neural cell adhesion molecule) and CD57 (myelin-associated glycoprotein) are expressed by a variety of different cell types including tissues of the peripheral nervous system (PNS) and CNS, as well as natural killer (NK) cells and neuroendocrine cells [265–267].

26.9.4 Endothelial Markers

Von Willebrand factor (vWF) is exclusively expressed by endothelial cells and is principally

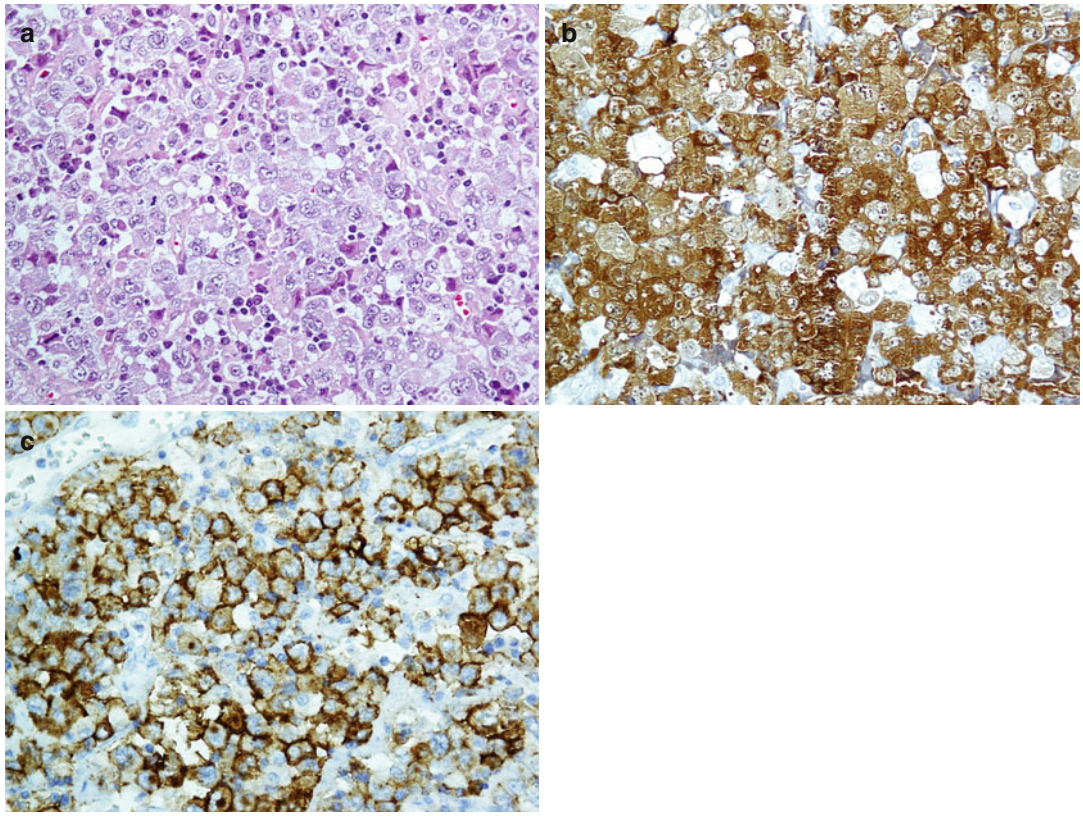

Fig. 26.42 Diffuse large B-cell lymphoma (ALK). Large anaplastic cells intermixed with lymphoplasma cells (**a**) are strongly positive for ALK (**b**) and EMA (**c**)

used to distinguish vascular neoplasms from their morphologic mimickers. Due to low sensitivity of vWF in detecting high-grade vascular neoplasms, other endothelial markers such as CD31, CD34, and FLI-1 have limited the routine use of vWF in the context of vascular tumors. Given similar sensitivity to CD34, CD31 is expressed by macrophages, being a more specific vascular marker than CD34. CD34 is expressed by bone marrow hematopoietic precursor cells and dendritic interstitial cells limiting its application in vascular tumors [268–271]. As a nuclear transcription factor, FLI-1 (Freund leukemia integration site) is an endothelial marker expressed in vascular tumors as well as ES/PNET and lymphoblastic lymphoma [57].

26.9.5 Fibrohistiocytic Markers

There are some nonspecific markers such as alpha 1-antitrypsin, muramidase (lysozyme), alpha 1-antichymotrypsin, cathepsin B, CD68, CD163, factor XIIIa, and the HAM 56 Ag which are expressed in melanomas, carcinomas as well as some sarcomas like MFH [272–278]. Therefore, application of these markers is limited and should be considered after ruling out other sarcomas with specific line differentiation.

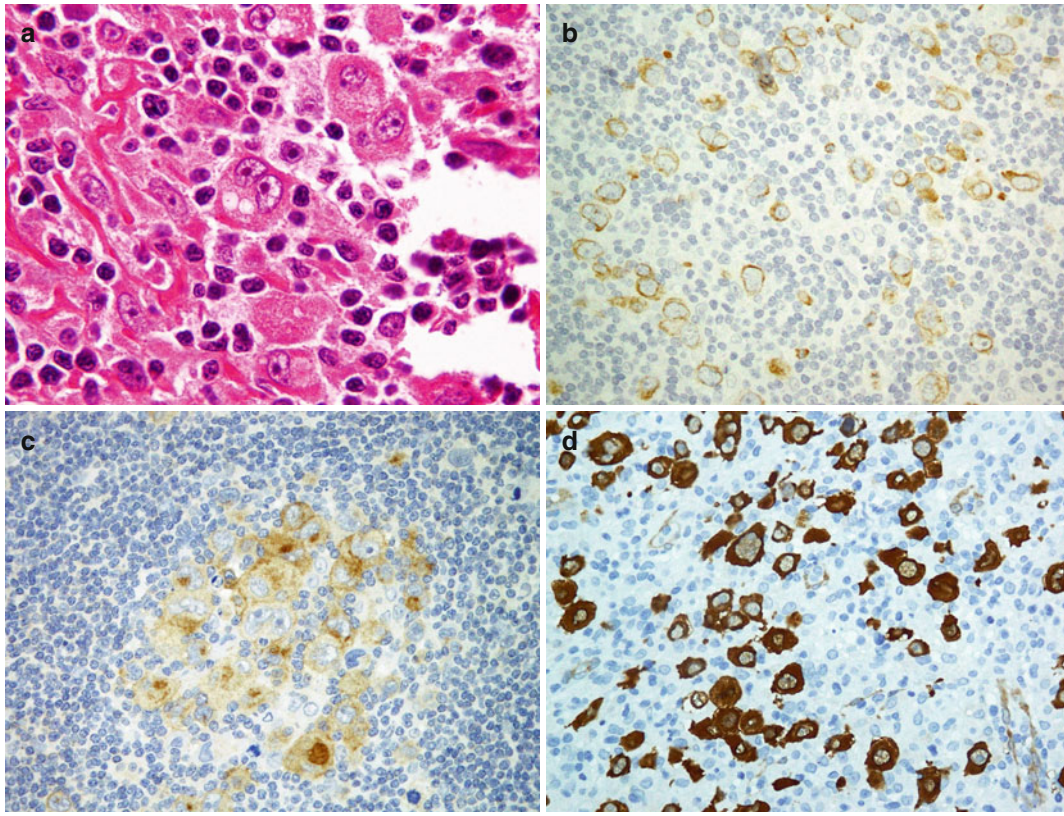

Fig. 26.43 Hodgkin lymphoma. Typical Reed-Stenberg cell with mirror binuclear feature of "Owl's eye" (**a**) weakly reacts with CD 15 (**b**) and CD30 (**c**) and strongly reacts with fascin (**d**)

Table 26.33 Immunohistochemical antibodies of normal mesenchymal tissues and related tumors

Soft tissue	Markers of soft tissue	Related tumor	Immunoreactive markers
Chondrocyte	S100, SOX9, vimentin	Chondrosarcoma	S100, vimentin, CD57, SOX9: sensitive marker for cartilaginous differentiation
Endothelial cells	Vimentin, CD31, CD34, FLI-1 D2-40 (lymphatic endothelium)	Angiosarcoma Lymphangiosarcoma	CD31, CD34, FLI-1 D2-40
Fibroblasts	Vimentin, CD10, CD99	Fibrosarcoma	Vimentin
Fibrohistiocyte	CD68, CD168, a1AT, cathepsin B, factor IIIA, HAM 56	Malignant fibrous histiocytoma	CD68
Lipocytes	Vimentin, S100 (variable), calretinin, MDM2, CDK4, CD-34	Liposarcoma	S100, MDM2, CDK4
Osteoblast	CD56, osteocalcin, osteonectin, vimentin	Osteosarcoma	Osteocalcin, collagen IV, CK, EMA, CD99, S100, desmin, SMA, factor 13
Nerve/Schwann cell	Vimentin, S100, CD56, CD57	MPNST	S100
Skeletal muscle	Desmin, myoglobin, CD56, GFAP	Rhabdomyosarcoma	Myogenin, myo-D1, PLAP, WT1
Smooth muscle	Desmin, NSE, SMA, MSA	Leiomyosarcoma	Desmin, SMA, MSA, h-caldesmon, collagen IV
Synovial cell	CD68, clusterin	Synovial sarcoma	CK, EMA, vimentin, CD68, CD99, E-cadherin, collagen IV

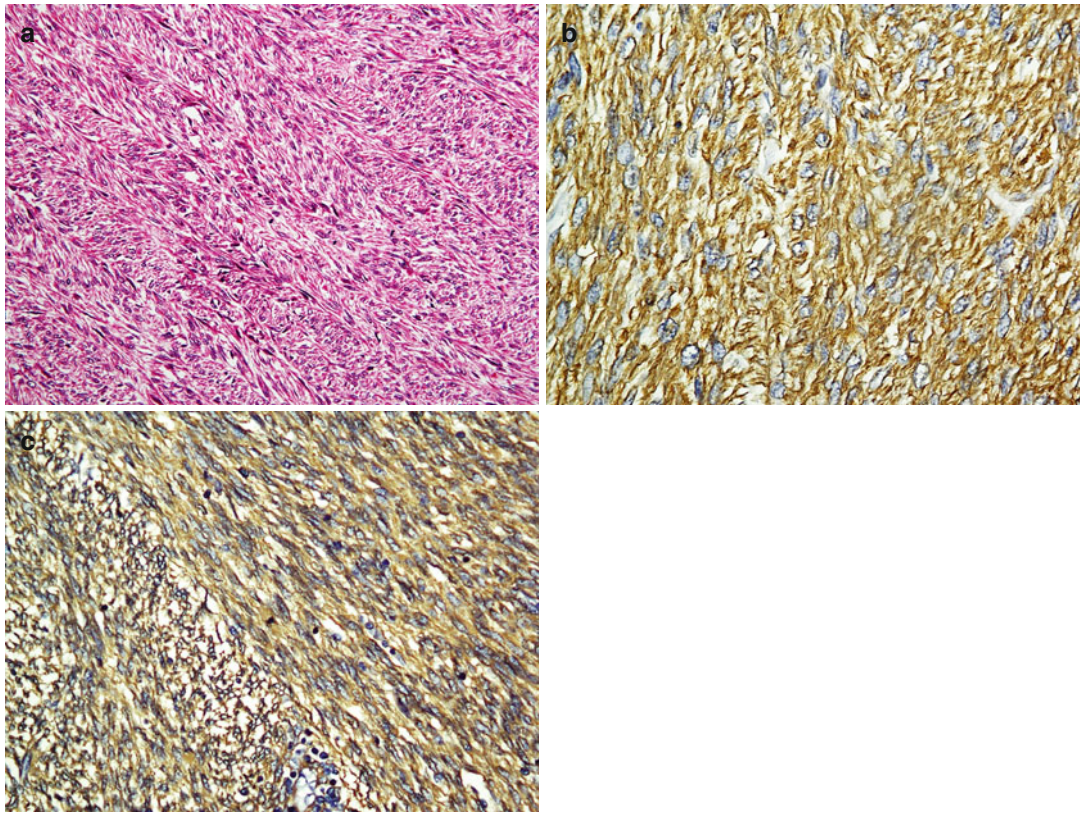

Fig. 26.44 Leiomyosarcoma. Spindle cells arranged in interlacing cross-striated fascicles (**a**) are immunostained with desmin (**b**) and h-caldesmon (**c**)

26.9.6 Lipocytic Markers

MDM2 (an inhibitor of p53 transcriptional activation) and CDK4 (a protein involved with cell cycle progression) are markers to separate dedifferentiated liposarcomas from other poorly differentiated sarcomas [279].

26.9.7 Chondrocyte Markers

Chondrocytes do not display specific markers and show reactivity with S100 and vimentin. Chondrosarcoma also exhibits reactivity with CD57 [280]. Being as a master regulator of chondrogenesis, SOX9 is a sensitive marker for cartilaginous differentiation distinguishing mesenchymal chondrosarcoma from other small blue round cell tumors [281].

26.9.8 Osteogenic Markers

Osteocalcin (a non-collagenous proteins) with approximately 70 % sensitivity is a completely specific marker for bone-forming tumors. In addition, osteonectin (a bone matrix glycoprotein participates in stromal mineralization) also has a sensitivity of 90 % and a specificity of 54 % in the diagnosis of osteoblastic neoplasms [282, 283]. These markers are rarely being used in routine diagnosis because the diagnosis of osteosarcoma is based on the presence of osteoid in the H&E-stained slides.

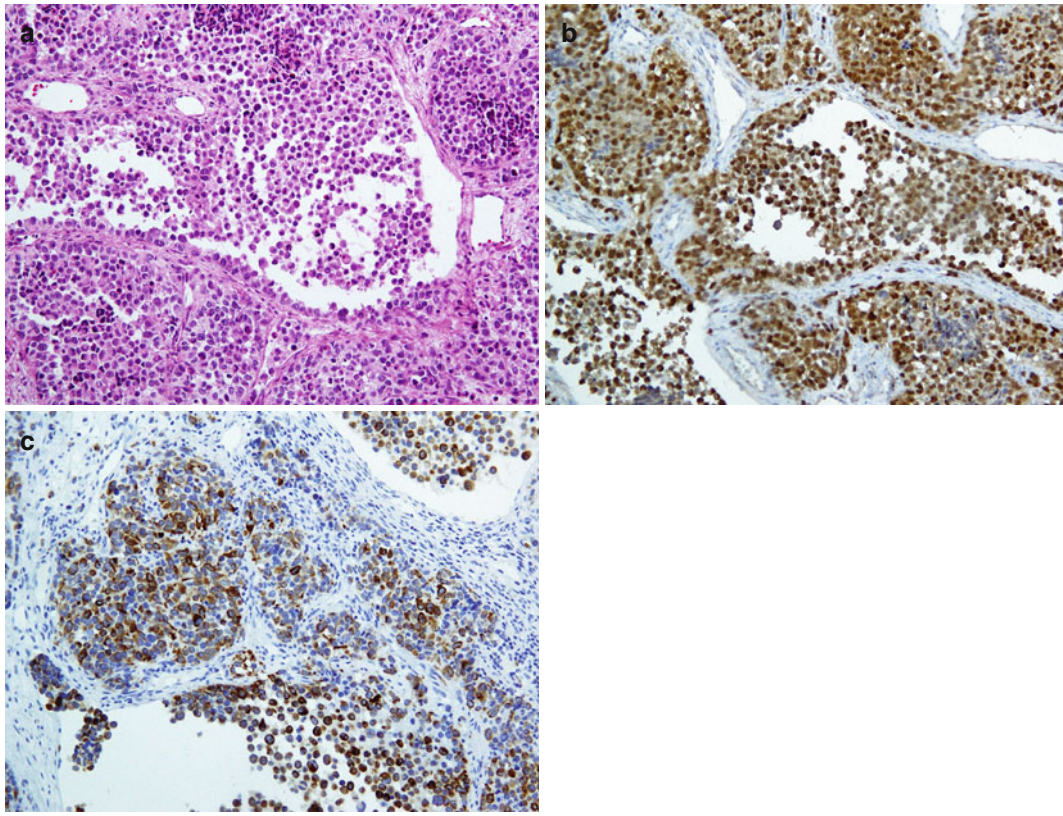

Fig. 26.45 Alveolar rhabdomyosarcoma. Large polygonal cells with alveolar pattern (**a**) are highlighted with myogenin (**b**) and desmin (**c**)

26.9.9 Unknown-Origin Soft Tissue Tumors

Ewing sarcoma/peripheral nerve sheath tumor (ES/PNET) comprises a prototype of small round cell neoplasms of bone and soft tissue exhibiting neuroectodermal features. As a product of the *MIC2* gene, CD99 is a cell surface transmembrane glycoprotein diffusely present in nearly all tumors (Fig. 26.46) [284]. Clear cell sarcoma (malignant soft part melanoma) shares markers of malignant melanoma such as S100, MART-1, HMB45, and tyrosinase [285]. Alveolar soft part sarcoma has been evaluated by presence of myo-D1 and myogenin [286, 287]. Desmoplastic small round cell tumor (DSRCT) is characterized by the coexpression of epithelial and mesenchymal markers [288]. The immunohistochemistry characteristics of these tumors are summarized in Table 26.34.

26.10 Immunohistochemistry of the Nervous System

The brain tumors are classified into two major groups: primary and metastatic. Primary brain tumors are further categorized into three major subtypes: neuroepithelial tumors (astrocytoma, oligodendroglioma, ependymoma, choroid plexus tumors, neuronal tumors, and pineal tumors), non-neuroepithelial tumors (meningioma, nerve

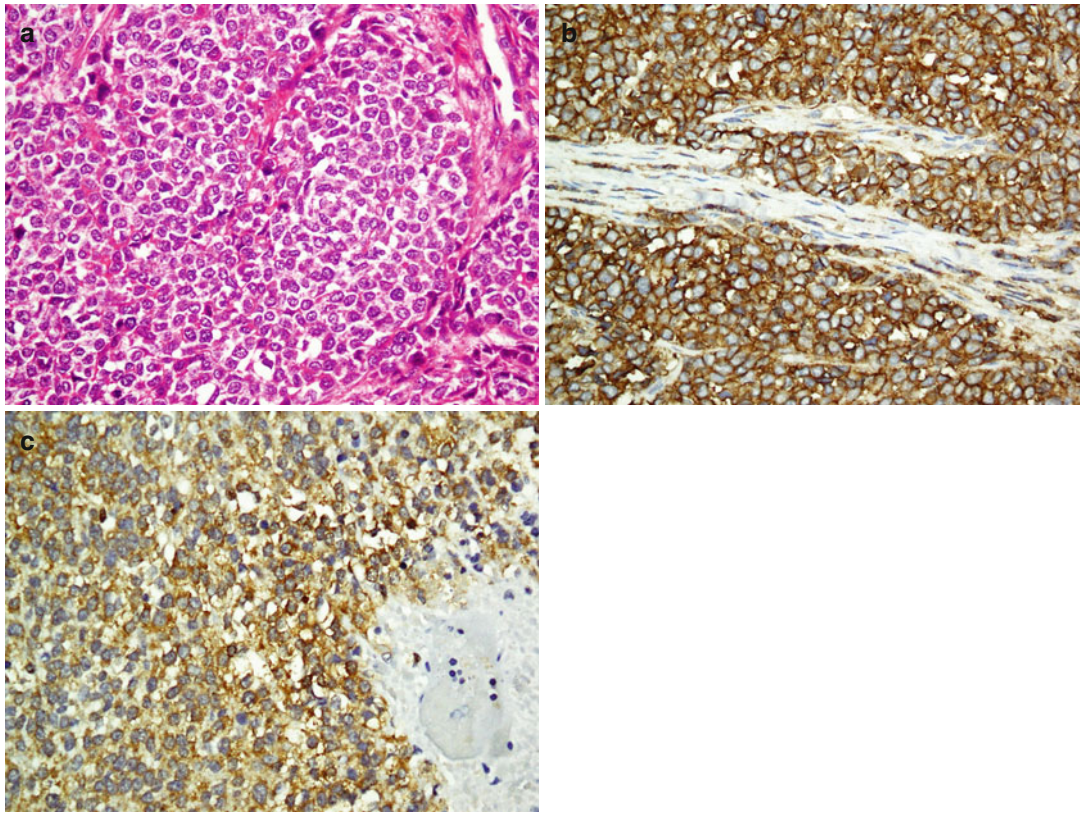

Fig. 26.46 Small round cell tumor (**a**). Immunoreaction with MIC2 (**b**) and NSE (**c**) antibodies supports the diagnosis of PNET

Table 26.34 Immunoprofile of unknown-origin soft tissue tumors: Ewing sarcoma/peripheral neuroectodermal tumor (ES/PNET), clear cell sarcoma (CCS), alveolar soft part sarcoma (ASPS), and desmoplastic small round cell tumor (DSRCT)

Panel antibodies	ES/PNET	CCS	ASPS	DSRCT
CD99/FLI-1	+	–	–	–
S100/HMB45/ MITF/Melan-A	–	+	–	–
TFE3	–	–	+	–
NSE	+	–	–	+
Desmin	–	–	–	+
CK/EMA	–	–	–	+
WT1	–	–	–	+

Refs. [284–288]

sheath tumors, lymphoma, chordoma, and germ cell tumors), and primitive undifferentiated tumors (medulloblastoma, pineoblastoma, ependymoblastoma, and PNET) [289–295]. Primary origin of metastatic carcinoma is determined by the use of immunohistochemical panel. Commonly used IHC Abs in primary CNS tumors are demonstrated in Table 26.35.

26.10.1 Neuroepithelial Tumors

Glial tumors (astrocytoma, oligodendroglioma, and ependymoma) usually react with glial fibrillary acidic protein (GFAP) [151, 152, 296]. Oligodendroglioma variably expresses GFAP and commonly reacts with Leu7 and S100 [297, 298]. Moreover, GFAP is present in other mixed glial and neuronal-glial tumors including oligoastrocytoma and ganglioglioma (Fig. 26.47) [296]. Neurocytoma and pineal tumors are GFAP negative

Table 26.35 Commonly used antibodies in primary CNS tumors

Antibody	Normal brain	Tumor
EMA	Epithelial, perineural, meningothelial cells	Meningioma, chordoma, medulloblastoma
GFAP	Glial cells	Glial tumors except oligodendroglioma, medulloepithelioma, choroid plexus tumor, ganglioglioma
Leu7 (CD57)	Oligodendroglial cells, Schwann cells,	Oligodendroglioma, schwannoma, neurofibroma, oligoastrocytoma
Neurofilament	Neuropil	Ganglion cell tumors, neurocytoma, pineocytoma, neurofibroma, medulloblastoma, PNET
NSE	Neuroectodermal and neuroendocrine cells	Neuroblastoma, hemangioblastoma, PNET, oligodendroglioma
S100	Glial cells, Schwann cells, dendritic and Langerhans cells, melanocytes, other mesenchymal cells	Gliomas, meningioma, schwannoma, neurofibroma, chordoma, craniopharyngioma, PNET, medulloblastoma, pineoblastoma, neuroblastoma, melanoma, chondroid tumors
Synaptophysin	Neuroendocrine cells, neuropil	Neurocytoma, ganglion cell tumors, pineocytoma, choroid plexus papilloma, medulloblastoma, pineoblastoma, neuroblastoma, PNET, oligodendroglioma, dysembryoblastic neuroepithelial tumor
Vimentin	Meningoendothelial cells, other mesenchymal cells	Meningioma, gliomas, chordoma, ependymoblastoma, hemangiopericytoma, ganglioglioma, embryonal tumors
Collagen IV	Ganglion cell, Schwann cell, other mesenchymal cells	Ganglion cell tumor, schwannoma, medulloblastoma/pineoblastoma

Refs. [151, 152, 296]

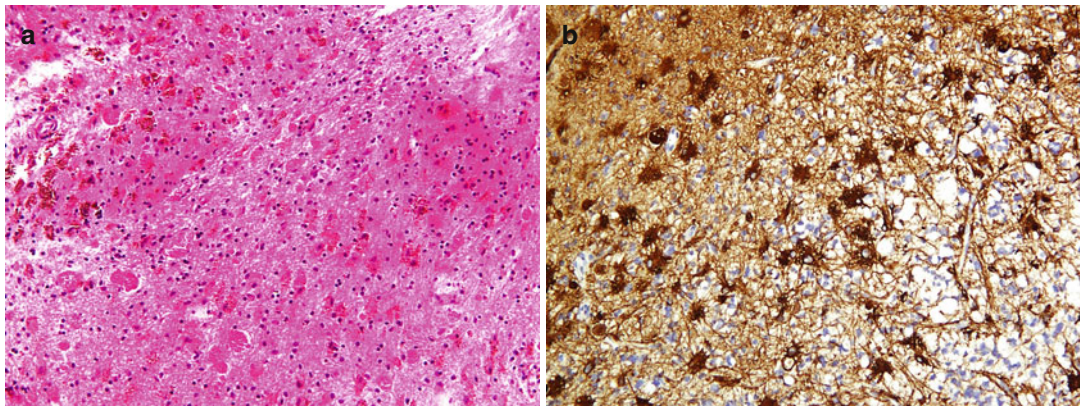

Fig. 26.47 Fibrillary astrocytoma with proliferation of atypical astrocytes (**a**) exhibits GFAP-positive cytoplasmic processes (**b**)

and synaptophysin positive. Among neuroepithelial tumors, choroid plexus tumors demonstrate reactivity with epithelial markers such as cytokeratin, CAM5.2, and EMA. Additionally, transthyretin, as a potential marker, and IGF-II, as a newer marker, are positive in choroid plexus tumors [299–301]. Pineal tumors are GFAP$^-$ and epithelial-negative tumors which exhibit reactivity with synaptophysin and neurofilament (Table 26.36).

26.10.2 Non-neuroepithelial Tumors

Among non-neuroepithelial tumors, meningiomas are positive for EMA which differentiates them from nerve sheath tumors and are negative for GFAP which distinguishes meningioma from gliomas. Schwannoma is distinct from glioma, meningioma, and neurofibroma by showing reaction to collagen type IV. Neurofibroma differs from schwannoma

Table 26.36 Immunopanel of neuroepithelial tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
GFAP$^+$, EMA$^-$, CAM5.2$^-$	Vim$^+$, NF$^+$, S100$^+$	Astrocytoma (Fig. 26.47)
	Leu7$^+$, NSE$^+$, S100$^+$	Oligodendroglioma
GFAP$^+$, EMA (R), CAM5.2 (R)	Vim$^+$, S100$^+$	Ependymoma
GFAP (S), EMA$^+$, CAM5.2$^+$	Laminin$^+$, SPN$^+$, S100$^+$, IGF-II$^+$	Choroid plexus papilloma
GFAP$^-$, EMA$^-$, CAM5.2$^-$	SPN$^+$, NF$^+$	Central neurocytoma
	SPN (S), NF (S), Collagen IV$^+$	Ganglion cell tumor
	NSE$^+$, SPN$^+$, NF (R)	Pineal tumor

Refs. [151, 152, 296–298, 302–310]
Note: *N* negative, *R* rare, *S* sometimes

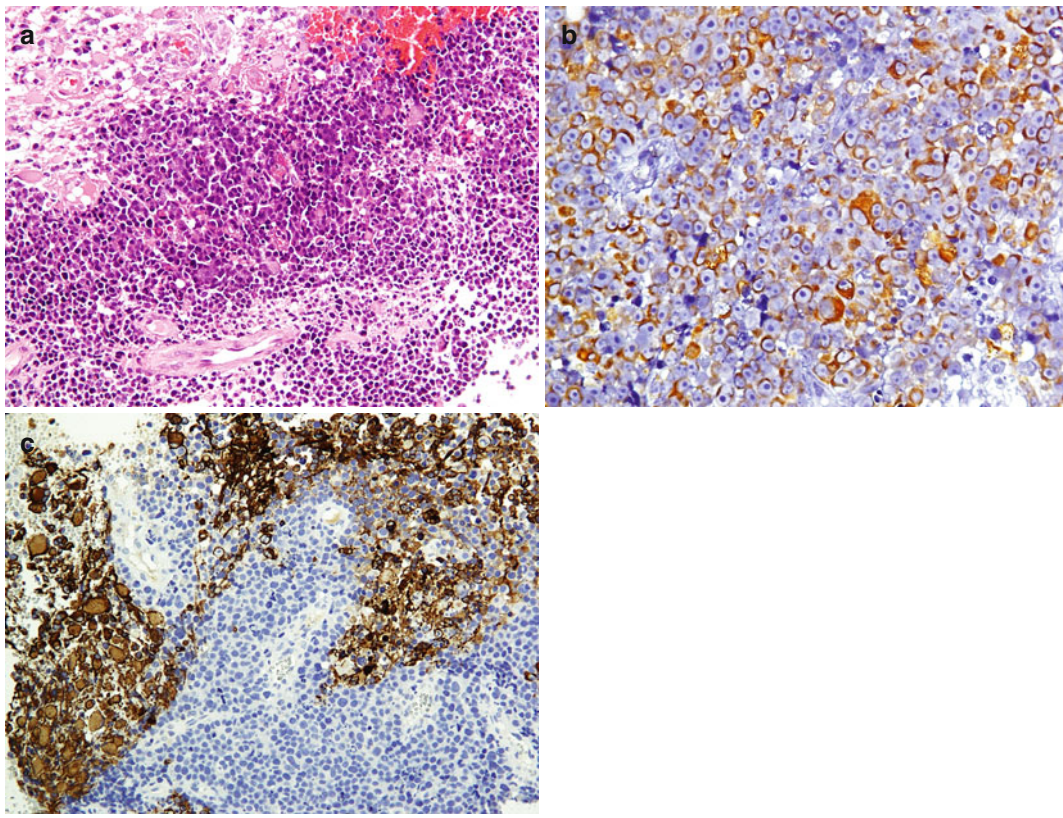

Fig. 26.48 Germinoma. (**a**) A tumor with relatively medium to large polygonal cells resembling an undifferentiated tumor surrounded by reactive astrocytes (*upper right corner*). Tumor cells react with PLAP (**b**) and reactive astrocytes stain by GFAP (**c**) (Courtesy of Dr. Taghi Ghiasi-Moghadam, Mashad, Iran)

by having neurofilament-positive axons. Primary and secondary brain lymphomas express LCA as a common marker and CD3 and CD20 as differentiating markers of T-cell- and B-cell-type lymphomas, respectively. Arising from notochord remnants, chordomas are malignant tumors along the axial skeleton recognized by characteristic physaliphorous cells with large intracytoplasmic vacuoles. Chordoma exhibits reactivity for CK and EMA as well as S100, whereas chondrosarcomas lack these features (CK/EMA negative and S100 positive). Primary germ cell tumors are found along the midline in the pineal and suprasellar regions which demonstrate immunostaining with placental alkaline phosphatase (PLAP), alpha-fetoprotein (AFP), beta-HCG, and CEA (Fig. 26.48) (Table 26.37).

Table 26.37 Immunopanel of non-neuroepithelial tumors

First-choice antibody panel	Second-choice antibody panel	Consistent with
Vimentin$^+$, S100+	EMA$^+$	Chordoma
Vimentin$^+$, S100 (R)	EMA (S)	Meningioma
Vimentin$^-$, S100$^+$	Leu7$^+$, collagen IV$^+$, GFAP (R)	Schwannoma
	Leu7$^+$, NF$^+$, EMA$^+$	Neurofibroma
Vimentin$^-$, S100$^-$	LCA$^+$, L26$^+$	Lymphoma
	PLAP$^+$, HCG$^+$, AFP$^+$	Germ cell tumor (Fig. 26.48)

Refs. [151, 152, 296–298, 311–316]
Note: *N* negative, *R* rare, *S* sometimes

Table 26.38 Immunopanel of primitive undifferentiated tumors

First-choice antibody panel	Second-choice antibody panel	Anatomic site	Consistent with
SYNP$^+$, S100$^+$	NF (R), GFAP (R),	Posterior fossa	Medulloblastoma
	Collagen IV$^+$, Vim (S), CD99$^-$	Pineal gland	Pineoblastoma
	NF (R), GFAP (R), Collagen IV$^-$, Vim$^-$, CD99 (S)	Anterior fossa	PNET
SYNP$^-$, S100$^+$	NF$^-$, GFAP (R), Collagen IV$^-$, Vim (S), CD99$^-$	Cerebrum, cerebellum	Ependymoblastoma

Refs. [151, 152, 296–298, 309, 317–322]
Note: *N* negative, *R* rare, *S* sometimes

26.10.3 Undifferentiated Tumors

Medulloblastoma, pineoblastoma, ependymoblastoma, and PNET are primitive undifferentiated tumors commonly located in the posterior fossa, pineal gland, periventricular area, and anterior fossa, respectively. Medulloblastoma, pineoblastoma, and ependymoblastoma differentiate from PNET by negative reaction for CD99. Ependymoblastoma can be distinguished from meduloblastoma/pineoblastoma/PNET by the absence of reactivity to synaptophysin and neurofilament (Table 26.38).

26.10.4 Proliferative Markers

MIB1 (Ki-67) is an Ab that detects proliferating cells in various phases of the cell cycle and is important in the grading of CNS tumors. It is used to predict patient outcome and distinguishes long and short time survivals in patients with glial tumors (Table 26.39 and Fig. 26.49). *p53* and *EGFR* overexpression can be defined immunohistochemically. Overexpression of p53 is associated with tumor progression in glioblastoma multiforme (GBM). EGFR overexpression correlates with poor prognosis in gliomas and is not present in low-grade gliomas. As a new therapeutic target, EGFR tyrosine kinase inhibitors are used for the treatment of GBM.

26.11 Immunohistochemistry of Pediatric Tumors

Solid pediatric tumors comprise a heterogenic group of variable entities with morphologies including small round cells, spindle cells, and polygonal cells. Small round cell tumors include neuroblastoma, rhabdomyosarcoma, Ewing sarcoma/PNET, desmoplastic small round cell tumor,

Table 26.39 Proliferative factor of MIB1 in some CNS tumors and correlation with survival (Fig. 26.49)

Tumor	MIB1 %		Survival
Astrocytoma	<2		80 %
	>2		20 %
Anaplastic astrocytomas	5–10		–
Glioblastoma multiforme	>10		–
Oligodendroglioma	<5		Longer survival
	>5		Shorter survival
Ependymal tumor	>5		Shorter survival
Choroid plexus papilloma	3.7		<6 % nonaggressive
Choroid plexus carcinoma	14		>6 % aggressive
Meningioma	*Ozen study*	*Abramovich study*	*Lanzafame study*
Benign (grade 1)	1.2	1	<1 % no recurrence
Anaplastic (grade 2)	2.3	5.5	>1 % recurrence
Malignant (grade 3)	6.7	12	
Medulloblastoma	50 %	–	

Refs. [151, 323–330]

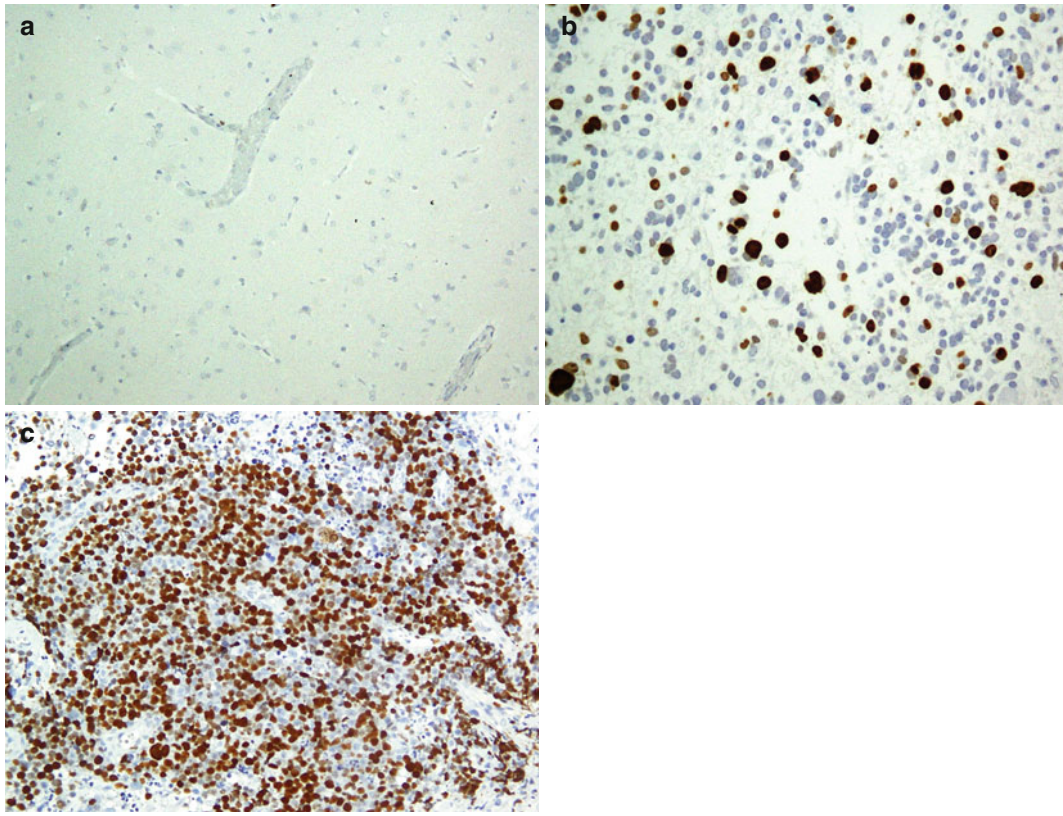

Fig. 26.49 Proliferating marker of Ki-67 is "nonreactive" in normal brain (**a**), 30 % reactive in astrocytoma (**b**), and 80 % reactive in germinoma (**c**)

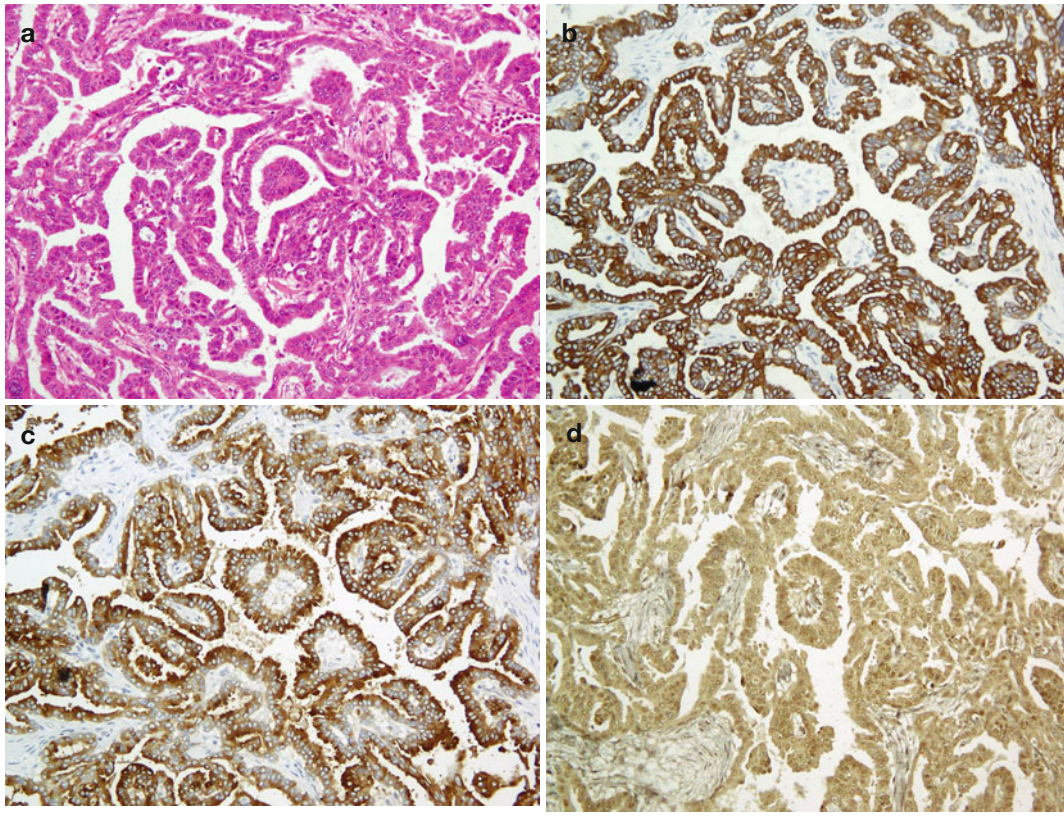

Fig. 26.50 Wilms tumor. Epithelial component with tubuloglandular structures (**a**) showing immunoreaction with CKAE1/AE3 (**b**), EMA (**c**), and WT1 (**d**)

Wilms tumor (Fig. 26.50), small cell osteosarcoma, lymphoma, and melanoma. Rhabdomyosarcoma, Wilms tumor, and melanoma also display spindle cell components or present as pure spindle cell tumor. Polygonal cell tumors of childhood comprise of rhabdomyosarcoma, malignant rhabdoid tumor, osteosarcoma, and melanoma [331, 332].

Frequently confused with primitive neuroectodermal tumors (PNETs), neuroblastoma is the most common malignant tumor of the posterior mediastinum in pediatric patients with morphology of small round cell tumor. Neuroblastoma has a predilection for adrenal glands and sympathetic ganglia, whereas PNETs are cholinergic tumors [333, 334]. Expression of CD44s and c-kit receptor correlates with favorable prognosis in a subset of neuroblastoma [335, 336]. Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma subclassified into embryonal, botryoid, alveolar, and spindle cell subtypes. Embryonal rhabdomyosarcoma (including botryoid), the most common type in childhood, usually displays small cell morphology, whereas the alveolar variant usually exhibits features of polygonal cells [337–340].

Initially regarded as an undifferentiated sarcoma of the bone and soft tissue, Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET) is now being classified as a small round cell tumor with varying degrees of neuroectodermal differentiation with pseudorosette formation [341]. Desmoplastic small round cell tumor is an aggressive, malignant tumor usually involving the abdominal or pelvic cavity of children or young adults with the morphology of small round cells arranged in nests and sepa-

Table 26.40 Immunopanel of pediatric tumors

First-choice antibody panel	Second-choice antibody panel	Additional antibody/histopathologic feature	Consistent with
AE1/AE3$^+$, CAM5.2$^+$, VIM$^+$	DES$^+$, WT1$^+$, EMA$^+$	SYN$^+$, CHG$^+$, NSE$^+$/ Small round cell	Wilms tumor
		SYN$^+$, CHG$^+$, NSE$^+$/ Polygonal cell	Malignant rhabdoid tumor
		SYN$^-$, CHG$^-$, NSE$^+$/ Small round cell	Desmoplastic small round cell tumor
AE1/AE3$^-$, CAM5.2$^-$, VIM$^+$	DES$^+$, MYOG$^+$, MyoD1$^+$	MSA$^+$, CD99$^\pm$, CK$^\pm$/ Small round/spindle/polygonal cell	Rhabdomyosarcoma
	DES$^-$, MYOG$^-$, MyoD1$^-$	CD45$^+$/ Small round cell	Lymphoma
		CD99$^+$, S100$^+$/ Small round/polygonal cell + osteoid	Osteosarcoma
		CD99$^+$/ Small round cell	ES/PNET
		S100$^+$, SYN$^+$, CHG$^+$, NSE$^+$/ Small round cell	Neuroblastoma
		S100$^+$, HMB45$^+$, MART1$^+$/ Small round/polygonal cell	Melanoma

Refs. [55, 348–362]

rated by a dense collagenized and desmoplastic stroma [288].

Wilms tumor (WT) or nephroblastoma is the most common pediatric neoplasm of the kidney derived from nephrogenic rests displaying divergent differentiation. The classic histopathologic pattern of WT consists of triphasic elements of blastemal, epithelial, and stromal components. Blastemal component is composed of small round cells exhibiting reactivity with vimentin and desmin. Epithelial component shows staining with cytokeratin, whereas stromal component demonstrates variable reactivity based on its differentiation pattern [342, 343]. Lacking a characteristic immunohistochemical profile, the diagnostic feature of osteosarcoma is the presence of osteoid which can be distinguished from other undifferentiated small round cell tumors [344, 345]. Originally described in the kidney and CNS, malignant rhabdoid tumor is a highly aggressive neoplasm of the childhood with a tendency of widespread metastases. Malignant rhabdoid tumor is a densely cellular tumor comprised of cords and sheets of polygonal cells with abundant eosinophilic cytoplasm and large eccentric nuclei containing prominent eosinophilic nucleoli [346, 347]. Table 26.40 displays an immunopanel to the diagnosis of common pediatric tumors.

26.12 Immunosurveillance, Immune Editing, Immune Constant of Rejection, Immune Contexture, and Immune Scoring of Cancers

Cancer is a complex disease involving cellular and molecular interactions between the tumor and the immune system [363]. The concept of *immunosurveillance*, first described by Lewis Thomas and Macfarlane Burnet, refers to the detection and destruction of tumor cells by the immune system [363–365]. This theory has been supported by the analysis of experimental and clinical tumor microenvironment data. The strongest argument for the existence of immunosurveillance is that immunodeficient hosts are

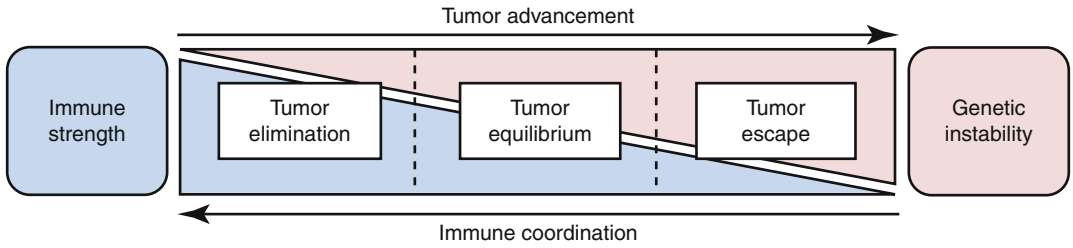

Fig. 26.51 Cancer-immune spectrum. The immunoediting theory describes how a tumor can evade from immune destruction and how the immune system restrains the tumor

associated with increased frequency of cancers. In addition, regression of primary and metastatic tumors has been attributed to immunologic mechanisms, but many other factors may have been responsible (e.g., hormonal, nutritional, or vascular). Tumor microenvironment is a complex milieu comprised of extracellular matrix and host cells, including mesenchymal, endothelial, and immune cells. During carcinogenesis process, the neoplastic cells constantly interact with the host cells, extracellular matrix, and bioactive molecules, which constitute the tumor microenvironment [366–368].

The concept of *cancer immunoediting*, proposed by a series of mouse model publications that immune deficiencies are associated with tumor aggressiveness, describes how the immune system encounters with tumor cells during tumorigenesis [369–372]. Immune cells engage to combat with cancer cells in three sequential phases: cancer elimination, cancer equilibrium, and cancer escape. In the elimination phase, the immune system clears most tumor cells; a population of immune-resistant tumor cells appears in the equilibrium phase; and finally, in the escape phase, the tumor develops strategies to evade immune destruction. The last phase is a consequence of immune exhaustion and inhibition or results from the emergence of tumor cell variants (Fig. 26.51).

It is now well known that innate and adaptive immune systems can promote tumor development and progression through immunosurveillance. However, there are many interactions between the innate immune cells [macrophages, neutrophils, mast cells, NK cells, and immature dendritic cells (DC)] and the adaptive immune cells [mature DC, B lymphocytes, T lymphocyte, and regulatory T cells (Tregs)]. Initially mediated by innate immunity, interaction between tumor cells and immune system develops and the tumor is eliminated through adaptive immune system activation [373, 374]. The immune-mediated tissue destruction process described by the concept of *immunologic constant of rejection* (ICR) includes the coordination of *interferon-stimulated genes (ISGs)* pathway and immune effector functions (IEFs) pathway. This constant demonstrates the activation of ISGs, recruitment of cytotoxic immune cells (primarily through CXCR3/CCR5 ligand pathways), and activation of the IEFs pathway (IEF genes; granzymes A/B, perforin) [375, 376].

The *immune contexture* is characterized as the density, type, location, and functional orientation of adaptive immune cells within the tumor which is essential to accurately define the impact of cancer prognosis [377–379]. Parameters of the immune contexture comprise of $CD3^+$ density, cytotoxic $CD8^+$ and memory $CD45RO^+$ T cells, their location at the tumor center (CT) and invasive margin (IM), combined with the quality of tertiary lymphoid structures (TLS) (Fig. 26.52). Evaluation of immune contexture in the clinical setting will provide prognostic and predictive benefits [377, 378].

In human, the presence of tumor infiltrating lymphocytes (TILs) has been reported as a favorable prognostic factor in many primary tumors. The high density of TILs associated with good

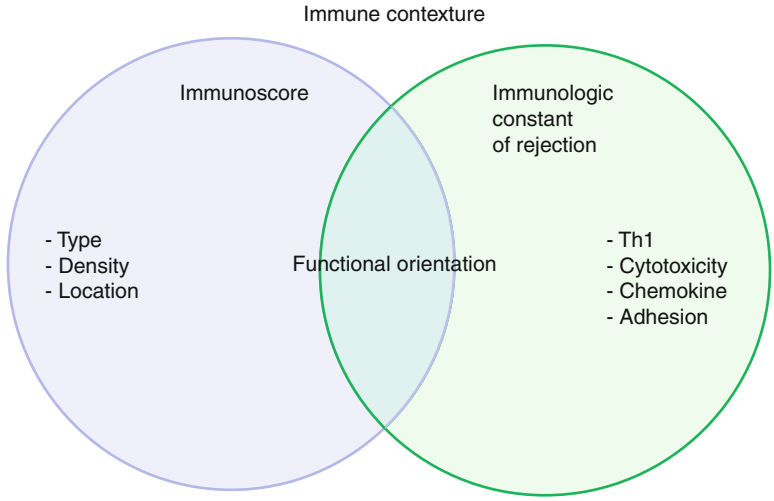

Fig. 26.52 The "immune contexture" at the background is defined by combination of immune variables associating the nature, density, functional orientation, and distribution of immune cells within the tumor. The "Immunoscore" and the "immunologic constant of rejection" are overlapped by functional orientation

prognosis has been well documented, not only to various organs of cancer origin (such as breast, colon, lung, head and neck, kidney, bladder, ovary, prostate) but also to various cancer cell types (adenocarcinoma, squamous cell carcinoma, large cell cancer, melanoma, etc.) [reviewed in 379–381] (Fig. 26.53). The quantification of TILs allowed defining a novel scoring system based on the densities of two lymphocyte populations ($CD3^+$ and $CD8^+$), both in CT and in IM of tumors. Based on the immune contexture, a standardized, simple, powerful immune scoring system (*Immunoscore*) was determinate. Immune classification of cancers provides a scoring system ranging from Immunoscore 0 to 4 and low to high densities of both lymphocyte populations in CT and IM of tumors (Table 26.41). The Immunoscore system has shown to have a prognostic significance superior to AJCC/UICC-TNM staging systems. Thus, incorporating the Immunoscore into traditional staging systems has an essential prognostic and predictive value [382, 383].

In 2012, an international task force was initiated to promote the Immunoscore in routine clinical settings as a new component of cancer classification, designated TNM-I (TNM-Immune) [384]. The purpose of the Immunoscore international task force was: (1) to validate the feasibility and reproducibility of the Immunoscore and (2) to validate the major prognostic and predictive power of the Immunoscore in colon cancer patients. In order to become globally applicable in routine clinical setting, evaluation of the Immunoscore must be pathology based, feasible in routine settings, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, and powerful [29, 384].

Multiple laboratory variables influence the validity and reliability of immunoscoring in the clinical setting which need to coordinate with distinct criteria. They are included in the complexity of quantitative IHC assay, variable protocols across laboratories, and immune cell analysis accompanied by uneven region selection criteria and variable ways to quantify TILs. An effort for harmonization and reproducibility of IHC method recommends laboratories to test the prognostic value of Immunoscore using the initial guidelines [383, 384]. It is also acknowledged that additional markers may be used to further refine the prognostic value of the Immunoscore.

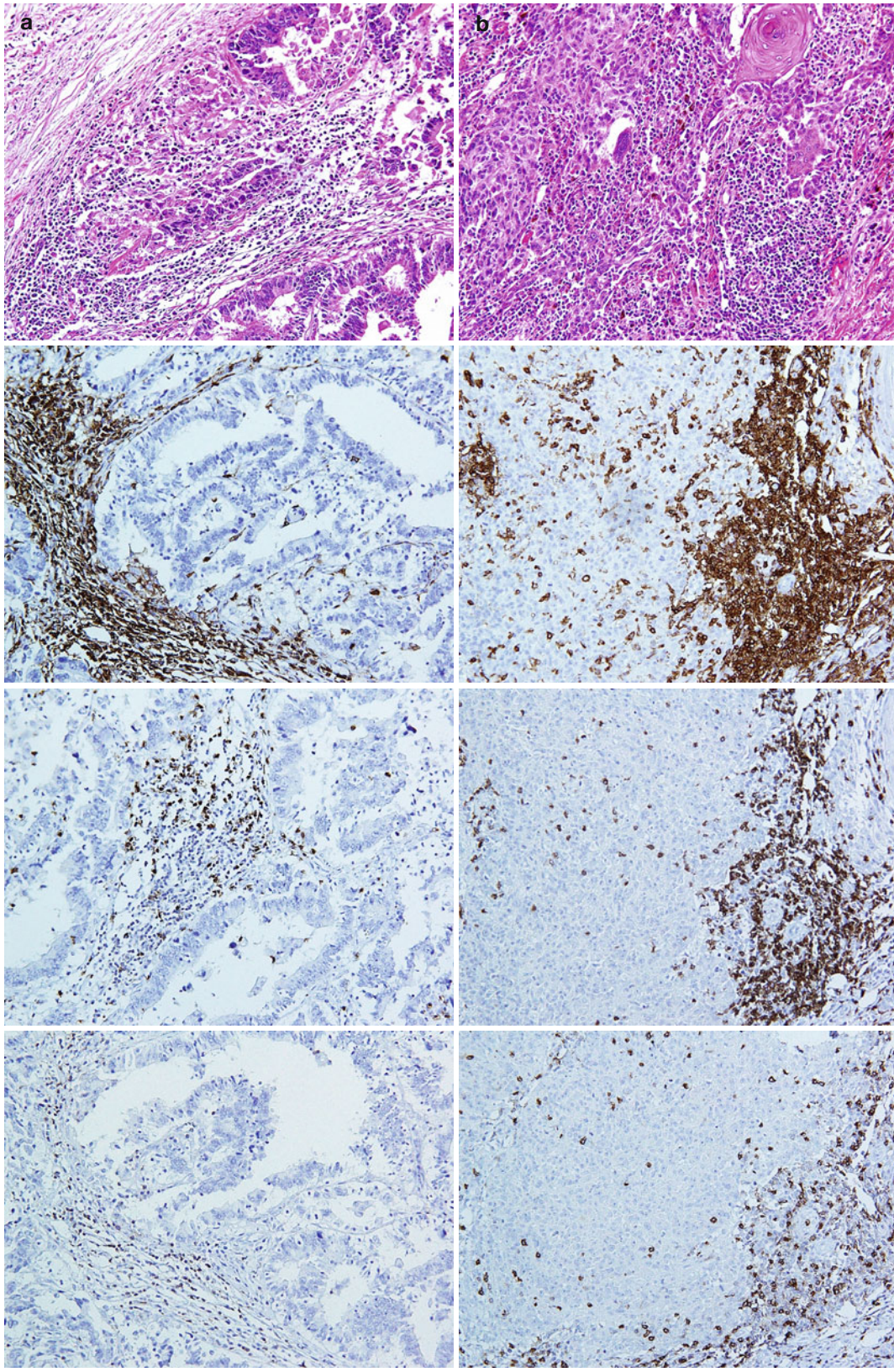

Fig. 26.53 (**a**) Colon adenocarcinoma and (**b**) skin SCC with surrounding TILs, immunostained with CD45RO, CD3, and CD8

Table 26.41 The characteristics of immune contexture, immunoscore, and immunologic constant of rejection

Concepts	Characteristic
Immune contexture	Type, density, location, and functional orientation of adaptive immune cells (Th1 cell, cytotoxicity, chemokine, adhesion)
Immunoscore	Standardized, simple, quantitative, routine test derived from the immune contexture
Type	$CD3^+$ T cell, $CD8^+$ T cell
Density	Quantification (cells/mm^2)
Location	Tumor center, invasive margin, tertiary lymphoid islets
Immunologic constant of rejection	Immune-mediated, tissue destruction processes
	(A) Interferon-stimulated genes pathway
	(B) Cytotoxic immune cells (primarily through CXCR3/CCR5 ligand pathways)
	(C) Immune effector functions pathway (IEF genes; granzymes A/B, perforin)

26.13 Concluding Remarks

Besides conventional histopathologic evaluation of various tissues, IHC has provided a significant aid in diagnosis, and its role is growing not only in arriving diagnosis but also for targeted therapies and predicting prognosis. Recently, various markers have been introduced which have therapeutic or prognostic value. Notably, it should be emphasized that IHC has some limitations and should be used in an appropriate setting by an experienced pathologist to avoid misdiagnosis. Additionally, a panel of related antibodies instead of single marker are needed to yield a correct and precise diagnosis.

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Index

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