Advances in Experimental Medicine and Biology 1263

Alexander Birbrair *Editor*

Tumor Microenvironment

State of the Science

Springer

Advances in Experimental Medicine and Biology

Volume 1263

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Alexander Birbrair
Editor

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Editor
Alexander Birbrair
Department of Radiology
Columbia University Medical Center
New York, NY, USA

Department of Pathology
Federal University of Minas Gerais
Belo Horizonte, MG, Brazil

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-030-44517-1 ISBN 978-3-030-44518-8 (eBook)
https://doi.org/10.1007/978-3-030-44518-8

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This book is dedicated to my mother, Marina Sobolevsky, of blessed memory, who passed away during the creation of this volume. Professor of Mathematics at the State University of Ceará (UECE), she was loved by her colleagues and students, whom she inspired by her unique manner of teaching. All success in my career and personal life I owe to her.

My beloved mom Marina Sobolevsky of blessed memory (July 28, 1959 – June 3, 2020)

Preface

This book's initial title was *Tumor Microenvriomen*t. However, due to the current great interest in this topic, we were able to assemble more chapters than would fit in one book, covering tumor microenvironment biology from different perspectives. Therefore, the book was subdivided into several volumes.

This book, *Tumor Microenvironment: State of the Science*, presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical and biological research. The chapters provide timely detailed overviews of recent advances in the field. This book describes the major contributions of different components of the tumor microenvironment during cancer development. Further insights into these mechanisms will have important implications for our understanding of cancer initiation, development, and progression. We focus on the modern methodologies and the leading-edge concepts in the field of cancer biology. In recent years, remarkable progress has been made in the identification and characterization of different components of tumor microenvironment in several organs using state-of-the-art techniques. These advantages facilitated identification of key targets and definition of the molecular basis of cancer progression within different tissues. Thus, the present book is an attempt to describe the most recent developments in the area of tumor biology which is one of the emergent hot topics in the field of molecular and cellular biology today. Here, we present a selected collection of detailed chapters on what we know so far about different aspects of the tumor microenvironment in various tissues. Ten chapters written by experts in the field summarize the present knowledge about distinct characteristics of the tumor microenvironment during cancer development.

Francesca Montenegro and Stefano Indraccolo from the University of Padua discuss the role of metabolism in the tumor microenvironment. Allan Tsung and colleagues from The Ohio State University describe the effects of neutrophil elastase and neutrophil extracellular traps in tumor microenvironment. Eleonora Timperi and Vincenzo Barnaba from Institut Curie update us with the relationship of viral hepatitis and the tumor microenvironment. Dima Dandachi and Fanny Morón from Baylor College of Medicine summarize current knowledge on the effects of HIV on the tumor microenvironment. Sherine F. Elsawa and colleagues from the University of New Hampshire address the importance of GLI2-mediated inflammation in the tumor microenvironment. Jason B. Fleming and colleagues from H. Lee Moffitt Cancer Center compile our understanding of stellate cells in the tumor

microenvironment. Ugo Testa and colleagues from the Istituto Superiore di Sanità talk about endothelial progenitors in the tumor microenvironment. Kavitha Gowrishankar and colleagues from the University of Sydney focus on the chimeric antigen receptors for the tumor microenvironment. Isabelle Cremer and colleagues from Sorbonne Université give an overview of toll-like receptors in the tumor microenvironment. Finally, Jie Hunter Huang and Jian Jian Li from the University of California, Davis expose multiple dynamics in tumor microenvironment under radiotherapy.

It is hoped that the articles published in this book will become a source of reference and inspiration for future research ideas. I would like to express my deep gratitude to my wife, Veranika Ushakova, and to Mr. Murugesan Tamilsevan, from Springer, who helped at every step of the execution of this project.

Belo Horizonte, MG, Brazil Alexander Birbrair

Contents

Contributors

Shereen A. Allam Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

Mahmud Angrini Institut National de la Santé et de la Recherche Medicale (INSERM), UMRS1138, Centre de Recherche des Cordeliers, Paris, France

Sorbonne Université, Paris, France

Vincenzo Barnaba INSERM U932, Institut Curie, PSL Research University, Paris, France

Istituto Pasteur, Fondazione Cenci Bolognetti, Rome, Italy

Center for Life Nano Science, Istituto Italiano di Tecnologia, Rome, Italy

Alexander Birbrair Department of Pathology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Germana Castelli Department of Oncology, Istituto Superiore di Sanità, Rome, Italy

Isabelle Cremer Institut National de la Santé et de la Recherche Medicale (INSERM), UMRS1138, Centre de Recherche des Cordeliers, Paris, France

Sorbonne Université, Paris, France

Dima Dandachi Division of Infectious Diseases, Department of Medicine, University of Missouri, Columbia, MO, USA

Sherine F. Elsawa Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

Jason B. Fleming Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center, Tampa, FL, USA

Kavitha Gowrishankar Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia

Rosemary Habib Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia

Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

Weiguo Han Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA

Hai Huang Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

Jie Huang Department of Radiation Oncology, University of California Davis, Sacramento, CA, USA

Stefano Indraccolo Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV – IRCCS, Padova, Italy

Jian Jian Li Department of Radiation Oncology, University of California Davis, Sacramento, CA, USA

NCI-Designated Comprehensive Cancer Center, University of California Davis, Sacramento, CA, USA

Kenneth Micklethwaite Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia

Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

Sydney Cellular Therapies Laboratory, Blood and Bone Marrow Transplant Unit, Department of Haematology, Sydney Medical School, Westmead Hospital, Sydney, NSW, Australia

Francesca Montenegro Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy

Fanny Morón Department of Radiology, Baylor College of Medicine, Houston, TX, USA

Adnan Nagrial Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

Amblessed E. Onuma Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

Elvira Pelosi Department of Oncology, Istituto Superiore di Sanità, Rome, Italy

David Roife Department of Surgery, University of South Florida Morsani College of Medicine, Tampa, FL, USA

Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center, Tampa, FL, USA

Bhaswati Sarcar Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center, Tampa, FL, USA

Ugo Testa Department of Oncology, Istituto Superiore di Sanità, Rome, Italy

Eleonora Timperi INSERM U932, Institut Curie, PSL Research University, Paris, France

Dipartimento di Scienze Cliniche Internistiche, Anestesiologiche e Cardiovascolari, Sapienza University of Rome, Rome, Italy

Allan Tsung Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

Aditi Varthaman Institut National de la Santé et de la Recherche Medicale (INSERM), UMRS1138, Centre de Recherche des Cordeliers, Paris, France

Sorbonne Université, Paris, France

Hongji Zhang Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

Department of Surgery, Union Hospital, Huazhong University of Science and Technology, Wuhan, People's Republic of China

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1 Metabolism in the Tumor Microenvironment

Francesca Montenegro and Stefano Indraccolo

Abstract

From a general perspective, in the context of solid tumors, we can distinguish metabolic alterations of cancer cells from those of the stroma. These two components interact with each other and with the extracellular matrix (ECM), and these interactions can take the form of either metabolic competition or metabolic symbiosis. The aim of this chapter is to overview the canonical metabolic alterations of tumor and stroma cells and to present specific examples of metabolic competition and symbiosis. We will also discuss the complexity and plasticity of metabolism, which pose indeed a serious threat to our ability to target selective metabolic features of tumor microenvironment with drugs. Finally, we will highlight some limitations of state-of-the-art techniques used to study tumor metabolism and propose some innovative solutions to investigate the clinical relevance of metabolic alterations for patient management and treatment.

F. Montenegro
Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy

S. Indraccolo (✉)
Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV – IRCCS, Padova, Italy
e-mail: stefano.indraccolo@iov.veneto.it

Keywords

Metabolism · Angiogenesis · Tumor · Stroma · Immune cell · Microenvironment · Glycolysis · Hypoxia · OXPHOS · Metabolic symbiosis · Metabolic competition · Glucose · Lactate · Glutamine · Lipids

1.1 Aim of the Chapter

Metabolic alterations represent one of the recognized hallmarks of cancer [25] and have been described in thousands of publications. Hereunder, we will briefly overview only the key metabolic traits of cancer cells, as this topic is covered by several updated and comprehensive reviews [15, 49]. From a general perspective, in the context of solid tumors, we should distinguish metabolic alterations of cancer cells from those of the stroma. The latter have attracted interest of the scientific community especially in the last decade, and include metabolic features of certain resident stromal cells, including endothelial cells [17], fibroblasts [69], and adipocytes [70], as well as those of mobile cells, such as lymphocytes, macrophages, and specialized subpopulations of myeloid cells [59]. These two components interact with each other and with the extracellular matrix (ECM), and these interactions

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_1

can take the form of either metabolic competition or metabolic symbiosis. In experimental models, distinct examples of metabolic interactions in the tumor microenvironment have been reported in the literature and will be reviewed here. When approaching tumor metabolism, a known obstacle is represented by its heterogeneity and plasticity [35]. Metabolic heterogeneity in tumors can be accounted for by (I) cancer cell autonomous factors, (II) local microenvironment factors such as hypoxia and acidosis, and (III) external factors, including diet, the microbiome, and certain drugs, which can generate signals that modulate metabolism in the tumor microenvironment. This chapter examines how metabolic traits of cancer are influenced by microenvironmental clues.

1.2 Metabolic Hallmarks of Cancer Cells

1.2.1 Glycolysis

Glucose is a key fuel source for many normal cells, despite the fact that cells can readily use different substrates. Normal cells generate much of their energy via mitochondrial oxidative phosphorylation (OXPHOS), whereas cancer cells often switch to aerobic glycolysis, a process where glucose is metabolized to pyruvate and ultimately converted to lactate [9, 37]. Aerobic glycolysis is an inefficient process to generate adenosine 5′-triphosphate (ATP), however, and the advantage it confers to cancer cells has been long unclear. It has been proposed that the glycolytic metabolism of cancer cells, and indeed all proliferating cells, has been selected to facilitate the uptake and incorporation of nutrients into the biomass (e.g., nucleotides, amino acids, and lipids) needed to produce a new cell [16, 63]. In fact, once in the glycolytic flux, glucose can be diverted into multiple branching pathways and can be used for the synthesis of glucose-derived macromolecules necessary for cell division. In a recent review by Kim & DeBerardinis, glycolysis has been considered a prime example of a convergent property of cancer cells, because it can be activated by many different oncogenic drivers or by hypoxia [33]. Convergent properties are stimulated by a large number of factors and are typically shared among diverse tumor types, as is the case for glycolysis.

1.2.2 Oxidative Phosphorylation

In addition to glycolysis, tumor cells engage other metabolic pathways to support cell proliferation and growth and it is now established that OXPHOS contributes to tumor initiation, progression, and metastasis. On the basis of the initial observation that breast cancer cells produce 80% of their ATP via mitochondrial-dependent metabolism [24], the concept “oxidative tumors” has been increasingly used to describe tumors characterized by ATP production through OXPHOS from glucose, fatty acids (FAs), or glutamine oxidation [47]. Mitochondrial metabolism is not only important for ATP generation but also to provide substrates for nucleotide, amino acid, and lipid biosynthesis through diversion of some intermediates into so-called cataplerotic pathways. One of the cataplerotic pathways is the reductive carboxylation of α-ketoglutarate (α-KG) to isocitrate, ultimately enabling citrate export into the cytosol and acetyl-CoA synthesis to fuel lipid anabolism [44]. Glutamine directly fuels this pathway when dysfunctional mitochondria diverge glucose away from acetyl-CoA production, or under hypoxic conditions and this reductive flux of glutamine-derived carbon is mainly dependent on the activity of the cytosolic isocitrate dehydrogenase 1 (IDH1) [42].

1.2.3 Amino Acid Metabolism

Glutamine plays a key role in many tumor types, since it can fuel OXPHOS, contribute to reductive carboxylation, and regulate NADPH production and redox balance, therefore affecting cell metabolic homeostasis [27]. The oncogene MYC stimulates mitochondrial glutamine catabolism by regulating expression of glutamine transporters and glutaminase, and tumors bearing MYC alterations are commonly glutamine-addicted [23, 67]. Serine and glycine are also critical for

cell proliferation and redox control in cancer cells, and high glucose levels enhance the glycolytic flux, providing precursors that can be channeled into serine biosynthesis [56].

1.2.4 Lipid Metabolism

Alterations in lipid metabolism are common in cancer, and many studies indicate that tumors often exhibit a lipogenic phenotype [41]. Part of these metabolic alterations has been attributed to the effects of hypoxia on lipid metabolism [1]. Specific examples include elevated rates of lipid synthesis accounted for by increased expression of various lipogenic enzymes, such as fatty acid synthase (FASN), which is strongly correlated with cancer progression [41, 72], or acetyl-CoA synthetase 2 (ACSS2), which contributes to cancer cell growth under low-oxygen and lipid-depleted conditions [58]. In certain tumor models, increased FA uptake through FA-binding proteins (FABPs) [4] or the FA channel protein CD36 [1] has been reported. Accumulation of lipid droplets in the cytoplasm of cancer cells is linked to hypoxia and confers cancer aggressiveness and chemoresistance [34]. Notably, certain studies indicate that LD accumulation and lipid desaturation are distinctive features of cancer stem cells, particularly in the case of ovarian cancer [36].

1.3 Metabolic Hallmarks of Stromal Cells

Cancers are highly diverse and, in addition to the metabolic heterogeneity of malignant cells, a broad spectrum of immune and nonimmune cell populations can be found in the tumor microenvironment. Among nonimmune cells, of particular biological relevance are cancer-associated fibroblasts (CAFs) and endothelial cells (ECs). CAFs can secrete various growth factors that sustain the proliferation of cancer cells, activate epithelial-mesenchymal transition (EMT), and exert also a proinflammatory and proangiogenic role in tumors, thus contributing to create the intratumoral vascular network [32]. The metabolism of CAFs has been pioneered by Dr. Lisanti and colleagues who proposed that tumor cells promote aerobic glycolysis in neighboring CAFs, a process referred to as the "reverse Warburg effect"[48]. This phenomenon represents an important metabolic hallmark of CAFs. Subsequently, CAFs secrete a bunch of metabolites, such as ketone bodies, lactate, and pyruvate, which in turn can be taken up by cancer cells and oxidized in the mitochondria for energy production [8]. Accumulating evidence suggests that CAFs promote tumor progression in certain tumors, such as breast cancer [39]. Lactate secretion from CAFs induces a local acidic microenvironment, which can enhance extracellular proteolysis and promotes the acquisition of drug resistance by tumor cells [13]. The metabolic coupling between CAFs and cancer cells relies upon unique MCT expression patterns: cancer cells in some models and cancer types express high level of MCT1, thus promoting uptake of lactate from MCT4-positive CAFs [62, 68]. However, other groups reported metabolic interactions between cancer cells and fibroblast in an opposite fashion, namely, between oxidative CAFs and glycolytic cancer cells [60]. These apparently contrasting results can be reconciled considering that the "reverse Warburg effect" could be a phenomenon presenting with variable intensities in different tumor types. Additional metabolic features of CAFs have been poorly investigated so far.

With regard to ECs, in the last years, Dr. Carmeliet and colleagues described in elegant studies key metabolic features of these cells [17, 64]. It has been demonstrated that tumor-associated ECs, and in particular tip cells, exploit glycolysis when they invade tissues. These studies implied that certain glycolysis inhibitors, such as the phosphofructokinase inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), could be used to contrast tumor angiogenesis [17]. Other studies from the same group reported on the role of fatty acid and glutamine metabolism in EC [26, 29]. Notably, antiangiogenic therapies exacerbate glycolysis in tumor cells [14, 45] and this could deplete glucose available for ECs, thus contrasting the bioenergetics requirements of angiogenesis. Type 2

pericytes represent another vascular component of the tumor microenvironment, as these cells – at variance with type 1 pericytes – can penetrate tumors and contribute to tumor angiogenesis [5]. However, in contrast to EC, the metabolic traits of type 2 pericytes and their interactions with surrounding cells remain substantially unknown and need to be investigated in future studies as these cells may provide a cellular target susceptible to signaling and pharmacological manipulation in treating malignancy.

While the paradigm of metabolic coupling between tumor and stroma has been best studied with CAFs, other stromal cell types may also engage in similar interactions with the growing tumor. In response to tumor-derived factors, adipocytes release free fatty acids, which can be taken up by cancer cells to sustain tumor growth via β-oxidation [46]. Moreover, metabolic reprogramming can modulate the function of infiltrating immune cells. Recent studies suggest that altered energy metabolism in tumor-associated macrophages (TAMs) can lead to distinct polarization states of these inflammatory cells. M1 macrophages preferentially engage glycolysis, whereas M2 macrophages predominantly rely on OXPHOS [7]. Macrophage metabolism has recently been shown to shape angiogenesis in tumors through a novel mechanism involving REDD1, an mTOR inhibitor induced by hypoxia [66]. The metabolism of tumor-infiltrating T lymphocytes will be summarized in the next section. Finally, interactions between cancer cells and components of the ECM also contribute to shape metabolic features of tumors, as reviewed elsewhere [33].

1.4 Metabolic Competition and Metabolic Symbiosis in the Tumor Microenvironment

The presence of different types of normal cells in the tumor microenvironment, each endowed with peculiar metabolic traits, forms the basis of intercellular interactions with cancer cells, which can take the form of either metabolic competition or symbiosis. Known mediators of these interactions include glucose, pyruvate, lactate, and fatty acids, although it is likely that additional metabolites will be described in the next years. In the microenvironment of highly glycolytic tumors, glucose availability could become a limiting factor and this metabolite could trigger metabolic competition between tumor cells and certain stromal cells, including EC and T lymphocytes. Cytotoxic T cells require glucose for proliferation and cytotoxic activity [3, 10, 28]; therefore, this form of metabolic competition could weaken immune response to cancer cells. A translational correlate of this hypothesis is that highly glycolytic tumors could be less responsive to immunotherapy, compared with poorly glycolytic tumors, although to the best of our knowledge, this has not been investigated in patients treated with immune checkpoint inhibitors (ICIs) so far. A similar type of competition could occur between cancer cells and ECs, and it might be speculated that angiogenesis is relatively impaired in highly glycolytic tumors, due to limited amounts of glucose required by angiogenic ECs. However, this hypothesis is at odds with the proangiogenic activity of lactate, the main catabolite of glycolysis which accumulates in the TME of highly glycolytic tumors [65]. Lactate has indeed a proangiogenic role [55], and it is expected that highly glycolytic tumors produce more lactate and more blood vessels, compared with poorly glycolytic tumors. These two alternative hypotheses are presented in Fig.1.1. Conceivably, new experimental studies involving modulation of glycolysis in tumor cells and evaluation of its impact on lactate production and tumor angiogenesis are required to clarify the relationship between glycolytic activity and tumor angiogenesis.

A different form of intercellular interaction in the TME is metabolic symbiosis. This has been described between tumor cells with different topographic localization, that is, in the context of hypoxic versus perfused areas of solid tumors [61] and can also be found in tumors treated with antiangiogenic drugs [2, 31, 52]. Metabolic

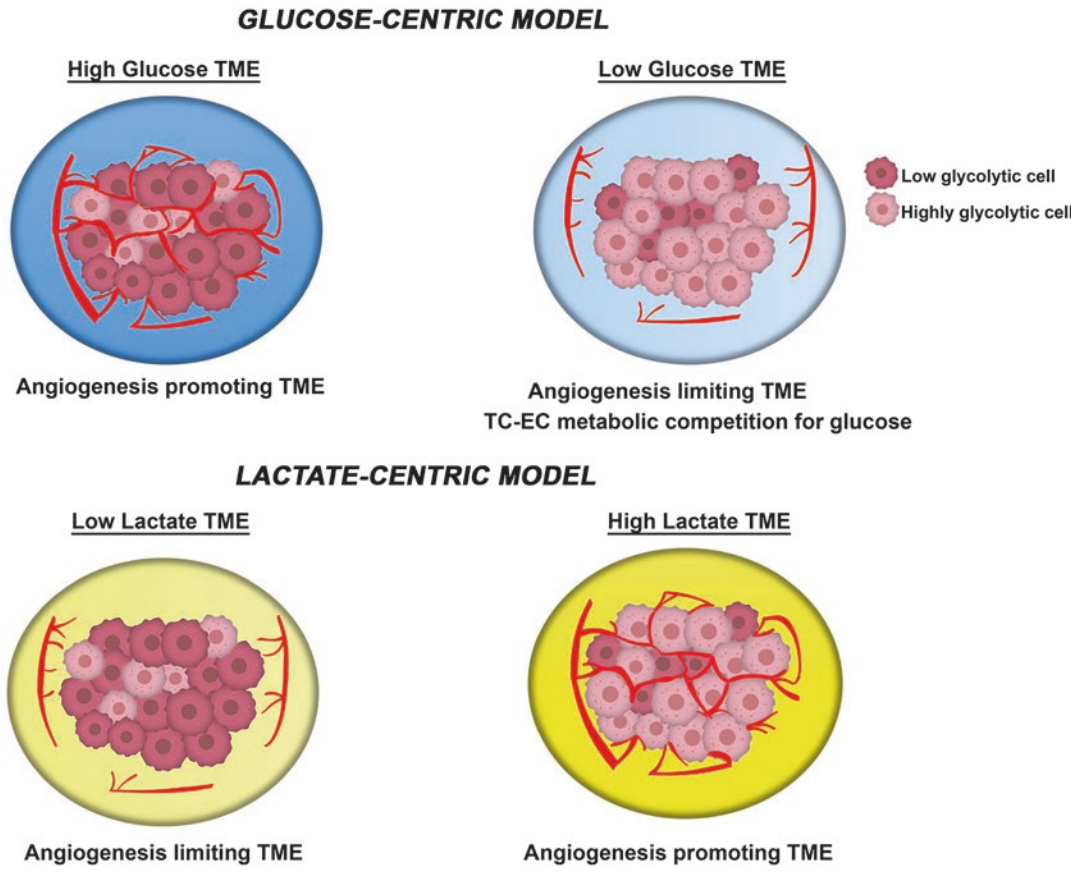

Fig. 1.1 Effects of glycolysis on tumor angiogenesis. Top panel shows a glucose-centric view of tumor angiogenesis. This model originates from the known metabolic switch to glycolysis of angiogenic EC and simulates the impact on angiogenesis of a glucose-rich (left) or a glucose-poor (right) TME. According to this model, competition for glucose could occur between cancer cells and ECs, and it might be speculated that angiogenesis is relatively impaired in highly glycolytic tumors, due to limited amounts of glucose required by angiogenic ECs. Bottom panel shows a lactate-centric view of tumor angiogenesis. Based on the well-known proangiogenic activity of lactate, it is expected that highly glycolytic tumors produce more lactate and more blood vessels, compared with poorly glycolytic tumors

symbiosis implies that lactate released by highly glycolytic tumor cells is picked up by tumor cells relying on OXPHOS, generally localized in oxygenized areas around tumor vessels. Conversely, pyruvate can be released by OXPHOS tumor cells and taken up via MCT1 or other carriers by glycolytic tumor cells. The finding that expressions of certain glycolysis-associated markers such as MCT4 are localized within defined areas in the context of tumor xenografts (Fig.1.2) supports the concept of metabolic symbiosis. In some examples, metabolic symbiosis takes place between cancer cells and stromal cells, such as fibroblasts and the key metabolite involved is lactate [30]. Finally, in the case of ovarian cancer and possibly other tumor types, which grow in fat tissues, a sort of symbiosis can be hypothesized between cancer cells and adipocytes in the tumor microenvironment [46]. From the tumor perspective, the common aim of these different forms of symbiosis is to achieve the best possible exploitation of nutrients which enter key metabolic pathway and support cancer proliferation and growth. It is likely that new studies will highlight additional forms of metabolic cooperation between stromal and cancer cells.

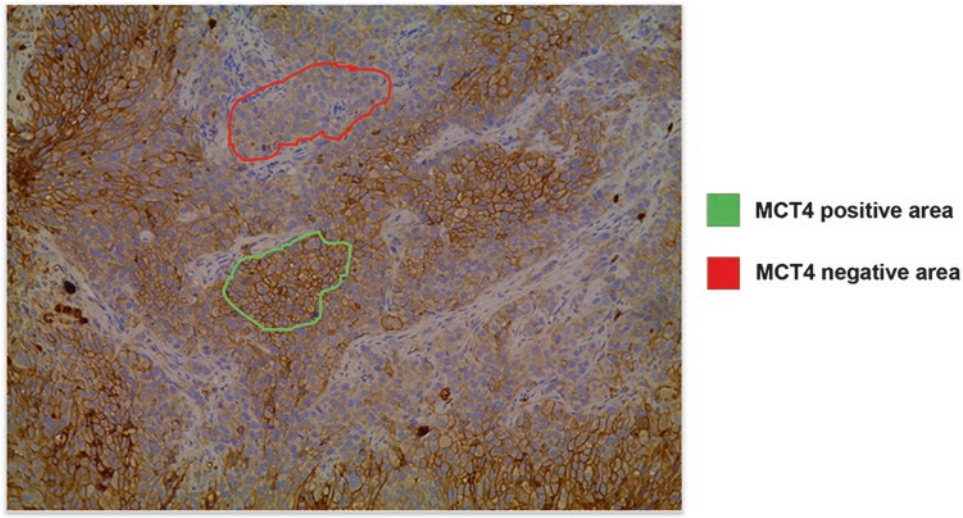

Fig. 1.2 Metabolic heterogeneity in an ovarian cancer xenograft. The picture shows MCT4 expression by IHC in an ovarian cancer xenograft. Labels indicate MCT4^{+}and MCT4^{-} areas within the same tumor. A 200x magnification is shown

1.5 Tuning the Metabolic Microenvironment of Tumors with Drugs or Diet

Understanding the critical mechanisms underlying metabolic adaptations in experimental tumor models may represent the basis for planning effective therapeutic interventions targeting metabolism in patients. However, the concept of metabolic therapy of cancer is not new. Various metabolic targets have been identified, but the approach has not been generally successful. The best example of successful exploitation of metabolic targets are certain drugs which inhibit de novo nucleotide synthesis, such as the antifolates methotrexate and pemetrexed, and antimetabolites such as 6-mercaptopurine, 6-thioguanine, 5-fluorouracil and capecitabine, which inhibit purine and pyrimidine biosyntheses. These drugs have shown significant therapeutic activity both in acute leukemia and certain solid tumors [38]. In contrast, other drugs targeting glycolysis including inhibitors of hexokinase (2-DG, lonidamine), phosphofructokinase (3PO), glyceraldehyde 3-phosphate dehydrogenase (3-BP), and lactate dehydrogenase (FX11, Oxamate) have either not yet been tested in clinical trials or have yielded negative results, including lack of efficacy and toxicity [12]. Recently, clinical trials investigated whether statins, which block HMGCoA reductase activity and may interfere with lipid metabolism both as systemic effect and in cancer, might exert anticancer effects, but results of a meta-analysis were negative [22]. Another active area of investigation regards metformin, a weak inhibitor of OXPHOS [54]. Many clinical trials involving combinations of metformin with chemotherapy are ongoing, but definitive results are not yet available. Moreover, in most of these clinical studies, patients were not stratified according to predictive biomarkers of response, despite some clues from preclinical work [6, 43]. An additional level of investigation regards diet. Many studies focused on the role of caloric restriction in cancer and in particular prior or concurrent to systemic therapy. The take-home message of most of these studies is that certain tumors – but not all tumors – might benefit from caloric restriction prior to and after chemotherapy. Various mechanisms have been envisioned

to explain this synergy, including induction of immunogenic cell death [51] but also diet-mediated inhibitory effects on certain key signaling pathways [19]. How caloric restriction modulates metabolism in the tumor microenvironment has not been investigated in detail so far. It might be possible that caloric restriction might improve therapeutic outcome in patients, but clinical trials are currently ongoing and it is premature to draw conclusions. Finally, the microbiota also sends metabolic signals to tumors [57]. One example is represented by butyrate, a metabolite produced by prokaryotic cells in the gut, which can affect tumor development [11].

1.6 Conclusions and Outlook

At the end of this short journey into metabolism in the tumor microenvironment, the reader might have the impression of an overwhelming complexity, arising from the simultaneous presence of various cell types endowed with different metabolic traits, as well as effects of acidosis or hypoxia, which introduce additional metabolic perturbations and the distant effects of metabolites carried in blood. This complexity and the plasticity of metabolism pose indeed a serious threat to our ability to target selective metabolic features of tumor microenvironment with drugs. Along this line, recent negative results from a phase 3 clinical study that combined the IDO1 inhibitor epacadostat with the anti-PD1 pembrolizumab remark the difficulties to translate into patients the knowledge from preclinical models [53].

A criticism which can help to move the field forward relates to the model systems we use. Although experimental mouse model systems remain fundamental to test hypothesis and advance understanding of the basic processes underlying metabolic reprogramming in cancer, in my opinion, more human studies are needed. Along this line, live cultures of freshly prepared human tumor slices represent one possibility [21], especially if combined with physiological culture conditions to reduce artifacts of commercial medium. Advanced technologies including metabolomics and metabolic flux analysis remain key to decode the heterogeneous metabolic preferences and dependencies of tumors in vivo, but they can be performed only in a very limited number of patients given the high costs of the equipment and the level of specialization of personnel involved in this type of analysis. Due to these constraints, these technologies will most likely be excluded from large randomized prospective clinical trials, which are typically designed to generate the most meaningful clinical information, that is, efficacy of a new therapy. I suggest that in parallel to these high-tech approaches, we should focus on "sentinel" biomarkers of essential metabolic processes, which can be easily analyzed in standard laboratories on archival FFPE samples. One possibility is represented by certain transporters or enzymes, which according to meta-analysis are key for the activity of the underlying metabolic pathway, such as MCT4 for glycolysis [20, 50] or GLS for glutamine metabolism [40]. Protein expression levels of these markers can be assessed by IHC, the signal digitalized and quantified by digital pathology techniques at a reasonable cost per sample. Integration of signal quantification into appropriate mathematical models can then be used to define best cut-off values in order to stratify patients into biomarker-positive or biomarker-negative and eventually investigate their prognostic or predictive value in large patients' cohorts. Although reduction of the complexity of a metabolic pathway to one or two sentinel markers might appear and likely is an oversimplification, there are other examples in oncology where expression of selective markers of complex biological processes turned out to be useful to take clinical decisions. Examples include expression of estrogen and progesterone receptors to predict response to hormonal therapy in breast cancer [18], and to some extent PD-L1 expression to predict the outcome of immunotherapy with ICIs [71]. We envision that similar attempts should be tried also for metabolism-associated markers in order to establish their clinical utility as prognostic or predictive biomarkers for cancer patients.

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2

Neutrophil Elastase and Neutrophil Extracellular Traps in the Tumor Microenvironment

Hai Huang, Hongji Zhang, Amblessed E. Onuma, and Allan Tsung

Abstract

Tumor-associated neutrophils (TANs) play a major role during cancer development and progression in the tumor microenvironment. Neutrophil elastase (NE) is a serine protease normally expressed in neutrophil primary granules. Formation of neutrophil extracellular traps (NETs), a mechanism used by neutrophils, has been traditionally associated with the capture and killing of bacteria. However, there are recent discoveries suggesting that NE secretion and NETs formation are also involved in the tumor microenvironment. Here, we focus on how NE and NETs play a key regulatory function in the tumor microenvironment, such as tumor proliferation, distant metastasis, tumor-associated thrombosis, and antitumor activity. Additionally, the potential use of NETs, NE, or associated molecules as potential disease activity biomarkers or therapeutic targets will be introduced.

Keywords

Antitumor · Biomarker · Cancer · Metastasis · N1 neutrophil, N2 neutrophil, DNase · NETosis · Neutrophil · Neutrophil elastase (NE) · Neutrophil extracellular trap (NET) · PAD4 · Therapeutic target · Tumor microenvironment · Tumor-associated neutrophils (TANs)

Abbreviations

CXCL	C-X-C motif chemokine
CXCR	C-X-C chemokine receptor
DNase	Deoxyribonuclease
LPS	Lipopolysaccharide
MPO	Myeloperoxidase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
PAD4	Protein arginine deiminase 4
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear neutrophil
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus

H. Huang · A. E. Onuma · A. Tsung (✉)
Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA
e-mail: allan.tsung@osumc.edu

H. Zhang
Department of Surgery, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

Department of Surgery, Union Hospital, Huazhong University of Science and Technology, Wuhan, People's Republic of China

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_2

TAN	Tumor-associated neutrophil
TGF-β	Transforming Growth factor-β
TLR	Toll-like receptor

2.1 Introduction of Tumor-Associated Neutrophils (TANs)

Neutrophils, also known as polymorphonuclear cells (PMNs), are the most abundant white blood cells in human circulation accounting for approximately 60% of all leukocytes [1]. They are the first immune cells to respond to inflammatory or infectious etiologies as they are crucial participants in the proper functioning of both innate and adaptive immune responses [2]. As part of their key role, neutrophils are the first line of immune defense against invading pathogens and also participate in the development of inflammatory cascades [3, 4]. Neutrophils have a unique polymorphic nucleus that is segregated into 3–5 lobules in humans, with each lobule having a diameter of approximately 2 μm [5]. In healthy individuals, most neutrophils are destined to be cleared even without ever performing their function. During infection, they have crucial roles in the clearance of microorganisms [4, 6].

Currently, there is increasing evidence demonstrating that not only do neutrophils play key roles in inflammatory diseases but also in cancer as neutrophils have both pro- and antitumor properties [7–9]. Neutrophils accumulate in many types of human and murine tumors and regulate nearly all steps of tumor progression. Neutrophils are involved in cancer through multiple mechanisms and have been implicated in almost all stages of the oncogenic process including tumor initiation, growth, proliferation, and metastases [10–13]. Human and mice studies have shown that there are two neutrophil phenotypes: antitumor N1 neutrophils and protumorigenic N2 neutrophils [14, 15]. Tumor-associated neutrophils (TAN) can be activated under various conditions resulting in antitumor and protumor functions [14]. Although the ability of TANs to promote or prevent cancer progression is not fully understood, we know that TANs consist of various polarization states, and the mechanism behind this polarization is ill defined. Here we aim to review the existing evidence of neutrophil recruitment, functions as well as the different regulators of neutrophil in the tumor microenvironment.

Neutrophils found within the tumor play a central role in inflammation and are attracted by CXCR2 ligands such as CXCL1, CXCL2, and CXCL5 [13, 16]. The initiation of tumor growth can be promoted by neutrophils through the release of reactive oxygen species (ROS), reactive nitrogen species (RNS), or proteases [17]. Neutrophils have been implicated in various cancers. Human colorectal cancer liver metastases and murine gastrointestinal liver metastases exhibit infiltration by neutrophils. The depletion of neutrophils in a murine liver metastases model has been shown to diminish metastatic growth through fibroblast growth factor 2 [18]. In human hepatocellular carcinoma (HCC) samples, overexpression of CXCL5 was well correlated with intratumoral neutrophil infiltration, shorter overall survival, and tumor recurrence [19]. The depletion of hepatic neutrophils via antibody has been shown to protect the liver from diethylnitrosamine (DEN)-induced HCC in murine models, since neutrophils stimulate hepatocellular ROS and telomere DNA damage [20]. In a mice flank tumor model, neutrophils contribute to the antitumor activity of TGF-b blockade [14]. Neutrophil-derived ROS are important regulators of protumorigenic γδ17 T cells that have been identified as immunosuppressive and antitumoral in a mice melanoma model [21].

Alternatively, neutrophil recruitment has been shown to be a key component of the antitumor efficacy of bovis Bacillus Calmette-Guerin treatment in bladder cancer, chemotherapy in mouse lung cancer, radiotherapy in several syngeneic mouse tumor models, rituximab and trastuzumab treatments in human non-Hodgkin's lymphoma, and breast cancer, respectively [22–26]. Activated TANs can also elicit antitumor functions either directly through lysis of tumor cells or by antibody-dependent cell-mediated cytotoxicity [23, 27, 28].

2.2 Introduction of Neutrophil Elastase in Health and Disease

Neutrophil elastase (NE) belongs to the family of serine protease normally expressed in polymorphonuclear neutrophils (PMNs) [29–31]. It plays a key role in numerous physiological and pathological processes, including antimicrobial defenses, inflammation, and cancer progression [2, 32]. During neutrophil degranulation or neutrophil extracellular traps (NETs) formation, NE is released into the extracellular space in a process known as NETosis. Not only is NE a necessary component of NETs, but activated NE is also required for NET formation [33]. Although NE can also be released from neutrophils independent of NET formation, and rapidly inactivated by plasma antiproteases, DNA-associated NE appears to retain its proteolytic activity for extended periods [34, 35]. NE has been implicated as a biomarker for the diagnosis and prognosis of inflammatory bowel disease as it has been detected in the colonic mucosa of patients with ulcerative colitis [36–40].

It has been shown that activated NE protects against infection by destroying pathogenic bacteria [4]. Mutations in the ELA2 gene encoding neutrophil elastase (NE) occur in most cases of severe congenital neutropenia as well as sporadic and autosomal-dominant cyclic neutropenia [41, 42]. The release of NE is responsible for the activation of epithelial protease-activated receptors, which leads to cell shrinking and reduction of barrier function [43]. NE and ROS are required for TNFα-primed neutrophils and antineutrophil cytoplasmic antibodies to cause increased pulmonary endothelial permeability and lung edema in a model of acute Wegener's granulomatosis [44]. In addition, NE is also important in the reciprocal coupling between innate immunity and coagulation necessary for thrombus formation [45]. NE has been shown to decrease the allostimulatory ability of human monocyte-derived dendritic cells [2]. Finally, how neutrophil elastase is integrated in the activation, regulation, and effector mechanisms of cancers continues to be explored [46].

2.2.1 Neutrophil Elastase in Primary Tumor Initiation and Growth

NE has been shown to have a protumorigenic role in breast, lung, prostate, and colon cancers [47–52]. Blocking the activity of NE in mouse models of numerous cancer types can significantly reduce the effect of neutrophils on tumor progression, and metastasis [47, 49, 50, 53]. NE promotes tumor growth in different ways either by increasing cancer cell proliferation, migration, invasion, or by inducing angiogenesis within the tumor microenvironment. NE may also contribute to tumorigenesis by inactivating tumor suppressors [47, 52, 54–57]. Also, there is increased expression of NE within the tumor and in circulation during tumorigenesis, which promotes tumor growth [58–60]. NE may directly induce tumor cell proliferation in both human and mouse lung adenocarcinomas by gaining access to an endosomal compartment within tumor cells where it degrades insulin receptor substrate-1 [47]. It has been shown that the production of NE from TANs may be involved in tumor invasion, which is associated with a poor prognosis in patients with non-small cell lung cancer. Lastly, NE may facilitate the invasion of cancer cell either by directly dissolving the tumor matrix or indirectly by activating a protease cascade [61].

NE released from activated neutrophils may also mediate PI3K-associated signaling pathway for tumor cell proliferation and promote the growth and progression of cancer cells. The treatment of esophageal cancer cell lines with NE induces the release of growth factors, including protransforming growth factor-alpha, platelet-derived growth factor-AA, platelet-derived growth factor-BB, and vascular endothelial growth factor. Furthermore, use of Sivelestat, a specific NE inhibitor, significantly inhibits the release of these growth factors [47, 57]. EMILIN1, a multidomain glycoprotein expressed in several tissues, exerts a crucial regulatory tumor suppression function through the engagement of α4/α9 integrins. This tumor suppressor function of EMILIN1was impaired through its cleavage by NE, a process that has been implicated in the digestion of sarcomas and ovarian cancers [54].

2.2.2 Neutrophil Elastase in Cancer Metastases

In human studies, NE has been associated with breast cancer metastasis, and the detection of tumor NE level might be helpful in selecting the appropriate individualized treatment for patients with breast cancer [51, 62]. The release of NE can also facilitate non-small cell lung cancer metastasis by degrading basement membranes and allowing egress of tumor cells into the circulation [63, 64]. ONO-5046.Na is a specific NE inhibitor that has been reported to reduce hepatic ischemia-reperfusion injury by inhibiting accumulation of neutrophils [65, 66]. Ischemia-reperfusion injury has been implicated in liver metastasis and ONO-5046.Na may reduce the burden of metastasis through reduction of ischemia-reperfusion injury [67].

2.2.3 Neutrophil Elastase as a Therapeutic Target in Cancer

It has been reported that NE released by TANs can be taken up by breast cancer cells, which could enhance their susceptibility to cytotoxic T lymphocyte lysis [60, 68]. In a colorectal cancer mouse model, a higher expression of active NE was detected in the tumor and giving the NE inhibitor Sivelestat inhibited tumor growth. Within human colorectal cancer tissue, increased amounts of NE expression were detected compared to the adjacent nontumor tissues. In addition, the serum NE concentration in colorectal cancer patients was significantly higher than that in the healthy controls, indicating that the NE levels in serum may also potentially be a diagnostic marker of colorectal cancer in patients [49]. Investigation of Sivelestat use for cancer therapy should be further explored as its safety and efficacy have been tested in treatment of patients with acute lung injury and acute respiratory distress [69, 70]. The NE inhibitors, AZD9668 and BAY-85-8501, were developed and studied in phase II clinical trials for pulmonary diseases. AZD9668 has the potential to reduce lung inflammation and the associated structural and functional changes in some other human diseases. BAY 85-8501 was shown to be safe and efficacious in a mouse acute lung injury model and recently is being tested in clinical studies for the treatment of pulmonary diseases [71, 72].

Taken together, NE could be of great value as a potential diagnostic marker or therapeutic target for cancer patients. Given that NE plays a key role in normal immune response to bacterial pathogens, there is a potential detrimental risk for infections if the drug is given chronically. Currently, there has been no clinical trial on the use of NE in cancer but in designing such trial, every step must be carefully considered in order to gain the greatest benefit [46].

2.3 Introduction of Neutrophil Extracellular Traps in Health and Disease

Neutrophils form neutrophil extracellular traps (NETs), which are large, extracellular, web-like structures composed of cytosolic and granule proteins that are assembled on a scaffold of decondensed chromatin [73]. The granular components are 25 nm in diameter and are normally stored in distinctive neutrophil granules that reside on the DNA backbone structure of NETs and provide antimicrobial activity [73, 74]. NETs can capture and kill bacteria, fungi, viruses, parasites and are thought to prevent bacterial and fungal dissemination [75–79].

NET formation, also known as "NETosis," describes the process by which neutrophils produce and release active NETs. It is important to note that NETosis is distinct from necrosis and apoptosis. This same process, which leads to neutrophil death, is also a mechanism of bacterial sequestration [74, 80–82]. During NETosis, the nucleus first delobulates, while the granules disappear, followed by membrane vesiculation (Fig. 2.1). After nuclear disintegration, the chromatin expands, allowing contact between granular and cellular components. Finally, the cytoplasmic membrane ruptures, releasing NETs into the extracellular space [83]. In summary,

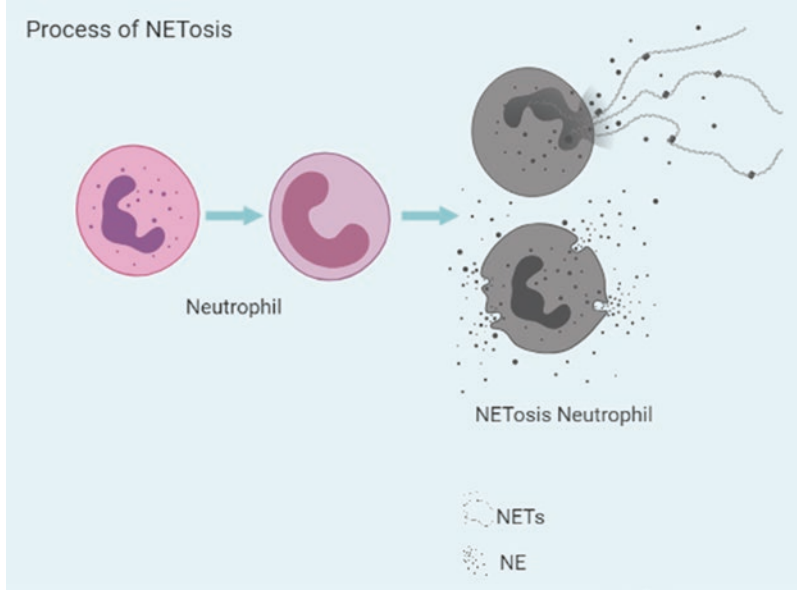

Fig. 2.1 Formation of neutrophil extracellular traps

NETosis is a form of neutrophil-specific cell death where neutrophils extrude extracellular fibers composed of chromatin and granule proteins that are characterized by the release of large web-like structures [84, 85].

NETs have been shown to contribute to the pathogenesis of immune-related diseases such as lupus nephritis when dysregulated [86–88]. Increasing evidence suggests that the process by which neutrophils produce and release NETs not only happens in infections such as sepsis, but also has key role in noninfectious diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), diabetes, atherosclerosis, acute lung injury, hepatic ischemia reperfusion injury, thrombosis, and chronic liver disease [87, 89–100]. In the past few years, there has been increasing evidence for a role of NETs in cancer [86].

2.3.1 Neutrophil Extracellular Traps in Primary Tumor Initiation and Growth

The first association of NETs in cancer was described in 2012 when neutrophils from tumor-bearing mice showed an increased propensity for NETosis upon stimulation with LPS [101]. NET-induced coagulation is a sequela of some malignancies and in an intestinal cancer model, coagulation and tumorigenesis was reduced by DNase [102, 103]. In a Lewis lung carcinoma model with hemorrhagic tumors, a large area of neutrophils and NET-like structures were found within the hemorrhagic tumors [104]. These hemorrhagic areas contained intact and primed hypercitrullinated neutrophils ready for NET formation [104]. In mouse insulinoma and breast cancer models, NET accumulation extends to the peripheral circulation, causing systemic inflammation and impaired vessel function in organs not infiltrated by tumor cells. DNase I treatment abolished these remote effects, suggesting that NETs mediate the collateral effects of tumors in distal organs [105]. Our group also recently found that blocking NETs can reduce the risk of HCC in the setting of non-alcoholic steatohepatitis (NASH) [100].

2.3.2 Neutrophil Extracellular Traps in Metastases

NETs have been implicated in cancer metastasis in the context of systemic infection. In a cecal ligation and puncture (CLP) model, microvascular NET deposition and consequent trapping of circulating lung carcinoma cells within DNA

webs were associated with increased formation of hepatic micrometastases. This effect was abolished by NET inhibition with DNase or neutrophil elastase inhibitor [106]. Our laboratory has reported that NET formation is accelerated immediately after major liver resection in patients with metastatic colorectal cancer. Circulating MPO–DNA levels, a NET biomarker, was associated with a significant increase in early metastatic recurrence. Mechanistic investigations in vitro indicated that mouse neutrophil–derived NET triggered HMGB1 release and activated TLR9-dependent pathways in cancer cells to promote their adhesion, proliferation, migration, and invasion [107]. These effects can be abrogated by inhibiting NET formation in mice via DNase I treatment or inhibition of peptidyl arginine deiminase type IV (PAD4). PAD4 is an essential enzyme in NET formation that catalyzes the citrullination of histones-H3, a critical step for chromatin decondensation and expulsion [97].

2.3.3 Antitumor Properties of Neutrophil Extracellular Traps

Increasing evidence suggests that NETs play a key role in cancer; however, the antitumorigenic and the protumorigenic roles of NET formation appear to depend on various conditions. For example, NETs induced within the vasculature by systemic bacterial infection or surgical stress facilitates cancer metastasis in the liver and the lung. In contrast, neutrophils that express high CD16 and low CD62 have shown increased migratory and NET-producing capacity and this unique feature correlates with better survival in patients with head and neck squamous cell carcinoma [108]. These neutrophils and NETs are presumed to have destroyed the tumor cells. Based on these reports, the functions of NETs and neutrophils seem to differ based on the type of cancer or the degree of progression [109]. Further studies are needed to characterize factors that influence NET production and the difference between various forms of NETS and neutrophils.

2.3.4 Neutrophil Extracellular Traps as a Therapeutic Target in Cancer

NETs are involved in tumor proliferation, distant metastasis, and tumor-associated thrombosis, strongly suggesting that NETs are a potential therapeutic target in cancer [109, 110]. Multiple studies have shown that DNase can digest NET formation, thereby reducing tumor proliferation, distant metastasis, and tumor-associated thrombosis [100, 102, 105–107]. In human studies, PAD4 has been shown to be overexpressed in some tumors [111, 112]. Inhibiting PAD4 by using PAD4 inhibitors or PAD4 KO mice significantly reduces tumor progression [100, 107, 113–115]. However, PAD4 inhibition or the use of DNase for cancer treatment in patients is still in the rudimentary stage. Additionally, it is unknown if inhibiting NET formation has a synergistic effect with current cancer treatments, such as chemotherapy, radiotherapy, molecule-targeted therapy, and immunotherapy [110]. More work is needed to investigate the relationship between NET and already established cancer treatments.

2.4 Conclusions

NE secretion and NET formation in the tumor microenvironment could be the initiators of disease or a side effect of the general overwhelming response of the immune system. There is potential for NE or NET-related molecules to be used as biomarkers and as targets for therapeutic intervention in cancer-related diseases. Although additional studies on the pathogenicity of NE and NETs in tumor microenvironment are still needed, important progress has been made to show that both NE and NETs may induce tumor proliferation, distant metastasis, and tumor-associated thrombosis (Fig. 2.2). The capacity of NE and NETs to potentiate or suppress inflammation may have a beneficial function in cancer and other pathologies. A better understanding of the function and impact of NE and NETs on health will enable the suppression of detrimental

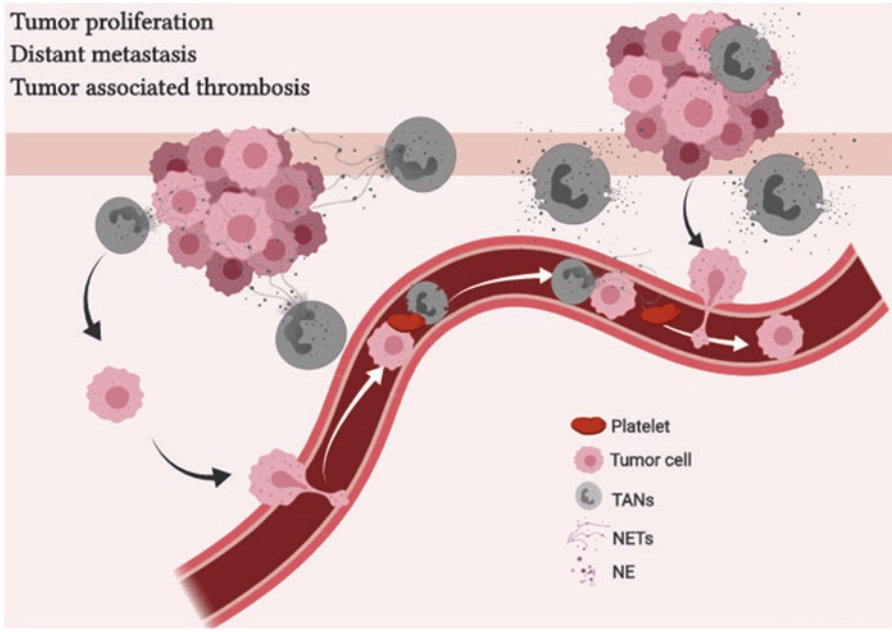

Fig. 2.2 Neutrophil elastase and neutrophil extracellular traps can induce tumor proliferation, distant metastasis, and tumor-associated thrombosis

attributes without interfering with beneficial ones and ultimately, and allow us to exploit NE and NETs to treat diseases. Continued investigations into the relevance of NE and NETs in disease will reveal new functions and shed further light on the role(s) of extracellular chromatin.

Conflicts of Interest The authors have nothing to disclose.

Funding This work was supported by the grants from the National Institute of Health, T32AI 106704-01A1 (AEO), CA214865-01 (AT) and GM095566-06 (AT).

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Viral Hepatitides, Inflammation and Tumour Microenvironment

3

Eleonora Timperi and Vincenzo Barnaba

Abstract

In this chapter, we discuss the role of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in the establishment of hepatocellular carcinoma (HCC), highlighting the key role of the multiple, non-mutually exclusive, pathways involved in the modulation of immune responses and in the orchestration of a chronic low-level inflammation state favouring HCC development. In particular, we discuss (*i*) HCC as a classical paradigm of inflammation-linked cancer; (*ii*) the role of the most relevant inflammatory cytokines involved (i.e. IL-6, TNF-α, IL-18, IL-1β, TGF-β IL-10); (*iii*) the role of T cell exhaustion by immune checkpoints; (*iv*) the role of the Wnt3a/β-catenin signalling pathway and (*v*) the role of different subsets of suppressor cells.

Keywords

Liver · Hepatic sentinel cells · HBV · HCV · Immunopathology · Tumour microenvironment · Chronic low-level inflammation · Immunoregulatory mechanisms · Cytokines · T cells · T cell exhaustion · Immune checkpoint blockade · Wnt/β-catenin · Suppressor cells · Hepatocellular carcinoma

3.1 Liver Anatomy and Role of the Immune System

The liver is a vital organ playing a central role in metabolic processes, such as the regulation and storage of energy and nutrients, digestion and detoxification. Due to its anatomy, and thanks to the portal and venous circulation, the organ is continually in contact and highly supplied with blood [1]. The organisation of the liver, efficiently structured by hepatocytes together with other differentiated cells, (i.e. hepatic stellate cells [HSCs], liver sinusoidal endothelial cells [LSECs], cholangiocytes and Kupffer cells [KCs]), results in lobules, the basal structures of the organ.

E. Timperi
INSERM U932, Institut Curie, PSL Research University, Paris, France

Dipartimento di Scienze Cliniche Internistiche, Anestesiologiche e Cardiovascolari, Sapienza University of Rome, Rome, Italy

V. Barnaba (✉)
INSERM U932, Institut Curie, PSL Research University, Paris, France

Istituto Pasteur, Fondazione Cenci Bolognetti, Rome, Italy

Center for Life Nano Science, Istituto Italiano di Tecnologia, Rome, Italy
e-mail: vincenzo.barnaba@uniroma1.it

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_3

The liver can be considered as an "immunological" organ, containing a wide range of resident immune cells playing essential functions in the maintenance of organ homeostasis [1]. Given its intrinsic role in detoxification, the liver is continually exposed to external agents, including diet products or commensal bacteria derived from the intestine via the portal vein, as well as infectious microorganisms derived from the systemic circulation via the arterial vein. Therefore, the immune surveillance in the organ is highly dynamic.

Resident innate immune cells comprising macrophages or KCs, natural killer (NK) cells, NKT cells, and dendritic cells (DCs) are considered the most important sentinels of the liver. Additionally, tissue resident memory T cells, which are normally homing cells without recirculating and promptly attacking pathogens at the site of infection, are also involved [2]. A key role also seems to be played by resident regulatory T cells (Tregs) that are highly specialised in maintaining tissue tolerance [3].

Under normal conditions, the innate immune sentinels are poor activators of adaptive immunity, but rather favour tolerance, through multiple (not mutually exclusive) mechanisms, guaranteeing survival of the organ. For instance, the continual exposure to sub-threshold lipopolysaccharide (LPS) stimuli from the intestine promotes the production of high IL-10 and low IL-12 levels by LSECs, KCs or tissue DCs favouring Th2-like rather than Th1-like responses, as well as the development of Tregs. The liver tolerance can be converted into immunity by the presence of infections or inflammatory cytokines that convert LSECs, KCs and DCs into powerful stimulators of resident NKT cells and T cells generally originating from mesenteric lymph nodes (LNs), ultimately leading to liver immunopathology: protection and recovery are dependent on appropriate levels of immunopathology. HSCs, also defined as liver pericytes, display important roles in health and disease: they ensure the storage of the majority of vitamin A in a healthy body, and represent the major source of fibrotic tissue in liver disease [4]. A different subset of pericytes (pericytes type 2) has been proposed to contribute to angiogenesis, improving perfusion in ischemic tissues, on the one hand, and metastasis in cancer, on the other hand [5].

However, immunopathology can degenerate into a state of chronic low-level inflammation generally maintained by chronic antigenic stimuli (such as those provided by persisting hepatotropic viruses), which represents the main substrate for hepatocellular carcinoma (HCC) development [3].

3.2 Mechanisms Causing Hepatocellular Carcinoma and Role of the Immune System

Numerous factors may induce the transformation of liver cells into cancer cells. Primary liver cancer is the sixth most common cancer worldwide [6] and its prevalence is especially increasing in developing countries, where almost 85% of cases occur. Among liver cancers, HCC is the most diffuse form; the second most prevalent is cholangiocarcinoma, while squamous cell carcinoma is considered a rare form with a high malignancy rate [6]. HCC (generally diagnosed in the age range from 55 to 65 years) is the fifth most common cancer in men worldwide (523,000 case per year), and the seventh in women worldwide (226,000 cases per year); most of the cases (more than 80%) occur in Asia and Africa [7].

The underlying mechanisms causing HCC comprise several factors. Alcohol is a strong risk factor for developing HCC. Indeed, chronic alcohol intake induces liver damage by alcoholic hepatitis, fatty liver disease and cirrhosis. HCC has also been demonstrated to correlate with non-alcoholic fatty liver diseases (NAFLDs) that are generally associated with a variety of metabolic disorders such as insulin resistance, obesity, diabetes mellitus type II and hyperlipidaemia [8]. Steatohepatitis, evaluated by the accumulation of the fat in the liver in at least 20–30% of patients, culminates in progressive liver disease with necroinflammation and fibrosis, resulting in cirrhosis in 10–20% of cases and, in the latest phase,

in HCC development [9]. A diet with an excess of saturated fats has been related to weight gain and NAFLD, in which an excess of dysfunctional adipose tissue and insulin resistance may generate the dysregulation of adiponectin production, and a consequent increase in secreted pro-inflammatory cytokines (e.g. tumour necrosis factor [TNF]-α, interleukin [IL]-6, leptin). The progressive accumulation of lipids in the hepatocytes can cause lipotoxicity, an excess of free radicals and an oxidation of fatty acids, inducing strong inflammation, peculiar to non-alcoholic steatohepatitis (NASH) [10].

However, most HCCs are associated with chronic liver diseases caused by HBV and HCV infections. Although HBV and HCV belong to two distinctive families, HBV, a double-stranded DNA (dsDNA) virus belonging to the Hepadnaviridae family, and HCV, a virus of the Flaviviridae family characterised by single-stranded RNA (ssRNA), the mechanisms responsible for HCC induction have common pathogenic mechanisms. Both viruses are poorly cytopathic: hepatocyte lysis is not due to a direct viral effect, but is caused by immune responses elicited in the attempt to clear viruses and establish immunity [3].

In particular, primary viral infections promptly (within few hours) cause inflammation by innate immunity [3], through their own pathogen-associated molecular patterns (PAMPs) (e.g. viral DNA or RNA sequences) or damage-associated molecular pattern (DAMP) signals (e.g. oxidative stress, necrotic products, and high-mobility group box 1 [HMGB-1]) generally derived from infected tissue cells. Both PAMPs and DAMPs are recognised by pattern recognition receptors (PPRs) (e.g. Toll-like Receptors [TLRs], RIG-I-like RNA helicases [RLH/RLRs], Nod-like Receptors [NLRs]), expressed by a wide range of immune cells, which, as consequence, can be activated to produce a storm of pro-inflammatory and anti-viral cytokines (including type I interferons [IFNs], TNF-α, IFN-γ). Simultaneously, the entry of the virus into hepatocytes promptly drives direct (granule- and death receptor-mediated) killing by NK and NKT cells, providing antiviral effects by producing pro-inflammatory cytokines as well. In addition, the recruitment and activation of innate immune cells are crucial steps for the delayed adaptive immunity to be mounted (emerging within 10–15 days upon the infection) through the priming of antigen-specific B and T cells, inducing strong antiviral responses and long-term immunological memory (Fig.3.1). Tissue-resident DCs represent the principal cells that connect innate and adaptive immunities, because, in an inflammatory context, they acquire the capacity to differentiate into a phenotype of professional antigen-presenting cells (APCs), and migrate into draining lymph nodes (LNs). Here, they can prime naïve B and T cells that, in turn, proliferate, differentiate and are proficiently recruited into the inflammatory sites of the virus-infected liver, where they can protrude with their pseudopods through the endothelial cell fenestrae in order to probe and search for infected hepatocytes and, ultimately, kill them [11]. Alternatively, naïve HBV-specific $CD8^+$ T cells can arrive in the lumen of liver sinusoids and can be primed directly in the HBV-infected liver through the contribution of third-part IL-2-activated KCs [12]. Collectively, the suppression of viral replication is mainly caused by the combination of specific anti-viral antibodies (produced by B cells), killing of virus-infected liver cells, and anti-viral inflammatory cytokines (produced by NK, NKT, $CD4^+$ and $CD8^+$ T cells, etc.) [3]. Effector $CD8^+$ T cells provide the most efficient protection against intracellular pathogens by the serial killing of infected hepatocytes, which is consequent to the direct antigen-specific T cell receptor (TCR) recognition of short viral antigenic peptides complexed with class I major histocompatibility complex molecules on infected cells. In addition, they can simultaneously provide bystander-indirect killing (to which the expression of activating NK receptors [e.g. NKG2D] contributes) of neighbouring infected or non-infected hepatocytes expressing the proper ligands of activating NK receptors [13]. At the same time, the selection of B cells with highly specific BCR takes place in the LNs, while the production of antibodies is not highly efficient in the first part of infection, somatic

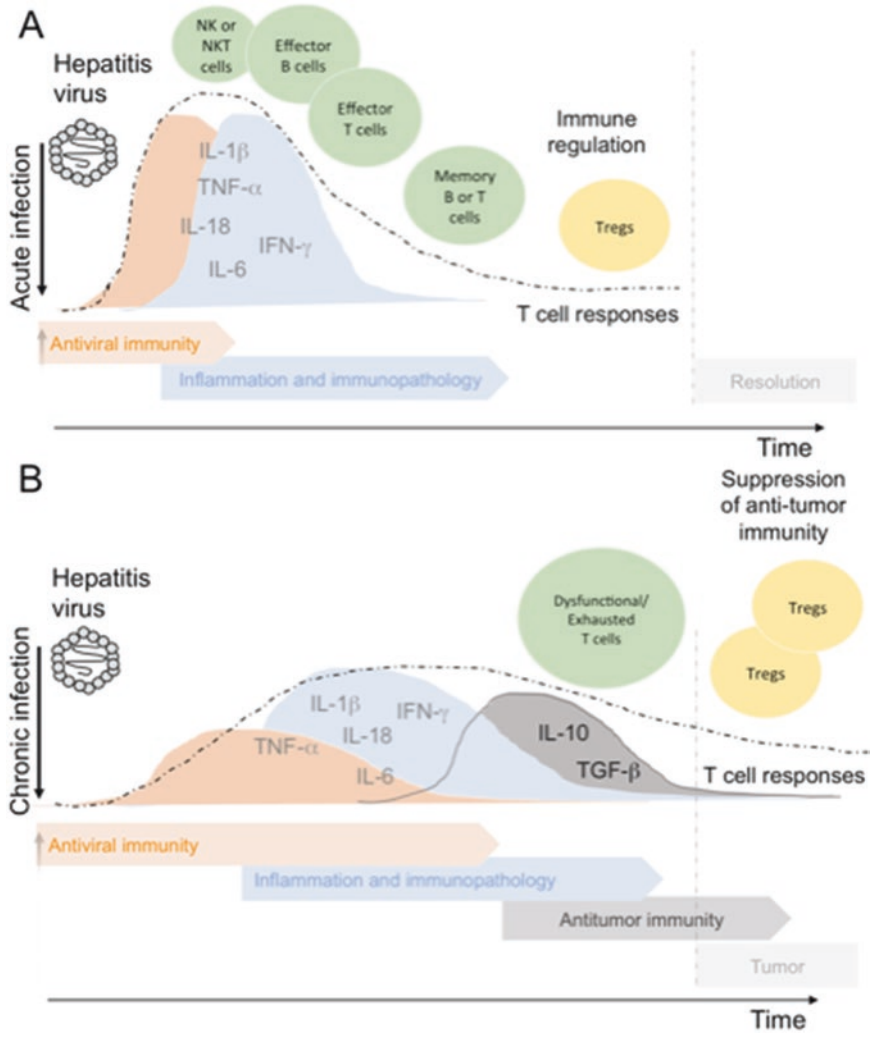

Fig. 3.1 Immune responses to HBV and HCV infections. (**a**) Acute infections are characterised by a phase of strong anti-viral immunity aimed at eliminating viruses from the host. A cytokine storm (IL-6, TNF-α, IL-1β, IL-18) is basically produced by innate immune cells, such as macrophages, DCs and HSCs. Consequently, the adaptive responses are mounted to both directly kill virus-infected cells by antigen-specific effector T cell responses and neutralise the virions by antibodies produced by antigen-specific B cells. IFN-γ production by T cells, as well as by NK and NKT cells, contributes to viral clearance. Finally, memory T and B cells are generated to guarantee the host protection against secondary infections. An immunoregulatory mechanism mediated by regulatory T cells (Tregs) results crucial for the resolution of immunopathology. (**b**) Under chronic infection conditions, characterised by a long phase of inefficient anti-viral immunity, and chronic inflammation and immunopathology, the persistence of hepatitis viruses is the key point. Indeed, effector T cells that are chronically stimulated by the persisting virus undergo consecutive steps of exhaustion (partially and then fully exhaustion) and, together with the parallel expansion of suppressive Tregs, establish a state of chronic low-level inflammation favouring, in the long term, a suppressive liver microenvironment and the development of HCC. In this context, the anti-tumour immunity response, mounted in an attempt to eliminate the cancer, will not result in the development of immunological memory, but will be non-protective, because of the immunosuppressive TME

hypermutations of germline V(D)J generate antibodies with higher specificity [14]. Whether virus-specific B or T cells are generated in tertiary lymphoid structures formed in a chronic inflammation liver is a crucial issue to investigate. Overall, these responses take place to

eradicate the virus in the organ through a state of robust inflammation, characterising the acute phase of infection (Fig.3.1a). During this phase, the adaptive response has the major role of clearing the viruses and building long-term protection by T and B cell memory. Immunological memory remains for almost a lifetime in the body, guaranteeing protection against secondary infections (Fig.3.1a).

If the activity of the immune system is crucial for the eradication of the virus, on the one hand, the virus can activate different mechanisms of immune escape to persist in the host, on the other. Indeed, although the infection and replication cycles are completely different between the two viruses, both HCV and HBV are able to persist in a chronic manner in the infected host. HCV is capable of escaping better than HBV, mainly due to the higher mutational rates [15, 16]. Since both HBV and HCV are not cytopathic per se, the difference in establishing viral persistence is principally based on different interactions of viruses with the immune system. Both viruses are able to escape innate immune controls (e.g. PPRs, interferon [IFN] type I-based immunity, and NK cell responses), suggesting that the capacity of HCV to escape adaptive immunity, which is significantly greater than HBV, is due to the higher rate of its persistence [17]. Indeed, HCV is characterised by a higher mutation rate than HBV (~4.0×10^{-19} HCV mutations vs. ~3.0×10^{-5} HBV mutations generated daily worldwide), which can result in the generation of >10^6 HCV copies each day in each infected individual; this may therefore allow the evasion of B or T cell recognition much more frequently than HBV [18, 19]. In the case of viral escape, a state of chronic low-level immunity/immunopathology is adapted in order to avoid excessive liver damage, on the one hand and excessive viral spread, on the other (Fig.3.1b). This fine balance results in a crucial equilibrium, allowing a long-lasting life of the infected host that can, in the long-term, undergo HCC development. Many findings have indeed demonstrated a direct connection between chronic inflammation and the development of cancer in both mouse and human cancers [20]. The process toward liver cancer development is characterised by an intermediate state of cirrhosis persisting in the liver for many years.

3.3 Inflammation Mediated by Cytokines in the Establishment of HCC

Although different factors can cause HCC, as mentioned above, the crucial role is played by inflammation. HCC is actually considered a classic paradigm of inflammation-linked cancer, since around 90% of HCCs result from injury and inflammation [21].

Inflammation is a complex biological response addressed to combat any dangerous agent/substance for the host (i.e. pathogens, damaged cells, irritants, alcohol, a high fat diet, toxins, etc.). The dominant feature of non-resolving inflammation in HCC is mainly characterised by the establishment of a state of chronic low-level inflammation mediated by the continuous recruitment of immune cells elicited by persisting viral or danger signals, and counterbalanced by various regulatory mechanisms addressed to avoid excessive liver damage. Indeed, the infiltration and activation of monocytes/KCs and immature myeloid cells produces a wide range of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β, IL-18) that, together with the activation of various signalling pathways (NF-κB, STAT3, Wnt/β-catenin), elicit the production of reactive oxygen and nitrogen species (ROS and NOS). At the same time, anti-inflammatory responses (i.e. TGF-β, IL-10) are mounted in the liver to counterbalance the excessive inflammation resulting in a perturbation of wound-healing responses, leading to the sequential steps of fibrosis, cirrhosis and HCC [22]. All of these reported cytokines are mainly produced by immune cells and, at a lower level, by liver cells (hepatocytes and LSECs expressing the related receptors).

Recent data suggested a peculiar compartmentalisation of cytokines according to the inflammatory or cirrhotic state [23]. Indeed, the balance between inflammatory (IFN-α, IL-27, IL-12, TNF-α) and anti-inflammatory (e.g. IL-10, TGF-β) cytokines was found to be consistently

favouring the former in highly inflamed not-cirrhotic (HBV- or HCV-infected) livers, whereas the latter is favoured in cirrhotic livers and tumour lesions. The cytokine milieu plays an essential role in supporting chronic low-level inflammation, culminating in cirrhotic phase and HCC development [23].

Liver injury is also triggered by the combination of direct cell killing and the production of large amounts of IFN-γ and TNF-α, initially mediated by NK cells and then by $CD8^+$ T cells elicited in an attempt to clear HBV- or HCV-infected hepatocytes, as well as by the activation of KCs representing a further cause of TNF-α-mediated liver damage [24]. A direct effect can also be played by molecular structures of the viruses. For example HBx, a small protein encoded by HBV, directly modulates mediators such as IL-8, ICAM-1, MHC complex, and activates the NF-kB and NFAT signalling pathways implicated in the production of IL-6, IL-8 and TNF-α [25].

IL-6 is one of the most studied cytokines in physiological and pathological processes of the liver. It can regulate the epithelial mesenchymal transition (EMT), which has a dominant role in HCC progression. Indeed, it promotes the mesenchymal phenotype of hepatocytes, such as motility invasion and resistance to apoptotic stimuli, supporting the metastatic phase of HCC. In the hepatic acute phase responses, KCs and LSECs are the main producers of IL-6, causing hepatocyte activation and the release of c-reactive protein, which helps innate immunity against infection. Elevated serum levels of IL-6 have been observed in patients with chronic HBV and HCV infections and are associated with a higher risk of developing HCC, as well as in HCC patients, associated with a poor prognosis [26–28]. IL-6-dependent oncogenic activity is exerted by downstream signals via Signal Transducer and Activator of Transcription 3 (STAT-3) phosphorylation [29]. The STAT-3 signalling pathway is a crucial step of the process: cyclooxygenase 2 (COX-2), the mediator of inflammation, has been demonstrated to activate STAT-3, not only promoting the direct transformation of hepatocytes, but also regulating the pro-tumorigenic functions of M2 tumour-associated-macrophages (TAMs) [30]. Furthermore, the activation of STAT-3 exerts an immunosuppressive effect on immune cells, counterbalancing anti-tumour immune responses [31].

TNF-α is another crucial cytokine involved in the regulation of HCC development. Many data have described the role of TNF-α in HCC mouse models. It has been demonstrated that TNF-α promotes HCC in inflammation-induced carcinogenesis models, where the in vivo administration of antibodies neutralising TNF-α decreases HCC growth [32]. TNF-α is also strongly involved in tumour promotion, invasion, angiogenesis, and metastasis, and is correlated with tumour growth, oxidative stress, and carcinogenesis [33, 34]. In line with these observations, the level of TNF-α has been found to be significantly higher in patients with cirrhosis and acute/chronic hepatitis compared with healthy subjects [35]. Moreover, TNF-α expression is mostly elevated in patients with recurrent HCC [36]. The major source of TNF-α in the liver is derived from TAMs, and the impact of TNF-α production on immune cells is well described. For instance, TNF-α induces the expression of TNF-related apoptosis-inducing ligand (TRAIL) on liver cancer cells, leading to the apoptosis of T cells [37]. It can also stimulate the up-regulation of inhibitory molecules (i.e. programmed cell death ligand-1 [PDL-1]) on both macrophages and DCs suppressing anti-tumour responses by $PD\text{-}1^+$ $CD8^+$ T cells [38]. Tregs are also highly susceptible to TNF-α, constitutively expressing TNFR-I and modulating TNFR-II expression during inflammation [39, 40]. Amongst its roles, TNF-α can induce the expression of OX40 (CD134), which belongs to the TNFR family, on Tregs, boosting their proliferation capacity and suppressive activity [23].

The IL-1 system comprises IL-1β and IL-18, which together are considered crucial mediators for host responses in infections and inflammation. Both IL-1β and IL-18 exist as inactivated forms (pro-IL-1β and pro-IL-18) in cells, and, upon inflammasome activation, can be released as active forms by the activity of caspase-1 (casp-1)[41, 42]. The close association between these cytokines and liver inflammation is well

described [43, 44]. In addition, the relationship between IL-1β and cancer [45], as well as the connection between IL-1β and NLR family pyrin domain containing 3 (NLRP3), promoting the occurrence and development of chronic liver diseases, has been well characterised [46]. IL-1β can also directly activate quiescent HSCs, which are involved in the formation of liver fibrosis and even cirrhosis, and induce the production of monocyte chemoattractant protein-1 (MCP-1/CCL2) and IL-8 (CXCL8) [47]. Additionally, in a mouse model of ethanol liver injury, a direct role of IL-1β in the regulation of steatosis, inflammation and fibrosis has been demonstrated. Notably, casp-1-knock-down mice are protected from developing fibrosis [48]. Accordingly, IL-1β serum levels are extremely augmented in patients with hepatitis, liver fibrosis, cirrhosis and primary HCC [36].

While a pro-tumorigenic role of IL-1β in HCC development has been clearly demonstrated, the function of IL-18 is controversial. A main biological difference between IL-18 and IL-1β is the ability of IL-18 to be a strong stimulator of IFN-γ production in the presence of IL-12 [49]. IL-18 has been demonstrated to increase the production of IFN-γ by tumour-infiltrating $CD8^+$ T cells in patients with non-small-cell lung cancers ex vivo, overcoming the suppressive mechanisms of exhaustion in the tumour microenvironment (TME) [50]. Also, it has been reported that NK cells can boost their anti-viral and anti-cancer activities, by blocking or knocking down the expression of IL-1 receptor 8 (IL1R8), which negatively regulates the effects of IL-18 and, therefore, represents a new immune checkpoint (IC) of NK cells [51]. These data suggest a positive role of IL-18 in the regulation of anti-viral and anti-cancer activities. However, although many findings have described the protective role of IL-18 in a number of tumour models [52], the function in human hepatitis and HCC is less clear. For example IL-18 can induce the up-regulation of TRAIL in HCC cell lines, promoting tumour evasion by inducing the apoptosis of activated T cells [37]. Additionally, high levels of circulating IL-18 were correlated with poor prognosis in HBV- or HCV-related HCC [53]. On the other hand, IL-18 serum levels were found to be increased in acute phase of HBV infection, and its expression correlated with the expression of IFN-γ in chronic HCV infection and cirrhosis [52, 54]. Consistent with a possible protective role of IL-18, the deletion of IL-18 receptor (IL18R1) correlates with enhanced tumour growth in HCC mouse models, suggesting that IL-18 exerts inflammation-dependent tumour-suppressive effects by promoting the differentiation, survival and activation of tumour-infiltrating T cells [52]. These contrasting data are likely due to the different stages of HCC, in which IL-18 shows its effects: pro-inflammatory during the immunity phase and anti-inflammatory during the late cirrhotic/tumour stages.

Macrophages and KCs represent the cells that are mainly involved in cytokine production during inflammation. TAMs, indeed, are central actors of cancer-related inflammation, since they are found at high proportions amongst infiltrating-tumour $CD45^+$ immune cells [55]. Beyond their ability to produce IL-6, IL-1β, TNF-α, IL-18 and other cytokines, they play a key role in angiogenesis, tissue remodelling and repair, wound healing, tumour invasion and metastasis. Indeed, they can produce growth factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), as well as metalloproteinases (MMPs) and COX-2. In this context, each mediator can be responsible for particular activities at different phases, leading to the development of HCC. For instance, both MMP-9 and MMP-2 are involved in growth and invasion, as well as in matrix remodelling and degradation at the invasive front of murine HCC [56–58]. VEGF is a critical player in liver HCC angiogenesis, through its effect on the proliferation of both endothelial and VEGF-A receptor-expressing cancer cells [59]. PDGF, which is considered an angiogenic factor, promotes the activation of cancer-associated fibroblasts (CAFs), HSC differentiation and extracellular matrix (ECM) production by myofibroblasts [60].

If liver injury is guided by the activity of several pro-inflammatory cytokines, on the one hand, the anti-inflammatory mechanisms occur,

on the other hand, to counterbalance the excessive inflammation. The contribution of TGF-β to the induction of HCC was first investigated in HCC patients. In early studies, an increase in TGF-β levels in both plasma and tumour tissues of HCC patients was reported [61, 62]. Moreover, the overexpression of TGF-β was associated with the shorter survival of HCC patients [63]. However, TGF- β can exert a dual role in hepatocarcinogenesis according to HCC stages [64]. For example, it can act as a tumour suppressor, mediating pro-apoptotic and anti-proliferative signals in precancerous states, whilst it promotes tumorigenesis through several mechanisms in established tumours [64]. The EMT process can be strongly promoted by TGF-β1, through the down-regulation of E-cadherin, which is one of the principal components of epithelial adherent junctions [65]. Additionally, TGF-β can induce the up-regulation of mesenchymal genes such as vimentin, the HCC-associated antigen CD147 and N-cadherin, all favouring the EMT process [66]. TGF-β can also increase tumour invasion by inducing the expression of α3β1 integrin [67]. In its soluble form, this can be a strong activator of HSCs, further supporting HCC growth in TME. Importantly, TGF-β limits anti-tumour responses as an immune suppressor, dampening the production of IFN-γ by $CD8^+$ T cells and inducing the production of IL-6 and IL-1β [68].

As well as TGF-β, IL-10 exerts immunosuppressive activities in HCC. Several studies have reported high levels of IL-10 in liver cancer patients, and high serum levels of IL-10 have been associated with poor prognosis in HCC patients [43, 69]. The up-regulation of PD-L1 on cancer cells and TAMs is strongly supported by IL-10, via NF-KB and STAT3 signalling pathways [70]. Furthermore, a crucial role of IL-10 is to support the induction and differentiation of Tregs expressing the X-linked transcription factor (FOXP3) in HCC; indeed, the high prevalence of Tregs has been found to be strongly associated with high levels of IL-10 in HCC patients [71]. Treg expansion is strongly correlated with the aggressiveness of HCC and the poor survival of patients [71].

Over the past few years, many studies have shown that, amongst the wide range of molecular players connecting inflammation and cancer, NF-kB, HIF-1a, STAT3 and Wnt/β-catenin exhibit key functions [72]. In the liver tissue, KCs, hepatocytes and HSCs can express these pathways and may be intimately connected with the cytokines described above [73]. For instance, activating mutations of the β-catenin-1 gene (*CTNNB1*), or inactivating mutations of the axis inhibition protein (*AXIN1*) and APC, can cause the Wnt/β-catenin signalling pathway to be triggered [74, 75]. Although HBV-related HCC shows low rate of mutations in the Wnt/β-catenin pathway compared with HCV-related HCC, data have demonstrated a close connection between Wnt/β-catenin and the promotion of HBV-related HCC [73]. In addition, many findings demonstrated a direct role in the transformation of cancer cells mediated by the Wnt/β-catenin pathway [76]. This pathway can further affect immune cell functions and immunosurveillance, indirectly facilitating tumour development, as described in more detail below [77].

3.4 Mechanisms Favouring HCC and Therapeutic Perspectives

Whilst the high production of several inflammatory cytokines released by immune cells, including IFN-γ, TNF-α, IL-6 and IL-1β, favours the resolution of acute inflammatory disease (e.g. acute HBV infection), the same pro-inflammatory cytokines, during chronic inflammatory diseases (e.g. chronic HBV or HCV infections), can synergistically promote carcinogenesis in combination with the production of reactive oxygen or nitrogen species by activated myeloid cells [72] (Fig.3.1a and b). Indeed, if the virus has been able to escape immune control, a state of chronic low-level immunopathology emerges through various and simultaneous immunoregulatory mechanisms (IRMs) fine-tuning immune responses. The selection of IRMs establishing chronic low-level inflammation/immunopathology has primarily been developed during the evo-

lutionary process to limit excessive liver inflammation, and thereby avoid the complete suppression of antiviral immune responses, ultimately allowing the long-term survival of the chronically infected host. However, in the long-term, chronic low-level inflammation results in the establishment of severe "side effects" including cirrhosis, organ failure and tumours [3]. These severe side effects represent the cost to pay for maintaining long-lasting survival.

Once the virus has been able to escape the immunosurveillance (HCV significantly more than HBV, mainly due to its higher mutational capacity, as described above), a cascade of IRMs intervenes, including the exhaustion mechanism, the production of immunosuppressive cytokines and the overwhelming function of Tregs. All of these mechanisms are the same as those taking place upon the resolution of an acute infection (e.g., acute HBV infection), in order to terminate the immune effector responses and favour the generation of memory immune cells. In contrast, in chronic infections or tumours, where persisting viral or tumour antigens cause long-term immune stimulation, IRMs dominate and limit the function of virus- or tumour-specific T cells, excluding the generation of memory cells (Fig.3.1b).

3.4.1 IRMs by T Cell Exhaustion

A pivotal IRM is caused by the sustained expression of various ICs (e.g., PD-1, CTLA-4, TIM-3, etc..) on T cells chronically stimulated by persisting viral or tumour antigenic stimuli; these T cells acquire a dysfunctional signature (low IFN-γ, TNF-α, IL-2 production) and undergo exhaustion in the long-term (Fig.3.2). ICs (e.g. PD-1 or CTLA-4), following engagement with their own ligands, which are strongly up-regulated by both lymphoid (e.g. PD-L1, B7.1 or B7.2) and non-lymphoid (e.g. PD-L1) cells during inflammation, deliver a series of negative signals, switching off the effector immune responses [78, 79]. The mechanisms whereby ICs provide partial or complete T cell exhaustion in chronic infections or tumours are an issue of intense study in order to identify new therapeutic strategies to block them, mainly by the usage of IC inhibitors (ICIs) (e.g. anti-PD-1 or anti-CTLA-4 monoclonal antibodies [mAbs]). ICIs have been demonstrated to be highly effective therapeutic agents, due to their capacity to unleash anti-tumour T cell responses, leading to a dramatic contraction of several metastatic tumours [80–82]. The Nobel Prize for Medicine 2018 was awarded jointly to James P. Allison and Tasuku Honjo "*for their discovery of cancer therapy by inhibition of negative immune regulation*," through immunotherapy with anti-CTLA-4 and anti-PD-1 mAbs [78, 79].

The availability of next-generation sequencing technologies has made it possible to define a complete "exhaustion map" in chronically stimulated T cells, by analysing their whole transcriptome (RNA-seq) and epigenome (Assay for Transposase-Accessible Chromatin using sequencing [ATAC-seq]). Multiple molecular mechanisms associated with exhausted T cells have been proposed, including (*i*) the demethylation of *Pdcd1* regulatory region, due to the sustained transcription of FoxO1 factor, leading ultimately to up-regulation of PD-1 [83, 84], or (*ii*) the sustained transcription of NR4A1 or TOX factors sequentially providing selective histone modifications, expression of exhaustion genes (e.g., *PDCD1* and *HAVCR2*), and overexpression of the related ICs (e.g. PD-1 and TIM-3) [85, 86]. According to the complete or partial expression of these epigenetics-driven molecular pathways (likely caused in turn by the increased or decreased duration of the viral or tumour antigenic stimuli), exhausted T cells can be divided into fully or partially exhausted T cells (Fig.3.2). This dichotomy is showing itself to be of great importance from a therapeutic point of view, because it provides molecular basis on the evidence that the partially exhausted (e.g., PD-1^{low}, T-bet^{high}, $Eomes^{mid}$, $CD39^{high}$, and CTLA-4^{+}) rather than the fully exhausted (e.g., PD-1^{high}, T-bet^{-}, $Eomes^{high}$, and $TIGIT^{+}$) T cells can be rescued by ICI therapies and re-acquire their immunosurveillance competences against tumours, and possibly in chronic viral infections as well [87] (Fig.3.2).

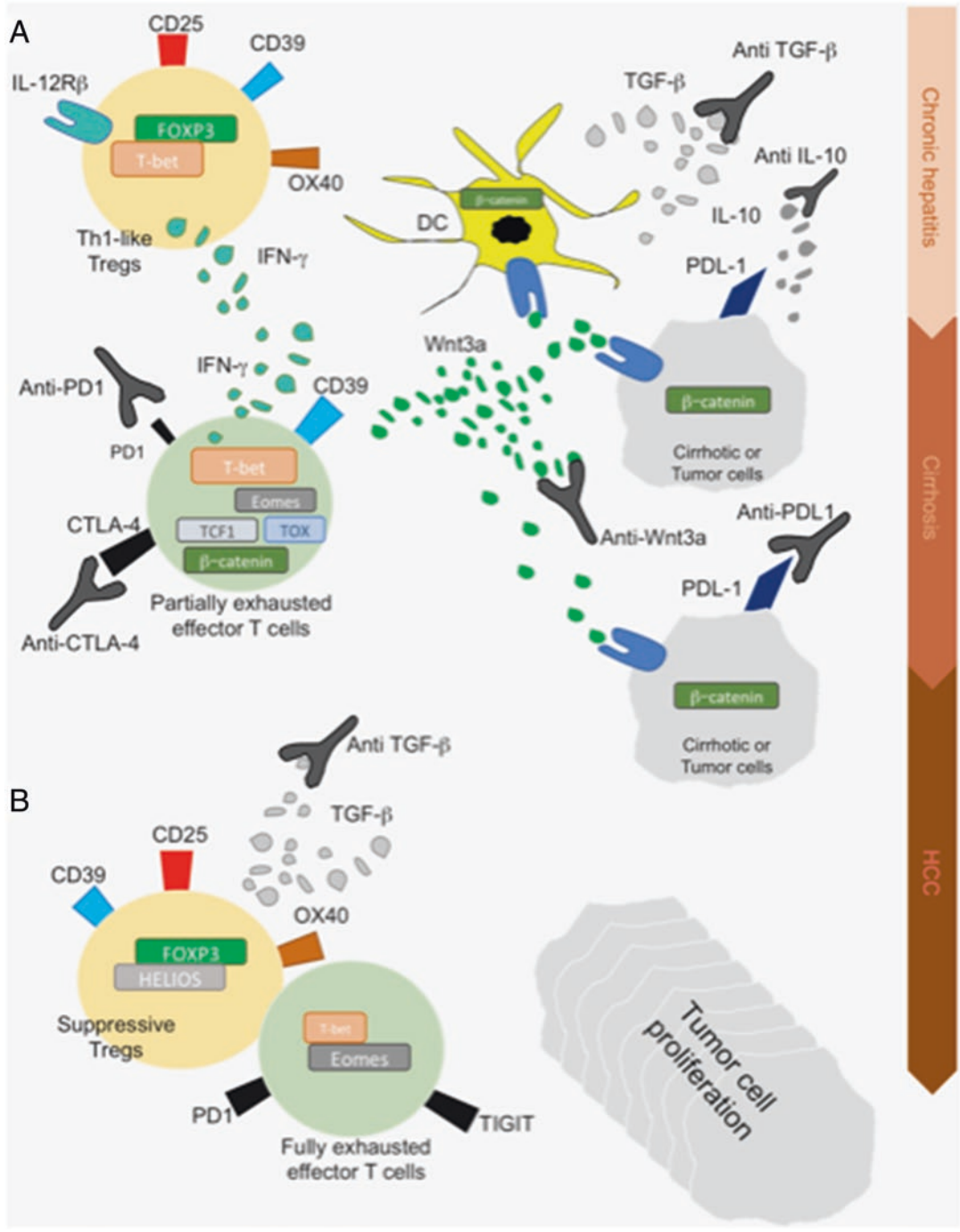

Fig. 3.2 Immune cell mechanisms in chronic inflammation and HCC

(**a**) Under conditions of chronic infection, a state of chronic low-level immunopathology adapts through various and simultaneous immunoregulatory mechanisms, including: (*i*) the induction of partially exhausted effector T cells (T-bet^{high}, Eomes^{low}, PD-1^{low}, CTLA-4^{high}, β-catenin^{+}, TCF1^{+}, CD39^{high}, TOX^{+}), which act by producing low level IFN-γ, limiting viral load and controlling tumour development for many years; (*ii*) the expansion of plastic Tregs (Th1-like Tregs: FOXP3^{+}, T-bet^{+}, CD39^{low}, Helios^{low}, OX40^{low}, IL-12Rβ^{++}) with the ability to produce IFN-γ, supporting chronic low-level inflammation; (*iii*) the Wnt-β catenin signalling pathway,

However, despite the current ICIs (in particular anti-PD-1 and/or anti-CTLA-4 mAbs) being highly effective therapeutic agents in various tumours, they are not efficient in all tumours, causing partial remission in several patients, or even serious adverse events (e.g. autoimmunity), inducing autoreactive T cells [88]. In this context, the therapeutic effects of nivolumab (anti-PD-1) in HCC have not provided the expected results to date; the recent phase III trial in unresectable HCC missed the primary endpoint [89]. Indeed, it failed to meet statistical significance in overall survival improvement between nivolumab and sorafenib (a well-known kinase inhibitor drug used for the treatment of various tumours including HCC) therapies. A possible reason for the therapeutic failure of nivolumab in HCC may be ascribed to the evidence that only 30% of HCC patients showed an inflamed ("hot") TME with high immune cell infiltration and high levels of PD-L1 and PD-1 [90], whereas the majority of them displayed a non-inflamed ("cold") tumour profile, with low or absent immune infiltration; these features are possibly associated with diminished response to anti-PD-1 therapy. Another (non-mutually exclusive) reason may be based on the hypothesis that the long-term antigenic stimuli (whose duration can be of several decades), provided initially by persisting viruses during the chronic infection phase, and then by tumour antigens as well, may advance fully exhausted T cells that would become non-susceptible to immunotherapy [87] (Fig.3.2b). Therefore, innovative therapeutic approaches are required in order to convert "cold" into "hot" TME, to target novel ICs expressed by exhausted T cells, and to neutralise soluble immunosuppressive agents secreted in the TME.

3.4.2 IRMs by the Wnt/β-Catenin Signalling Pathway

Several immunosuppressive molecules (the identity of which is currently being investigated by analysis of the tumour secretome in our laboratory) can be secreted in TME and contribute to the development of HCC. Amongst the multitude of secreted proteins associated with TME- or pre-tumour microenvironment (e.g. cirrhosis), besides the cytokines described above, interesting candidates whose blockade may have a beneficial therapeutic effect in cancer include the molecules involved in the Wnt/β-catenin signalling pathway (Fig.3.2a). They are abundantly expressed in various TMEs, can induce intracellular signals including carcinogenesis [76], and can also regulate multiple stages of T cell activation [91] and prevent the intratumoral migration of conventional (c)DCs [92].

In recent studies, both Wnt3a and β-catenin were overexpressed by tumour- and non-tumour-infiltrating $CD4^+$ or $CD8^+$ T cells, which displayed a dysfunctional effector phenotype in both HBV- or HCV-related HCC and CRC patients [93]. These cells were, however, defined as partially exhausted $CD4^+$ or $CD8^+$ T cells, because they performed discrete effector functions (in terms of IFN-γ and TNF-α production, as well as cytotoxicity function), despite

Fig. 3.2 (continued) which induces partially exhausted effector T cells via third-party DCs, on the one hand, and directly acts on tumour cells inducing and supporting cell transformation, on the other hand, and (*iv*) the production of immunosuppressive cytokines (i.e. TGF-β or IL-10) by various types of cells in the attempt to repair inflammatory damage. In this equilibrium phase, several therapeutic strategies can be beneficial, comprising: (*i*) ICIs, such as anti-PD-1, anti-PDL-1, anti-CTLA-4 mAbs addressed to revert partially exhausted T cells into effector T cells; (*ii*) the blockade of soluble pro-tumorigenic factors (e.g., by anti-Wnt3a mAb), reducing the levels of soluble Wnt3a in the TME, and ultimately inhibiting the pro-tumour and immunoregulatory effects of Wnt3a and (*iii*) reduction of the pro-tumorigenic activity of immunosuppressive cytokines, such as TGF-β and IL-10. (**b**) In the absence of these therapeutic strategies, more severe immunosuppressive mechanisms develop including (*i*) the differentiation of partially into fully exhausted T cells ($T\text{-}bet^{low}$, $Eomes^{high}$, $PD\text{-}1^{high}$, $CTLA\text{-}4^{low}$, $TIGIT^+$), decreasing the ability to mount protective anti-tumour immune responses and supporting tumour escape and (*ii*) the expansion of suppressive Tregs ($FOXP3^+$, $Helios^{high}$, $CD39^{high}$, $OX40^{high}$) supporting a pro-TME by inhibiting anti-tumour T cell responses. Generally, these inefficient responses can hardly be rescued by the current ICIs, and require innovative therapeutic strategies (e.g. by neutralising TGF-β produced by Tregs, or new molecules secreted in the TME and that are being defined)

being unable to control tumour progression (Fig.3.2a). These partially exhausted β-catenin$^+$ T cells up-regulated the β-catenin targets Tcf1 and Axin2, presenting features that are intermediate between memory and exhausted T cells, which expand upon IC blockade [94]. Collectively, these data suggest that Wnt3a may deliver β-catenin-dependent signals, generating partially exhausted (Wnt3a$^+$, β-catenin$^+$, T-bet^{low}, PD-1^{low}, Tcf1$^+$) T cells, which would represent candidate target cells for immunotherapy. Mechanistically, Wnt3a blockade indirectly restored anti-tumour T cell functions, by neutralising Wnt signals into β-catenin$^+$cDCs, and thus converting tolerogenic into stimulatory DCs with the consequent rescue of anti-tumour T cell activity; this ultimately led to a significant decrease of the tumour mass in mouse CRC or HCC models [95] (Fig.3.2a). Recently, another component of the Wnt family, Wnt1, has been demonstrated to induce immunologically "cold" TME by dampening cDCs in lung carcinoma [96]. These data suggest that blocking of the Wnt/β-catenin signalling pathway might be a promising immunotherapy approach, by rescuing not only anti-tumour immune responses, but also anti-viral immune responses in chronic viral infections.

3.4.3 IRMs by Suppressor Cells

Critical IRMs for both systemic and tissue immune homeostasis are provided by the emergence of functional Tregs expressing FOXP3 [97, 98], which can be either committed in the thymus (thymus-derived Tregs) or induced in the periphery (iTregs) [99]. Tregs can prevent the differentiation of autoreactive T naïve cells into detrimental effector cells in the periphery (avoiding thus autoimmunity), and can also stop or limit the excessive immunopathology by self- or non-self-reactive T effector cells through a wide range of immunosuppressive mechanisms [100, 101]. Tregs (particularly tissue-resident Tregs) also perform tissue-protective activities, for instance, by promoting tissue repair and systemic metabolism [102]. These activities are beneficial in resolving acute inflammatory diseases by promoting tissue health, but become detrimental in chronic inflammatory diseases, because they contribute to organ failure via the persisting tissue repair mechanisms, resulting in severe fibrosis, cirrhosis and HCC development.

Tregs are expanded in HCC and also in cirrhosis as a result of its peculiar plasticity committed at an epigenetic level [23]. Indeed, in the inflammatory liver contexts (e.g. chronic HBV or HCV infections), Tregs are exposed to strong inflammatory cytokines (i.e. IFN-γ and IL-12), which deliver signals favouring the methylation of TSDR, and, as a consequence, the generation of Tregs with unstable FOXP3, poor suppression function, low OX40, CD39 and Helios, and high T-bet and IFN-γ expressions (Th1-like Tregs) (Fig.3.2a). They contribute to inflammation by IFN-γ production, on the one hand, and by establishing the state of chronic low-level inflammation by its mild suppression function, on the other. A vital immunological "compromise" is thus established, allowing the control of immunopathology and avoiding the excessive suppression of protective immunity [103]. This compromise may also take place because of the contribution of a counter-suppression mechanism, whereby Treg proliferation and function is controlled and limited through the PD-1/PD-L1 interaction [104]. In contrast, in "cold" TME (HCC and cirrhosis as a premalignant condition), Tregs convert into frank suppressor cells; in the absence of strong inflammatory cytokines (i.e. IFN-γ, IL-12), they express the demethylated form of TSDR stabilising FOXP3, high OX40, CD39 and Helios expression, and a lack of T-bet and IFN-γ (committed suppressing Tregs) (Fig.3.2b). They strongly suppress immune responses and facilitate tissue repair mechanisms, thereby contributing to the development of HCC.

TGF-β, a well-known immunosuppressive cytokine, has been shown to play an essential role in establishing immunological tolerance, principally via its critical role in both inducing iTregs to acquire the capacity to produce TGF-β themselves, and transdifferentiating iTregs into Th17 cells [105]. The TGF-β/SMAD pathway is a central regulator in chronic liver disease, contributing to all stages of disease progression from

initial liver injury through inflammation and fibrosis to cirrhosis, EMT and HCC [106] (Fig.3.2b). To date, efficient systems to inhibit Treg suppression in order to restore immunosurveillance are not available for clinical use, despite some attempts having been made to deplete Tregs at least partially in cancer patients, for instance, through targeting CD25, CTLA-4 or CCR4 on Tregs [107–109]. TGF-β, which is produced by various cell types including iTregs, monocytes/macrophages (e.g. KCs) and platelets, may be a promising candidate for neutralisation in order to convert "cold" into "hot" TME. Recently, stromal TGF-β signalling has been identified as a determinant of immune exclusion in TMEs, and the combination of TGF-β neutralisation and immunotherapy has been shown to induce complete responses in mouse models [110, 111]. These data paved the way to designing novel highly potent TGF-β inhibitors in order to selectively target principal oncogenic TGF-β isoforms and reverse TGF-β-mediated immunosuppression, thereby rendering tumours sensitive to IC blockade in pre-clinical models [112]. In addition, therapeutics that target TGF-β production or block its action are in early clinical trials (including phases I/II in glioma, non-small lung cell lung cancer, melanoma, renal cancer, HCC, etc.) and have shown promise to support the rationale of combining it with current ICIs in future clinical trials [113]. In addition, data on the metabolic profile of activated Tregs suggest that metabolic drugs may preferentially modulate Tregs compared to other T cells [114].

A second suppressor cell population providing an IRM during chronic HBV or HCV infection and which may potentially contribute to HCC progression is the suppressor $CD8^+$ T cell subset representing, historically, the most ancient population with suppression function described [115]. It has been demonstrated that virus-specific $CD8^+$ T cells can dampen virus-specific responses via the production of a powerful immunoregulatory cytokine, IL-10, thus contributing to the establishment of chronic low-level inflammation, representing a crucial substrate for HCC development [116, 117] (Fig.3.2b). If these viruses are intrinsically capable of directly regulating *IL-10* gene expression, as shown for the *Mycobacterium tuberculosis* [118, 119], this is an interesting issue to investigate. Altogether, these data suggest a rationale to block IL-10, even in combination with the current ICIs, to revert immunosuppression and enhance anti-viral or anti-tumour immune responses in chronic HBV or HCV infections and HCC.

Finally, some specialised myeloid cells can take the form of suppressor cells. For instance, it has been proposed that granulocytic myeloid–derived suppressor cells (gMDSCs) can be recruited into inflamed (HBV-infected) livers, where they produce the arginase-1-cleaving L-arginine, which is an essential fuel for T cell proliferation [120, 121]. The impoverished T cells can be restored by various compensatory pathways, including the increased uptake of amino acids, transferrin and phenylalanine, contributing thus to the establishment of chronic low-level inflammation and possibly resulting in HCC development in the long term. Under these conditions, it is tempting to hypothesise a rationale to counterbalance the immunosuppressive gMDSC effects, by the therapeutic administration of the compensatory molecules required to restore T cells, even in combination with the current ICIs, and enhance anti-viral or anti-tumour immune responses.

3.5 Concluding Remarks

In conclusion, the persistent HBV or HCV infections can undergo HCC, through the intervention of multiple, non-mutually exclusive, pathways involved in the modulation of immune responses favouring the establishment of a status chronic low-level inflammation. Therefore, HCC can be defined as a classical inflammation-linked cancer that is supported by the co-evolution of three major mechanisms: (*i*) the viral persistence; (*ii*) the chronic effect by a wide storm of inflammatory cytokines (i.e. IL-6, TNF-α, IL-18, IL-1β, TGF-β, IL-10); (*iii*) the failure of immunosurveillance principally due to T cell exhaustion by ICs, Wnt3a/β-catenin signalling pathway and different subsets of suppressor cells.

Acknowledgements This work was supported by the following grants: Associazione Italiana per la Ricerca sul Cancro (AIRC) (progetti "Investigator Grant" [IG]-2014-17 id. 15199 and IG-2017-22 id. 19939 to VB); The Accelerated Award 2018 (Project Id.22794); Ministero della Salute (Ricerca finalizzata [RF-2010-2310438 and RF 2010-2318269]); Fondazione Italiana Sclerosi Multipla (FISM) onlus (cod. 2015/R/04); Fondo per gli investimenti di ricerca di base (FIRB)-2011/13 (no. RBAP10TPXK); Istituto Pasteur Italia – Fondazione Cenci Bolognetti (grant 2014-2016); International Network Institut Pasteur, Paris – "Programmes Transversaux De Recherche" (PTR n. 20-16).

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Effects of HIV on the Tumor Microenvironment

4

Dima Dandachi and Fanny Morón

Abstract

Oncomodulatory viruses can affect the tumor microenvironment (TME) by triggering inflammation, suppressing apoptosis, initiating angiogenesis, altering tumor metabolism, and stimulating tumor cell signaling pathways, leading to tumor growth, proliferation, and invasion. The higher incidence of malignancies among people with HIV (PWH), despite the widespread use of antiretroviral therapy (ART), suggests a more complex relation than HIV-associated immune deregulation. Viral cooperation can have synergistic effect on tumorigenesis. The most relevant oncogenes involved in viral cooperation include the HIV-1-related Tat and Vpu genes, EBV LMP-1 and EBNA-2 genes, and Kaposi's sarcoma herpesvirus (KSHV) KIE2, Rta, and LANA genes. The TME in HIV-related malignancies is highly angiogenic and characterized by high microvessel density compared to sporadic cases. Tat protein, found in patients with HIV infection regardless of their immune status, has been widely implicated in the increased angiogenesis and has been a target of interest for therapeutic strategies. Similarly, HIV-1 matrix protein p17 can be detected in the plasma and tissues of PWH, including those treated with ART. Studies have found that p17 can cause dysregulation of the biological activity of different immune cells, is involved in aberrant angiogenesis, and exhibits an IL8 chemokine activity, activating multiple intracellular signaling pathways, promoting angiogenic responses in endothelial cells, and forming capillary like structures. In addition, several studies have demonstrated difference in the cellular immune components within the TME in patients with or without HIV infection, as well as cases in pre- and post-ART era. In this chapter, we review the existing literature about the role tumor microenvironment plays in the pathogenesis of HIV-related malignancies. Understanding the functions of each component of the TME and determining how these cellular and noncellular components contribute to tumorigenesis will impact the advancement of interventions and treatment in clinical oncology among PWH.

D. Dandachi
Division of Infectious Diseases, Department of Medicine, University of Missouri, Columbia, MO, USA
e-mail: dandachid@health.missouri.edu

F. Morón (✉)
Department of Radiology, Baylor College of Medicine, Houston, TX, USA
e-mail: fmoron@bcm.edu

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_4

Keywords

Angiogenesis · Computed Tomography (CT) · HIV-1 · HIV-associated Burkitt lymphoma · HIV-associated DLBCL · HIV-associated Hodgkin's Lymphomas · HIV-related Kaposi sarcoma · Magnetic Resonance Imaging (MRI) · Matrix protein p17 · Oncogenes · Oncomodulatory viruses · Primary Central Nervous System Lymphoma (PCNSL) · Tat protein · Tumor microenvironment · Tumorigenesis

4.1 Effects of HIV on the Tumor Microenvironment

4.1.1 Introduction

The major hallmarks of cancer include sustaining proliferative signaling and indefinite cycles of proliferation by evading growth suppressors and immune destruction, resisting apoptosis, activating invasion and metastasis, inducing angiogenesis, reprogramming of energy metabolism, genome instability and mutation, and tumor-enhanced inflammation. The tumor microenvironment (TME) contributes to the acquisition of cancer hallmarks and is a key factor in tumor initiation, progression, and metastasis [23]. The naïve stroma, in healthy individuals, functions as an important compartment to maintain the physiological homeostasis in normal tissues and has even anticancer properties through regulating immunosuppression and suppressing carcinogenesis. However, in the presence of cancer cells, the adjacent TME is transformed by various stimuli into a pathologically active niche that can support, potentiate, and accelerate tumor progression, and a protective milieu that limits the activity of anticancer treatment [11].

The pathogenesis of cancer is largely dependent on the interactions between cancer cells and its local and systemic microenvironmental components. Tumor and the microenvironment interactions are bidirectional and constant. The tumors can influence the microenvironment, and the microenvironment can regulate the cancer growth and evolution. Certain tumor-microenvironment interactions may initiate and drive circular chains of tumor progression–enhancing events known as vicious cycles [40, 42]. The essential elements of the TME include cancer-associated fibroblasts, myofibroblasts, neuroendocrine cells, adipose cells, immune and inflammatory cells, the blood and lymphatic vascular networks, and the extracellular matrix (ECM) [18, 40]. Understanding the functions of each component of the TME and determining how these cellular and noncellular components contribute to tumorigenesis will impact the advancement of interventions and treatment in clinical oncology.

Recent studies have established a link between several viruses and human carcinogenesis by a number of different mechanisms. Some of these viruses are essential to cancer development such as cervical cancer and human papillomavirus (HPV). Other oncoviruses contribute to cancer development like hepatitis B (HBV) and C viruses (HCV) and liver cancer. A third group of viruses exhibit indirect carcinogenic factors. Human immunodeficiency virus type 1 (HIV-1) is a good example of indirect carcinogenesis by immune suppression, leading to the activation of other latent tumor virus infections such as Epstein–Barr virus (EBV)-associated B-cell lymphoma [44]. Oncomodulatory viruses can affect the TME and influence the tumor behavior by triggering TME inflammation, suppressing apoptosis, initiating angiogenesis, stimulating tumor cell signaling pathways leading to tumor growth, proliferation and invasion, and altering tumor metabolism [4].

It is important also to note that while some viruses act as single tumor-promoting agents through a set of genes that are involved in oncogenesis, more evidence is emerging about the role of multiple viral cooperation to induce malignancy. Viral cooperation can be defined as the mechanism by which coinfecting viruses have synergistic or regulatory effects on tumorigenesis. It targets cancer cells as well as immune cells and nonimmune cells that form the TME. The concept of viral cooperation is particularly important in patients with HIV infection.

Besides the indirect cooperation of HIV and other oncogenic viruses through immune dysregulation, there is growing evidence of direct cooperative role of HIV-1 in tumorigenesis independent of its immunosuppressive effects. In addition, the possibility of being infected with more than one oncogenic virus is higher among people with HIV (PWH). The most relevant oncoviruses and corresponding genes involved in viral cooperation include HIV-1 (Tat, Vpu genes), EBV (latent membrane protein LMP-1, EBNA-2 genes), and Kaposi's sarcoma herpesvirus (KSHV), also known as Human gamma herpesvirus 8 (HHV-8) (KIE2, Rta, and LANA genes) [14]. In this chapter, we review the existing literature about the role tumor microenvironment in the pathogenesis of HIV-related malignancies.

4.1.2 The Role of Tumor Microenvironment in HIV-Associated Malignancies

The introduction of antiretroviral therapy (ART) has dramatically improved the outcomes of people with HIV (PWH). As the survival of people with HIV infection increases, malignant disease has been a major cause of death in this population [5]. HIV-related lymphomas are the most common malignancies in patients with HIV after the introduction of ART. However, the decline in cases of HIV-related diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL) has been less than what is expected, which suggests that the relation between HIV and lymphoma is more complex than HIV-associated immune deregulation. In addition, the rate of HIV-HL (Hodgkin lymphoma) is increasing despite the introduction of ART [39].

4.1.3 The Tumor Microenvironment in HIV-Associated DLBCL (HIV-DLBCL)

DLBCL is the most common form of HIV-associated lymphomas. Patients with HIV-DLBCL as compared to sporadic DLBCL have higher frequency of extranodal disease and prominent association with EBV and have worse prognosis [39]. Few studies have compared the TME between sporadic DLBCL and HIV-related DLBCL and found significant differences.

First, the TME in HIV-DLBCL is highly angiogenic and characterized by high microvessel density compared to sporadic cases. Angiogenesis is regulated by many angiogenic stimulators and inhibitors. Vascular endothelial growth factor (VEGF) is a primary factor in this process. An increase in VEGF expression has been seen across solid and hematological malignancies. However, VEGF must bind to the tyrosine kinase receptors endothelial growth factor receptor (VEGFR)-1 or VEGFR-2. Tat protein, an HIV-1 gene product, has been widely implicated in the increased angiogenesis in acquired immune deficiency syndrome (AIDS)-related malignancies, among other functions, and has been a target of interest for therapeutic strategies. Tat protein is found in patients with HIV infection regardless of their immune status [35].

HIV infection infects mainly CD4 T lymphocytes, dendritic cells, and macrophages, and does not target B-cells. However, it was established that a soluble form of Tat protein can be released from HIV-infected cells, has the capability to penetrate uninfected B-cells, and to work extracellularly on the microenvironment. This mechanism will not require direct infection of the tumor cells. Lazzi et al. demonstrated positive immunostain with anti-Tat monoclonal antibody in tissue sections of all 21 cases of AIDS-related lymphoma examined and only 2 had HIV DNA detected in cancer cells by PCR [27]. The presence of Tat protein in the DLBCL TME has been demonstrated in other studies as well [28, 39].

It has been established that Tat protein modulates VEGF and targets VEGFRs. The Tat-rich arginine and lysine sequence is similar to the sequences of potent angiogenic factors such as basic fibroblast growth factor (FGF), VEGF [37]. Similarly, to the proper ligand VEGF, Tat can bind with high affinity and activate downstream signaling at the VEGF receptor to the VEGFR-2/KDR, through phosphorylation, which in turn will increase angiogenesis. On the

other hand, VEGFR inhibitors blocked the binding of Tat to this receptor further support that binding occurs at the same sites as for the authentic VEGF [1, 27]. Moreover, Nyagol et al. also observed significant increase in microvessel density and CD34 expression, which is a marker for microvessel density, in HIV-1-positive DLBCL, suggesting that Tat might have a wider angiogenic role, related to the increased endothelial cell proliferation and augmented microvessel density [32].

Tat protein is not the only protein implicated in HIV and angiogenesis. HIV-1 matrix protein p17 is secreted by HIV-infected cells, can be detected in the plasma and tissues, and accumulates in lymph nodes germinal centers of PWH, including those treated with ART. Studies have found that p17 can cause dysregulation of the biological activity of different immune cells after an interaction of its functional epitope (AT20) with receptors expressed on target cells [8]. The hypothesis of p17 being involved in aberrant angiogenesis has been supported by the presence of p17 deposits in the nucleus of endothelial cells. Furthermore, p17 exhibited an IL8 chemokine activity binding with high affinity to IL-8-related receptors CXCR1 and CXCR2, activating multiple intracellular signaling pathways, promoting angiogenic responses in endothelial cells, and forming capillary-like structures [41].

EBV is strongly associated with DLBCL in patients with HIV. Some studies have shown a role for EBV in angiogenesis. EBV life cycle alternates between latent and lytic stages, although there is evidence that latent and lytic genetic expression can coincide. EBV-associated cancers were thought to be related to latency and long-lasting persistency. However, studies demonstrated that a subset of tumor cells contains the lytic form and that the lytic cycle gene expression enhanced VEGF translation or secretion and directly contributed to angiogenesis and carcinogenesis [24, 30]. EBV can also trigger the angiogenesis process by the EBV oncoprotein LMP1, which can induce VEGF through induction of COX2. EBV can also activate the alpha subunit of the hypoxia-inducible factor 1 (HIF1) through Siah proteins, which in turn will induce VEGF [26]. In addition, HIV-secreted p17 has also been shown to upregulate LMP1 expression. In the study by Taylor et al., high angiogenic activity in HIV-DLBCL correlated with EBV positivity, suggesting a relationship among HIV, EBV, or both and angiogenesis [39].

The immunohistochemical prognostic markers for sporadic DLBCL were not predictive in HIV-DLBCL, suggesting differences in the pathophysiology. Given the effect of HIV on cell mediated immunity, studies looked at the cellular immune components within the TME in patients with or without HIV infection, as well as cases in pre- and post-ART era. HIV-DLBCL was characterized by increased hyperproliferation and c-Myc rearrangements, fewer CD3+ T lymphocytes, markedly reduced T helper (CD4+) and T regulatory (FOXP3+) cells, and a disproportionately higher accumulation of CD8+ T cells. Tumor-infiltrating cytotoxic T lymphocytes (CTLs), identified by staining for TIA1, perforin, and granzyme B (GrB), demonstrated that TIA1+ CTL were more frequent among HIV-negative patients, whereas granzyme expression was significantly higher in HIV-DLBCL. In addition, cases expressing viral antigens EBV-related LMP and/or HIV-related p24 antigen contained a higher number of CD8+ T cells and cytotoxic T cells [39].

Whereas early initiation of ART should improve immunosurveillance and reduce the incidence of LMP1-positive, HIV-DLBCL cases without viral antigens appear able to avoid immunologic reaction and likely require additional strategies to improve immune surveillance [28]. In the post-ART era, Taylor et al.[39] showed that the number of CD4+ T cells was higher in specimens but still reduced when compared with sporadic DLBCL. There was no significant difference in the expression of other lymphocytic markers. Only a weak correlation was observed between blood CD4+ T-cell counts and those seen within the TME. A possible explanation would be that blood lymphocytes represent only 2% of the total lymphocyte pool and their composition differs from that within the organs [16].

PD1 and its ligand PD-L1 mediate an inhibitory pathway on activated T cells characterized

by diminished cytotoxicity and cytokine production, and increased apoptosis. Tumors can choose the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) immune checkpoint pathway to avoid host immune surveillance. T-cell exhaustion, present in chronic viral infections, is involved in cancer immune evasion seen in various cancers including DLBCL. In the study by Taylor et al. [39], CD279 (PD1) expression was seen on a higher proportion of HIV-DLBCL than in sporadic cases. CD274 (PD-L1) expression was similar in HIV-positive and HIV-negative cases. These findings correlated with other studies. For instance the TME in anal SCC and HIV status did not correlate with PD-L1 expression in anal SCC [43]. Similarly, another study evaluating the immune competence in patients, with or without HIV head and neck cancers, found no significant difference between cases and controls in PD-1 and PD-L1 expression. Overall, 62% had high PD-1 expression and 82% expressed PD-L1 within the TME [33]. These studies supported clinical investigations of PD-1/PD-L1 checkpoint inhibitors, irrespective of HIV status and that PWH should not be excluded from cancer immunotherapy regimens.

In a direct comparison of DLBCLs in cases with or without HIV infection, activated B-cell was substantially more common in HIV-infected (83%) than in HIV-uninfected (54%) cases. In the same study, EBV was detected in 63% of DLBCLs in HIV-infected cases, occurring almost exclusively in activated B-cell-DLBCL (74%) and rarely detected in sporadic DLBCLs (3%) [31]. These results were consistent with other studies demonstrating a coinfection rate with EBV or HHV-8 in 60% of the cases. Specimens that were positive for LMP1 and/or p24 contained a markedly higher number of GrB+ CTL than cases negative for either viral antigen. The authors concluded that cytotoxic cell infiltration of HIV-DLBCL appears to be dependent partially on the presence of LMP1 or p24 viral antigens [39].

Macrophages are a major target of HIV and a source of virus production. As viral reservoirs, macrophages play an important role in the tumorigenesis of HIV-related lymphoma and tumor progression. Overall, infected macrophages were found in 39% of samples, typically from ART-naive patients. HIV replication strongly represses CD163 expression, orientating macrophages toward a proinflammatory phenotype in HIV infection. However, there was coordinate expression of CD68 and CD163 in the HIV-DLBCL microenvironment, suggesting that the tumor-associated macrophages are alternatively activated (M2). Huysentruyt et al. found that 60% of HIV-associated lymphomas had macrophages stained for intracellular p24 protein, thus suggesting a role for these infected cells in the development of these tumors, possibly by their proinflammatory activities [25].

4.1.4 Imaging Primary Central Nervous System Lymphoma (PCNSL) in PWH

The differences in the TME among patients with HIV are also reflected in the differences, between patients with or without HIV infection, observed in imaging. PWH are at a higher risk of developing PCNSL, the most common histology type being DLBCL [13, 21]. PCNSL is one of the most common brain tumors in PWH and is an AIDS-defining malignancy [6, 20].Computed tomography (CT) and Magnetic resonance imaging (MRI) are the most commonly used imaging modalities. On noncontrast CT, brain lesions of PCNSL are of a similar or peripherally higher attenuation to the density of the brain gray matter (whiter) with associated surrounding edema of lower density on noncontrast CT (darker) (stars on Fig. 4.1). On contrast-enhanced CT, most lesions reveal enhancement, which can be solid or more commonly around the periphery in a "ring pattern" (arrow on Fig. 4.1b).

Although CT is usually the initial imaging study done, MRI is the modality of choice to evaluate PWH and brain pathology. PCNSL lesions in PWH differ when compared to their immunocompetent, sporadic non-EBV-associated counterpart dramatically on MRI [13]. PWH are more likely to have multiple lesions, hemorrhage, and necrosis within the tumor as

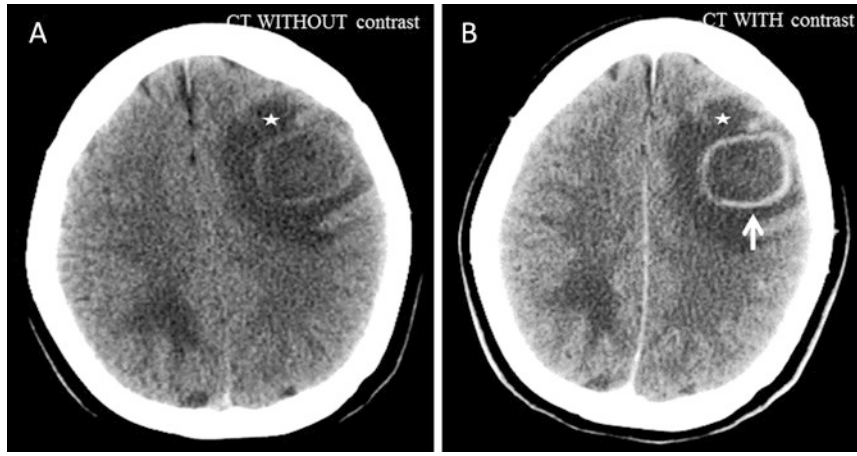

Fig. 4.1 Axial plane CT without contrast (**a**) and postintravenous contrast injection (**b**) show a rounded lesion in the left frontal lobe with an enhancing-hyperdense ring (arrow on **b**) and prominent surrounding hypodense edema (star on **a** and **b**). A subtle second lesion is seen in the right posterior parietal lobe

well as a pattern of contrast in the periphery "ring enhancement" along the solid component of the tumor on T1-weighted images (T1WI) (arrows on Fig. 4.2a). The lesions can be of similar, lower or higher signal intensity to the normal brain on T2-weighted images (T2WI). On diffusion-weighted images (DWIs), the lesions show increased signal intensity with low signal on the corresponding apparent diffusion coefficient maps (ADC). This corresponds to restricted diffusion or limited motion of the molecules of water within the solid component of the tumor (higher concentration of cells) [12]. Characteristically, the lesions are located in the deep structures of the brain (the deep gray nuclei and periventricular regions).

Tumors composition can further be evaluated by creating volumes of the different components of the tumor from the MRI, separating the central necrosis, the solid marginal enhancing tumor, and the perilesional edema; these correspond to the imaging phenotypes or radiophenotypes (color-coded on Fig. 4.2b, c, e, f). More specifically, the solid tumor, the area of enhancement (arrows on Fig. 4.2a and yellow on color coded fig), reflects the area of higher cellularity and increased vascularity with leakage of the contrast material, corresponding to the angiogenic nature of the tumor and vascular endothelial growth factor (VEGF) expression as previously described in more detail in this chapter. The area of increase signal intensity (white or bright) around the tumor on fluid attenuation inversion recovery (FLAIR) and T2WI sequences (stars on Fig. 4.2d and green on color-coded fig) correspond to edema and swelling, which correlates with previously mentioned macrophages proinflammatory phenotype in HIV infection. The central area of necrosis does not enhance and corresponds to liquefied, nonperfused tumor with dead cell (red on color-coded Fig. 4.2).

Routinely performed MRI additionally contains a large array of quantitative radiological data about features or characteristics of the tumors that is not being used, because they are beyond human visual perception. The evaluation of these radiologic features – characteristics from the images through data – and characterization algorithm corresponds to radiomics. Quantitative macroscopic imaging features (radiophenotypes) are indirectly linked to microscopic tissue heterogeneity, microscopic composition, microenvironment, and molecular profile (genotype); large-scale artificial intelligence efforts are currently being made to further exploit this area of research [38].

Metabolic imaging can also aid in the diagnosis of PCNSL. Thallium-201 and 99Tc (m)-sestamibi single photon emission computed tomography (SPECT) scans and positron emis-

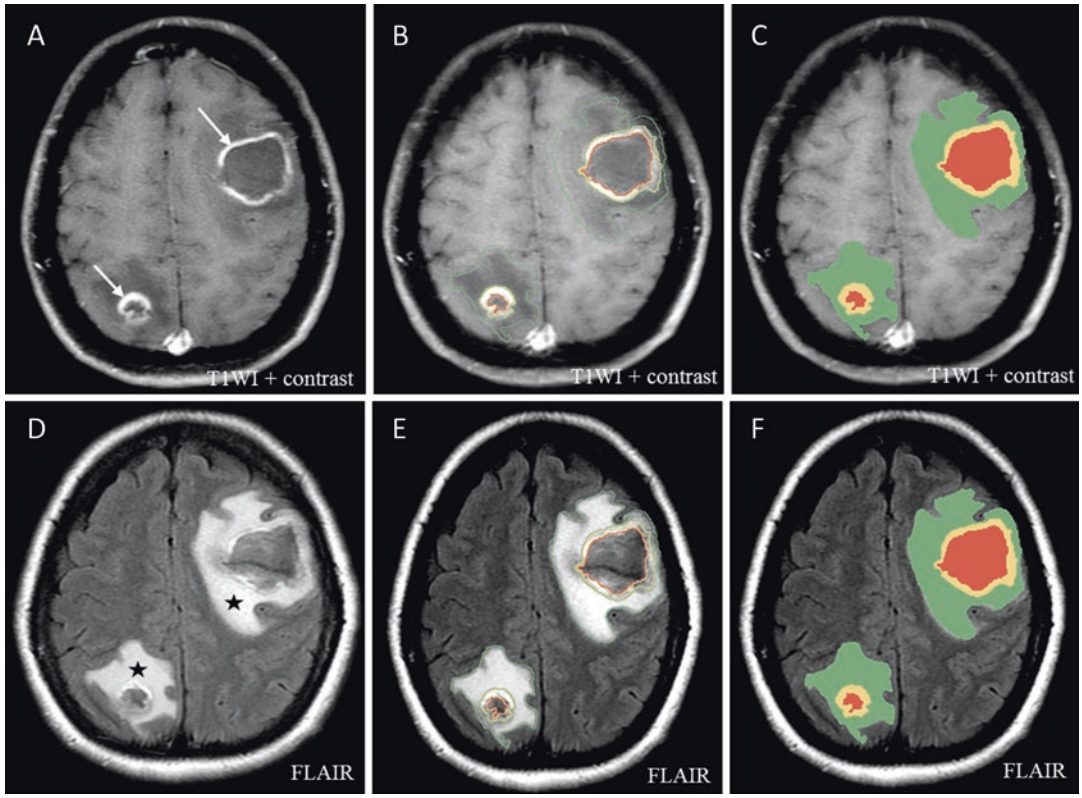

Fig. 4.2 Axial plane MRI, T1WI with contrast (**a**, **b**, **c**) shows the 2 peripherally enhancing tumors (arrows on **a**) better than the CT on Fig.4.1. Additionally the 3 components of the tumor can be separated with color-coded labels to *edema*, *solid tumor*, and *central necrosis*, creating the radiophenotypes (overlapped on **b** and **c**) that can then be used for feature analysis and indirectly obtain information about the tumor microscopic composition and environment with artificial intelligence models. The axial FLAIR (**d**, **e**, **f**) sequence at the exact same level as the T1WI clearly depicts the surrounding *edema* (stars on **d**) better than the T1WI on **a**. It allows very accurate segmentation of the *edema* volume (**e**, **f**)

sion tomography (PET) help differentiate PCNSL from other brain tumoral and infectious lesions (particularly from toxoplasmosis infection). PCNSL lesions shows increased isotope uptake on thallium SPECT and increased glucose utilization on 18-F fluorodeoxyglucose (FDG)-PET studies. Other advanced MRI modalities include MR perfusion; PCNSL demonstrates increased perfusion; and MR spectroscopy demonstrates increased choline metabolite, reverse choline/creatinine ratio, and markedly decreased N-acetyl-aspartate (NAA). Often the combinations of these modalities help confirm the diagnosis. Future imaging research is needed to close the outcome gap between PWH-related PCNSL and non-HIV population. Imaging modalities play a critical role in treatment plan, prediction of outcome, planning targeted therapies, and monitoring treatment response in neuro-oncologic patients [22].

4.1.5 HIV-Associated Burkitt Lymphoma

PWH are at increased risk for Burkitt lymphoma (BL), NHL B-cell malignancy. BL is not usually associated with EBV. BL occurs after a chromosomal translocation rearranging the c-MYC oncogene and the IgH heavy chain gene. This rearrangement is believed to be the result of an erroneous *IgH* class switch recombination (CSR).

IgH CSR is driven by exposure to various B-cell stimulatory cytokines and is mediated by the activity of activation-induced cytidine deaminase (AICDA), a DNA-modifying enzyme [7]. HIV infection is characterized by dysregulation of cytokine pathways, overproduction of B-cell stimulatory cytokines leading to chronic B-cell hyperactivation, and AICDA DNA modification errors and translocations. Several studies demonstrated an association between elevated serum levels of B-cell stimulatory molecules [IL6, IL10, CXCL13, and soluble CD23 (sCD23)] and immune activation molecules (sCD27, sCD30, sCD44, and IgE) in patients with HIV-associated lymphomas. Tat protein activated the expression of the AICDA gene. In vivo studies also found AICDA overexpressed in peripheral blood B-cells from PWH. In the presence of Tat, there was an increased rate of colocalization between IGH and MYC in B-cells nuclei and DNA damage was observed concomitantly in both MYC and IGH, followed by DNA repair by nonhomologous end-joining. These changes could be a plausible cause for the increased incidence of BL with PWH [36].

4.1.6 HIV-Associated Hodgkin's Lymphomas

In 20–40% of classical Hodgkin lymphoma (HL) cases, Hodgkin and Reed–Stenberg cells carry a monoclonal infection by EBV. In these cases, EBV shows a latency type II infection pattern with the expression of LMP-1. In PWH, Hodgkin and Reed–Stenberg cells almost always harbor EBV genome and express the viral oncoprotein LMP-1, resulting in different pathogenic pathways including genetic alterations, and interactions with critical microenvironmental components [10].

A significant difference in the TME of HIV-positive HL cases compared to HIV-negative cases is the reduction in number of CD4 T lymphocytes, stable number of CD8 cells, and inverted CD4/CD8 proportion. Similarly, a reduced number of functional natural killer cells and CD20+ B cells are seen in HIV-related HL. The matrix protein p17, encoded by HIV, can affect the immune cells, induce angiogenesis and lymphangiogenesis, and produce a microenvironment that might trigger tumor growth and maintenance as suggested by vitro and in vivo studies [17]. Recent data showed that a variant p17, called S75X, induces cell growth by triggering MAPK/ERK and PI3K/AKT pathways [3]. In addition, p17 promoted lymphangiogenic activity also on human lymph node–derived lymphatic endothelial cells (LN-LECs) relying on activation of an autophagy-based pathway. On the other hand, pharmacological and genetic inhibition of autophagy inhibits p17-triggered lymphangiogenesis. Also, the vasculogenic activity of p17 was totally inhibited in autophagy-incompetent mice [29, 34].

4.1.7 HIV-Related Kaposi Sarcoma

PWH have an increased incidence of Kaposi's sarcoma (KS). Although HHV8 or KSHV infection is necessary and often precedes the development of KS, HHV8 causes cancer only in defined populations including PWH. The pathogenesis of HIV-related KS is multifactorial. KS is an angioproliferative tumor of the skin, mucosa, and infrequently the viscera, characterized by presence of spindle cells of endothelial origin. The detection of HHV8 viral DNA in KS lesions provides some evidence supporting the role of HHV8 in the pathogenesis of KS. Mounting evidence suggests viral cooperation between HHV8 and HIV. Both viruses exert positive transcriptional effect on the other virus. HHV8 induced HIV replication in CD4 T cells, leading to increased viral load and more profound immune deficiency. Conversely, expression of Tat protein resulted in reaction of HHV8 [14]. HIV protein Tat was found to play a major role in the pathogenesis of HIV-related KS by increasing angiogenesis through activating VEGFR-2. In mice, basic FGF and Tat protein had synergistically modulated the VEGF and induced angiogenesis and histological changes seen in KS [19]. In addition, Tat stimulates macrophage and activated CD8+ T Lymphocyte production of IFN-γ,

which has angiogenic properties [9]. Tat protein also mimics the effects of the extramedullary matrix proteins fibronectin and vitronectin and increases the expression of matrix metalloproteinases. Inflammatory cytokines induce endothelial cells to acquire the phenotype and functional features of KS spindle cells [15].

Viral products from HHV8 have angiogenic activities, including chemokines, proliferative factors. The viral homolog of IL-6 has the potential to stimulate B cell growth and accelerate angiogenesis via VEGF-A induction [2]. The coinfection of HIV with HHV8, the synergy between Tat protein and cytokines, and the effect of Tat protein on tumor growth could partly explain the higher incidence and the more aggressive KS seen in PWH.

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GLI2-Mediated Inflammation in the Tumor Microenvironment

5

Weiguo Han, Shereen A. Allam, and Sherine F. Elsawa

Abstract

The tumor microenvironment (TME) plays an important role in the development and progression of cancer and has been shown to contribute to resistance to therapy. Inflammation is one of the hallmarks of cancer implicated in disease phenotype. Therefore, understanding the mechanisms that regulate inflammation in cancer and consequently how inflammatory mediators promote cancer progression is important for our understanding of cancer cell biology. The transcription factor GLI2 was initially identified as a member of the Hedgehog (HH) signaling pathway. During the last decade, studies have shown a novel mechanism of GLI2 regulation independent of HH signaling, where GLI2 consequently modulated several cytokine genes in the TME. These studies highlight a novel role for GLI2 as an inflammatory mediatory independent of HH stimulation. This chapter will discuss canonical and noncanonical pathways of GLI2 regulation and some of the downstream cytokine target genes regulated by GLI2.

Keywords

Bone marrow · GLI · GLI2 · Inflammation · TME · Tumor microenvironment · Waldenstrom macroglobulinemia

5.1 Introduction

Cancer cells are surrounded by a local microenvironment (tumor microenvironment; TME) that enables them to survive and persist within the host. This supportive microenvironment is composed of cellular and noncellular factors. The cellular component includes a variety of cells such as immune cells (B cells, T cell, myeloid cells, among others) and nonimmune cells, while the noncellular components include cytokines and growth factors that mediate the cross-talk between tumor cells and cells in the TME as well as the extracellular matrix. In cancers involving the bone marrow, such as Waldenstrom macroglobulinemia (WM), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM), the bone marrow microenvironment also involves fibroblasts, mesenchymal cells, endothelial cells, adipocytes, bone-related cells such as bone-related cells such as osteoblasts and osteoclasts. The interaction between the TME and cancer cells is important for the development and maintenance of cancer cells and is therefore an inte-

W. Han · S. A. Allam · S. F. Elsawa (✉)
Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, NH, USA
e-mail: sherine.elsawa@unh.edu

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_5

gral part of cancer cell biology. This interaction can also promote resistance to therapy. Therefore, understanding the mechanisms by which the TME promotes cancer cells is important for the development of therapies that not only target cancer cells, but also target the TME. This chapter will focus on a novel role for the transcription factor GLI2 in regulating the expression of various inflammatory cytokines in the TME, independent of signaling from the Hedgehog (HH) pathway.

5.2 Inflammation and Cancer

A correlation has long been observed between incidences of cancer and the presence of inflammation [34]. The tumor microenvironment is a major site of cytokine and chemokine production by immune and nonimmune cells. These cytokines serve different functions in the TME. Many of them promote an inflammatory TME. Tumor-promoting inflammation is a characteristic observed in many cancers [38] where inflammation plays an essential role in cancer cell initiation, proliferation, invasion, and metastasis. Indeed, an inflammatory microenvironment is now considered as a hallmark of cancer [38, 64]. The quantitative evidence linking inflammation to cancer growth now shows that inflammation has a direct link to tumor growth. An estimated 15–20% of all cancer-related deaths stem from inflammation and secondary infections [64]. Tumor-promoting cytokines, which are produced by immune/inflammatory cells, induce genes that stimulate cell proliferation and survival, and activate transcription factors such as NF-kB, STAT3, and AP-1. The activation of transcription factors is considered a major tumor-promoting mechanism [35]. NF-κB has also been reported as a key molecule that is involved in inflammation, tumor promotion, and tumor progression [48, 49]. NF-κB can regulate the expression of many genes, which can suppress tumor cell death, stimulate cycle progression, and enhance epithelial-to-mesenchymal transition (EMT) (which plays an important role in tumor invasiveness). In addition, NF-κB can provide newly emerging tumors with an inflammatory microenvironment that supports the progression of cancer cells, their abilities to invade surrounding tissues, angiogenesis, and metastasis [60]. Therefore, NF-κB can impact cancer cells directly and indirectly through regulation of expression of inflammatory cytokines, which ultimately affects cancer cell biology.

There are a variety of cytokines produced by immune cells that are involved in tumor development and progression. Among these cytokines, tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) have been reported, as outlined below. TNF-α produced by tumor cells or inflammatory cells in the TME can promote tumor metastatic growth, and this promotion depends on the NF-κB activation in tumor cells [61]. TNF-α can also cooperate with other proinflammatory cytokines and, together, they contribute to tumor promotion. The enhanced production of the tumor-promoting cytokines IL-6 and TNF-α causes hepatic inflammation and activation of STAT3 to promote hepatocellular carcinoma (HCC) development in mice [70, 71]. These studies report that TNF-α, as a proinflammatory cytokine produced by both host and tumor cells, plays an important role at different stages of cancer. The inflammatory cytokine IL-6 can mediate the differentiation of lymphocytes, promote cell proliferation, and support cell survival despite apoptotic signals [39, 47]. IL-6 plays an important role in normal B cell survival and regulates immunoglobulin secretion by normal B cells in response to antigenic stimulation [52, 53]. The role of IL-6 is also evident in hematological malignancies, particularly B-cell neoplasms, where IL-6 is produced by both malignant B cells and bone marrow stromal cells [27, 28, 44]. In the plasma cell cancer multiple myeloma (MM), IL-6 plays an important prosurvival role for MM cells [1, 2, 4, 30, 40]. IL-6 signaling through IL-6R/STAT3 contributes to the pathogenesis of MM [9], and blockade of the IL-6R/STAT3 signaling pathway leads to the induction of apoptosis in MM cells [15]. Additionally, IL-6 secreted by bone marrow stromal cells in MM TME can enhance the interaction between MM cells and the TME and promote the proliferation of MM

cells [16]. In the bone marrow TME, IL-6 has been reported to be regulated by the chemokine CCL5, at the level of both gene expression and protein secretion in stromal cells [28]. This CCL5-mediated regulation of IL-6 occurred through the chemokine receptor CCR3 and resulted in activation of the PI3K/AKT signaling pathway. Ultimately, this increased the expression of the transcription factor GLI2, which regulates IL-6 expression and secretion. IL-6 then induces immunoglobulin (Ig) secretion by malignant B cells within the surrounding bone marrow microenvironment [28]. Subsequent studies showed that the IL-6Rα subunit, which binds IL-6 to activate the IL-6 signaling pathway, is a downstream target of the transcription factor GLI2 in malignant B cells [44]. This regulation was found to promote IgM secretion in the B-cell lymphoma Waldenstrom macroglobulinemia (WM) [44].

The inflammatory environment surrounding developing cancer cells is often proliferated by leukocytes. Leukocyte populations are attracted to the chemokines and cytokines made by the tumor cells [20, 32]. The development of better cell markers makes it possible to detect specific immune cells and identify their cytokine secretion profiles. This has led to the finding that almost every tumor type, including the tumor microenvironment, contains a variety of immune cells. The presence of these cells varies, with certain detection only possible through cell type–specific antibodies, while others are easily identified with standard staining techniques. Traditionally, inflammation caused by the immune cells was considered to be a method to enable the body to fight and eliminate cancer cells. There is supporting and increasing evidence that this inflammatory immune response affects the growth of certain tumors.

The continued study of inflammation and cancer has also produced evidence that certain tumors benefit from the inflammatory response, and that cells of the immune system may aid in the development of certain cancers. Research into inflammation as it relates to the development and progression of cancer has provided abundant evidence that bioactive molecules in the inflamed tumor microenvironment can be utilized by tumor cells [55]. This utilization can lead to the development of the well-known hallmarks associated with cancer. Tumor cells may use cytokines for unchecked proliferation, survival factors to limit apoptosis and autophagy, enzymes to aid in the creation of new blood vessels, and other signals that trigger the activation of epithelial-to-mesenchymal transition (EMT) and metastatic traits [23]. Inflammation has been observed in the earliest stages of tumor growth, and it has been demonstrated that this inflammation can directly benefit a tumor cell's development into full-blown cancer. Additionally, certain cells of the immune system contain reactive oxygen species (ROS) that can be released into the tumor microenvironment [34]. These reactive molecules are mutagenic by nature and can accelerate the genetic development of cells into fully malignant cancer cells [35].

5.3 Hedgehog (HH) Signaling

The Hedgehog (HH) signaling pathway is an integral mechanism of embryonic development, cell maintenance, and tumor development [83]. There are two transmembrane proteins that are involved in and control the HH signaling pathway: Patched-1 (PTCH1), which acts as HH signaling ligand-binding receptor, and Smoothened (SMO), which is the signal transduction component of HH receptor. In the absence of HH ligands, PTCH1 receptor blocks the function of SMO and maintains it in an inactive state. However, if any of the three HH ligands (Desert, Indian, and Sonic HH) binds with PTCH1, the inhibition of SMO by PTCH1 is alleviated, thereby allowing SMO to become active. This inhibition of SMO then initiates and transduces signaling, resulting in the activation of GLI transcription factors [26, 43, 85, 86].There are three members of the GLI family of transcription factors (GLI1-3) that act together in response to signaling from Hedgehog (HH) and other signaling inputs, resulting in the regulation of target genes' expression and, ultimately, a change in cellular activities [81]. In response to HH signaling,

GLI1 and GLI2 are known transcriptional activators, whereas Gli3 is a transcriptional repressor [83].

As the downstream components of HH signaling, GLI proteins are posttranslationally modified and are normally suppressed/sequestered in the cytoplasm where they are prevented from translocating to the nucleus. This inactivation is achieved by interaction with cytoplasmic proteins, including the proteins Fused and Suppressor of Fused (Sufu). Activation of the HH signaling pathway initiates a signaling cascade that leads to activation of GLI proteins and their release from the GLI–Fused–SUFU complex. This allows GLI proteins to translocate to the nucleus to regulate gene expression. Nuclear GLI proteins proceed along the transcription factor function and activate target gene expression, including PTCH and GLI genes. Some other target genes are regulated, including those that are involved in controlling cell proliferation, such as cyclin D, cyclin E, Myc, and components of the EGF pathway and, in angiogenesis, such as components of the platelet-derived-growth-factor and vascular-epithelial-growth-factor pathway [26]. Cytokines in the BM microenvironment are also targets of GLI and regulate malignant cell growth and survival [27, 36, 44, 65, 93]. HH-GLI signaling pathway is also involved in cancer development. Mutations in Sonic HH have been reported to cause basal cell carcinomas in mice, suggesting that HH may have a role in tumorigenesis in humans [68]. For example, constitutively active mutations of SMO have been found in basal cell carcinoma, and GLI1 was originally identified in human glioma [51]. Moreover, ectopic expression of transcription factor GLI1 in the embryonic frog epidermis results in tumor development and induces formation of basal cell carcinoma [21]. Similarly, overexpression of GLI1 in mice induces basal cell carcinoma as well as other follicle-derived neoplasias, such as trichoepitheliomas, cylindromas, and trichoblastomas [67]. In addition, overexpression of GLI2 in the skin also induces basal cell carcinomas in mice [33].

5.4 GLI2-Mediated Regulation of Inflammation in the TME

In response to activation of the HH signaling pathway, GLI2 target genes are genes that regulate cell cycle progression, cell proliferation, and cell survival [66]. As such, GLI2 plays a significant role in the development of cancer in the context of HH signaling. However, several studies implicate GLI2 in the regulation of inflammatory cytokines and cytokine receptors in the TME (Fig. 5.1). This GLI2-mediated regulation of cytokines in the TME can promote cancer cell biology and can result in the spread or the maturation of cancer cells [27, 31, 36]. The cytokine transforming growth factor beta 1 (TGF-β1) is directly linked to the expression of GLI2 across various healthy and cancerous cell lines in humans [24]. TGF-β induces the expression of GLI2 in human cell types, including normal fibroblasts and keratinocytes, as well as various cancer cell lines. This induction is rapid, independent of HH signaling and requires SMAD transcription factors, which are specific to TGF-β1/TGF-β receptor (TGF-βR) signaling. This evidence suggests that TGF-β1 is a strong transcriptional inducer of GLI2 [24], highlighting a novel signaling pathway that can regulate GLI proteins through TGF-β/TGF-βR signaling independent of HH signaling. This finding is significant as inhibition of HH signaling may not be an efficient way to inhibit these transcriptional effectors. Therefore, a thorough understanding of additional pathways that can regulate these proteins is important for the development of novel inhibitors for GLI rather than HH. There is also evidence that GLI2 modulates the expression of TGF-β1 by modulating the promoter of TGF-β1 [31]. GLI2 was found to modulate TGF-β1 expression in $CD4^+$ T cells by regulating the TGF-β1 promoter [31]. Furthermore, this GLI2-mediated regulation of TGF-β1 occurred via direct GLI2 binding to the TGFβ1 promoter and modulating its transcriptional activity. This has implications for cancer cells, as regulatory T cells are associated with a poor prognosis in many cancers, and understanding their regulation and the potential role of GLI2 in these cells will be

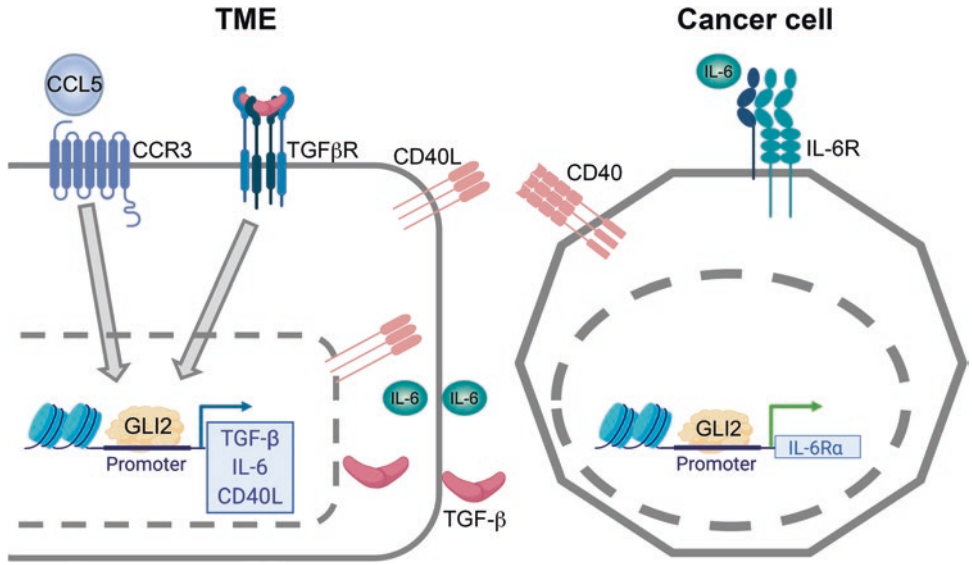

Fig. 5.1 GLI2-mediated inflammation in the TME. GLI2 can be activated by several mechanisms that are independent of HH signaling. CCL5/CCR3 signaling and TGF-β/TFG-βR signaling pathways regulate the expression and cellular localization of GLI2. Several downstream inflammation targets of GLI2 have been identified in TME, including TGFβ, IL-6, and CD40L. GLI2 regulates these targets expression by modulating their promoters' activities in the TME. Receptors for these cytokines are expressed on cancer cells and cells in the TME. The regulation between GLI2 and TGF-β is bidirectional: TGF-β signaling can activate GLI2 and GLI2 can also regulate TGF-β expression in the TME. In the TME, IL-6 and CD40L bind to their respective receptors on the surface of cancer cells to further initiate their own signaling cascades and ultimately lead to activation and proliferation of cancer cells. GLI2 was also shown to directly regulate IL-6Rα by modulating its promoter in cancer cells. Figure made with BioRender.com

important. The expression of GLI2 can also be regulated by signaling through the CCL5/CCR3 receptor in the TME [27]. In bone marrow malignancies, such as multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and the non-Hodgkin lymphoma (NHL) Waldenstrom macroglobulinemia (WM), bone marrow stromal cells play an important role in malignant B cell biology by secreting a variety of cytokines that are used by malignant cells to survive and proliferate [7, 12, 13, 18, 28, 45, 54]. We have reported that CCL5 levels are elevated in the sera of WM patients [27]. This elevated CCL5 can signal through the CCR3 receptor to regulate the expression of GLI2 in the TME through the PI3K/AKT signaling pathway. This results in the activation of NK-κB, which directly modulates the promoter activity and expression of GLI2 [27]. In addition to this PI3K/AKT regulation of GLI2 in the TME, there is support for the regulation of GLI2 by PI3K/AKT in cancer cells where overexpression of AKT in renal cell carcinoma increased GLI2 expression [94]. Therefore, in addition to canonical HH signaling, recent studies have identified TGF-β/TGF-βR signaling and CCL5/CCR3 signaling as novel pathways that can modulate GLI2 expression in the TME. In these studies, inhibition of HH signaling with the SMO inhibitor Cyclopamine had no effect on the regulation of GLI2 [27, 94]. These studies suggest that targeting HH is not the only mechanism to inhibit GLI functions, and directly targeting GLI therapeutically may be a beneficial strategy rather than targeting HH.

As mentioned earlier, in response to noncanonical HH signaling in the TME, GLI2 was shown to modulate a novel set of downstream cytokine targets. This includes TGF-β [31], IL-6 [27], IL-6Rα [44], and CD40L [36]. This GLI2-mediated regulation of cytokines was shown to exert a biological effect on cancer cells. For example, in response to CCL5/CCR3 signaling,

the regulation of GLI2 expression modulated the expression of IL-6 [27, 28]. IL-6 is a cytokine with a well-established role in normal and malignant B cells [52, 53]. Therefore, the identification of GLI2 as a novel regulator of IL-6 highlights an indirect role for GLI2 in modulating normal and malignant B cell biology. In the TME, GLI2-mediated regulation of IL-6 results in the regulation of IgM secretion by malignant B cells. Coculture of bone marrow stromal cells with GLI2 knockdown and malignant B cells results in reduced IL-6 secretion and IgM secretion both in vitro and in vivo [27]. Therefore, the regulation of inflammatory IL-6 by GLI2 in the TME modulates cancer cell biology. A direct role for GLI2 in modulating IL-6 signaling was also reported. In malignant B cells, GLI2 knockdown resulted in reduced IgM secretion [44]. Because IL-6 is known to modulate immunoglobulin secretion, an investigation of the mechanism by which GLI2 modulates IgM secretion identified the IL-6 receptor (IL-6R) as a novel transcriptional target of GLI2 [44]. Therefore, GLI2 performs a dual role in IL-6-mediated regulation of Ig secretion—one by regulating IL-6 expression and secretion in the TME and another by regulating the expression of the IL-6R on the malignant cells. Both scenarios described a novel role for GLI2 in regulating the expression of cytokine/cytokine receptor targets IL-6 and IL-6R. Additional screening of GLI2 cytokine targets in the TME identified CD40L as a novel GLI2 target gene. CD40L is a homotrimer that belongs to the tumor necrosis factor (TNF) gene family [76]. It is expressed by a variety of cells including activated T cells and platelets, monocytes, NK cells, B cells, endothelial cells, and stromal cells [29, 62, 76]. CD40L is expressed on the cell surface but can also be cleaved to release a soluble biologically active form [36, 78]. Engagement of the CD40 receptor on the surface of normal and malignant B cells by the CD40L activates several transcription factors including AP-1, NFAT, and NF-kB [80] and leads to increased growth and survival of several B cell malignancies [19, 36, 84]. Therefore, understanding the regulation of CD40L is essential to understanding malignant B cell biology. GLI2 was found to directly modulate the expression of CD40L in the TME [36]. This increased CD40L promotes malignant B cell activation and proliferation [36]. GLI2 was also found to increase the soluble form of CD40L in the TME, whereas conditioned media from bone marrow stromal cells expressing an active form of GLI2 increases malignant B cell proliferation [36]. Further investigations identified the CCR3-PI3K-AKT pathway to modulate this GLI2-CD40L pathway in the TME [36]. Therefore, these studies highlight a novel role for GLI2 in modulating an array of cytokines including TGF-β, IL-6, CD40L, and IL-6R, which ultimately contributes to malignant B cell biology by promoting malignant cell growth, survival, and Ig secretion. Future studies investigating the role of GLI2 in modulating cytokine genes in other malignancies will be important for understanding the role of GLI2 in regulating inflammation in the TME. Additionally, a small-molecule antagonist for GLI (GANT61) was developed and can inhibit GLI1/GLI2 expression and activity. A preclinical evaluation of GANT61 will be necessary to delineate its utility in cancer patients. Furthermore, evaluating the efficacy of targeting GLI using GANT61 to target the TME will allow us to target GLI2-mediated inflammation in the TME and therefore reduce the effect of inflammation on cancer cells. A recent study showed that targeting IL-6 in the TME reduced tumor growth and Ig secretion by lymphoma B cells [37]. Perhaps, targeting GLI2 in the TME will reduce IL-6 and other cytokines, and combining it with therapies that target cancer cells such as Ibrutinib may be beneficial in reducing the effect of the supportive TME on cancer cell biology.

5.5 Therapeutic Implications

The role of HH signaling pathway is well established in embryonic development and in cancer [88]. Inhibition of Smoothened (SMO), the signal transduction subunit of the Hedgehog (HH) receptor, initially identified GLI2 as a transcriptional activator and has been shown to abrogate leukemia stem cell dormancy and reduce the dor-

mant leukemic stem cell burden using a clinical antagonist [75]. This suggests that SMO inhibition can provide therapeutic efficacy for cancer patients. As a result, several small-molecule inhibitors for SMO have been developed and tested in the clinical setting to treat solid tumors and hematological malignancies either as single agents or in combination with other chemotherapeutic agents [17, 92]. To date, two Smoothened inhibitors (Vismodegib and Sonidegib) have gained FDA approval, but are limited to locally advanced and metastatic basal cell carcinoma patients [73, 90]. However, the use of these inhibitors as single agents in cancer patients has not elicited the anticipated clinical responses. This may be in part due to resistance to SMO inhibitors [3, 22, 42, 79, 90], which have been identified by several studies in animal models [5, 90, 92]. One of the mechanisms of resistance is in other signaling pathways that are SMO independent, which can regulate the activity of GLI proteins, the effectors of this pathway. There is also evidence supporting a role of GLI2 inhibition in basal cell carcinoma [46]. Therefore, although the activity of GLI proteins through SMO is disrupted by the use of SMO inhibitors, these proteins remain active via other pathways such as PI3-K-mTOR, BET bromodomain proteins, PDE4, CCR3, and TGF-bR signaling pathways [6, 11, 24, 27, 36, 82, 87, 88].

Direct inhibition of GLI has gained interest and shows promise to target these transcription factors and their target genes. In malignant pleural mesothelioma, inhibition of GLI by siRNA, or a novel small-molecule GLI inhibitor, suppressed tumor cell growth dramatically both in vitro and in vivo. Furthermore, inhibition of GLI exhibited better cytotoxicity than inhibition of Smoothened, suggesting that inhibition of GLI function could be a strong and effective novel approach to treat malignant pleural mesothelioma [58]. In acute myeloid leukemia, inhibition of GLI by the GLI antagonist GANT61 causes growth arrest and apoptosis in human myeloid leukemia cell lines [69]. Direct targeting of GLI2 also reduced medulloblastoma cell survival [10]. A screen for small-molecule antagonists of GLI-mediated transcription revealed two molecules that can selectively inhibit GLI-mediated gene transactivation by acting in the nucleus to block GLI function. One of these molecules was found to interfere with GLI1 DNA binding in living cells. More importantly, these compounds efficiently inhibited in vitro tumor cell proliferation and successfully blocked cell growth in an in vivo xenograft model using human prostate cancer cells [56]. Arsenic compounds were found to induce developmental defects that are dependent on HH signaling [14, 25, 63]. In an effort to circumvent the problems associated with SMO resistance, arsenic trioxide (a chemotherapeutic agent used for acute promyelocytic leukemia) was found to antagonize HH pathway by targeting GLI proteins through blocking the ciliary accumulation and levels of GLI2 [50]. However, the role of arsenic trioxide as a GLI inhibitor has not been evaluated in the clinical setting.

There is ample research to support the role of the TME in cancer and targeting the TME may be an effective therapeutic strategy for cancer patients. The inflammatory cytokine IL-6 has a well-established role in several malignancies, particularly B cell malignancies. Several monoclonal antibodies targeting IL-6 or IL-6 receptor (Tocilizumab) were developed and tested in several diseases including malignant disease. In multiple myeloma (MM), targeting IL-6 in combination with other therapies enhanced therapeutic outcomes compared with chemotherapeutic agents alone [41, 74, 78]. In a xenograft model of WM, administration of Tocilizumab to tumor-bearing mice that were xenografted with cancer cells and cells from the TME reduced tumor growth and IgM secretion [37]. Targeting IL-6 was also investigated in autoimmune diseases to target chronic inflammation and was shown to provide clinical benefits (ref autoimmune papers). Therefore, targeting inflammatory mediators such as IL-6 can be successful, although it was only investigated in the context of the TME in WM, and to a lesser extent in MM (since IL-6 is provided in both an autocrine and paracrine manner in MM). IL-6 itself is a GLI2-target gene in the TME. Disrupting the cross-talk between cancer cells and cells in the TME using IL-6-based therapies showed promising results in tumor-bearing mice. However, IL-6 therapy alone did not affect mice survival [37], which leads to the

question of whether we can switch from targeting the genes regulated by GLI2 to targeting the transcription factor GLI2 itself.

Currently, several studies focus on targeting different transcription factors that play critical roles in cancer biology. Hypoxia-inducible factor 1 (HIF-1) regulates genes that are involved in cancer biology, including angiogenesis, cell survival, glucose metabolism, and invasion. In addition, in preclinical studies, the inhibition of HIF-1 activity has marked effects on tumor growth. Therefore, HIF-1 can be used as a potential therapeutic target [77]. Forkhead O (FOXO) transcription factors play an essential role in the regulation of cellular functions such as cell cycle arrest, cell death, and protection form stress stimuli, and inactivation of FOXO protein is associated with several neoplasms including breast cancer, prostate cancer, glioblastoma, rhabdomyosarcoma, and leukemia. Moreover, clinical studies have shown therapeutic benefits from using drugs like paclitaxel, imatinib, and doxorubicin that activate FOXO targets [91]. The transcription factor NF-ƙB is frequently in tumor cells and contributes to aggressive tumor growth and resistance to chemotherapy and radiation during cancer treatment. Accumulating evidence shows that induction of chemoresistance and radioresistance is mediated by several genes regulated by NF-ƙB, and the inhibition of NF-ƙB increases the sensitivity of cancer cells to chemotherapeutic agents and radiation exposure. Therefore, targeting NF-ƙB may be a potential therapeutic strategy to overcome chemoresistance and radioresistance in cancer treatment [57]. In addition, transcription factors from the STAT family (STAT3 and STAT5) are important players in human cancers and are validated targets for therapeutic intervention [59, 72].

5.6 Future Directions

While significant effort has focused on developing inhibitors for the HH pathway that target SMO, these inhibitors will likely only be effective therapies in neoplasms that are responsive to HH ligand stimulation. As evidence accumulates to show an HH-independent mechanism of regulation of GLI2, and consequently a role for GLI2 in regulating inflammation, novel inhibitors of GLI2 such as GANT61 and arsenic trioxide might be therapeutically beneficial to target GLI2-mediated inflammation. While directly targeting GLI2 in cancer cells may provide therapeutic efficacy, future studies focusing on targeting GLI2 to target inflammation in the TME will provide insight into the therapeutic utility of targeting this axis in cancer patients. Combined targeting of cancer cells, and cells in the TME (or molecules such as cytokines), which mediate the ability of cancer cells to survive and resist therapy, may prove to be a successful strategy for cancer patients.

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Stellate Cells in the Tumor Microenvironment

6

David Roife, Bhaswati Sarcar, and Jason B. Fleming

Abstract

As tumor microenvironments share many of the same qualities as chronic wounds, attention is turning to the wound-repair cells that support the growth of cancerous cells. Stellate cells are star-shaped cells that were first discovered in the perisinusoidal spaces in the liver and have been found to support wound healing by the secretion of growth factors and extracellular matrix. They have since been also found to serve a similar function in the pancreas. In both organs, the wound-healing process may become dysregulated and lead to pathological fibrosis (also known as cirrhosis in the liver). In recent years there has been increasing attention paid to the role of these cells in tumor formation and progression. They may be a factor in initiating the first steps of carcinogenesis such as with liver cirrhosis and hepatocellular carcinoma and also contribute to continued tumor growth, invasion, metastasis, evasion of the immune system, and resistance to chemotherapy, in cancers of both the liver and pancreas. In this chapter we aim to review the structure and function of hepatic and pancreatic stellate cells and their contributions to the tumor microenvironment in their respective cancers and also discuss potential new targets for cancer therapy based on our new understanding of these vital components of the tumor stroma.

Keywords

Hepatic stellate cell · Pancreatic stellate cell · Cirrhosis · Fibrosis · Hepatocellular carcinoma · Pancreatic adenocarcinoma · Metastasis · Alpha-smooth muscle actin · Vitamin A · Desmoplasia · Epithelial-mesenchymal transition · Sonic hedgehog

6.1 Introduction

Stellate cells have a long history in the records of pathology, as they were first identified in the liver by the pathologist Karl Wilhelm von Kupffer and described in a research letter in 1876. Kupffer described star-shaped cells that were stained with

D. Roife
Department of Surgery, University of South Florida Morsani College of Medicine, Tampa, FL, USA

Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center, Tampa, FL, USA
e-mail: David.Roife@moffitt.org

B. Sarcar · J. B. Fleming (✉)
Department of Gastrointestinal Oncology, H. Lee Moffitt Cancer Center, Tampa, FL, USA
e-mail: Bhaswati.Sarcar@moffitt.org; Jason.Fleming@moffitt.org

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_6

gold chloride in the perisinusoidal spaces in the liver, which he termed "sternzellen." These were later independently discovered by Toshio Ito as fat-storing cells of the liver and termed "Ito cells," but the two discoveries were not unified until Wake et al. confirmed the two cells to be the same in studies on the lamprey liver [1, 2]. Hepatic stellate cells (HSCs) have been studied under various names such as perisinusoidal cell, pericyte, lipocyte, interstitial cells, fat-storing cells, Vitamin A-storing cells, or Ito cells [3]. This clarification was a catalyst for the progression of research on hepatic stellate cells and their paramount role in liver physiology. Not to be confused with resident liver macrophages, also known as "Kupffer cells," hepatic stellate cells can be identified with storage of Vitamin A in the cytoplasm and are in fact the largest reserve of Vitamin A in the body [4]. Upon encountering signals from damaged hepatocytes and leukocytes, HSCs differentiate into an activated phenotype, losing Vitamin A storage droplets and gaining expression of alpha-smooth muscle actin (αSMA), taking on a contractile myofibroblast-like phenotype in order to deposit extracellular matrix (ECM) protein and heal areas of tissue damage, but this may also result in fibrosis [5, 6]. Activated HSCs also express markers such as desmin, glial fibrillary acidic protein (GFAP), and vimentin and begin to deposit extracellular matrix proteins such as fibronectin and collagen I/collagen III [7]. HSCs can be distinguished from myofibroblasts by their expression of cytoglobin, which is not expressed on myofibroblasts [8]. HSCs secrete cytokines and growth factors that contribute to the survival and regeneration of hepatocytes [6]. As part of their wound-healing abilities, HSCs have also been shown to be capable of phagocytosing apoptotic bodies, an activity that also contributes to fibrosis [9]. In chronic liver damage, ECM deposition becomes dysregulated in a positive feedback loop where excess ECM leads to liver fibrosis, distortion of hepatic vasculature, and eventually resulting in cirrhosis and portal hypertension [10]. Unlike most cancers, hepatocellular carcinoma (HCC) is usually preceded by this extensive fibrosis, rather than developing a desmoplastic reaction following carcinogenesis, and HCC is much more common in patients with liver cirrhosis than noncirrhotic patients.

Pancreatic stellate cells (PSCs) were not identified until 1982 by Watari et al. by observing mouse pancreatic cells with a phenotype featuring characteristic Vitamin A storage previously seen in HSCs in the liver [11]. However pancreatic stellate cells did not become a major target of research until methods were published to isolate and culture them 16 years later by Apte et al. [12] Since then PSCs have been found to be a major component of the source of pancreatic fibrosis in diseases such as pancreatitis and also the fibrotic component of pancreatic tumors [13–16]. Pancreatic stellate cells have been found to be akin to hepatic stellate cells, suggesting that they share a common progenitor in embryological development [17]. They share a similar star-shaped phenotype and also act as repositories for Vitamin A. They can be identified by their expression of desmin, glial fibrillary acidic protein (GFAP), vimentin, nestin, nerve growth factor (NGF), and neural cell adhesion molecule (NCAM) [18]. When they are activated, they exhibit a loss of the cytoplastic Vitamin A droplets and gain expression of alpha-smooth muscle actin (αSMA), taking on a myofibroblast-like phenotype, just as with HSCs. A major function of PSCs is to synthesize and secrete ECM proteins as well as the production of the degrading enzymes matrix metalloproteases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs) [19]. However, unlike in hepatocellular carcinoma, pancreatic ductal adenocarcinoma (PDAC) contains and typically features a more desmoplastic tumor, suggesting some differences in the ways PSCs respond to the tumor microenvironment [20]. In this chapter, we aim to review the ways in which HSCs and PSCs contribute to the tumor microenvironment in cancers of the liver and pancreas.

6.2 Hepatic Stellate Cells in Tumors of the Liver

In the primary hepatic malignancies hepatocellular carcinoma and cholangiocarcinoma (CCA), stellate cells have been identified as a major component of the tumor stroma [21]. As a major reparative cell in liver injury, HSCs secrete a multitude of cytokines and growth factors in order to support the liver during injury, and chronic injury may result in progressive terminal liver fibrosis, also known as cirrhosis. As most cases of hepatocellular carcinoma in Western countries arise in the setting of liver cirrhosis, the HSC has become implicated in carcinogenesis as well. Cytokine crosstalk between HSCs and hepatocytes and inflammatory signals from infiltrating inflammatory cells is likely to blame for dysregulation of hepatocyte growth and resulting cancer. As HCC is the most common form of primary liver tumor (90%), most research of HSCs in cancer is in the setting of HCC and that is what this review will focus on.

Activated HSCs produce a variety of growth factors and cytokines that promote the proliferation of normal and neoplastic liver cells. HSCs are known to secrete hepatocyte growth factor (HGF), epidermal growth factor (EGF), and TGFα/TGFβ, which stimulate hepatocyte proliferation [22]. These factors not only stimulate hepatocyte growth and division but also cause activation and proliferation in hepatic stellate cells through an autocrine fashion. Platelet-derived growth factor (PDGF) is secreted by HSCs, and this creates fibrosis in the liver parenchyma that is often seen before the development of hepatocellular carcinoma [23]. TGFβ causes an increase in HSC membrane expression of the PDGF receptor, which acts as a potent growth factor for HSCs, creating an auto-feedback loop that drives the progression of fibrosis [23]. TGFβ has been identified as a major proliferative signal for HCC cells that is secreted by HSCs [24]. TGFβ in association with laminin-5, both produced by HSCs, has been shown to induce the process of epithelial-to-mesenchymal transition (EMT) in HCC cells which is known to be essential to carcinogenesis [25]. Mikula et al. demonstrated that TGFβ secreted from HSC cells were crucial in the progression of hepatocyte neoplasia by inducing more paracrine TGFβ from the hepatocytes, causing cellular accumulation of beta-catenin, and the loss of E-cadherin cell-cell contacts between hepatocytes, which are hallmarks of EMT [24]. Conditioned media from cultured HSCs increases the proliferation of HCC cells, and in vivo models show that implanting HSCs at the same time as HCC cell increases tumor growth and invasiveness. The conditioned media was found to stimulate HSCs through activation of the MAPK/ERK pathway and increasing the transcription factor NFkappaB, all of which are essential for cell growth [26]. Activated HSCs also produce SDF-1 (aka CXCL12) and support hepatocyte growth through the SDF-1/CXCR4 axis. Aberrant signaling through the SDF-1/CXCR4 pathway can dysregulate hepatocyte growth by inducing the EMT process and creating dysplastic cells [27–29]. HSCs have also been shown to be capable of being pluripotent progenitor cells. Kordes et al. demonstrated a subpopulation of HSCs that express the stem/progenitor cell marker CD133 that were capable of differentiating into not only the typical myofibroblast-like cell such as an activated HSC but also endothelial-like cells and hepatocyte-like cells in vitro [30].

HSCs also have been shown to allow neoplastic cells to survive in harsh environmental conditions associated with solid tumors. Solid tumors often harbor areas that are hypoxic due to outgrowth of the tumor from feeding vessels and reliance on disorganized neovasculature, and they are often acidic in nature due to excessive glycolysis such as the Warburg effect [31, 32]. HSCs are sensitive to hypoxia and secrete factors that induce angiogenesis, and they stabilize new vessels during angiogenesis through direct contact [33]. HSCs have been found to secrete vascular endothelial growth factor (VEGF) and this in turn helps hypoxic cancer cells establish new blood supplies. In vivo orthotopic HCC models demonstrate higher levels of VEGF in tumors that were derived from injecting cancer cells and HSCs at the same time, and conditioned media from HSCs elicited tubule formation in

endothelial cell assays in a VEGF-dependent manner [34]. HSCs also combat hypoxic conditions by expression of hypoxia-inducible factor-1α (HIF-1α) and this is controlled by the hedgehog signaling pathway [35]. Coculturing HCC cell lines with HSCs under hypoxic conditions showed increases in proliferation, migration, and resistance to apoptosis through signaling with PDGF-BB, a subtype of PDGF [36, 37]. Acidic environments naturally accompany hypoxia in the tumor microenvironment, and hepatic stellate cells have been shown to allow the tumor to adopt to the acidic pH commonly found in hepatocellular carcinoma by becoming activated at low pH through ERK1/2 phosphorylation, which results in improved migration and subsequent metastasis for cocultured cancer cells, and these effects were inhibited by culture in the presence of a proton-pump inhibitor [38]. In the hypoxic and acidic liver tumor, HSCs act as instruments vital for survival of the tumor cells.

HSCs participate in immunomodulation of the tumor microenvironment and may elicit contradictory pro- and anti-inflammatory effects. HSCs attract leukocytes such as monocyte/macrophages and neutrophils by secreting macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1), and platelet-activating factor (PAF), which serve to perpetuate the inflammatory response and contribute to fibrogenesis [6, 39, 40]. HSC conditioned media also stimulated the production of the proinflammatory cytokine IL-8 in HCC cells, which acts in turn to attract more leukocytes and HSCs [26]. They increase tissue infiltration by monocytes and neutrophils, enhancing the inflammatory response to initial detection of a liver injury [41]. HSCs are also able to present antigen via CD1, MHC I, and MHC II to NKT, CD4, and CD8 T helper cells. The presentation of antigen elicits a T-cell response that enhances local inflammation and fibrosis, especially in the event of CD8+ T-cell activation, making the environment more conducive to tumorigenesis [42]. On the other hand, HSCs are also able to create an immunosuppressive environment which may help tumors evade recognition by the immune system. HSCs have been shown to attract myeloid-derived suppressor cells (MDSCs) [43, 44]. The MDSCs, in turn, prevent activation of T-helper cells and increase the population of regulatory T-cells (Treg), all of which enable continued evasion of the immune system by the neoplastic cells. Activated HSCs also produce the immune checkpoint B7-H1 on the cell membrane, which is related to programmed death ligand-1 (PDL-1). B7-H1 binds to PD1 receptors on T-cells and inhibits the T-cell response, affording the tumor cells another route to evasion [45].

Hepatic stellate cells also aid HCC cells in resistance to chemotherapy. One of the only chemotherapeutics for hepatocellular carcinoma, the tyrosine kinase inhibitor sorafenib, is commonly met with resistance and disease progression. Studies have shown sorafenib to only offer 2.8 months of added overall survival versus no treatment, and while the initial disease control rate is 30–43%, resistance commonly occurs at around 6 months of treatment [46, 47]. Azzaritti et al. found that HSCs, through secretion of the laminin (Ln)-322, not only enhance HCC migration and invasion but also confer resistance to sorafenib [48]. Song et al., in coculture experiments with HCC cell spheroids, found that coculture with HSCs conferred strong resistance to sorafenib and cisplatin [49]. Conditioned media from activated HSCs has also been shown to create resistance of cholangiocarcinoma cells to chemotherapy such as 5-fluorouracil and gemcitabine in vitro [50]. Therefore targeting stellate cells may make HCC more sensitive to chemotherapeutics.

HSCs have also been implicated in tumor formation in cholangiocarcinoma (CCA), so HSC growth factors can act in a similar way to biliary epithelial cells compared to hepatocytes. CCA patient samples that had higher levels of HSC-related markers such as high αSMA, desmin, and GFAP were associated with a poor prognosis [51]. In vitro and in vivo data suggest that HSCs induce proliferation, migration, and invasion of CCA cells through signaling with the hedgehog pathway [50–52]. A vital mechanism for this tumorigenic crosstalk in cholangiocarcinoma is the release of the growth factor SDF-1 by HSCs

and its subsequent binding to CXCR4 expressed by CCA cells, causing tumor cell proliferation and migration [29, 53]. Okamoto et al. also described the presence of angiotensin II in patient samples of intrahepatic cholangiocarcinoma and found it to be effective in causing HSC activation and concomitant increases in secreted SDF-1, which then elicited features of epithelial-to-mesenchymal transition (EMT) in cholangiocarcinoma cells [54]. Taken together it is clear that HSCs have an important role in the progression of cholangiocarcinoma and not only hepatocellular carcinoma.

In addition to primary liver tumors such as hepatocellular carcinoma and intrahepatic cholangiocarcinoma, HSCs have been implicated in liver metastases from distant organs. Many cancers have a predilection for metastasizing to the liver, including breast, colorectal, esophageal, lung, melanoma, pancreatic, and gastric cancer [55]. Stephen Paget was the first to propose the "seed and soil" hypothesis, in which he postulated that anatomical drainage patterns were not the lone factor in determining where cancers metastasize. The analogy "When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil" describes his rationale for his observations in a series of patients with metastatic breast cancer [56]. HSCs may be one of the factors preparing the "congenial soil" of the liver. Tumor cell binding to distant sites is the first step in the initiation of a metastatic tumor, and HSCs provide binding sites through multiple methods. In in vitro experiments, Antoine et al. demonstrated that activated HSCs express E-selectin, and that under hypoxic conditions, this is released into the supernatant as E-selectin ligand (ESL-1) and retains cell-binding ability. These binding sites for both leukocyte and tumor cells may be an important factor generation of a metastatic tumor [57]. In pancreatic adenocarcinoma, which commonly metastasizes to the liver, tumor cells release exosomes that are taken up by resident liver macrophages, also known as Kupffer cells, which in turn release TGFβ that causes activation of HSCs which then release fibronectin. This deposition of fibronectin and creation of a profibrotic environment traps more hematopoietic leukocytes and forms a premetastatic niche which allows a metastasis a place to anchor and grow [58]. PDAC cells also prepare the liver for metastasis by secretion of tissue inhibitor of metalloproteinases-1 (TIMP-1) which is another route for activation of HSCs by binding the CD63 receptor and subsequent production of SDF-1 [59]. There has also been evidence that quiescent HSCs are able to maintain a quiescent phenotype in metastatic PDAC cells and that transformation to activated myofibroblasts promotes metastatic outgrowth [60]. The aggressive primary skin cancer melanoma also has a tendency to metastasize to the liver, and in vitro experiments have found that conditioned media from melanoma cell lines increased the proliferation and migration of HSC cell lines, upregulated production of VEGF and inflammatory cytokines COX-2 and PGE2, and that cell lines derived from metastatic melanoma had greater proliferative effects than cell lines from primary melanomas. This implies that metastatic melanoma cells may initiate liver metastases by attracting HSCs which in turn create stroma for which to support tumor growth [61, 62]. Uveal melanoma, the aggressive ocular version of malignant melanoma, has a high likelihood of metastasis to the liver and interactions between uveal melanoma and HSCs to create a proinflammatory environment and promotes metastatic progression [63]. Colon cancer is another tumor that often metastasizes to the liver. Animal models of colorectal liver metastasis have suggested that HSCs surround metastatic liver deposits and that they secrete SDF-1, HGF, and PDGF, which also has been shown to increase proliferation and resistance to apoptosis in colon cancer cell lines [64, 65].

6.3 Pancreatic Stellate Cells in Tumors of the Pancreas

Similar to hepatic stellate cells in morphology and function, pancreatic stellate cells have also become known to be an important mediator in pancreatic fibrosis and response to cancer development. Apte et al. demonstrated that acti-

vated PSCs were a major source of ECM proteins as evidenced by αSMA mRNA and collagen mRNA being restricted to the tumor stroma and that they were the main source of collagen in the tumor stroma of PDAC [15]. As with HSCs in HCC, PSCs may also play a role in cancer development in the pancreas, as PSCs have been found to surround pancreatic intraepithelial neoplastic lesions (PanINs), a precursor lesion to PDAC [66]. However there continues to be debate on whether the role of PSCs and their resulting fibrosis in PDAC is protective or deleterious to the patient, and conflicting findings have been reported in the literature. There is no clear answer at this time, and it is likely that both possibilities are true at different stages of the disease process [67].

History of alcohol abuse and tobacco use have been linked to a higher risk of developing PDAC. Alcohol was demonstrated to directly activate PSCs in in vitro experiments, but the effect is more striking in combination with byproducts of tobacco smoking. PSCs have nicotinic acetylcholine receptors, and results from culture experiments combining ETOH and byproducts of tobacco smoking showed synergism in markers of activation [68]. Therefore PSCs may play a role in the higher risk of developing PDAC with a history of alcohol and tobacco abuse.

PSCs secrete a variety of growth factors and ECM proteins in order to support the neoplastic stroma of pancreatic cancer. This interaction goes both directions, as PDAC cells also can activate PSCs which creates a positive feedback loop. Cancer cells produce signals such as TGFβ, PDGF, and fibroblast growth factor (FGF) in order to stimulate ECM secretion and proliferation of PSCs [69]. PDAC cells also produce cyclooxygenase-2 (COX-2) which causes proliferation of PSCs [70]. PSCs secrete a multitude of growth factors such as insulin-like growth factor, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and TGFβ1, all of which can stimulate cancer cell proliferation [71]. In addition to proliferation, PSCs may contribute to PDAC cell invasiveness by promoting epithelial-to-mesenchymal transition (EMT). Coculture experiments of PDAC cell lines and PSCs show decrease of epithelial markers and increase in migratory capability [72]. Secretions from PSCs have also been found to inhibit PDAC cell apoptosis [73]. In vivo experiments have shown that injection of PSCs along with PDAC cell lines results in larger tumors and greater metastatic events than injecting the PDAC cells alone. These studies, both heterotopic and orthotopic models of coinjection, showed an increase in tumor stroma compared to injection of PDAC cells alone, which better recapitulates the human PDAC tumor histology, as PDAC cell-line only tumors often have little associate stroma [69, 73–75]. Lumican is an extracellular proteoglycan that regulates collagen production and development [76]. We have previously shown that pancreatic stellate cells secrete the proteoglycan lumican into the stroma, and this molecule may have a protective role in the tumor microenvironment, as patients with lumican expression in the stroma had a better prognosis than patients without such expression [77, 78]. There is also a functional heterogeneity in the population of PSCs in the tumor stroma, as Ikenaga et al. have shown that a population of CD10+ PSCs in patient samples was associated with positive nodal metastases and shorter survival time, and these cells increase tumor growth and invasiveness in vitro [79].

The PDAC stroma is poorly perfused and has low oxygen tension, so in order for the cancer cells to survive, they are thought to be protected by the actions of PSCs. Hypoxia may be one of the factors that turn the microenvironment from restraining the tumor into enabling invasion and metastasis. PSCs have a dual role in this situation as they simultaneously work to relieve hypoxia by attempting to establish neovascularization by secretion of VEGF; however, their abnormal deposition of ECM creates continued fibrosis that prevents adequate perfusion [80]. PSCs have also been shown to increase PDAC cell invasion under hypoxic conditions compared to normoxic controls; this signaling which may direct PDAC cells to migrate away to better perfused areas also leads to the creation of metastases [81]. Autophagy is a method by which cells degrade

intracellular components to survive in times of metabolic stress, and it has been found that PSCs undergo autophagy in the tumor microenvironment and it contributes to their acquisition of an activated phenotype [82]. Hypoxia has also been shown to induce autophagy in PSCs and also increased the secretion of ECMs and VEGF [80, 83, 84]. Production of the protective ECM protein lumican is decreased under hypoxic conditions [85].

PSCs participate in immunomodulation of the tumor microenvironment by producing factors that influence local inflammation, such as TGFβ, TNFα, connective tissue growth factor (CTGF), monocyte chemoattractant factor-1 (MCF-1), IL-1β, IL-6, IL-8, IL-15, and RANTES [18, 86, 87]. Cytokines such as IL-8 attract neutrophils, and it has been found that neutrophils can, in turn, activate PSCs by the production of neutrophil extracellular traps (NETs), which continues the cycle of stromal activation and support of the cancer cells' proliferation [88].(p) PSCs also contribute to cancer cell immune avoidance by the production of galectin-1, which has been shown to induce apoptosis of CD4 and CD8 T-cells [89]. Galectin-1, which has also been shown to be highly overexpressed in PDAC stroma and animal models of PDAC cells coinjected with galectin-1-depleted PSCs, showed decreased tumor and metastasis formation [90]. Similarly, galectin-3 which is highly expressed by PDAC cells has been found to activate PSCs and stimulate proinflammatory cytokines such as IL-8 [91].

PSCs also aid in PDAC resistance to treatment by both chemotherapy and radiotherapy. Coculture experiments of PSCs and PDAC cells have shown that the presence of PSCs in culture decreased the level of apoptosis caused by the common chemotherapeutic gemcitabine [92]. There are multiple mechanisms that have been discovered for PSCs' role in chemoresistance. Zhang et al. demonstrated that PSCs protect PDAC cell lines from gemcitabine-induced apoptosis through secretion of SDF-1α and induction of FAK-AKT and ERK1/2 signaling pathways which resulted in an autocrine loop of proinflammatory cytokine IL-6 expression [93]. Another method PSCs confer gemcitabine resistance to PDAC cell lines by a mechanism reliant on HES-1 which is a component of the Notch signaling pathway [94]. In addition to paracrine signals, PSCs may protect PDAC cells by the physical sequestration of chemotherapeutic drugs. Using the KPC genetic mouse model and in vitro cell lines, Hessman et al. discovered that PSCs and cancer-associated fibroblasts (CAFs) scavenge gemcitabine from the tumor microenvironment leaving less active metabolite available for activity against cancer cells [95]. Liu et al. demonstrated that a ECM protein secreted by PSCs, periostin, is vital to this chemoresistance, making it an attractive target for new treatments [96]. Erkan et al. demonstrated that periostin increased markers of PSC activation and ECM secretion, and they also confer protection to chemotherapy and radiotherapy in vitro through paracrine signaling and activation of the Akt and MAPK pathways [97]. Mantoni et al. also found that PSCs can protect PDAC cells from radiation therapy through paracrine signaling in coculture in vitro experiments, and this protection was dependent on signaling via β1-integrin [98].

PSCs not only play a role in the local tumor microenvironment, but they have also been found to contribute to metastatic events in PDAC. Patients staining positive for activated PSC markers such as αSMA, desmin, and MMP2 in PDAC tumor stroma had a higher rate of lymph node metastases than patients who were negative for these staining patterns [99]. Multiple in vitro studies have shown that PSCs increased migration and invasion of PDAC cells through various secreted factors, which is the prerequisite for progression to metastasis [72, 73, 100–102]. Using a sex mismatch animal model, Xu et al. determined that PSCs accompany metastatic PDAC cells to distant sites, where they may aid in establishment of ECM and neovasculature [103]. Animal experiments with fluorescently labeled cells have shown PSCs associated with metastatic PDAC cells at every site of metastasis, which suggests that PSCs are necessary for the formation of metastases [104]. While it has never been shown for PSCs to travel in the circulation alone, it has been postulated that PSCs can travel along with circulating tumor cells (CTCs) in tumor micro-

emboli that have been found in human patients and animal models [105]. It is possible for PDAC cells to recruit PSCs from the circulation if they do indeed circulate alone, as was demonstrated by Zhang et al. in in vitro and in vivo experiments where PDAC exosomes from cell lines were able to recruit PSCs from the blood, so the cancer cells can recruit a supportive microenvironment in which to continue to grow [106]. Another mechanism for PSCs in metastasis is through the expression of galectin-1, which induces SDF-1 secretion through autocrine signaling, which in turn increases invasion and metastasis in in vitro and in vivo models [107]. Metastasis of PDAC is also by way of perineural invasion of tumor cells, and PSCs have been shown to facilitate perineural invasion by secretion of HGF and activation of the HGF/c-met pathway [108]. In addition to metastasis, PDAC has the ability to recur locally in approximately 20% of cases [109]. Recurrence may be influenced by the presence of cancer stem cells that are resistant to treatment [18]. PSCs were shown to induce the expression of stem cell-like genes, ABCG2, nestin, and LIN28, in pancreatic cancer cell lines during indirect coculture experiments [110]. It is clear that pancreatic stellate cells are important players in metastasis and both local and distant recurrence of pancreatic adenocarcinoma.

6.4 Pericytes

There is a ubiquitous population of stellate-like cells that surround blood vessels in all vascular tissue, referred to as pericytes. Hepatic stellate cells and pancreatic stellate cells are thought to be the pericytes in the liver and pancreas, respectively [111, 112]. Like stellate cells, pericytes generally express desmin, vimentin, glial fibrillary acidic protein (GFAP), and nestin [112]. Pericytes have been shown to encircle vascular endothelial cells along the length of blood vessels and interact with the blood vessel through signals and depolarization of nervous fibers, thereby affecting blood flow through capillaries [113]. Pericytes have a critical role in the central nervous system (CNS) by regulating blood vessel formation, maintaining the blood-brain barrier, and control of cerebral blood flow [114]. The pericyte population is heterogeneous, and two subtypes have been identified by Birbrair et al. Type 1 pericytes ($Nestin^{-}/NG2^{+}$) are fibrogenic and adipogenic in old and diseased skeletal muscle, while Type 2 pericytes ($Nestin^{+}/NG2^{+}$) generate new muscle tissue after injury [115]. As regulators of angiogenesis, pericytes have an important role in the tumor stroma. In a mouse model of glioblastoma, Type 2 pericytes, but not Type 1, were demonstrated to be recruited for tumor vessel formation [116]. Pericytes have been shown to be recruited to the tumor stroma in animal models of pancreatic cancer under the control of SPARC (secreted protein acidic and rich in cysteine). Mice that lacked SPARC had decreased pericyte populations in the stroma and reduced ECM proteins and reduced microvessel density, but an increase in permeability and perfusion with less hypoxia. The pancreatic tumors in these mice grew faster and exhibited more metastases, suggesting pericyte recruitment may act to restrain cancer growth [117, 118]. It has been suggested that pericytes and stellate cells arise from the same progenitors during development [119]. As a strategy to modulate the tumor microenvironment, it will be important to target the spectrum of mesenchymal cells including pericytes and stellate cells as well as other resident immune cells.

6.5 Targeted Therapies

Due to the central role of stellate cells in fibrosis in the liver and pancreas, and the hypothesis that this fibrosis is supporting cancer growth while inhibiting effective treatment, novel strategies to specifically target stellate cells are being developed.

There have been several pathways found to be present in activated stellate cells in the tumor stroma and targeted therapies have shown promising results in preclinical studies. Hwang et al. found that PSC proliferation was dependent on the hedgehog pathway, and by using a hedgehog pathway inhibitor AZD8542, tumor growth was

restrained, and these findings were also seen in models of prostate and colon cancer [75]. Another hedgehog inhibitor, cyclopamine, was also found to be effective in stopping metastases in a PDAC animal model and showed an additive effect in decreasing tumor size with gemcitabine [120]. Another target specific to stellate cells has been found to be tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Administering TRAIL in vitro has been shown to cause apoptosis of activated hepatic stellate cells in culture and also decrease ECM production [121]. HSCs express the receptor neuropilin-1, which was originally identified in central nervous system research as an axonal guidance protein. Recently a group has found that HSCs use this receptor as part of their activation process, and that silencing the receptor attenuates this response and inhibits the response of local tumor cells [122]. Recently a PSC-derived factor Dickkopf-3 (DKK3) has been found to stimulate PDAC growth, metastasis, and resistance to chemotherapy through a NFκβ-dependent pathway, and that inhibition of this PSC growth factor increased survival in an animal model, and this worked synergistically with immune checkpoint inhibition [123].

A growing trend has been the repurposing of existing medications as some of them have been found to have unexpected anticancer effects far removed from their original intention. Metformin, a commonly used diabetes medication, confers a better outcome to patients with PDAC, and the possible mechanism is that it has been found to reduce ECM accumulation and decrease activation of PSCs in cell line and animal models [124]. Recently a study has shown the anti-estrogen drug tamoxifen, commonly used in treatment for breast cancer, to be effective in halting the activation of HSCs through inhibition of the G-protein-coupled receptor [125]. Losartan, a common hypertension medication that works through the inhibition of angiotensin, has been found to reduce the density of PSCs in the tumor stroma in animal models and to reduce the collagen content of tumor, thereby improving drug and oxygen delivery [126]. Bisphosphonates, drugs which have been used to inhibit osteoclast-mediated bone resorption in order to treat osteoporosis, have also been demonstrated to inhibit PSC activation and proliferation, as well as reduce tumor size and fibrosis in an animal model, resulting in more efficacy of nab-paclitaxel treatment [127].

There are many small-molecule multikinase inhibitors on the market approved for the treatment of various cancers; however, they have not been used thus far in PDAC. In vitro assays of the MEK inhibitor trametinib and the PI3K/mTOR inhibitor dactolisib showed efficacy in preventing PSC proliferation; however, the tyrosine kinase inhibitor regorafenib did not affect proliferation, while it did still suppress PSC production of IL-6 and TGFβ1. Interestingly, the combination of dactolisib and trametinib showed an additive effect [128]. These results suggest that further study is warranted in the use of kinase inhibitors in PDAC.

Hepatic and pancreatic stellate cells characteristically store Vitamin A under quiescent conditions and lose their Vitamin A storage droplets during activation. In PDAC, for example, the patient may become deficient in Vitamin A due to a decrease in pancreatic secretions with resultant decrease in absorption of fat-soluble vitamins which includes Vitamin A. Froeling et al. showed that restoration of Vitamin A stores by supplementation with isoforms of retinoic acid induced PSC quiescence in vitro and also decreased tumor proliferation in a KPC mouse model [129]. Guan et al. also demonstrated that activated stromal cells from PDAC patients could be inhibited by retinoic acid treatment, which decreased EMT in cancer cells and decreased IL-6 secretion by the stromal cells [130].

A theory behind the lack of effectiveness of chemotherapeutics is that the dense tumor stroma limits perfusion and hence limits drug delivery to tumor cells nested in the stroma, so loosening the stroma would possibly improve drug delivery and anticancer effect. One such drug, pirfenidone, inhibits desmoplasia by decreasing the proliferation of PSCs and reducing the deposition of ECM in pancreatic tumors. In animal studies, it made chemotherapeutic treatment with gemcitabine more efficacious compared to either pirfenidone or gemcitabine alone, likely due to

increased penetration of the chemotherapeutic through the loosened stroma [131]. Certain chemotherapy drugs themselves have been found to target the stroma. Von Hoff et al. reported that nanoparticle albumin-bound paclitaxel (nab-paclitaxel, Abraxane) found depletion of the desmoplastic stroma in a patient-derived xenograft mouse model, and the resulting depletion of stromal cells resulted in an increase in concentrations of gemcitabine in the tumor [132]. This improved drug delivery with stromal depletion may account for some of the clinical benefit seen in the positive clinical trial of giving nab-paclitaxel with gemcitabine versus gemcitabine alone, with median overall survivals of 8.5 months and 6.7 months, respectively [133].

Thermal ablation is a treatment commonly used as a local treatment option for HCC tumors in the liver; however, this treatment is hampered by common recurrence due to incomplete thermal ablation of all viable tumor cells. Zhang et al. demonstrated that conditioned media from HSC enhanced proliferation, EMT, and invasion of HCC cells treated with a sublethal exposure of heat, and that this effect was mediated by periostin. Treatment with the Vitamin D analog calcipotriol blocked the secretion of periostin from HSCs, and it demonstrated an additive effect when used with cisplatin, making this target attractive as an adjuvant therapy after thermal ablation of HCC [134].

Some naturally available compounds in vegetation, phytochemicals, have also been studied in the context of targeting the tumor stroma. Curcumin, which has been studied for its various anticancer and anti-inflammatory effects, has also been shown in vitro and in vivo to decrease activation in HSCs, by way of protecting them from oxidative stress and inhibiting their release of inflammatory cytokines [135–138]. Ellagic acid found in a variety of fruits and nuts and embelin found in a Japanese herb used in traditional medicine were both shown to decrease proliferation and increase apoptosis in PSCs as well as PDAC cell lines [139]. PSCs have also been shown to express cannabinoid receptors, and activation of the cannabinoid receptor inhibited the activated PSC phenotype, inhibited growth, and decreased secretion of IL-6 and MCP-1 as well as decreased fibronectin, collagen I, and αSMA levels [140].

A growing number of studies have found that supplementation of pancreatic cancer patients with polyunsaturated omega-3 fatty acids, such as DHA and EPA, provides clinical benefit such as improved liver and pancreas function postoperatively, and weight gain and improved quality of life in the palliative setting, with a low incidence of side effects [141–143]. In a recent phase II study of omega-3 supplementation, Arshad et al. showed that it decreased levels of circulating cytokines known to be secreted by PSCs/CAFs including IL-6, IL-8, TGFβ, and FGF. While it was a small study, the patients that showed decreases in these levels have better survival times [144]. A follow-up mechanistic study elucidated that treatment with DHA/EPA augmented gemcitabine-induced growth inhibition in PDAC cell lines and PSCs in vitro, with PSCs showing the greatest differences, through inhibition of PDGF-BB secretion [145].

PSCs have been shown to undergo autophagy in the PDAC microenvironment, so targeting this process with autophagy inhibitors is one strategy to inhibit PDAC growth. Endo et al. showed that inhibition of autophagy with chloroquine or siRNAs inhibited PSC activation and also decreased PDAC growth in an animal model [82].

Despite many promising studies showing that inhibition of stellate cell interactions also inhibits tumor growth in preclinical models, the relationship is complicated by conflicting evidence that depleting the stroma may not be the solution after all. Ozdemir et al. used a transgenic mouse model in which αSMA+ myofibroblasts (activated pancreatic stellate cells) could be depleted and demonstrated that stromal depletion at either the PanIN stage (precancerous) or invasive PDAC stage resulted in more aggressive tumor phenotypes, with less differentiation, more hypoxia, greater EMT changes, cancer stem cells, and decreased animal survival. There was also a stronger immunosuppressive environment characterized by a greater number of Treg cells in stroma-depleted tumors; also these tumors did not respond to gemcitabine treatment [146]. In a

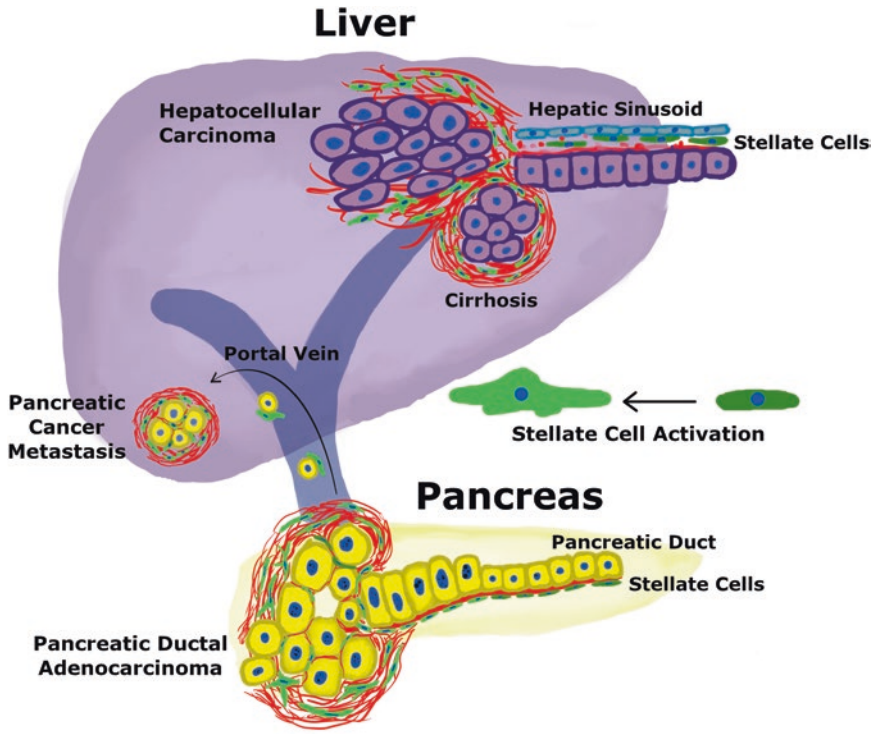

Fig. 6.1 Stellate cells in the progression of hepatocellular carcinoma (HCC) and pancreatic ductal carcinoma (PDAC). Top: Quiescent hepatic stellate cells (qHSCs; dark green) are pictured in the space of Disse located between the hepatic sinusoids and hepatocytes. Responding to stress signals, activated hepatic stellate cells (aHSCs; light green) deposit extracellular matrix (ECM; red), which results in cirrhosis and contributes toward the progression of HCC formation. Bottom: Quiescent pancreatic stellate cells (qPSCs; dark green) are shown lining the normal pancreatic duct along pancreatic ductal epithelial cells. As pancreatic ductal epithelial cells become dysplastic (pancreatic intraepithelial neoplasia; PanIN), they are supported by activated pancreatic stellate cells (aPSCs; light green). These aPSCs deposit ECM (red) and create the heavy desmoplastic reaction seen in pancreatic tumors. aPSCs also can accompany metastatic PDAC cells through the portal vein circulation to form liver metastases

complementary study, Rhim et al. deleted the sonic hedgehog (Shh) ligand in a transgenic mouse model and this resulted in tumors with deficient stroma as expected. As in the previous study, the Shh-depleted mice developed more aggressive tumors that were less differentiated, with increased vascularity, and also confirmed this with long-term pharmacological Shh inhibition [147]. These findings were surprising given all of the preceding data about the role of PSCs as accomplices of PDAC cancer development and survival and suggest that the tumor stroma has dual roles in both supporting and restraining cancer growth (Fig. 6.1).

Blocking signaling pathways involved in pericyte signaling has also shown promise in preclinical models. The blockade of VEGFR2 with a targeted antibody slowed tumor growth in mouse models of human PDAC, and this resulted in less tumor vessel density and reduced pericyte accumulation [148]. In mouse models of glioblastoma, targeting pericytes with the thymidine kinase inhibitor ibrutinib improved the efficacy of chemotherapy [149]. Therefore targeting this population of stellate cells and pericytes will be crucial in not only removing growth-enhancing support but also removing the protective effects they confer against chemotherapeutics (Table 6.1).

Table 6.1 Functional features and cytokines of activated stellate cells

Stellate cell activation features	Stellate cell immunomodulatory signals	Stellate cell growth factors
Loss of Vitamin A droplets	Macrophage colony-stimulating factor (M-CSF)	Transforming growth factor-β (TGFβ)
+ α-smooth muscle actin	Platelet-activating factor (PAF)	Platelet-derived growth factor (PDGF)
+ Phagocytosis	Monocyte chemoattractant protein-1 (MCP-1)	Connective tissue growth factor (CTGF)
+ Antigen presentation	Angiotensin II	Fibroblast growth factor (FGF)
+ Proliferation	Interleukin-1	Hepatocyte growth factor (HGF)
+ Migration	Interleukin-4	Nerve growth factor (NGF)
+ Extracellular matrix production	Interleukin-6	Stromal-derived factor-1 (SDF-1)
	Interleukin-8	Vascular endothelial growth factor (VEGF)
	Interleukin-13	Stem cell factor (SCF)
	Interleukin-15	
	Chemokine ligand 5 (CCL5/RANTES)	
	Endothelin	
	Tumor necrosis factor-α (TNFα)	
	Cyclooxygenase-2	
	Nitric oxide	
	Galectin-3	

6.6 Conclusion and Future Directions

In liver and pancreatic tumors, evidence continues to mount that stellate cells are key players in cancer initiation, progression, invasion, and metastasis. They have been shown to allow cancer cells to survive in harsh conditions and protect them from chemotherapy and radiotherapy. The cumulative data is suggesting that targeting this cell population in addition to the tumor cells themselves can result in strides toward more effective cancer treatments. However as stromal elements also seem to restrain cancers while supporting their survival, modulating the function of the stroma is likely to be more effective than the overall depletion of the stroma. Since stellate cell identification and isolation methods have been developed, many new targets involving the stellate cell-cancer cell relationship have been coming to light in recent years and research continues to accelerate. Strategies may include reprogramming the stellate cells to decrease fibrosis by inducing quiescence, improving drug delivery and sensitivity, and alleviating the immunosuppressive environment created by the tumor stroma. Harnessing control of the body's normal cells such as the stromal cells and immune populations is likely the key for obtaining durable cures from cancer, rather than relying on targeting neoplastic cells with heterogeneous mutations and behaviors.

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Endothelial Progenitors in the Tumor Microenvironment

7

Ugo Testa, Elvira Pelosi, and Germana Castelli

Abstract

Tumor vascularization refers to the formation of new blood vessels within a tumor and is considered one of the hallmarks of cancer. Tumor vessels supply the tumor with oxygen and nutrients, required to sustain tumor growth and progression, and provide a gateway for tumor metastasis through the blood or lymphatic vasculature. Blood vessels display an angiocrine capacity of supporting the survival and proliferation of tumor cells through the production of growth factors and cytokines. Although tumor vasculature plays an essential role in sustaining tumor growth, it represents at the same time an essential way to deliver drugs and immune cells to the tumor. However, tumor vasculature exhibits many morphological and functional abnormalities, thus resulting in the formation of hypoxic areas within tumors, believed to represent a mechanism to maintain tumor cells in an invasive state.

Tumors are vascularized through a variety of modalities, mainly represented by angiogenesis, where VEGF and other members of the VEGF family play a key role. This has represented the basis for the development of anti-VEGF blocking agents and their use in cancer therapy: however, these agents failed to induce significant therapeutic effects.

Much less is known about the cellular origin of vessel network in tumors. Various cell types may contribute to tumor vasculature in different tumors or in the same tumor, such as mature endothelial cells, endothelial progenitor cells (EPCs), or the same tumor cells through a process of transdifferentiation. Early studies have suggested a role for bone marrow-derived EPCs; these cells do not are true EPCs but myeloid progenitors differentiating into monocytic cells, exerting a proangiogenic effect through a paracrine mechanism. More recent studies have shown the existence of tissue-resident endothelial vascular progenitors (EVPs) present at the level of vessel endothelium and their possible involvement as cells of origin of tumor vasculature.

Keywords

Cancer · Tumor microenvironment · Tumor progression · Metastasis · Tumor vasculature · Angiogenesis · Vasculogenesis · Vascular mimicry · Hypoxia · Vascular endothelial growth factor · Cancer therapy · Angiogenic agents · Antiangiogenic agents · Endothelial progenitors · Endothelium · Vessel wall

U. Testa (✉) · E. Pelosi · G. Castelli
Department of Oncology, Istituto Superiore di Sanità, Rome, Italy
e-mail: ugo.testa@iss.it; elvira.pelosi@iss.it; gramana.castelli@iss.it

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_7

7.1 Endothelial Progenitors and Tumor Angiogenesis

The blood vasculature is a closed circulatory system that is formed by arteries, veins, and capillaries; the inner layer of these vessels is formed by a single layer of endothelial cells. The physiological role of endothelial cells is not limited to the creation of a barrier between blood and tissues but is related also to the control of organ growth, regeneration, and stem cell niche. Furthermore, growing evidences indicate that endothelial cells are specialized according to the specific needs of the tissues that they supply, showing distinct barrier properties, angiogenic potential, and metabolic rate, all necessary to support specific organ functions [107].

The vascular system derives from the differentiation of mesodermal stem cells into angioblasts, embryonic endothelial progenitors. The vascular system first appears in the embryo as a highly branched network formed by primitive vessels composed by endothelial cells [25]. Embryonic endothelial cells acquire then an identity as arterial, venous, lymphatic, or hemogenic and then further specialize in an organotypic-dependent manner. The hemogenic endothelium is essential for the generation of the primitive and definitive hematopoietic system [17, 124].

Endothelial progenitor cells (EPCs) are present in adult life. Two types of EPCs have been reported: one of non-hematopoietic origin, called endothelial colony-forming cell (ECFC) able to generate true endothelial cells, resident in vessel wall but present also at very low levels in the peripheral blood and directly participating to the regeneration of endothelium following injury or ischemic damage [73], and another of hematopoietic origin, called myeloid angiogenic cells (MACs), resident in the bone marrow, generating monocytic cells supporting angiogenesis through paracrine mechanisms [10]. Growing evidences suggest that ECFCs may play a key role in reparative processes. ECFCs display a hierarchy of clonal proliferative potential and in experimental models display a pronounced postnatal vascularization ability in vivo (Table 7.1). Because of these properties, ECFCs represent a promising cell source for revascularization of damaged tissue. ECFCs are detectable in peripheral blood and were enriched in the cell fraction $CD34^+/CD133^-/CD146^+$; however, these cells are not generated by the bone marrow.Therefore, ECFCs are not detected in the bone marrow, and $CD34^+/CD133^-/CD146^+$ cells present in this tissue fail to generate ECFCs [171] (Table 7.1).

Emerging evidence supports the existence of specific endothelial cells with stem cell properties within blood vessels in the postnatal period. These cells exhibit the properties described for ECFCs and are present in the endothelium of vessels in a quiescent state: in response to injury, these quiescent endothelial stem/progenitor cells are activated and undergo a vasculogenic process [111, 180]. There are some remarkable differences between ECFCs and mature endothelial cells. Interestingly, a recent study showed that ECFCs possess a high sprouting activity, a property related to angiogenesis [164]. In fact, Sturtzel and coworkers showed that the forkhead box transcription factor (FOXF1) is selectively expressed in ECFCs compared to mature endothelial cells; FOXF1 expression had a strong impact on the sprouting capabilities of endothelial progenitor cells, as supported by the observation that FOXF1 overexpression in endothelial cells induces the expression of NOTCH2 receptors and induces sprouting [164]. Thus, this study supports a key role for endothelial progenitors as the mediators of both vasculogenic and angiogenic processes. A second recent study further supports the capacity of ECFCs to promote angiogenic sprouting, related to the synthesis and release of Cytokine-Like 1 (CYTL1): this proangiogenic factor is induced by hypoxia and promotes in both ECFCs and mature endothelial cells angiogenic sprouting [151]. Furthermore, CYTL1 promotes vessel formation in animal models comparable to VEGF-A [151]. Thus, these recent studies support a possible role of endothelial progenitor cells not only as promoters of vasculogenesis but also as mediators of angiogenic processes.

Endothelial cells are heterogeneous, and significant differences have been detected between arteries and veins, large and small vessels, and different microvascular beds in various organs.

Table 7.1 Five different mechanisms of neovascularization in tumors have been identified: (1) vascular co-option, (2) angiogenesis, (3) vasculogenesis, (4) vascular mimicry, and (5) endothelial cell transdifferentiation

Vascular co-option
Vascular co-option is a nonangiogenic process through which tumor cells utilize preexisting tissue blood vessels to support tumor survival and proliferation.
Vessel co-option was observed in various tumors growing at the level of various tissues, including in the lungs, brain, liver, and lymph nodes.
In many tumors, vascular co-option is an initial mechanism of development of tumor vasculature.
The identification of vascular co-option requires a detailed histopathological analysis of tumor specimens and cannot be discriminated from angiogenesis on the basis of the simple analysis of microvessel density.
Mechanisms driving vessel co-option are largely unknown; tumor cell invasion and tumor cell adhesion seem to be involved in vessel co-option.
It is believed that vessel co-option represents a mechanism of resistance to current antiangiogenic therapies.
Angiogenesis
Angiogenesis implies the development of new vessels from preexisting ones. There are two types of angiogenesis:
Sprouting angiogenesis, involving an enzymatic degradation of the capillary basement membrane with a weakening of endothelial cell-cell interaction, proliferation of endothelial cells, and sprouting toward an angiogenic stimulator.
Intussesceptive angiogenesis: an existing vessel is split into two new vessels by cellular reorganization.
Tissutal hypoxia and matrix metalloproteinase are two major drivers of angiogenesis. VEGF-A is the most important and common growth factor signal for angiogenesis.
Angiogenesis is stimulated by VEGF which activates the proliferation of endothelial cells: endothelial cells degrade the extracellular matrix and migrate toward the VEGF gradient; a new blood vessel is then guided through sprouting by tip cells, expressing high levels of VEGF receptors; VEGF induces the expression of the
NOTCH Delta-like ligand 4 (DLL4) on activated endothelial cells; DLL4 activates NOTCH receptors on adjacent cells, which adopt the stalk endothvelial phenotype and form new vessel lumen.
Due to abnormal growth factor levels and to other microenvironmental influences, tumor vessels formed by angiogenesis are abnormal and display multiple functional abnormalities.
Vasculogenesis
Vasculogenesis implies a process, very active during embryonic life, by which endothelial progenitor cells differentiate into endothelial cells and promote new vessel formation.
Human endothelial colony-forming cells (ECFCs) are identified as endothelial progenitors that can be isolated from umbilical cord blood, peripheral blood, and vascular endothelium; ECFCs are not of bone marrow origin and are physiologically resident at the level of vessel wall.
ECFCs are distinguished from mature endothelial cells for their capacity of forming functional blood vessels in vivo upon transplantation.
Endothelial cells resident at the level of vessel walls are heterogeneous and are composed by endothelial vascular progenitors (EVPs), with self-renewing capacity, present in a quiescent state, and capable of endothelial colony formation in vitro and of endothelial regeneration in vivo; transit-amplifying (TA) and mature (D) endothelial cells, not having self-renewal and endothelial-regenerating capacity.
Several signaling pathways control the proliferation and differentiation of ECFCs, including NOTCH, VEGF, WNT, SHH, and CXCR4.
Vascular mimicry
Vascular mimicry is a process through which tumor cells form functional vessel-like structures independent of VEGF and of typical modes of angiogenesis.
There are two types of vascular mimicry:
Type I: tubular type, composed of non-endothelial cell-lined blood tubes and cancer cells lining the surface of the channels
Type II: patterned matrix-type, comprising a basement membrane rich in fibronectin, laminin, and collagens, surrounded by tumor cells
High tumor vascular mimicry is associated with high tumor grade, shorter survival, invasion, metastasis, and poor prognosis.
Initially described in aggressive human intraocular and metastatic melanoma, vascular mimicry has been observed in a variety of cancers, including breast, lung, and ovarian cancers; osteosarcoma; gastric cancer; and bladder, colorectal, and hepatocellular cancers.

(continued)

Table 7.1 (continued)

The mechanisms driving vascular mimicry are largely unknown. Some studies suggest that vascular mimicry is initiated by tissue signals triggering peculiar differentiation pathways of cancer stem cells.
Endothelial cell transdifferentiation
This mechanism implies the transdifferentiation of tumor cells into an endothelial cell phenotype.
In some tumors, such as glioblastomas, a part of tumor vessels is composed by endothelial cells generated by the transdifferentiation of cancer stem cells into endothelial-like cells. This transdifferentiation is related to the high plasticity of the cancer stem cell population. AKT-mediated upregulation of WNT5A plays an important role as a driver of endothelial transdifferentiation of glioma stem cells.
In some hematological neoplasia, such as chronic myeloid leukemia, myelofibrosis, and lymphomas, there is evidence that a part of endothelial cells pertain to the leukemic clone. It was hypothesized that these diseases could derive from the malignant transformation of a hemangioblastic progenitor.

However, at the moment, it is largely unknown how each organ determines the specialization and functional properties of its endothelium. Intrinsic and microenvironmental mechanisms contribute to human endothelial cell heterogeneity. A better understanding of the cellular and molecular mechanisms underlying endothelial heterogeneity is of fundamental importance.

The discovery of EPCs, their purification and expansion in vitro opened the way to potential clinical applications for the use of these cells in clinical applications for the regeneration of damaged or injured endothelium and for the understanding of angiogenic mechanisms underlying various pathological conditions, such as tumors.

The mechanisms involved in tumor vessel generation are heterogeneous and imply different biological processes occurring in different tumors or in the same tumor (Fig. 7.1 and Table 7.1). The basic mechanisms of tumor vessel generation involve vasculogenesis and sprouting angiogenesis (Fig. 7.1 and Table 7.2). Vasculogenesis is a process that implies the de novo formation of blood vessel as a consequence of endothelial progenitor cell differentiation and represents the process of vessel generation during initial stages of embryonic and fetal development; sprouting angiogenesis implies the generation of new vessels from preexisting blood vessels. Vasculogenesis revquires the recruitment of EPCs in the tumor microenvironment, orchestrating their differentiation into mature endothelial cells and the generation of new vessels. Angiogenesis by vessel sprouting includes the specification of endothelial cells into tip and stalk cells, orchestrated by a variety of physical, chemical, and biological mechanisms [33], with a key role played by VEGF [9] and NOTCH signaling pathways; endothelial cells present at the vascular front are induced to become tip cells and may be activated by microenvironmental stimulations promoting vascular sprouting. In addition to these more commonly adopted mechanisms for new blood vessel generation by tumor cells, two additional mechanisms were observed in tumors: vascular mimicry and intussusception (Fig. 7.1 and Table 7.2). Vasculogenic mimicry implies the biological process adopted by some cancer cells with an aggressive phenotype to generate vascular-like structures without the presence of endothelial cells: in this process, tumor cells line tumor vessels and thus contribute to the formation of vessel-like structures (Fig. 7.1 and Table 7.2) [56]. Vascular intussusception or intussusceptive microvascular growth was defined as a process of intravascular growth consisting in the splitting of a preexisting vessel into two new vascular structures. Intussusceptive microvascular growth was reported in some tumors, including mammary, colorectal, and melanoma tumors [201].

In addition to the abovementioned mechanisms, there is now growing evidence that tumors can grow also through a mechanisms by which tumor cells infiltrate normal tissue, exploiting existing vessels to sustain their growth: this mechanism is known as vascular co-option and implies that in these nonangiogenic tumors, no new vessels are seen [42]. Only a minority of tumors grow in a purely nonangiogenic manner,

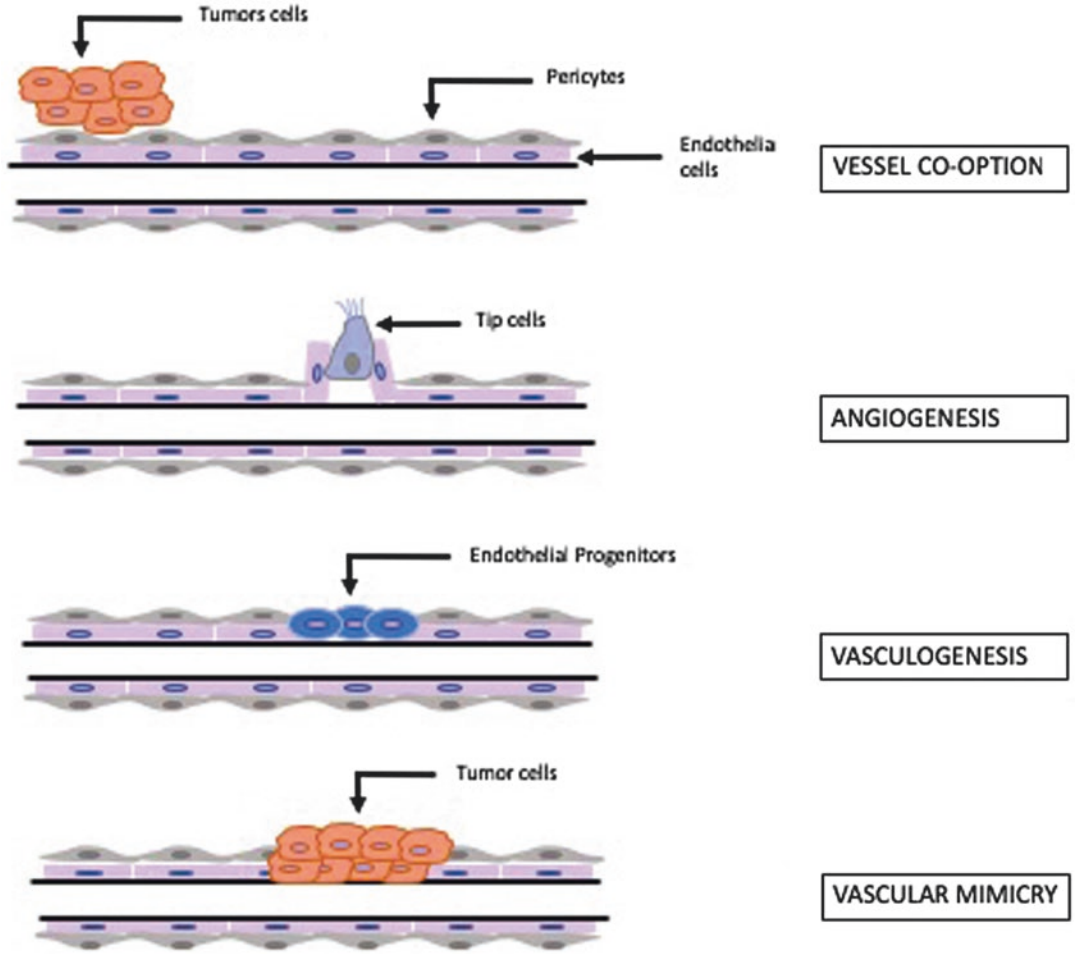

Fig. 7.1 Schematic representation of different patterns of tumor vasculature
Vascular co-option does not imply new vessel formation and is the process of taking over or incorporating the existing vasculature by developing tumor cells
Angiogenesis: new blood vessels branch out of preexisting ones via a process called angiogenesis. The formation of new vascular wall is a multistep process involving the migration of tip endothelial that with their extension creeps into the connective tissue and then the proliferation and subsequent migration of cells at the base (stalk endothelial cells)
Vasculogenesis: new blood vessel formation due to the proliferation and differentiation of vessel wall-resident endothelial progenitor cells (endothelial vascular progenitors)
Vascular mimicry is a process in which tumor cells create their own vessels without or in cooperation with endothelial cells, assuming the role and the properties of endothelial-like cells

while many tumors contain a mixture of angiogenic and nonangiogenic areas [42]. Both angiogenic and nonangiogenic areas have been commonly seen in primary and secondary lung tumors [20] and in breast cancer liver metastases [54]. Experimental and computational analyses of the dynamics of vessel co-option during tumor progression and in response to antiangiogenic treatment in gliomas and brain metastases provided evidence that vessel co-option is a mechanism of antiangiogenic resistance and that tumor progression can be efficiently blocked only by therapies inhibiting both angiogenesis and co-option [179].

Tumor progression from a benign to a malignant condition is usually associated with an angiogenic switch, characterized by the development of a vascular network actively growing and

Table 7.2 Phenotypic, functional properties and cellular origin of MACs (monocytoid angiogenic cells) and ECFCs (endothelial colony-forming cells)

Phenotypic or functional marker	ECFC	MAC
Cellular origin	Vessel wall	Bone marrow
In vitro tube formation	+	+
In vivo vessel formation	+	–
Clonal replating capacity	+	–
Expansion capacity in vitro	+	–
In vivo homing to hypoxic tissues	+	+
Paracrine capacity of sustaining angiogenesis	+	+
CD45	–	+
CD14	–	+
CD115	–	+
CD31	+	+
CD146 (MCAM)	High	Low
CD144 (VE-Cadherin)	+	–
CD105	+	+
vWF (von Willebrand)	+	–
CD34	+	–
CD133	–	+
CD117	–	+
VEGF-R1	+	+
VEGF-R2	+	+
TIE-2	+	+
CXCR4	+	+
CD157	+	?
AcLDL uptake	+	+
UEA-1 lectin binding	+	+
ALDH	+	+

displaying infiltrative properties [39]. The pattern of vascularization is highly variable in various tumors, depending on the tumor type, grade and stage, the anatomical location, and the microenvironment composition and distribution of proangiogenic and antiangiogenic growth factors [39].

Tumor vessel formation involves the formation of highly disorganized vessel networks, morphologically and functionally abnormal [26]. Tumor vasculature displays numerous abnormalities, being tortuous, irregular, and nonhomogeneous and characterized by weak junctions between endothelial cells, resulting in augmented leakiness facilitating extravasation of cancer cells [76]. Tumor neovascularization plays an essential role in tumor biology by promoting tumor growth, metastasis, and resistance to chemotherapy. The proximity of cancer cells to blood vessels within a tumor is a major determinant of tumor phenotypic and metabolic diversification [90]. Furthermore, endothelial cells adapt to the tumor microenvironment and display a hyperglycolytic metabolism, shunting glycolytic intermediates to nucleotide synthesis [27].

Tumor-associated endothelial cells not only provide oxygen and nutrient supply to tumors but regulate also tumor aggressiveness, converting naïve tumor cells to chemoresistant tumor cells with stemlike properties [22]. The blocking of the interactions between tumor cells and endothelial cells at the level of perivascular tumor niches may represent a strategy to bypass chemoresistance of these "protected" tumor cells [24].

The mechanisms driving tumor angiogenesis are heterogeneous and involve different molecular and cellular processes. The common initial driving force of tumor angiogenesis is represented by the tumor microenvironment, composed by a variety of cell types that under the influence of growth factors and cytokines activate their metabolic and functional activities and promote various types of angiogenic mechanisms, triggering an "angiogenic switch" to allow the tumor to progress [184]. Mechanical (stress relaxation) and chemical (hypoxia) properties are critical factors in tumor microenvironment driving tumor angiogenesis and tumor progression promoting tumor cell invasion and migration [93, 94]. Some of the mechanisms of tumor angiogenesis, involving the generation of new vessels by vasculogenesis, imply endothelial progenitor cells. These vasculogenic mechanisms are poorly defined, and some additional confusion is related also to the unprecise definition of endothelial progenitor cells, englobing both true endothelial progenitor cells (ECFC) generating in their differentiation an endothelial cell progeny and myeloid cell progenitor cells (myeloid angiogenic cells, MACs) generating a monocytic angiogenic cell progeny exerting an indirect proangiogenic activity through paracrine mechanisms. Thus, the tumor angiogenic mechanisms may involve the recruitment in the tumor microenvironment of either tissue-resident ECFCs or

of circulating ECFCs or of bone-marrow-derived MACs. Initial studies in experimental mouse models have suggested that tumor angiogenesis requires the migration of endothelial progenitor cells from the bone marrow initially in the blood and then in the tumor microenvironment, promoting in this site tumor vascularization (reviewed in [38]). It is now evident that these studies describe the contribution of some bone marrow-derived myeloid cells to tumor angiogenesis. Subsequent studies have defined in various mouse models the specific proangiogenic role exerted by Tie-2^+ monocytes/macrophages; Tie-2 inhibitors block the recruitment in the tumors of Tie-2^+ monocytes and reduce tumor angiogenesis [39]. However, other studies have shown that mouse embryonic endothelial progenitor cells are able to home at the level of tumor, trapped by hypoxia-originated signals, promoting a process of active vasculogenesis [177]. This mechanism of vasculogenesis in adults was also supported by recent studies showing that tissue hypoxia through HIF-1-dependent and HIF-1-independent mechanisms promotes postnatal vasculogenesis through the recruitment of EPCs, their clusterization and integration in existing microvasculature [18].

The study of the involvement of EPCs in tumor angiogenesis is further complicated by the presence of numerous altered phenotypes of tumor endothelial cells. An example is given by the observation that tumor endothelial cells exhibit an increased aldehyde dehydrogenase (ALDH) expression: these $ALDH^{high}$ tumor endothelial cells have proangiogenic properties, express stem/progenitor cell genes (CD90, Oct-4, MDR-1), and show drug resistance [67]. High ALDH expression in some endothelial cells is induced by tumor-conditioned medium [67]. Furthermore, the heterogeneity of tumor endothelial cells between low- and high-metastatic tumors suggests in the latter ones the involvement of EPCs in the mechanisms sustaining tumor angiogenesis [122].

The study of endothelial progenitor cells in adult life remained closed within these last years within a scientific dilemma, with some studies supporting the presence and the functional contribution to reparative vascular processes of bone-marrow-derived EPCs and other studies showing that true EPCs are present in the vessel wall, do not derive from the bone marrow, and are capable of regenerating damaged vessels. A recent study was of fundamental importance to offer a reasonable explanation for this intriguing dilemma. Thus, Singhal et al. have used irradiation-based myeloablative and non-myeloablative mouse models to explore the cells responsible for vascular regeneration. The results of this study unequivocally showed that the pre-existing endothelium is the source of cells capable of promoting vascular regeneration [156]. However, if endothelial cell proliferation is impaired by irradiation, myeloid cells were recruited from the bone marrow by the damaged endothelium and actively contribute to vessel wall reconstitution [156]. These findings support a model of vascularization in adults implying the cooperation between endothelial-derived and bone marrow-derived progenitors. This model must be carefully considered for its important implications for our understanding of tumor neovascularization processes.

The present paper reviews the study of tumors in which some evidences support a role for EPCs in the mechanisms underlying tumor vascularization.

7.2 Endothelial Progenitors in Melanoma

In melanoma, angiogenesis is implicated in tumor progression and metastasis, and microvessel density is an adverse prognostic factor [80]. A recent meta-analysis of all current studies on this topic shows that increased MVD in melanoma is associated with reduced DFS, but not with reduced OS [132]. More recent studies suggest that vascular proliferation index (VPI) could represent a more sensitive prognostic index to capture and to measure the expanding vasculature than the standard microvessel density evaluation [68]. The rate of intratumoral blood vessel proliferation at the level of metastatic sentinel lymph nodes was inversely associated with overall sur-

vival of melanoma patients [129]. In a recent study, Hugdahl and coworkers analyzed a large set of melanomas for MVD and VPI at the level of primary tumor sites and at the level of locoregional metastatic sites [62]. A significant impact of VPI on melanoma survival was observed; furthermore, VPI strongly correlated with high-grade characteristics of the primary tumor in terms of tumor thickness, tumor ulceration, mitotic count, and presence of necrotic areas. Interestingly, VPI was lower in locoregional metastases than in corresponding primary tumors, suggesting that activated angiogenesis is more critical for primary tumors than for metastatic tumors; in contrast, MVD was higher in metastatic sites than in primary ones [62]. A tentative explanation at least for lymph node metastases could be related to the presence in these tissues of a highly vascularized network, not requiring active neoangiogenesis to sustain tumor growth but predominantly co-option mechanisms [62]. The ensemble of these observations supports neoangiogenesis as an aggressive component of melanoma microenvironment, required to sustain tumor development and progression.

Few studies support a direct role of endothelial progenitors in tumor vascularization.

In this context, particularly relevant was a recent study by Donovan and coworkers in a model of orthoptic melanoma. Fate-mapping analysis of endothelial cell populations showed a consistent heterogeneity of endothelial cells present in the growing melanomas, characterized by the very early infiltration of endovascular progenitors in growing tumors [43]. Importantly, delivery of melanoma cells with EVP resulted in the formation of larger tumors, whereas tumor cells delivered with D (mature) endothelial cells resulted in smaller tumors [43]. In the tumoral microenvironment, EVPs reactivated the expression of SOX18 transcription factor and initiated a vasculogenic process, progressing through various stages of endothelial differentiation, including TA cells and D cells, generating mature endothelial cells with arterial, venous, and lymphatic subtypes [43]. Molecular profiling by RNA sequencing of purified endothelial cell populations supported that EVPs are quiescent progenitors: EVPs present in the tumors or in the wall of the aorta share common molecular signature [43]. Importantly, anti-VEGF-A therapy was unable to target EVP cells; in contrast, conditional ablation of NOTCH signaling alters EVP. Endothelial-specific ablation of Rbpj, a direct protein interactor of SOX18B, depleted the EVP population and strongly inhibited metastasis. These important observations support the view that EVPs are major drivers of tumor vascularization and cannot be significantly inhibited by commonly used anti-VEGF antiangiogenic agents.

This interesting study was in part based on a previous study showing that, on the basis of flow cytometry detection of common endothelial markers, three subpopulations of endothelial cells can be identified among cells characterized by expression of an endothelial marker (VE-Cadherin) and absence of a pan-hematopoietic marker (CD45) [53, 130, 131]. Lineage tracing experiments have shown that the $CD31^{-}$/VEGF-$R2^{low}$ cell population is heterogeneous and composed by three different subpopulations: Endothelial Vascular Progenitors (EVPs), displaying a self-renewing capacity, present in a quiescent state and capable of endothelial colony forming capacity in vitro and endothelial-regenerating capacity in vivo; Transit-Amplifying (TA) and Mature (D) endothelial cells not possessing self-renewal capacity [130, 131]. Gene expression studies show a remarkable difference between EVP and TA/D cells; SOX18 is essential for differentiation of EVP cells [130, 131]. Single-cell transcriptomic studies showed the existence of crucial regulatory gene expression networks specific for EVPs, including significant metabolic gene networks, and higher mitochondrial content and activity [104].

Studies in experimental models further support a role for SOX18 in promoting tumor angiogenesis in murine melanoma models. In fact, in these murine models, SOX18 induction represents a key step in mediating melanoma lymphangiogenesis and metastasis, as supported through experiments of genetic ablation of SOX18 in these tumors [46, 47].

These observations have strongly supported the development of small molecules acting as

SOX18 inhibitors and exerting a potent antiangiogenic activity on tumor vasculature [52, 125]. These observations support the clinical study of SOX18 inhibitors, although the still limited understanding of the biological effects of SOX transcription factors on endothelial cell biology suggests a great prudency in the clinical translation of SOX18 inhibition [84]. A small mechanistic trial in which SOX18 inhibition will be initiated following diagnosis of melanoma and sentinel lymph nodes collected few weeks later the start of therapy will be performed with the aim of studying changes in vascularization [69].

Other studies have shown that elevated numbers of tumor-associated macrophages (TAM) in the tumor microenvironment are often associated with poor prognosis in melanoma and these cells activated by amoeboid melanoma cells play an essential role in tumor progression through a secretory cross talk with tumor microenvironment, promoting abnormal vascularization [57]. Adrenomedullin, clearly expressed by infiltrating TAMs in human melanomas, is a key mediator of angiogenesis in these tumors [32]. Previous studies have shown that (i) adrenomedullin/cyclic AMP pathways induce NOTCH activation and differentiation of arterial endothelial cells from vascular progenitor cells [193]; (ii) adrenomedullin augments the angiogenic potential of ECFCs [66]; and (iii) adrenomedullin augments proliferation and migration of endothelial progenitor cells [186].

7.3 Endothelial Progenitors in Lung Cancer

The lung is unique for having two different sources of blood: the systemic bronchial vasculature from the aorta and the pulmonary circulation of deoxygenated blood from pulmonary artery. The pulmonary circulation has a limited angiogenic capacity and is not able alone to sustain lung tumor growth, while the bronchial circulation possesses angiogenic properties and is exploited by lung tumors. In fact, studies in murine lung cancer models have shown that although the existing pulmonary circulation can supply the nutritional and metabolic needs for initial tumor development, further growth of the tumor requires angiogenesis from the highly proliferative bronchial circulation [48]. The circulation of human lung tumors was explored by contrast-enhanced computed tomography scanning. Yuan and coworkers reached the conclusion that the bronchial circulation is dominant, and the tumor circulation was related to the tumor size [192]. Nguyen-Kim and colleagues, using a comparable methodology, confirmed that tumor perfusion was related to tumor size and to the histological subtype [119].

The essential role of angiogenesis for lung cancer growth is supported by observations in naturally occurring tumors and in experimental models of lung cancerogenesis. A recent experimental model of lung cancer tumorigenesis is initiated by lung cancer stem cell (LCSC) expansion [96]. In this model, the expansion of LCSC pool promotes angiogenesis through secretion on angiogenic growth factors, such as placental growth factor (PlGF) by LCSCs [96]. This observation supports the view that the tumor cells responsible for tumor development trigger the angiogenic switch required to sustain tumor growth.

The analysis of the pattern of vascularization of a large set of primary non-small cell lung cancers through immunostaining of endothelial cells showed four distinct patterns of tumor vascularization – basal, papillary, and diffuse – implying destruction of normal lung tissue and generation of new vessels; a fourth pattern, called alveolar, was observed in 16% of cases and is characterized by the lack of parenchymal destruction and the absence of new vessel formation and, therefore, can be considered as a nonangiogenic subtype [134]. There is consistent variation in the differentiation of tumor vasculature in lung carcinoma, as evidenced through labeling of endothelial cells for CD31 and LH39 antigens. Highly angiogenic lung carcinoma exhibits an immature endothelial phenotype, while low angiogenic lung cancers have a mature endothelial phenotype [79]. These findings were confirmed and extended by Passalidou and coworkers showing that in non-oncogenic lung cancer, endothelial

cells were characterized by positivity for the late endothelial marker LH39, while in the more frequent angiogenic tumors, only a minority of vessels express LH39 [128]. The angiogenic or nonangiogenic phenotype of primary lung cancers is in large part dictated by the properties of tumor cells. Thus, nonangiogenic tumors have higher levels of genes involved in mitochondrial metabolism, mRNA transcription, protein synthesis, and the cell cycle; angiogenic tumors have higher levels of genes coding for membrane vesicles, integrins, remodeling, angiogenesis, and apoptosis [71]. A recent study on a large set of lung cancer specimens confirmed these initial observations and led to conclude that both angiogenic and nonangiogenic lung cancers are characterized by a comparable hypoxia/HIF and VEGF expression, but angiogenesis does not ensue in nonangiogenic tumors in which hypoxia triggers a metabolic mitochondrial response which allows tumor growth without triggering angiogenesis; in angiogenic tumor in which the expression of genes involved in mitochondrial metabolism is more limited, hypoxia together with tissue remodeling and inflammation triggers an angiogenic response [1].

The functional integrity and homeostasis of lung tissue is maintained by lineage-restricted stem cells [70]. Alvarez and coworkers were among the first investigators to provide evidence about the existence of vascular resident progenitor cells, able to differentiate into ECs and capable of extensive proliferative activity [6]. These endothelial progenitor cells are involved in tissue endothelial repair mechanisms: in fact, in models of lung injury induced by chronic hypoxia [120] or LPS administration [82], tissue-resident endothelial progenitors increase, became highly proliferative, and substantially contribute to pulmonary vascular repair in vivo. The analysis of LPS-treated mice showed a transient decrease of the number of pulmonary vascular endothelial cells, associated with an increase of pulmonary vascular endothelial cells undergoing a process of endothelial-to-mesenchymal transition (EndMT) [166]. Interestingly, EndMT endothelial cells expressed c-kit and CD133 and had increased vasculogenic capacity, aldehyde dehydrogenase (ALDH) activity, and expression of drug-resistant genes, all properties typically observed in progenitor cells [166].

Sekine and coworkers identified $CD31^{+}CD45^{-}CD133^{+}$ as a rare population of vascular-resident endothelial cells exhibiting properties of endothelial progenitor cells, with consistent in vitro and in vivo angiogenic capacities; these cells are locally resident endothelial progenitor cells expressing other progenitor cell markers, such as ALDH positivity, and do not derive from the bone marrow [155]. Immunofluorescence studies indicate that this population of $CD133^{+}$ endothelial progenitors is preferentially resident in the arterial vasculature at the level of large vessels [155]. $CD133^{+}$ endothelial progenitors have been reported also in human pulmonary arteries [50].

C-kit^{+} lung endothelial cells act as endothelial progenitors under homeostatic or repair conditions, but do not contribute to lung epithelium [100]. Xu and coworkers described two types of vascular endothelial progenitors in the adult mouse lung: $CD45^{-}/CD31^{+}/VEGFR2^{-}$/LSP (lung side population) cells corresponding to bipotent progenitors of endothelial and smooth muscle and $CD45^{-}/CD31^{+}/VEGFR2^{+}$/LSP acting as late endothelial progenitors [188]. Lineage tracking experiments have shown that maintenance and repair of the lung endothelium does not involve contributions from bone-marrow-derived endothelial progenitor/precursor cells [123].

Alphonse and coworkers have reported that human fetal and neonatal rat lung contain ECFCs with robust proliferative capacity, secondary colony formation on replating, and de novo blood vessel formation in vivo when transplanted into immunodeficient mice [4]. These lung-resident EPCs can be isolated by enzymatic dispersion of human lung samples by enzymatic tissue digestion and selection of CD31-positive cells by magnetic-activated cell sorting and plating in vitro in endothelial-specific conditions [5].

Vessel wall endothelial progenitor cells were identified on the basis of their capacity to efflux Hoechst 33342 dye, a property displayed by

many stem cell populations [116]. Because of this property, these cells have been termed side population (SP) cells for their typical appearance in flow cytometry, being identified as a cell subpopulation at the side of main population (MP) cells [116]. These cells, called EC-SP, have the capacity to form in vitro colonies of endothelial cells and display in vivo potent angiogenic activity when assayed by transplantation in an ischemic limb [116]. These EC-SP cells were clearly detected in the lung vasculature and give a major contribution to new blood vessels in lung tumors [117]. These cells, whose origin is not at the level of the hematopoietic system, proliferate in lung tumors, and the frequency of this population clearly increases in the tumoral tissue, compared to normal lung tissue [117], survived to angiogenic drugs, and remained in the tumor tissue [117]. Other evidences in favor of a possible role of endothelial progenitor cells in mediating angiogenic mechanisms derive from the study of a KRAS-driven mouse model of lung cancer in which endothelial expression of the microRNAs (miRNAs) miR-143/145 promoted angiogenesis-induced tumor growth [41]. miR-143/145 were expressed at the level of a minor subpopulation of lung endothelial cells, seemingly endothelial progenitors [41]. miR-143/145-deficient mice displayed a reduced endothelial proliferation and reduced endothelial tip cells [41]. Lung tumor development was more limited in miR-143/145-deficient mice, where the tumors exploit co-option mechanisms of the normal lung vasculature, compared to WT mice exploiting neoangiogenic mechanisms to sustain lung tumor growth [41]. Another study showed that the NAD-dependent protein deacetylase Sirtuin 1, a master regulator of angiogenesis, increases vessel density and stimulates the growth of Lewis lung carcinoma xenografts through a mechanism involving downregulation of delta ligand 4 (DLL4)-NOTCH signaling and deacetylation of the NOTCH1 intracellular domain in lung cancer-derived endothelial cells [187]. These effects of SIRT1 on lung tumor angiogenesis are seemingly mediated by a stimulatory effect on the angiogenic properties of endothelial progenitor cells [182].

Although these experimental models supported a key role of neoangiogenic mechanisms operating in lung cancer, studies on primary human lung cancers have provided evidence that angiogenic mechanisms are heterogeneous in these patients and a subset of patients with non-small cell lung cancer exhibit nonangiogenic growth patterns [105].

Targeting angiogenesis was a therapeutic strategy intensively explored in NSCLC, but the utility of this approach remains limited. The addition of bevacizumab (mAb anti-VEGF) to platinum-based chemotherapy in first-line setting induces a modest benefit. Some antiangiogenic therapies have been approved in the second line by the FDA (bevacizumab or ramucirumab (anti-VEGF-R2)) or by the European Union (nintedanib anti-VEGF-R1, anti-VEGF-R2, anti-VEGF-R3, PDGFRA, FGF-R1), but the extent of the improvements at the level of PFS and OS were modest and must be balanced against the expected toxicities and the cost associated with these agents. These limited clinical benefits are related to therapy-induced blood vessel alterations, with consequent increased hypoxia which can worsen the tumor microenvironment and induce treatment resistance. However, in spite of all these limitations and the existence of intrinsic mechanisms of resistance to current antiangiogenesis inhibitors of NSCLCs, combination therapies targeting multiple mechanisms fueling neovascularization could bypass resistance to standard antiangiogenic therapy. In this context, a recent study provided evidence that targeting of the proangiogenic peptide Apelin (APLN), an endogenous ligand of the G-protein-coupled cell surface receptor APLN receptor, together with a standard antiangiogenic drug, such as sunitinib, in lung adenocarcinoma models, diminished tumor growth, blood vessel density, and vessel abnormality within the tumor microenvironment and consequently hypoxia and tumor resistance [173]. Apelin levels are elevated in lung adenocarcinomas and were associated with elevated microvessel densities [16]; apelin

elevation in these tumors seems to be related to the downmodulation of miR-195, directly targeting Apelin [200]. Apelin levels are heterogeneous in lung adenocarcinomas, and Apelin-high tumors have a poorer prognosis than Apelin-low tumors [200].

7.4 Endothelial Progenitor Cells in Multiple Myeloma (MM)

MM is the second most common hematologic cancer after non-Hodgkin's lymphoma, with a higher incidence over 60 years. Bone marrow angiogenesis is a constant hallmark of MM progression and has prognostic potential. Microvessel density in the bone marrow clearly increases with disease progression from monoclonal gammopathy to smoldering gammopathy and to primary and relapsing MM [139]. The pathophysiology of MM-induced angiogenesis implies production of angiogenic cytokines both by cells of the marrow microenvironment and by tumor plasma cells [89, 174].

Several studies suggest that an increased angiogenic activity mediated by EPCs could contribute in a relevant way to the increased angiogenesis observed in MM. Thus, Vacca and coworkers provided evidence that EPCs isolated from MM patients exhibit increased angiogenic activity in vitro showing rapid capacity to form capillary networks and in vivo inducing the generation of numerous new vessels [175]. Zhang et al. reported an increased number of EPCs among peripheral endothelial cells isolated from MM patients; their number was significantly decreased following thalidomide treatments [196].

The increased number/activity of EPCs in multiple myeloma suggests that vasculogenesis may contribute to new vessel formation in this disease. A study by Moschetta and coworkers investigated the role of vasculogenesis in a mouse model of MM [115]. This study showed an early mobilization of EPCs from BM to PB, followed by recruitment at the level of MM-colonized niches; the use of EPC-defective $ID1^{+/-}$, $ID3^{-/-}$ mice showed that MM progression is strictly dependent upon EPC trafficking, due to the supportive role of endothelial cells for the growth of MM cells [115]. Importantly, angiogenic dependency of MM occurs at early and not at late stages of MM development; in line with this finding, early targeting of EPCs with anti-VEGF-R2 antibody at early stages, but not at all late stages of disease progression, inhibited MM development [115]. This study supports the idea of inhibiting vasculogenesis at an early stage of disease (smoldering MM) in future clinical trials. Vasculogenic activity in myeloma may be supported by some growth factors present in the tumor microenvironment: (a) pleiotrophin, a developmental cytokine highly expressed in MM [30, 191], promotes transdifferentiation of monocytes into vascular endothelial-like cells and through this mechanism promotes tumor-induced vasculogenesis [31]; (b) ephrin receptor A3 (EphA3) is markedly overexpressed in MM endothelial cells and acts as a proangiogenic factor [21]; (c) NOTCH signaling, a crucial pathway for embryonic vasculogenesis [2], is increased in MM microenvironment and sustains the increased vasculogenic/angiogenic activities observed in this tumor, and its targeting resulted in a marked inhibitory effect on tumor angiogenesis [148]. Importantly, recent studies have shown that NOTCH signaling in the bone marrow microenvironment promotes EPC expansion and in vivo vasculogenic activity of EPCs [74, 85].

The treatment of multiple myeloma is a complex medical problem, related to the difficulty to completely eradicate malignant plasmocytes and their precursors. Recent developments have provided significant improvements in the treatment of these patients based on autologous bone marrow transplantation when possible or drug combinations involving the association of corticosteroids with immunomodulating agents (thalidomide, lenalidomide, pomalidomide) and either proteasome inhibitors (bortezomib, carfilzomib, or ixazomib) or monoclonal antibodies (daratumumab, anti-CD38) or histone deacetylase inhibitors (panobinostat). Interestingly, immunomodulatory agents, such as lenalidomide or thalidomide, exert many biological effects, including an antiangiogenic effect on MM and, when administered with other drugs, contribute

to a significant antitumor effect [141]. One example of the clinical utility of this triplet drug combination is given by a recent study: lenalidomide plus dexamethasone and daratumumab (anti-CD38 mAb) significantly improved both PFS and OS of newly diagnosed MM patients ineligible for autologous stem cell transplantation [49].

The value of inhibiting angiogenesis in MM as a therapeutic tool is supported also by two recent studies. A phase I/II study explored evofosfamide, a hypoxia-activated prodrug without or with bortezomib in patients with relapsed/refractory MM: although the number of patients exhibiting CR and PR was not high, disease stabilization was observed in >80% of patients, resulting in a prolonged overall survival of 11.2 months [91].

Recent studies have explored the antitumor effects of melphalan-flufenamide (mel-flufen), a dipeptide prodrug of melphalan: the intracellular conversion of mel-flufen to melphalan is mediated by the enzyme aminopeptidase N [29]. Mel-flufen was more active than melphalan in inducing apoptosis of myeloma cell and in vitro and in vivo exerted a potent inhibitory effect on angiogenesis [162]. A phase I/II study (OP-104 Anchor study) is evaluating safety and efficacy of mel-flufen and dexamethasone in combination with either bortezomib or daratumumab in patients with relapsed/refractory MM, and an early analysis showed preliminary encouraging data about the antitumor efficacy, particularly in patients treated with mel-flufen and daratumumab [138]. The phase I/II study OP-106 Horizon showed that mel-flufen has promising activity in heavily pretreated refractory-stage RRMM patients, in whom the majority of available therapies have failed [144].

7.5 Endothelial Progenitor Cells in Myeloproliferative Disorders

The study of EPCs and of angiogenesis in myeloproliferative disorders is particularly important because in these tumors, the endothelial lineage could be directly involved in the neoplastic transformation and pathological endothelial cells could be directly involved in the neoplastic transformation and could contribute to tumor development promoting the formation of a pathological marrow niche. Myeloproliferative neoplasms (MPNs) are clonal hematopoietic cancers characterized, in their chronic phase, by overproduction of differentiated hematopoietic cells pertaining to one or more lineages The Philadelphia chromosome-negative are the most frequent diseases among the myeloproliferative disorders and include three different main diseases: polycythemia vera (PV), essential thrombocytopenia (RT), and myelofibrosis (MF) [118, 176]. All MPNs arise from the malignant transformation of hematopoietic stem cells (HSCs) that acquire a growth advantage over normal HSCs, leading to their expansion and generation of all myeloid-related lineages [112]. These disorders have frequent disease-related complications, mainly represented by venous and arterial thromboses, hemorrhages, and transformation to acute myeloid leukemia [176]. In MPNs, in >95% of cases, mutations that affect and drive tumor development occur in a mutually exclusive manner in one of three different genes: *JAK* (Janus Activating Kinase 2), *CALR* (calreticulin), or *MPL* (myeloproliferative leukemia virus, corresponding to the thrombopoietin receptor); while the *JAK2V617F* mutation activates three different membrane receptors (erythropoietin receptor, granulocyte colony-stimulating factor receptor, and thrombopoietin receptor), *CALR* or *MPL* mutants affect only thrombopoietin receptor activation [176]. The different consequences of these three different mutations help to understand the reasons of their different disease association: *JAK2V617F* is associated with PV, ET, and PMF, while *CALR* and *MPL* mutations are exclusively associated with ET and PMF.

Studies performed in the last years have supported a possible direct implication of the endothelial lineage in the pathological processes involving the development of *JAK2V617F*-mutated MPNs [169].

Initial studies have provided conflicting results: thus, an initial study based on the in vitro assay of endothelial progenitor cells showed that

JAK2V617F mutation was detected in CFU-ECs (colonies of monocytoid proangiogenic cells), but not in E-CFCs (colonies formed by true endothelial cells) [135]. However, Sozer and coworkers reached a different conclusion through the analysis of the liver endothelial cells of patients with Budd-Chiari syndrome and *JAK2V617F*-positive hematopoiesis showing a positivity of the endothelial lineage for *JAK2V617F* mutations [160, 161]. According to these findings, it was hypothesized that *JAK2V617F* neoplasia may derive from a common cell of origin for both hematopoietic and endothelial cells.

Teofili and coworkers have subsequently explored this issue and have analyzed the molecular features of EC-CFCs generated from 22 MPN *JAK2V617F*-positive patients and showed that in 45% of cases, endothelial cells present in these colonies are positive for this disease-specific marker [168]. These findings were corroborated by two additional important observations: all patients displaying molecular abnormalities at the level of the endothelial lineage experienced thrombotic complications; endothelial cells bearing *JAK2V617F* mutations showed a pronounced Stat3/Stat5 activation [168]. In line with these observations, $CD34^+$ cells isolated from *JAK2V617F*-positive MPN patients generate in vivo in immunodeficient mice both mutation-positive and mutation-negative endothelial cells [160, 161]. Furthermore, endothelial cells displaying *JAK2V617F* mutation were observed in >60% of myelofibrosis patients at the level of splenic capillaries and splenic vein [147].

Other recent studies have developed animal models to directly assess the consequences of *JAK2V617F* expression at the level of endothelial cells. Thus, Guadall et al. have developed an induced pluripotent stem cell strategy to compare *JAK2* mutant to *JAK2* wild-type endothelial cells [60]. The results of this study showed that *JAK2V617F*-mutant endothelial cells exhibit a pro-inflammatory and pro-thrombotic phenotype and display pro-adherent features [60]. Guy et al. have explored the role of endothelial cells that express *JAK2V617F* mutant through an in vitro model of endothelial cells expressing *JAK2V617F* and in vivo model of mice with endothelial-specific *JAK2V617F* expression [61]. Endothelial cells expressing *JAK2V617F* have a pro-adhesive phenotype associated with increased P-selectin expression and increased propensity to thrombotic events [61].

Another set of studies provided evidence that the development of myeloproliferative syndromes requires *JAK2V617F* expression in both HSCs and endothelial cells. Two lines of evidence support this conclusion: (a) no difference was noted between WT and *JAK2V617F* Lin^-/Kit^+ progenitor cell proliferation when cocultured on WT endothelial cells while a relative growth advantage over the corresponding WT progenitor cells when cocultured on *JAK2V617F*-bearing endothelial cells [97]; and (b) competitive marrow transplantation experiments provided evidence that the *JAK2V617F*-bearing vascular niche promotes clonal expansion of *JAK2V617F* HSCs but not normal HSCs [195]. Another study showed that *JAK2V617F* expression in the niche leads to HSC radioresistance, which could be responsible for the high frequency of disease relapse in patients undergoing allogeneic stem cell transplantation for MPNs [98].

7.6 Tumor Angiogenesis in Lymphoma

Several studies have shown an association between increased angiogenesis and more aggressive tumor behavior in different lymphoid neoplasms, including large B-cell lymphoma [23], mantle cell lymphoma [178], and follicular lymphoma [172].

Intriguingly, a study by Streubel and coworkers suggested that 15 to 85% of the microvascular endothelial cells in the B-cell lymphomas bear lymphoma-specific chromosomal translocations [163]. This observation may be explained either assuming the existence of a common lymphoid-endothelial malignant progenitor cell or of a lymphoid progenitor with a peculiar differentiation plasticity or by cell fusion or gene transfer events [163].

Among the various lymphomas, particularly interesting is the SOX11-positive mantle cell lymphoma (MCL), characterized by increased angiogenesis that could be ascribed to neoangiogenic mechanisms. MCL is an aggressive B-cell lymphoma, genetically characterized by the presence of the translocation t(11;14)(q13;q32) inducing overexpression of cyclin D1. MCLs can be subdivided according to SOX11 expression into SOX11-negative characterized by more frequent leukemic non-nodal disease, classic morphology, more frequent CD23 expression, and lower Ki67 proliferation index and SOZ11-positive, characterized by conventional nodal disease and aggressive phenotype. SOX11 is not expressed in normal B-lymphocytes, and its expression characterizes a part of MCLs, where it acts as a key transcription factor interfering with B-cell differentiation program and promoting tumor cell-microenvironment interactions that favor tumor growth [15]. Experimental studies have shown that SOX11 expression is associated with the induction of an angiogenic switch, characterized by increased expression of angiogenic-related gene expression signatures and induction of tumor vasculature [127]. Petrakis and coworkers directly analyzed primary tumors and showed that MVD was much higher in SOX11-positive MCLs than in SOX11-negative MCLs [133]. Studies in zebrafish support a key role of SOX11 as a key regulator of vascular development, particularly involved in sprouting angiogenesis during development [150].

7.7 Angiogenesis in Malignant Gliomas

Glioblastomas are the most common and aggressive brain tumors. These tumors are characterized by their resistance to chemotherapy and radiotherapy and by the abundant and abnormal vasculature. High-grade gliomas have been classified as one of the solid tumors' most vascular, and vessel proliferation is a typical feature of glioblastomas [19]. One pathological feature that distinguishes glioblastoma multiforme from lower-grade glial tumors is represented by the presence of microvascular proliferation.

Glioblastoma is characterized by abundant and abnormal neovascularization giving rise to functionally and structurally abnormal vessels, with the generation of hypoxic, necrotic tumor regions. The mechanisms generating the neovascularization of glioblastomas are complex and multiple and certainly heterogeneous in their individual contribution from one tumor to another: (i) *vascular co-option* which is a mechanism operating during the early phases of glioblastoma development and implies the migration of tumor cells, forming cuffs around preexisting normal vessels, and must be considered as a strategy to increase blood flow before the development of mechanisms of adaptation of the tumor vasculature [42]; (ii) *sprouting angiogenesis*, consisting in the generation of capillaries from preexisting blood vessels, through a mechanism implying endothelial cell proliferation triggered by tumor hypoxia, inducing VEGF production and resulting in the development of an abnormal vascular network with dilated blood vessels, abnormal branching, arteriovenous shunts, and vascular leakiness [95]; (iii) *release of angiogenic growth factors from tumor cells*, particularly from glioma stem cells, with consequent stimulation of new vessel formation [13]; (iv) *vasculogenesis*, induced by the migration (triggered by hypoxia and VEGF) and differentiation of bone marrow endothelial progenitors (MACs) acting at the tumor sites as catalyzers of vasculogenic processes, seemingly involving local endothelial progenitors cells (Du et al. [44, 51]); the vasculogenic mechanisms are particularly active in glioblastoma recurrence after radiotherapy, where the vasculogenic response is not promoted by VEGF but by the cytokine stromal-derived factor-1 (SDF-1) [87, 167]; (v) *vasculogenic mimicry*, a process in which the same tumor cells form functional vessel-like networks, providing an alternate mechanism to support tumor growth, a phenomenon correlated with tumor grade, poor outcome, and aggressive phenotype [99] and triggered by growth factors released in the tumor microenvironment [101, 102]; and (vi) *transdifferentiation*

of glioma stem cells into endothelial cells or pericytes [63].

The mechanism of glioma stem cell transdifferentiation seems to represent a peculiar mechanism of tumor oncogenesis observed in glioblastomas. Using tumor biopsies, Wang and coworkers [181] and Ricci-Vitiani and coworkers [143] identified a subset of endothelial cells lining tumor vessels, carrying the same genetic alterations, as well as malignant glioma cells. Furthermore, the $CD133^+$ tumor population includes a subset of vascular endothelial-cadherin (CD144)-positive population showing features of endothelial progenitor cells and capable of generating mature endothelial cells [143, 181]. These tumor-derived endothelial cells comprise from 20 to 90% of the tumor vasculature, have tumor-specific genetic abnormalities, and do not respond to inhibitors of VEGF or bFGF; furthermore, these cells are preferentially localized at the level of deep hypoxic tumoral areas, thus suggesting that hypoxia is a major driver in their genesis. Mei and coworkers have reported the occurrence of tumor-derived endothelial cells in 47% of a group of 64 glioblastoma patients [113]. In the cases displaying the endothelial transdifferentiation, tumor-derived endothelial cells accounted for 14–18% of total vessels [113].

Importantly, cancer stem cells isolated from primary glioblastomas are able to transdifferentiate to endothelial cells in vitro [143, 181]. Soda et al. identified a subpopulation of endothelial cells within glioblastomas, expressing both endothelial-specific and tumor-specific markers [157]. In a more recent study, De Pascalis and coworkers showed that the fraction tumor-derived endothelial cells increased in glioblastomas recurring after radiotherapy [40]. These findings were further extended by a study of Cheng and coworkers providing evidence that glioma stem cells are able to generate also the vascular pericytes, cells normally of mesenchymal origin, which surround blood vessels; however, these authors provided strong evidence that glioma cancer stem cells do not differentiate to form endothelial cells [34]. Thus, the data of these studies are contradictory, suggesting the origin from tumor cells of either endothelial cells or of pericytes.

Golebiewska et al. have confirmed the existence within glioblastomas of an endothelial subpopulation of side cells, $CD133^+/CD31^+$ cells [58]. However, at variance with the previous studies, this $CD133^+/CD31^+$ cell population is non-tumorigenic [58].

Subsequent studies have explored the cellular and molecular mechanisms responsible for the differentiation of glioma stem cells into endothelial progenitor cells. In this context, particularly relevant was a study by Hu et al. based on a model of glioma stem cells obtained through the transformation of neural stem cells with mutant TP53 and constitutively expressing active AKT: these transformed cells exhibited an increased EC cell signaling and the augmentation of a subpopulation of $CD133^+/CD144^+$ cells [72]. Particularly, using these cells, it was shown that AKT activation upregulates WNT 5A to drive glioma-like EC differentiation of GSCs [72], resulting in the development of tumor invasive growth properties. Interestingly, clinical data showed higher glioma-specific endothelial and WNT5A expression in peritumoral and recurrent glioblastomas than in matched intratumoral and primary glioblastomas, respectively [72].

Other studies have shown that the adenosine A3 receptor is a regulator of the differentiation of glioma stem cells to endothelial cells under hypoxic conditions [146]. β1,4-Galactosyltransferase (β1,4GalTV) is highly expressed in glioma cells and contributes to the increased expression of highly branched N-glycans observed in these tumor cells; β1,4GalTV stimulates transdifferentiation of glioma stemlike cells into endothelial cells by activation of NOTCH1 signaling [37]. Finally, ETSA-variant 2 (ETV2), a master regulator of endothelial cell development, induces in glioma stem cells, in a VEGFA-independent manner, the repression of proneural genes and the activation of vascular genes; in primary glioblastomas, high ETV2 expression was associated with a particularly negative prognosis [198]. Interestingly, bone morphogenetic protein 9 (BMP9) counter-

acts the process of glioblastoma cell transdifferentiation into tumor-derived endothelial cells, in both in vitro and in vivo xenotransplantation models [137].

Other experimental studies support a major role of glioma stem cells in monitoring and regulating the angiogenic response of glioblastoma. Oligodendrocyte precursors (OPCs) expressing OLIG2, present at the level of the subventricular zone, can serve as the tumor progenitors of high-grade gliomas, including glioblastoma [92]. Using an experimental model of EGFRvIII-driven, TP53-negative murine glioma that can grow as OLIG2$^+$ or OLIG2$^-$, Griveau et al. have shown that (i) OLIG2$^+$ gliomas grow by invasion of the parenchyma by single-cell vessel co-option and (ii) OLIG2$^-$ gliomas grow as perivascular clusters, leading to disruption of the blood-brain barrier and innate immune cell activation [59]. Importantly, WNT7 expression in OLIG2$^+$ cells is required for vessel co-option, and WNT inhibition enhances the response to temozolomide therapy [59].

Glioblastoma tumor cells, including the stem-like cell population, invade into the brain parenchyma, engaging a migration along blood vessels. The interaction between glioma cancer stem cells and endothelial cells at the level of tumor perivascular niches is of fundamental importance for the promotion of tumor invasion and for new vessel formation, two properties that strictly intertwined [77, 194].

Therapy with currently used antiangiogenic agents blocking VEGF-A is used in the treatment of high-grade gliomas but is of limited benefit. A meta-analysis of randomized controlled trials showed that bevacizumab when combined with chemotherapy improved PFS, but not OS; furthermore, patients treated with bevacizumab-containing therapy reported increased objective response rate, but more treatment-related adverse events [190]. The same conclusions were reached by a recent *Cochrane Database of Systematic Review* showing that the use of antiangiogenic therapy does not significantly improve overall survival in newly diagnosed people with glioblastoma [7]. Similarly, there is lack of evidence of a survival advantage for antiangiogenic therapy over chemotherapy in recurrent glioma [7]. The limited benefits of antiangiogenic therapy in glioblastomas are related to two different tumor-related mechanisms: resistance and indifference. The resistance mechanism implies that the tumor, after an initial response, rapidly relapses acquiring an enhanced tumor invasion capacity [28, 126] or producing alternative angiogenic growth factors [152].

Large-scale clinical trials have shown the incapacity of bevacizumab, an anti-VEGFA monoclonal antibody, to significantly improve overall survival of high-grade glioma patients. However, in spite of the absence of benefit related to anti-VEGFA blocking therapy, glioma patients can be subdivided into a nonresponder and a responder group, the responders being characterized by a transient clinical benefit. This differential response to anti-VEGFA blocking therapy implies the existence of a heterogeneity of the mechanisms underlying tumor angiogenesis in glioblastomas. A recent study suggested that this heterogeneity could be related to the heterogeneity of the expression of SOX17 at the level of high-grade glioma tumor vessels: high Sox17 expression correlated with poor survival, early recurrence, and abnormal tumor vessels (Kim et al. [86]).

Currently, there are only two drugs approved for systemic treatment of glioblastoma: temozolomide, approved for the treatment of newly diagnosed glioblastoma, and bevacizumab, approved for the treatment of recurrent glioblastoma. Few studies have explored the effect of these two drugs on the aberrant neovascularization pattern of glioblastoma. In this context, particularly interesting was a study carried out by Xue et al. showing in an orthoptic mouse model transplanted with human glioblastoma cells that an increase in vascular mimicry was observed after bevacizumab administration, while an increased microvessel density was observed after temozolomide administration [189]. Similar conclusions were reached by Obad and coworkers exploring the morphology and the function of tumor vessels in two human glioblastoma xenografts treated with bevacizumab, as investigated by emission tomography; the results of this study

provided evidence that bevacizumab normalized vascular morphology in the period time investigated but failed to improve vascular function [121].

These studies clearly indicate the limitations of drugs, such as bevacizumab, as therapeutically efficacious agents for glioblastoma treatment. Even the exploration of new antiangiogenic treatments was at the moment unsuccessful. Thus, angiopoietin 1 and angiopoietin 2 inhibition with blocking peptidobody Trebananib was ineffective as monotherapy and failed to enhance the ability of VEGF blockade to improve the outcomes of patients with recurrent glioblastoma [140]. Recent data from a phase II trial involving patients with relapsed glioblastoma following chemoradiotherapy showed a slight but significant superiority of the antiangiogenic tyrosine-multikinase inhibitor regorafenib over lomustine chemotherapy, with an improvement of median overall survival from 5.6 months with lomustine to 7.6 months with regorafenib (Lombardi et al. [103]). These findings, if confirmed in future clinical trials, support the identification of a new antiangiogenic drug active in relapsing glioblastoma patients.

Apelin (APLN) is an endogenous peptide ligand for the G protein-coupled receptor APJ/AGTRL1/APLNR and is widely expressed throughout the human body. ELABELA was identified as a second endogenous APLNR ligand. APLN and ELABELA represent a double spatiotemporal system to control APLNR signaling. APLN acts as a proangiogenic peptide during embryonic development playing an essential role in promoting the migration of embryonic angioblasts, expressing APLNR, to midline under the stimulation of ELABELA and, through this mechanism, exerts an essential role in the control of vasculogenesis [65]. APLN was identified by mass spectrometry as one molecule present in endothelial secretome and found expressed in glioblastoma specimens in close proximity to blood vessels [64]. APLN was shown to be essential for the survival and expansion of glioma stem cells, as supported by experiments with specific inhibitors of APLNR signaling [64]. Mastrella and coworkers showed that APLN is selectively expressed in glioblastoma, compared to normal brain tissue; furthermore, APLN expression was decreased by anti-VEGF therapy, but tumor invasion was increased [109]. In glioblastoma xenotransplantation models, APLN and APLNR upregulation occurred in association with angiogenic switch [109]. Using a mutant form of the natural APLN peptide (APELIN-F13A), a reduction in both angiogenesis and tumor invasiveness and a potentiation of the efficacy on anti-VEGF therapy were shown [109]. These observations support a potential role of targeting of APLNR as a strategy to overcome resistance to VEGF blockade in glioblastoma. Finally, a recent study showed the abundant expression in high-grade gliomas of the poorly characterized APELIN early ligand A (APELA): high APELA expression was associated with poor patient survival and correlates with glioma grade [55]. APLN targeting could represent, used alone or in combination with anti-VEGF therapy, as a potentially new interesting approach in glioblastoma treatment. However, many questions remain to be carefully explored, as pointed out by Amoozgar et al. [8]. First, it remains to be explored the efficacy of APLN targeting on vascular function and vasogenic edema in glioblastoma patients. Second, it remains completely to be explored the possible effect of APLN targeting on the immunosuppressive microenvironment of glioblastoma. This point is justified by previous studies showing that in experimental tumor models the normalization of tumor vasculature induced by APLN targeting improves the efficiency of antitumor immune therapy [83]. On the other hand, studies on the mechanisms of resistance of cancer patients to anti-PD-1 immune check inhibitors showed the presence of APLNR mutations with consequent loss of function: restoration of APLNR function through JAK/STAT1 signaling is essential for IFNγ-mediated antitumor responses, including T-cell trafficking through tumor vasculature [130, 131].

The possible improvements of response of glioblastoma tumors to immunotherapy with immune check inhibitors are particularly important because the clinical efficacy of PD-1/PD-L1

blockers in glioblastoma is not significant and the combination of anti-PD-1 agents with bevacizumab did not show better efficacy over bevacizumab alone [183]. The recent demonstration in recurrent glioblastoma patients that neoadjuvant anti-PD-L1 treatment with continued therapy after surgery improved patient survival compared to patients receiving adjuvant, post-surgical PD-1 blockade alone [35], supports future studies aiming to improve immunotherapy response through a vascular normalization and a consequent better trafficking of immune effectors.

The large majority of studies on glioblastomas, as well as in other tumors, were based on the idea to target endothelial cells as a tool to inhibit or normalize tumor-associated vasculature; an alternative cell target could be represented by pericytes. Pericytes are multipotent perivascular cells playing a key role within the glioblastoma microenvironment to support tumors during all their stages of development [149]. The biological role of pericytes in the regulation of blood-brain barrier permeability and in promoting angiogenesis and tumor growth represents a strong support for their therapeutic targeting. Furthermore, a recent study showed that a glioblastoma subtype, characterized by the presence of isocitrate dehydrogenase 1 mutations, is associated with a reduced pericyte coverage, seemingly related to the downmodulation of several angiogenic growth factors [165]. The blood-tumor barrier is major obstacle for drug delivery to malignant brain tumors; eliminating the blood-tumor barrier by targeting of glioma stem cell-derived pericytes though inhibition of the BMX kinase with Ibrutinib consistently enhanced the chemotherapeutic efficacy of drugs with poor blood-tumor barrier penetration [199].

7.8 CD276: A Target of Both Tumor Cells and Tumor Vasculature

In 2007, Seaman and coworkers have made a fundamental observation in the context of a study aiming to identify genes whose pattern of expression allows to distinguish physiological and pathological angiogenesis: CD276 (also known as B7-H3) was the most differentially expressed cell surface tumor-specific endothelial marker identified in this study [153]. CD276 is a member of the B7 family of immunoregulatory molecules whose expression can be induced on T, B, and dendritic cells activated by different types of inflammatory cytokines. This first study showed CD276 expression on the tumor vasculature in colon, lung, breast, esophageal, and bladder cancers; CD276 expression was observed also on tumor cells [153].

Various clinical studies have shown the impact of CD276 expression on tumor vasculature for many cancers. Thus, tumor cell and tumor vasculature expression of CD276, observed in 46% of tumor specimens, predicts survival in clear cell renal cell carcinoma [36]. These findings were confirmed by a study on metastatic clear cell renal cell carcinoma patients undergoing cytoreductive nephrectomy and interferon (IFN) treatment, showing that patients with B7-H3 expression levels $\leq$16% were associated with an improved OS, while those with high B7-H3 expression had a poor survival [114]. The large majority of ovarian cancers express CD276 at the level of tumor cells; CD276 was also expressed in the endothelium of tumor vasculature in 44% of patients, largely pertaining to late-stage high-grade serous tumors (Zhang et al. [197]). Tumors with CD276-positive tumor vasculature display a reduced OS and a higher incidence of recurrence (Zhang et al. [197]). In Merkel cell carcinoma, an aggressive cutaneous malignancy whose pathogenesis and prognosis are related to the integrity of the host immune system, CD276 expression is colocalized with CD31, indicating a localization at the level of tumor vasculature: positivity for CD276 correlates with aggressive clinicopathological parameters and predicts a poor prognosis [11].

Given the pattern of expression of CD276 in many tumors, CD276 is also a promising ultrasound molecular imaging target. In this context, studies carried out in breast tumors showed that antibodies anti-CD276 conjugated with indocyanine green (ICG) allowed the imaging of prostate

cancers, with labeling of both tumor neovasculature and tumor tissue, sparing the normal mammary tissue or benign neoplastic lesions [12, 185].

The expression of CD276 at the level of both tumor cells and tumor vasculature offers the unique opportunity through the targeting of this molecule to destroy both tumor cells and their associated vasculature. Thus, Seaman and coworkers have shown that a monoclonal antibody reacting with both human and mouse CD276 conjugated with pyrrolobenzodiazepine injected in mouse transplanted with human tumors destroys both tumor cells and tumor vasculature, resulting in tumor eradication [154]. The type of drug conjugated with anti-CD276 is important because some drugs are unable to kill tumor-associated endothelial cells because these cells express high levels of the drug transporter P-glycoprotein (MDR-1). In fact, tumor endothelial cells resulted to be resistant to drugs such as monomethyl auristatin E [154]. Interestingly and importantly, CD276 was found to be expressed at the level of ECFCs, but not of MACs [158]. siRNA expression silencing experiments indicated a role for CD276 in ECFCs, showing that this protein promotes cell proliferation and migration (by targeting the extracellular matrix) but inhibits tube formation and exerts antiapoptotic, antioxidant, and antisenescence function in endothelial progenitor cells [158]. Additional experiments supported the view that CD276 stimulates endothelial progenitor cell proliferation and inhibits their angiogenic activity.

CD276 is a potentially interesting target for human cancer immunotherapy. Monoclonal antibodies anti-CD276, conjugated or not with cytotoxic drugs, are under clinical evaluation [136]. In preclinical models, CD276-specific monoclonal antibodies and antibody-drug conjugates displayed antitumor activity against $CD276^{+}$ tumor cells in xenograft mouse models. Phase I clinical studies showed a good safety profile (NCT01099644, NCT02381314 and NCT02982941). A recent dose I study showed an interesting application of radiolabeled anti-CD276 mAb by convection-enhanced delivery in diffuse intrinsic pontine glioma [159].

Recent preclinical studies have shown the consistent antitumor potential of chimeric antigen receptor T cells (CAR-Ts) for targeting CD276 in solid tumors. Thus, Du and coworkers developed CAR-Ts for targeting CD276, which effectively controlled tumor cell growth in vitro and in orthoptic, metastatic, and patient-derived xenograft models of pancreatic cancer, ovarian cancer, and neuroblastoma [45]. Appropriate 4-1BB co-stimulation promotes lower PD-1 expression in B7-H3.CAR-Ts and superior antitumor activity when targeting tumor cells, such as pancreatic cancer, which constitutively express PD-L1 [45]. Importantly, using mAbs that cross-react with murine CD276, it was shown that B7-H3.CAR-Ts control tumor growth in syngeneic tumor model without any evident toxicity; this lack of toxicity could be related to the observation that CD276 is expressed also in many normal tissues, but at a level significantly lower than that observed in tumor cells and in tumor vasculature [45]. Another recent study provided evidence that B7-H3.CAR-Ts mediate significant antitumor activity in vivo against various pediatric tumors, including osteosarcoma, medulloblastoma, and Ewing sarcoma; the strong efficacy of CAR-Ts in these tumor models is largely related to the high surface target antigen density on tumor tissues, including tumor vasculature, with an activity diminished against target cells that express low levels of CD276 [106].

CD276 targeting offers also the opportunity to overcome the immunosuppressive effects of tumor microenvironment and to enhance anti-PD-1/PD-L1 immune checkpoint inhibitors in cancer therapy in a model of metastatic lung cancer [14].

7.9 Conclusion

An increased understanding of the mechanisms of vessel formation and their regulation in tumors is strictly necessary to the development of novel agents targeting tumor vasculature. The investigation of neovascular processes observed in various tumors indicates that the pathways to neovascularization are complex and imply vari-

ous cellular and molecular mechanisms. It is still unclear which cells are at the source of newly formed vessels in tumors. Growing evidences indicate that the adult endothelium is heterogeneous and is composed by mature, transition, and progenitor endothelial cells. Evidences limited to few tumors support the view that these vascular-resident endothelial progenitors could represent the cells of origin of tumor vasculature. However, the understanding of the heterogeneity of endothelial cells is of recent acquisition, and its application to the study of tumor vasculature is very limited. Future studies are strictly required to define the implications of endothelial cell heterogeneity to the understanding of the cellular origin of vessel network in tumors.

Tumor neovascularization is a complex process involving five different, in part interlinked, pathways: vascular co-option, angiogenesis, vasculogenesis, vascular mimicry, and endothelial cell transdifferentiation. This heterogeneity is further amplified by phenomena of spatiotemporal heterogeneity, implying that tumors may utilize different mechanisms of vasculature formation in a spatially variable and temporally dynamic fashion. Angiogenesis was considered the predominant pathway and is triggered by hypoxia and by the release of hypoxia-regulated growth factors pertaining to the VEGF family. Thus, various agents targeting VEGF signaling entered clinical use, such as antibodies targeting VEGF (bevacizumab) or VEGF-R2 (ramucirumab), a VEGF trap (aflibercept), and various VEGF receptor tyrosine-kinase inhibitors (nintedanib, pazopanib, regorafenib, sorafenib, sunitinib, and vatalanib). The big hopes for the therapeutic antitumor efficacy of antiangiogenic drugs have been not fully met; these agents have shown to improve the progression-free survival and overall survival only in certain cancers, including renal cell carcinoma, colorectal carcinoma, and hepatocarcinoma; however, no improvements in overall survival have been documented in patients with brain tumors, pancreatic adenocarcinoma, prostate cancer, breast cancer, and melanoma. Furthermore, biomarkers linked to the evaluation of antiangiogenic effects, such as MVD and VEGF levels, did not adequately predict a response to antiangiogenic agents [81, 88, 142]. The reasons of tumor resistance to antiangiogenic growth factors are multiple and involve stroma-driven mechanisms of drug resistance and the production of angiogenic growth factors different from VEGF [81, 88, 142]. However, growing evidences indicate that some tumors are resistant because their tumor vasculature is not dependent upon angiogenesis, but upon nonangiogenic mechanisms such as vasculogenesis, vascular mimicry, or vascular co-option [81, 88, 142].

No clinically validated predictive biomarkers allow the identification of cancer patients who have elevated probability to respond to antiangiogenic therapies. Histopathology is probably the best approach to distinguish between the various pathways of tumor neovascularization and may represent a precious tool in future studies for stratification of patients for different types of antiangiogenic therapy.

Recent studies suggest the existence of a possible therapeutic interaction between antiangiogenic agents and cancer immunotherapy. This approach was based on the observation that the functionally aberrant tumor vasculature contributes to the immunosuppressive tumor microenvironment generating a physical barrier to T cells' infiltration. This conclusion is supported by experimental studies showing that anti-PD-1 therapy sensitized and prolonged the efficacy of antiangiogenesis inhibitors and increased vessel normalization [3, 78, 170]. The clinical rationale of this approach is supported by recent results obtained in clear cell renal cell carcinoma (RCC). RCC is characterized by a hyperangiogenic state related to the overproduction of VEGF, as a consequence of the von Hippel-Lindau gene inactivation. VEGF signaling targeting with bevacizumab or sunitinib was effective in the majority of RCC patients; however, the development of resistance limits the impact of this therapy. A phase II and a phase III trials showed improved progression-free survival for metastatic RCC patients treated with atezolizumab (anti-PD-L1) plus bevacizumab

compared to those treated with subutinib (11.2 months versus 7.7 months) [110, 145].

Although at the moment the clinical impact of antiangiogenic agents for cancer therapy is limited, there is a reasonable hope that a better understanding of the mechanisms leading to tumor vascularization; the development of drugs targeting vasculogenesis, vascular mimicry, and vascular co-option; and a better stratification of patients could consistently improve the antitumor efficacy of this therapeutic approach in the future. The development of these new antiangiogenic treatments will meet the general philosophy proposed by Jain in 2001 [75] that alleviating hypoxia at the level of the tumor through a normalization of tumor microenvironment may represent a general paradigm for improving cancer treatment using conventional (chemotherapy or radiotherapy) and novel therapy (targeted therapy or immunotherapy) [108].

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Chimeric Antigen Receptors for the Tumour Microenvironment

8

Rosemary Habib, Adnan Nagrial, Kenneth Micklethwaite, and Kavitha Gowrishankar

Abstract

Chimeric antigen receptor T (CAR-T) cell therapy has dramatically revolutionised cancer treatment. The FDA approval of two CAR-T cell products for otherwise incurable refractory B-cell acute lymphoblastic leukaemia (B-ALL) and aggressive B-cell non-Hodgkin lymphoma has established this treatment as an effective immunotherapy option. The race for extending CAR-T therapy for various tumours is well and truly underway. However, response rates in solid organ cancers have been inadequate thus far, partly due to challenges posed by the tumour microenvironment (TME). The TME is a complex structure whose role is to subserve the persistence and proliferation of tumours as well as support their escape from immune surveillance. It presents several obstacles like inhibitory immune checkpoint proteins, immunosuppressive cells, cytokines, chemokines, stromal factors and adverse metabolic pathways. CAR structure and CAR-T therapies have evolved to overcome these obstacles, and we now have several novel CARs with improved anti-tumour activity demonstrated in xenograft models and in some clinical trials. This chapter provides a discussion of the evolution of CAR-T therapies to enable targeting specific aspects of the TME.

Keywords

Tumour microenvironment (TME) · Chimeric antigen receptor · CAR · Adoptive cell therapy (ACT) · Cancer · Immunotherapy · Tumour immunology · Genetic engineering · Tumour metabolism · T-cell therapy · Checkpoint receptors/checkpoint inhibitors/

R. Habib
Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia

Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

A. Nagrial
Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

K. Micklethwaite
Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia

Crown Princess Mary Cancer Centre, Westmead Hospital, Sydney, NSW, Australia

Sydney Cellular Therapies Laboratory, Blood and Bone Marrow Transplant Unit, Department of Haematology, Sydney Medical School, Westmead Hospital, Sydney, NSW, Australia

K. Gowrishankar (✉)
Westmead Institute for Medical Research, The University of Sydney, Sydney, NSW, Australia
e-mail: Kavitha.Gowrishankar@sydney.edu.au

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_8

programmed cell death 1 receptor · Chemokine/chemokine receptor · T-cell homing · Stroma · Vasculature · NK-CAR

8.1 Introduction

Advances in synthetic immunology and genetic engineering have resulted in the evolution of immunotherapy. The advent of chimeric antigen receptor (CAR) expressing T cells to treat haematological malignancies has introduced the term "cell therapy" into the vernacular. While other forms of immunotherapy, which can include immunomodulatory drugs, cytokines, cancer vaccines, check point inhibitors and oncolytic viruses, have all played crucial roles in treatment, it is cell therapy with genetically modified T cells that has dramatically altered the way we design treatment.

While checkpoint inhibition with anti CTLA-4 and anti-PD1 inhibitors brought immunotherapy to the forefront during the first half of this decade, adoptive cell therapy (ACT) has dramatically revolutionised cancer treatment in the latter half of the decade. Cell therapy has surpassed all other forms of immunotherapy, with a 113% growth in the number of active agents being trialled between September 2017 and September 2018 [140]. ACT is the production and expansion of tumour-specific T cells ex vivo that are then infused into the patient. ACT is broadly divided into three types of therapy which include tumour infiltrating lymphocyte (TIL) therapy, transgenic T-cell receptor (TCR) therapy and chimeric antigen receptor (CAR) therapy.

TIL therapy involves isolating lymphocytes from within tumours, culturing them in the laboratory, expanding them and reinfusing them back into patients. Transgenic TCRs are created by genetically engineering T cells to express a T-cell receptor specific for a single peptide (specific to either tumour antigen or viral peptide). CAR is a completely synthetic fusion protein, generated by the combination of an extracellular domain against a tumour antigen (typically from an antibody molecule) and intracellular domains taken from signalling components of the T-cell receptor complex. CAR-expressing T cells are generated or manufactured in the laboratory and can be infused into patients as "living drugs".

CAR-T therapy has excited clinicians and patients alike with tremendous responses, especially for haematological malignancies, with CAR-T cell products (Kymriah™ and Yescarta™) targeting the protein CD19, being approved for clinical use for acute B-cell lymphoblastic leukaemia (B-ALL) and diffuse large B-cell lymphoma (DLBCL) [24, 72, 84, 85, 95, 116]. At the time of writing this chapter, there were 402 CAR-T cell clinical studies that were actively recruiting worldwide (clinicaltrials.gov), reflecting the promise that this technology holds. However, CAR-T cells are not the panacea that are successful for all malignancies. For solid tumour malignancies, several factors including tumour intrinsic factors and heterogeneity and more importantly the hostile tumour microenvironment (TME) act as significant hurdles.

At a glance

Chimeric Antigen receptor- CAR

A synthetic fusion protein usually consisting of a part of an antibody (Ab) targeting a tumour antigen and a part of a T-cell receptor responsible for intracellular signalling.

CAR-T cell

A T-cell genetically engineered to express a Chimeric Antigen Receptor.

TME

Tumour microenvironment that consists of the tumour cells, suppressive surrounding immune cells, secretory molecules including cytokines, chemokines, the stroma and vasculature than encase the tumour.

The role of the immune system in imparting a pro-tumour effect was first recognised in 1972.

Accelerated growth of sarcoma tumours was observed in mice which were inoculated with spleen cells compared with the sarcoma tumours in immunosuppressed mice [114]. The TME provides a supportive cellular and stromal environment which enables tumour cells to persist, proliferate and evade the immune system [37, 48]. However, the TME in parallel can hamper effective treatment responses against the tumour, due to physical stromal and metabolic barriers, immunosuppressive cells and a suppressive cytokine milieu, which are not conducive for effective immune responses [34, 82]. In addition, upregulation of checkpoint receptors and their ligands is frequently observed [90, 107]. These factors can impede the actions of therapeutic agents, including infused CAR-T cells. Some of the important components of the TME include cytokines such as TGF-β, IL-10 and IL-6, negative regulatory inhibitory receptors (including CTLA4, PD1, TIM3 and LAG-3) and ligands (PD-L1, Galectin 9) and immunosuppressive cells (such as myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs), T regulatory cells (T Regs) and neutrophils) [107]. Strategies for optimising CAR-T cell therapy need to take these factors into account to enable efficient homing of the CARs to the tumour, followed by effective clearance of the tumours while overcoming these hurdles.

The reader is directed to other chapters within this textbook for a detailed description of each component of the TME. This chapter will address the important question – how can we extend the benefits of CAR-T therapy to overcome the hostile TME? The CD19 CAR paradigm has taught us that infusing effector cells capable of long-term memory and persistence would be required for a sustained response [131]. The primary goal is thus to manufacture and expand CAR-T cells in optimal conditions to result in pure populations of effector cells, devoid of exhausted phenotypes. However, there are several additional factors which require to be considered while designing CARs for solid tumour malignancies. This chapter will focus on the basics of CAR structure and the evolution of CAR designs to enable targeting specific aspects of the TME.

8.2 Chimeric Antigen Receptors (CARs)

A CAR or chimeric antigen receptor is a synthetic fusion protein, comprised of domains taken from various diverse proteins, to make a functional novel chimeric protein or chimeric antigen receptor (CAR). Typically, single chain (heavy and light) variable regions from an antibody (i.e. specific for an antigen) are fused with the ζ activation domain from the CD3 molecule (Fig. 8.1). When the DNA encoding the CAR is expressed in T cells, it results in the formation of CAR-expressing T cells or CAR-T cells. These cells can then be infused into the patients as therapeutic drugs.

A native T-cell receptor (TCR) requires the α and β subunits to interact with the intracellular CD3ζ (zeta) chain for functional activity. It recognises a given antigen presented by an

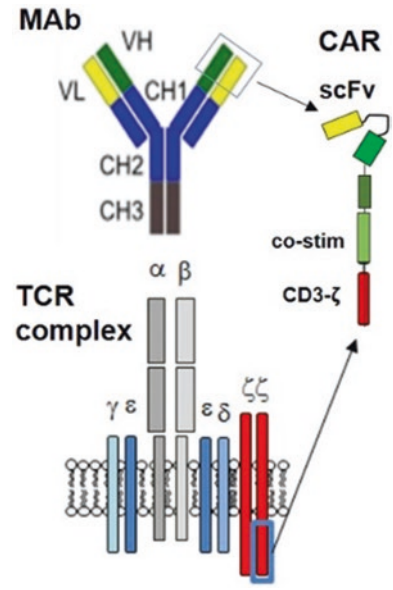

Fig. 8.1 Chimeric antigen receptor (CAR). Variable regions from the heavy (VH) and light chain (VL) from a monoclonal antibody (MAb) are fused to the ζ domain from the CD3 molecule (that makes up the T-cell receptor (TCR) complex) to result in a chimeric protein. This illustration incorporates an additional costimulatory domain from another protein (either CD28 or CD137) and a transmembrane region connecting the scFv to the signalling domain to depict a typical chimeric antigen receptor

antigen-presenting cell on a HLA molecule [106]. For an effective immune response, the T-cell receptor engages costimulatory proteins like those belonging to the immunoglobulin superfamily of which CD28 is the most common or of the TNF receptor family such as CD137 (4-1BB) or OX40. Through the collaborative action of these proteins, a T-cell response is subsequently generated.

CAR-T cell therapy is unique as it functions in a HLA-independent manner because the extracellular region (single chain variable, scFv) can bind to a tumour-specific antigen (TAA) [125] and directly activate the downstream intracellular signalling domains.

8.2.1 Evolution of CARs

Irving and Weiss demonstrated the significance of the ζ chain in translating ligand binding to signal transduction and resultant downstream effects, a mechanism that was demonstrated in the absence of the other CD3 linked chains γ, δ and ε [50]. It was Eshhar who first designed a CAR in 1990, now recognised as the first-generation CAR [29]. The chimeric T-cell receptor was named "T body" and comprised an extracellular single chain variable (scFv) antibody against hapten initially combined with the CD3 ζ chain. They demonstrated that attaching the heavy and light chains (VH and VL, respectively) of antibodies to signalling regions of the T-cell receptor complex maintained the functional activity of T cells. This allowed for HLA-independent antibody specificity and resulted in the effective transmission of a signal that mediated the cytotoxic action of the T cells [36]. This fusion protein was able to secrete IL-2 in response to the soluble antigen (TNP-BSA) [29]. First-generation CAR-T cells carrying a single signalling domain demonstrated antiviral effects in a randomised phase II trial in HIV but did not result in a cure [26].

The importance of costimulation for T-cell function was recognised, and incorporating a CD28 domain resulted in a second-generation CAR construct [2, 126]. Thus, a second-generation CAR-T cell has three key components to the synthetic receptor, the scFv region (comprised of VH and VL of an immunoglobulin) that primarily aids in the recognition of a cell surface-expressed antigen, a costimulatory domain (CD28, 4-1BB, DAP10, OX40 or ICOS) and the CD3 ζ chain [126].

The successful CARs against CD19 that have been approved by the FDA for clinical use (Kymriah™ and Yescarta™) are second-generation CARs. In a meta-analysis of phase I clinical trials comparing the efficacy of second-generation CD19 CAR-T cells for the treatment of B-cell malignancies [162], it was noted that the persistence of CAR-T cells with the 4-1BB costimulatory domain (19-BBz) was longer (6 months) than that of the CAR-T cells with the CD28 (19-28z) costimulatory construct (typically 1–4 months), despite comparable efficacy with evidence of complete remission in patients with ALL when using either treatment [162]. A study of the kinetics and persistence of these two different generation CAR-T constructs confirmed that 19-28z CAR-T cells display explosive activity early with rapid tumour killing compared with 19-BBz CARs. While not as potent as 19-28z, 19-BBz persisted for longer and could therefore result in equivalent tumour elimination over time [166]. The median in vivo persistence of a 19-28z CAR is 30 days, while it is 168 days for the 19-41BBz with the latter displaying a broad range of persistence times ranging from 20 to 617 days [85, 109, 131].

To take advantage of the qualities of both costimulatory domains, third-generation CARs were generated with the aim of combining the early cytotoxicity of 19-28z cells and the durability of 19-BBz cells for optimal anti-tumour activity. Third-generation CARs are composed of two costimulatory molecules, typically CD28 and 4-1BB, connecting the scFv portion to the CD3 ζ intracellularly (19-28BBz) [167]. Several different third-generation constructs targeting various tumour antigens were tested in preclinical and clinical studies in both haematological and solid organ malignancies; however, they failed to dem-

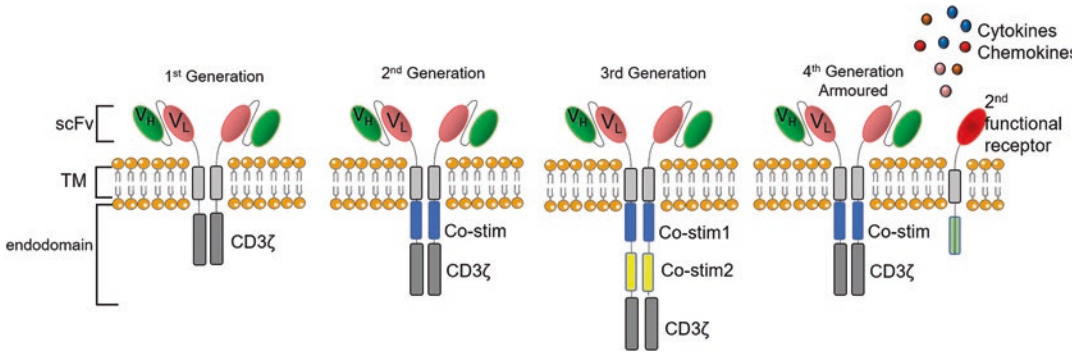

Fig. 8.2 Evolution of CARs. First, second, third and fourth generation of chimeric antigen receptors. The heavy and light chain variable regions (scFv) of an antibody making up the extracellular region of receptor are linked to the activation ζ domain from CD3 to generate a first-generation CAR. A prototype was originally described as the "T-body". Incorporation of additional costimulatory domains such as CD28 or CD137(4-1BB) or OX-40 along with the CD3ζ results in a second-generation CAR. Third-generation CARs can include several costimulatory domains linking the extracellular portion of the CAR to CD3ζ. Fourth-generation CARs are armoured with additional features – the ability to secrete cytokines or chemokines or express additional functional receptors (e.g. a neutralising antibody)

onstrate superior effectiveness [44, 58, 141]. Kinetic studies demonstrated an initial rapid anti-tumour response of 19-28BBz; however, overall efficacy was low due to poor persistence ([166]; Fig. 8.2). Hence, most of the CARs currently being designed and trialled are of the second-generation type with a single costimulatory domain.

8.2.2 Armoured CARs

With the challenges posed by solid tumour malignancies, it was imperative to develop multifunctional CARs. In addition to being cytotoxic, fourth-generation CARs, e.g. armoured CARs (some are also referred to as TRUCKs – for T cells redirected for universal cytokine-mediated killing), with additional capacity to secrete cytokines (IL-12 or IL-18) were developed ([18, 66, 156]; (Fig. 8.2). IL-12 and IL-18 are key anti-tumour cytokines that have pleiotropic effects. IL-18 enabled CARs to undergo improved expansion and persistence in vivo which resulted in enhanced anti-tumour activity [3].

IL-12 is crucial for the clonal expansion and cytolytic function of CD8+ T cells. However, IL-12 can also be associated with severe toxicity when administered systemically as monotherapy. Earlier versions of these CARs were designed to allow for regulated IL-12 secretion under the control of the activation inducible NFAT (nuclear factor of the activated T cell) response element [19]. Other studies also showed that CAR-T cells secreting IL-12 demonstrated enhanced cytotoxic activity against MUC-16 antigen in ovarian carcinoma preclinical mouse models [67, 157]. IL-12 can modulate the TME, by increasing the total endogenous CD8+ cells, polarising macrophages towards a pro-inflammatory M1 phenotype, reprogramming MDSCs and augmenting mature dendritic cell differentiation and activation [3, 156, 157]. IL-12-secreting CARs targeting the vascular endothelial factor receptor have also shown superior anti-tumour activity [17].

IL-7 is another important cytokine that is known to play a significant role in the establishment of central memory subset of T cells. CAR-T cells that have the capacity to secrete IL-7 in combination with a chemokine CCL19 acted like cellular vectors to recruit regulatory molecules to the microenvironment, resulting in increased recruitment of dendritic cells and T

cells, and showed superior activity and tumour clearance of several cancer cell lines in murine studies [1].

Armoured CAR-T cells have entered phase I and II clinical trials, which are currently underway targeting GD2 in neuroblastoma (NCT02992210), Nectin-4/FA in nectin-4 positive solid organ cancers (NCT03932565), PSMA in urothelial bladder cancer (NCT03185468) and folate receptor-α also in urothelial cancers (NCT03185468). Armoured CARs also have the potential to reduce manufacturing and treatment costs allowing for a broader and extended application of CAR-T therapy in solid organ cancers.

8.3 The Tumour Microenvironment Poses Significant Barriers

The barriers of the hostile TME that must be overcome by CAR-T cell therapies are threefold: firstly, trafficking barriers posed by a dysregulated chemokine and chemokine receptor axis and physical barriers posed by the stroma and the tumour vasculature; secondly, the challenges created by the immunosuppressive cells and their cytokines and inhibitory checkpoints; and thirdly, the hypoxic nature and nutrient deficiency of the TME (Fig. 8.3). The latter two are directly responsible for the limited survival and cytotoxic

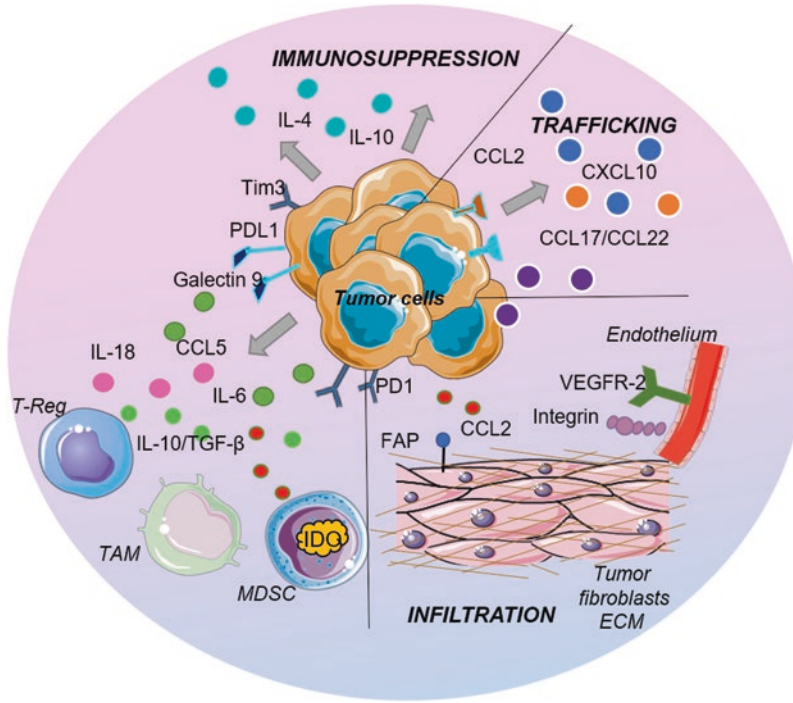

Fig. 8.3 The tumour microenvironment. Immunosuppressive environment is created by the monocyte-derived suppressor cells (MDSC), tumour-associated macrophages (TAM) and T-regulator cells (Treg). The schematic indicates some of the cytokines and chemokines seen surrounding the tumour mass, secreted either by the tumour cells or the suppressive cells. In addition, the tumour cells express a variety of inhibitory receptors and ligands (e.g. PD-1, Tim3, PD-L1, Galectin9). Trafficking barriers are generated by dysregulated chemokine gradients, while the stromal fibroblasts and endothelium with associated proteins (e.g. enhanced vascular endothelial growth factor (VEGF) and its receptor, fibroblast-associated protein FAP, heparan sulphate proteoglycans) pose significant hurdles for effective T-cell infiltration

actions of CAR-T cells, while the former prevents access of T cells to the tumour. Several novel next-generation CARs have been designed and tested to overcome these barriers and are discussed below. These various designs are brought together and displayed in a hypothetical CAR-T cell schematic that is given in Fig. 8.4.

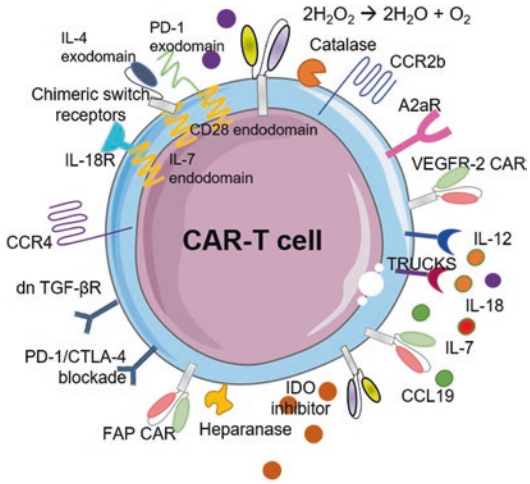

Fig. 8.4 Schematic of CAR-T cell with features to overcome TME barriers. Illustration of some of the additional features added to conventional CAR-T cells to increase functionality, as discussed in the text. A single synthetic receptor with a scFv targeting a tumour-associated antigen can be complimented by expressing chemokine receptors like CC2b and CCR4 to overcome the chemokine gradient and help in the trafficking of T cells into the microenvironment. Other armoured CARs can have the ability to secrete various cytokines and chemokines like IL-12, IL-7, CCL19 or inhibitors to IDO. Forced expression of additional CARs and proteins like heparanase and catalase can help overcome stromal and metabolic barriers. Antibodies against checkpoint inhibitors and their ligands such as anti-PD1/PD-L1 antibody expressed on the surface of the CAR-T cells have been effective in overcoming the inhibitory effects, while dominant negative receptors (dnTGF-βR) can titrate out the suppressive cytokines like TGF-β. Novel switch receptors designed to express a fusion protein of extracellular regions of IL-4 or PD-1 with intracellular signalling domains from IL-7 or CD28 that have shown tremendous promise in preclinical studies are also indicated

8.3.1 Trafficking Barriers and CARs Expressing Chemokine/ Chemokine Receptors

Chemokines are a family of small signalling proteins and play crucial roles in migration of cells during embryogenesis, in directing immune cells (adaptive and innate) during tissue injury and inflammation to assist with wound healing and in promoting or inhibiting angiogenesis [5]. However, cancer cells secrete chemokines in an aberrant manner which helps promote tumour proliferation and metastasis as part of the hallmark inflammatory process of cancer [38]. In addition to upregulating key signalling pathways like the MAPK (RAS-RAF) and pAKT pathways as reported in melanoma and pancreatic cancer, several cancer cells have a high degree of mutations [83], e.g. VHL mutation in renal cell cancer and TP53 mutation, while several others activate oncogenes like MYC as seen in pancreatic cancer [5]. These in turn can lead to increased or decreased levels of chemokines being secreted into the microenvironment, causing an alteration in the gradient. This can directly impede CAR-T cells that naturally do not have corresponding receptors to enable migration towards the tumour site. Alternatively, the chemokine environment can attract immunosuppressive cells like the tumour-associated macrophages (TAMs) which in turn can often overwhelm effector T cells after they have entered the tumour milieu [23].

Chemokine receptors control migration in response to chemokine gradients (towards a high concentration of the chemokine) (Fig. 8.5). The chemokine receptor family is a large group of G-protein-coupled receptors, located on all cell types in the human body including immune, epithelial and endothelial cells. There are more chemokine ligands than there are receptors, and their interaction is tightly regulated to maintain homeostasis. With the exception of CXCL10, most chemokines promote tumour progression, angiogenesis and metastasis [39] and control the movement of immune cells.

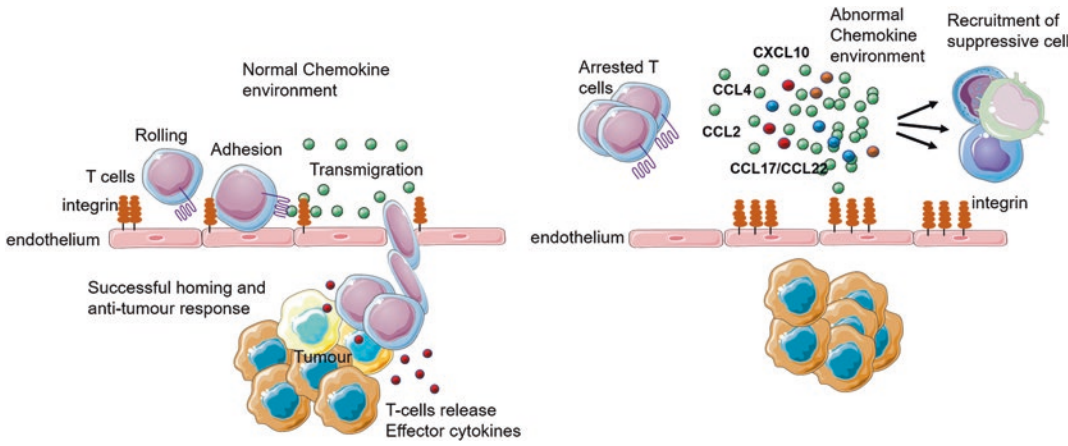

Fig. 8.5 Chemokine gradients can act as trafficking barriers. The recruitment of T cells to the tumour requires a balanced chemokine milieu with the T cells expressing the relevant receptors. The transmigration of T cells enables the traversing of the endothelium to reach tumour cells for a successful effector response, release of effector cytokines and cytolytic granules resulting in tumour clearance. In a dysregulated chemokine gradient, T cells lack the corresponding receptors and are arrested. They fail to adhere to the endothelium and migrate towards the tumour cells. Moreover, the chemokines serve to attract other suppressive immune cells like macrophages, T regulatory cells and monocyte-derived suppressors which further overwhelm the T cells. In addition, the hypoxic environment caused by the accumulation of tumour cells can increase expression of some integrins on the endothelium and on recruited cells, increasing interaction with VEGF, leading to disruption of angiogenesis and inhibition of apoptosis and causing further barriers to effector T-cell function

Tumour cells can secrete CXCL1, CXCL8 and CXCL12. TAMs can release chemokines including CCL1–3, CCL5 and CXCL1, CXCL10 and CXCL12 to facilitate abnormal neovascularisation, stroma formation, tumour growth and metastasis. Fibroblasts can secrete CXCL6, CXCL12, CXCL14, CCL2, CCL5 and several interleukins [56, 63, 98]. A positive association between TAM levels and high CCL2 expression was observed in breast cancer that predicted poor survival in patients [146]. Using breast cancer models, inhibition of CCL2 improved radiotherapy responsiveness and reduced metastatic spread [88]. In prostate cancer, CCL2 correlated with multiple roles including growth promotion and migration; however, anti-CCR2 monoclonal antibody therapy was not effective in reducing the rate of prostate cancer growth [88, 159]. However, using CAR-T cells to effectively overcome the high levels of CCL2 was demonstrated in models of neuroblastoma in CARs targeting the antigen GD2. Introducing the chemokine receptor, CCR2b, which can target the CCL-2 chemokine in the same CAR-T cells created dual functional CAR-T cells which resulted in increased trafficking and enhanced anti-tumour activity in xenograft models of the disease [23].

Treatment of adult T-cell leukaemia was attempted by targeting the chemokine receptor CCR4 using a monoclonal antibody. A phase II clinical trial demonstrated safety and clinical efficacy in patients with ATL, with an objective response rate of 50% including eight complete responses [51]. Following this demonstration, CARs expressing CCR4 were able to overcome the suppressive activity of CCL17 and CCL22 and demonstrated potent antigen-dependent cytotoxicity in a murine xenograft model of T-cell leukaemia, showing promise for clinical application [112]. Forced expression of CCR4 on CAR-T cells targeting CD30 in Hodgkin lymphoma also resulted in increased trafficking to the tumour sites and tumour clearance [28].

CXCR3 is one of the main chemokine receptors that are expressed on TILs in melanoma, colorectal cancer and breast cancer. It is crucial for the transmigration of T cells across the vascular endothelium and into the tumour space which can express high levels of CCR3. Strategies to express CXCR3 will play important roles in

influencing CAR-T cell transmigration into tumours and anti-tumour activity. Preliminary studies on CD8+ T cells expressing CXCR3 have demonstrated improved survival and effector function [45]. PD1 immunotherapy was shown to increase IFN-γ and CXCL10 at the tumour sites which corresponded with increased cytotoxic T-cell trafficking to melanoma metastatic sites [111]. Recently CXCR2-expressing anti-αvβ6 integrin CAR-T cells were shown to migrate more efficiently towards IL-8 containing microenvironments and showed improved anti-tumour activity in xenograft models of ovarian and pancreatic cancer [153]. Understanding the various chemokines in the milieu will enable the designs to express the corresponding receptors on the CAR-T cells.

To add to the complexity, tumours can also express their own chemokine receptors including CCR1-4, CCR7, CCR9, CX3CR1 and CXCR4 that aid in their migration across the endothelium to distant metastatic sites [115]. Allowing for secretion of chemokines by T cells would enable trafficking towards metastatic lesions. This was demonstrated with the expression of CCL19 in combination with cytokines which resulted in improved immune cell infiltration and CAR-T cell survival [1]. "Self-driving" CARs co-expressing a chemokine or chemokine receptor, in addition to the chimeric receptor targeting the TAA, will be important to increase the homing capacity and to overcome trafficking hurdles.

8.3.2 Vascular Barriers and CARs to Overcome VEGF

Tumour angiogenesis can be highly disorganised resulting in abnormal vascular networks, increased interstitial fluid pressure, vascular hyper-permeability and abnormal pericyte support [108]. A well-known feature of rapidly dividing cancer cells is the presence of a central necrotic core that results from fast-growing cells exceeding their diffusion limit for oxygen [10]. Cells within this core have defective apoptosis and are selected for through mutational pressures, with p53 being the most commonly observed mutation. Very few immune cells exist within the core, with TIL numbers being the lowest compared with other regions of the tumour. The ensuing tissue hypoxia induced by tumour growth leads to the formation of hypoxia-inducible factor 1 (HIF-1) that can bind to the promoter of the vascular endothelial growth factor (VEGF) gene to upregulate its transcription [108].

VEGF is the main stimulator for tumour angiogenesis and, along with VEGFR-2, is upregulated in many cancers particularly metastatic colorectal cancer. VEGF overexpression is associated with poor prognosis in many cancers including colorectal, gastric, pancreatic, breast, hepatocellular and ovarian carcinoma. VEGF induces an immunosuppressive microenvironment by disrupting the maturation of dendritic cells, upregulating Treg cells and downregulating CD8+ T cells ([132]; Fig. 8.6). FasL is also upregulated in response to VEGF, leading to CD8+ T-cell apoptosis while sparing Treg cells [129]. Clinical responses to targeted anti-VEGF antibodies have been observed in renal and colorectal cancers, owing to improved anti-tumour T-cell recruitment and efficacy [132].

Armoured CAR-T cells directed against VEGFR-2 in combination with IL-12 secretion altered the effect of MDSCs in the TME and improved anti-tumour effects for five different cancer types in murine models [17]. Promising preclinical results were disappointing when translated into a phase I/II study of "CAR-T Cell Receptor Immunotherapy Targeting VEGFR2 for Patients with Metastatic Cancer" (NCT 01218867). While this preliminary study failed to demonstrate objective responses in 22 out of the 24 participants, targeting VEGF is still crucial if effector T cells need to overcome the vasculature. Recent reports described the importance of targeting VEGFR3, and it is likely that further CARs targeting the VEGF/VEGF receptor axis will be developed.

Integrins are other proteins that are expressed highly on tumour cells and endothelial cells of the vasculature. CAR-T cells targeting integrin

αvβ3 (echistatin-containing CARs) and αvβ6 have shown tremendous responses in preclinical studies for ovarian, breast and pancreatic cancers [32, 151, 153].

8.3.3 CARs to Help Traverse the Stroma

The stroma creates a physical barrier that impedes the penetration of CAR-T cells into solid organ cancers. In many solid organ tumours including breast, pancreas and gastric cancers, the stroma can comprise >80% of the tumour mass. In such tumours, normal cells are replaced by fibrous material creating a desmoplastic stroma with increased amounts of collagen, proteoglycans, hyaluronic acid and chondroitin sulphate. The main components of the stroma are mesenchymal (including fibroblasts and myofibroblasts), endothelial and inflammatory cells. Their combined role is to lay the extracellular matrix (ECM), collagen and the vascular network [121].

The degree of fibrosis caused by the abnormal cytoarchitecture of a tumour stroma correlates with poor prognosis [59]. Biologically, it is one of the factors responsible for increased metastatic potential of tumour cells. This is seen in pancreatic cancer, one of the deadliest cancers with a 5-year mortality of >95% in advanced stages, where a high degree of stromal fibrosis is directly attributed to invasion and metastases. Its histopathology is characterised by changes in the ECM leading to a highly desmoplastic stroma and loss of the normal organisation of laminin and type IV collagen. The high tensional force induced by its stroma and the projection of laminin and collagen contribute to the enhanced migratory and metastatic capacity of the cancer cells [59]. This is believed to prepare "metastatic niches" or landing sites for metastatic spread [69].

Fibroblasts are the predominant cells in the stroma and are involved in crosstalk with other cells. In cancer, fibroblasts are constitutively activated with resultant enhanced pro-tumour activity [31, 60]. Fibroblasts existing within the tumour stroma are known as cancer-associated fibroblasts (CAFs) (Fig. 8.6). CAFs originate from a variety of cells, including endothelial cells and bone marrow-derived precursor cells [60]. CAFs

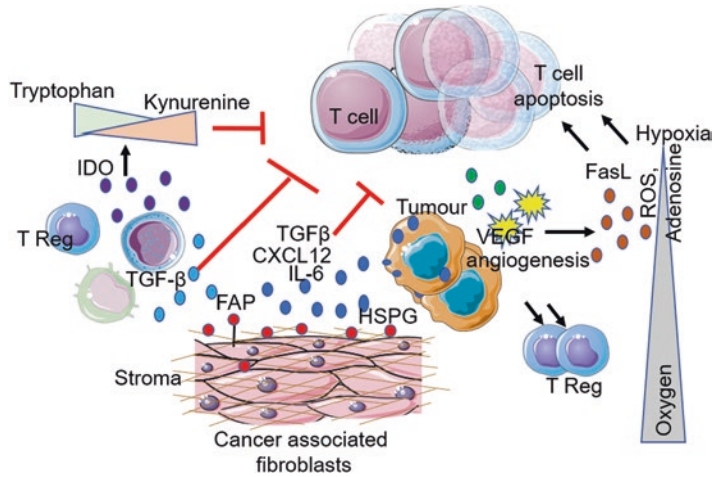

Fig. 8.6 Factors influencing T-cell function and survival/death. The cells within the TME can secrete inhibitory factors like transforming growth factor (TGFβ) and indoleamine 2,3-dioygenase (IDO) that impede T-cell function. IDO helps in the conversion of tryptophan to kyurenine and is a rate-limiting enzyme in tryptophan catabolism. The depletion of tryptophan inhibits effector T-cell proliferation. The stromal fibroblasts and heparan sulphate proteoglycan (HSPG) are strong barriers to T-cell migration and limit access to the tumour cells. Vascular endothelial factor can induce expression of the death ligand FasL causing T-cell apoptosis in Fas receptor expressing cells while sparing and recruiting inhibitory Tregs. Nutrient deprivation and hypoxic conditions result in reactive oxygen species and adenosine which can all be detrimental to T-cell function and survival

contribute to the ECM stiffness and lead to a physical barrier to CAR-T cells attempting to gain access to the tumour. In addition, they enhance tumour angiogenesis, producing a pro-tumour inflammatory environment (by increasing IL-6 and Tregs), and induce chemotherapy resistance. CAFs also release TGF-β to drive tumour progression (via activation of SMAD complexes or regulation of the MAP kinase and PI3K/Akt pathways) [31].

CAFs are regarded as heterogeneous and express a variety of cell surface proteins including α-SMA, fibroblast activation protein (FAP), FSP1, VIMENTIN and PDGFR α and β [31]. Further, their phenotypes are altered depending on their location within the TME [103]. For instance, in mouse and human pancreatic ductal adenocarcinoma, α-SMA high surface expressing CAFs reside close to tumour cells, while those distant to the cells expressed low levels of α-SMA and secreted IL-6, a pro-inflammatory cytokine. Interestingly however, FAP and PDGFR surface expression remained unchanged [103]. FAP is unique to CAFs; it is seen in >90% of CAFs associated with epithelial carcinomas and has a very low expression on normal fibroblasts. Thus, FAP represents an attractive target for adoptive cell therapy.

Currently FAP and heparan sulphate proteoglycans are being targeted with CAR-T cells in the hope to overcome the stromal barriers for T-cell therapy. Targeting the stroma is in theory a plausible idea as it is common to most solid organ tumours, and downregulation of CAFs could improve anti-tumour activity and assist in delivering CAR-T therapies to the TME. Early preclinical studies with anti-FAP CAR-T cells in 2008 demonstrated efficacy in xenografts of lung, pancreas and head and neck cancer in immunodeficient mice [105]. This was confirmed by another independent study which also verified anti-tumour activity of an anti-FAP CAR in preclinical murine studies [144].

Other reports using second-generation anti-FAP CD28/CD3ζ CAR-T cells in mice with malignant pleural mesothelioma (MPM) demonstrated antigen-specific activity and resulted in increased IFN-γ secretion with a reduction in tumour cell proliferation [130]. A phase I clinical trial is currently underway to assess the safety and feasibility of local administration of anti-FAP CAR-T cells in patients with malignant pleural mesothelioma, and results are currently awaited (NCT01722149; clinicaltrials.gov). Yet another phase I trial with local injection of a fourth-generation CAR construct targeting both FAP and Nectin-4 has recently commenced for recruitment, for patients with Nectin4-positive advanced solid organ tumours (NCT03932565). Briefly, Nectin-4 is a TAA that is overexpressed in certain types of breast, bladder, lung and pancreatic cancers and minimally detected on the cell surface of healthy tissues [77]. In 2018, anti-Nectin-4 monoclonal antibody (enfortumab vedotin) received FDA breakthrough treatment for bladder cancer.

Heparan sulphate proteoglycans (HSPGs) are a type of glycosaminoglycan that also play an important role in tumour growth. They bind to fibroblast growth factor ligands and receptors to enhance tumour growth. CAR-T cells can lose their ability to breakdown HSPGs and to generate effective responses against solid organ cancers, a deficiency that was recognised for CAR-T therapy developed for neuroblastoma. To overcome this, heparanase (HSPE) was co-expressed in CAR-T cells targeting GD2, which resulted in significantly greater tissue penetration compared with CARs that lacked HSPE (4.6% vs. 0.1%, respectively) and improved their in vitro activity (66% vs. 13%, respectively) and survival in xenograft models of neuroblastoma [11].

8.3.4 Cells and Cytokine Milieu and CARs to Overcome the Suppressive Factors

Several key immunosuppressive cells exist within the TME which can overwhelm CAR-T cells preventing their function and transmigration into the tumour. They include T regulatory cells, MDSCs, TAMs and tumour-associated neutrophils. These

cells are responsible for the release of immunosuppressive factors, notably TGF-β, cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), VEGF, PDGF, IL-4 and IL-10 [94, 152, 168].

TGF-β has a pleiotropic effect that is predominantly inhibitory to T cells and favours tumour survival and growth. TGF-β acts through its receptors TGF-βRI, TGF-βRII and TGF-βRIII and activates transcription of several genes, through signalling proteins Smad 3 and 4. Given its widespread effects within the body, inhibiting TGF-β could have devastating adverse effects including autoimmune diseases and could disrupt physiological angiogenesis and muscle growth [160]. Therefore, controlled and targeted inhibition of specific TGF-β or its receptors could allow for improved T-cell activity while minimising toxicity.

In 2018, preclinical efficacy of an anti-PSMA CAR-T cell expressing a dominant negative TGF-βRIII (dnTGF-βRIII-TA-Pbbz) in prostate cancer murine models was demonstrated [65]. There was an increase in CAR-induced lysis of the cell lines expressing both PSMA and TGF-β through increased secretion of Th1 and Th2 cytokines, enhancing T-cell persistence and long-term proliferation. This lentiviral transduced CAR-T cell has been translated into clinical trials in men with refractory castrate-resistant prostate cancer (NCT 03089203) [65, 96]. An abstract presented at the American Society of Clinical Oncology (ASCO) demonstrated safety in two of the cohorts in this trial with the side effect being cytokine release syndrome (release of IL-6) that was successfully managed with the administration of tocilizumab (an IL-6 receptor antibody) [96]. Another phase I clinical trial of dnTGF-βRIII in EBV-related T-cell lymphoma is also being trialled (NCT 01140373) [65].

High IL-4 levels in the TME lead to reduced effector T-cell proliferation, promote stromal fibrosis and support tumour growth. IL-4 was targeted using a chimeric "switch receptor" method whereby the extracellular domain of the IL-4 receptor was fused to the stimulatory intracellular signalling domain of IL-7. Thus, binding of IL-4 to the CAR-T cell leads to activation via the IL-7 intracellular domain. Hence, instead of inhibiting T-cell responses, IL-4 binding can now enhance proliferation and function of the CARs. The chimeric switch receptor CAR demonstrated activity in an in vitro model of pancreatic ductal adenocarcinoma [87]. Hence, while inhibiting the suppressive cytokine milieu (e.g. IL-4, TGFβ, IDO) is crucial, designing alternate and combinatorial strategies to mine the positive effects of cytokines like IL-12 and IL-7 is equally important.

A CAR which co-expressed an anti-GD2 receptor and a constitutively expressed cytokine receptor (C7R) that triggered IL-7 signalling led to enhanced anti-tumour activity and improved T-cell proliferation and persistence in xenograft models of neuroblastoma and glioblastoma [133]. In another example, in a xenograft model of neuroblastoma, an EBV-specific CAR construct co-expressing IL-7Rα and anti-GD2 receptor demonstrated improved CAR-T cell proliferation and resistance to regulatory T cell-induced immunosuppression with resultant improved cytotoxic activity [113]. Direct secretion of IL-7 in combination with CCL19 also showed superior anti-tumour effects against several types of solid tumour cells in murine models [1]. In this case, an increase in infiltrating T cells and dendritic cells into the tumour was observed, suggesting that CAR-T cells collaborated with the endogenous immune cells to exert the anti-tumour effects. It is known that IL-7 is likely to aid in T-cell memory responses against tumour cells.

Indoleamine-pyrrole 2,3-dioxygenase (IDO) is an enzyme that catabolises tryptophan (that is required for T cells) converting it into kynurenine. It also increases Treg activity which in turn suppresses anti-tumour function of CD4+ and CD8+ T cells ([101]; Fig. 8.6). The idea of blocking IDO-induced MDSC function has gained momentum in the treatment of solid organ malignancies to overcome the immunosuppressive tumour milieu. IDO inhibitors such as epacadostat have shown some promise in phase I studies either alone or in combination with PD1 inhibi-

tors. Pretreatment with fludarabine and mafosfamide altered the levels of tryptophan and downregulated Treg cells in xenograft models of lymphoma expressing IDO, thus improving the activity of CAR 19 cells suggesting a beneficial role for targeting and reducing IDO. This paved the way for generating armoured CARs that can directly secrete IDO inhibitors [100].

8.3.5 Strategies for Checkpoint Receptors

Inhibitory receptors and ligands within the TME can dampen the anti-tumour immune response [145]. The inhibitory proteins including the checkpoint receptors (which include PD-1, Tim3, Lag3, CTLA-4) are upregulated on both tumour cells and on a variety of immune cells like Tregs, MDSCs and immature dendritic cells. The inhibitory ligands include PDL-1/PDL-2. Galectin9 are also frequently known to be overexpressed on the tumour cells. The ligands can bind to their corresponding receptors on the T cells and inhibit effector function [165]. Many of these ligands signal through SHP-1 and SHP-2 (Src-homology 2 domain (SH2)-containing phosphatases 1 and 2) pathways to mediate their inhibitory effects [78].

Indeed, several CAR-T cells including those against CD19, HER2, mesothelin, CEA and GD2 have been reported to acquire an "exhausted" phenotype by upregulating the receptors corresponding to the checkpoint/inhibitory ligands [9, 55, 158]. Gene expression profiling comparing the immune infiltrate of the TME in pre- and post-CD19 CAR-T cell therapy of patients with non-Hodgkin lymphoma enrolled in the multicentre ZUMA-1 clinical trial [97] showed a significant upregulation of RANTES (CCL5), CTLA-4, PD-1 and LAG-3 in post-treatment biopsies [33]. This phenomenon of modulation or immunoediting has also been demonstrated in mesothelioma mouse models exposed to CAR-T cell therapy [16, 89].

To overcome the negative effect of PD-1/PDL-1 within the TME, various types of CAR-T cells have been developed. Dominant negative CARs against PD1 and SHP2 showed improved activity in preclinical models [4, 13, 15]. Three early-phase clinical trials of an anti-CD19 and dnPD-1 CAR demonstrated improved persistence and a clinically significant response in patients with DLBCL with bulky tumours [15]. Dual anti-PDL-1 or dnPD1 CARs co-expressing anti-mesothelin receptors demonstrated improved cytotoxic activity in mouse models [16]. Similar enhanced activity was demonstrated in a bicistronic CAR which secreted an anti-PDL1 antibody to neutralise PDL-1 while also targeting carbonic anhydrase IX (CAIX), significantly reducing T-cell exhaustion, which subsequently translated into a fivefold reduction in tumour cell growth in a renal cell cancer mouse model [137]. Switch receptors have also been generated against a truncated PD-1 extracellular domain substituted with the activating intracellular domain of CD28 to decrease CAR exhaustion and enhance positive intracellular effector signalling, resulting in anti-tumour activity in preclinical models [76].

In a first proof of principle study, Rupp et al. utilised CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-mediated gene editing tool) mediated gene editing to knockdown PD-1 on CD19 CAR-T cells with resultant improvement in CAR-T cell cytotoxic activity against PDL-1 high tumours in mouse models [124]. The reader is referred to other reviews for an in-depth understanding of CRISPR [52, 154]. CRISPR/Cas9-mediated PD-1 knockdown also demonstrated significantly improved function in xenograft models with CAR-T cells targeting mesothelin [47, 118].

LAG 3 (lymphocyte activation gene 3, CD223) functions synergistically with PD-1 to induce anergy in activated T cells [164]. LAG-3 gene knockdown using CRISPR/Cas9 produced CAR-T cells with equal functional activity compared with non-gene-edited CAR-T cells in murine mouse models [164]. Dual blockade of both checkpoints may enhance the therapeutic activity of CAR-T cells.

CTLA-4 (cytotoxic T lymphocyte antigen-4) is constitutively expressed by Treg cells and possesses a high affinity for the ligands CD80/CD86 (B7-1 and B7-2) compared with CD28. While CD28 is fundamentally expressed as a costimulatory molecule in T cells, CTLA-4 is upregulated from intracellular compartments during an immune response and competes with CD28 for binding to CD80/CD86. The dampening of the immune response mediated by CTLA-4 action was what led to the original discovery of checkpoint inhibition which served to keep the immune response under check, preventing over-activation and autoimmune reactions [150]. shRNA-mediated downregulation of CTLA-4 in CD19 CARs in conjunction with CD80 costimulation displayed significantly better in vitro expansion and anti-tumour activity indicating the advantages of targeting checkpoint molecules like CTLA-4 [22].

T-cell immunoglobulin mucin-3 (TIM-3 orHAVCR2) is also involved in immune tolerance and exhaustion of activated T cells, with the ability to terminate Th1 effector responses [40]. TIM-3 is thought to be responsible for the development of resistance to anti-PD1 checkpoint inhibitors [68]. It is induced in the TME of mouse models of acute myeloid leukaemia (AML) after prior CAR-T cell treatment [61]. Dual blockade of TIM-3 and PD-1 improved the function of TILs and could equally enhance anti-tumour effects of CAR-T cells [127].

CD276 or B7-H3 is another checkpoint molecule with high homology to PD-L1 and is expressed highly on several cells within the TME in addition to being expressed on tumour cells (e.g. renal cell cancer, NSCLC, colorectal carcinoma). Overexpression of B7-H3 is associated with poor prognosis. A phase I study using a monoclonal antibody (enoblituzumab or MGA271; NCT01391143; clinicaltrials.org) in advanced refractory solid organ cancers overexpressing B7-H3 provided promising results, and recently, CARs targeting B7-H3 demonstrated regression of tumours in preclinical studies of paediatric sarcomas [81].

It is important to target one or several checkpoint receptors and/or their cognate ligands to overcome the suppressive barriers posed by them. We already have promising results from various CAR-T cells that have targeted PD1/PD-L1 showing significant improvements in preclinical models and in some clinical trials. The next few years will no doubt explore the possibilities of targeting several receptors using CARs and comparing them with the combination checkpoint inhibitor therapy used in clinical practice [92, 155].

8.3.6 Manipulating the Metabolic Barriers of the TME with CAR-T Cells

In the early twentieth century, Otto Warburg observed that cancer cells had increased rates of glycolysis despite having oxygen levels that could foster the oxidative phosphorylation pathway, a concept which he termed the Warburg effect [71]. The consumption of glucose could result in an anaerobic microenvironment not conducive for anti-tumour immune responses, posing significant metabolic hurdles. CAR-T cells need to be empowered with additional features to overcome the metabolic hurdles to result in an effective clearance of the tumour cells.

8.3.6.1 Hypoxia, Acidosis and Nutrient Deprivation

The hypoxia-inducible factor, HIF-1, not only stimulates angiogenesis but also activates the glycolytic pathway leading to the accumulation of lactic acid and acidosis [71]. Acidosis suppresses immune effector functions and upregulates Treg function, allowing for tumour proliferation and metastasis. It promotes M2 polarisation (macrophage differentiation) and MDSC generation, further impeding cytotoxic T lymphocyte activity. Oxidative stress caused by increased reactive oxygen species (ROS) places additional metabolic stresses on effector T cells trying to survive in the TME [71, 81].

The hypoxic TME has been targeted in the generation of "self-decision-making" CAR-T cells with the addition of a HIF domain to the antigen-specific CAR-T constructs [57]. HIF-"decision-making" (or Smart T cells) is activated only in a hypoxic microenvironment. The HIF-CAR construct utilises the hypoxia signal in the TME, resulting in target-specific function under hypoxic conditions compared with normoxic conditions in vitro [57].

Another consequence of the Warburg effect is the generation of high levels of hydrogen peroxide and superoxide reactive oxygen species (ROS) within the TME. Catalase is an enzyme that breaks down ROS and reduces intracellular oxidative stress within T cells. A dual-CAR construct with catalase (CAR-CAT) was engineered in cells co-expressing an scFv against either CEA or HER2 [75]. The CAR-T cells retained proliferative capacity when cultured with hydrogen peroxide, maintained cytotoxicity against the relevant antigens and exhibited improved short- and long-term persistence. In addition, a "bystander effect" was noted in the ability of the catalase to improve the function of bystander T and NK cytotoxicity in enhancing tumour elimination [75].

During hypoxia, factors that induce effector T-cell death are upregulated, fostering an immunosuppressive environment akin to wound healing. These include FasL which results in Fas-mediated apoptosis of CD8+ T cells (while sparing the Treg cells) (Fig. 8.6).

Adenosine is another important component of the TME that acts as a negative regulator of immune cells in order to promote the healing of injured tissues. It is upregulated in tissue hypoxia and signals through its receptors A2a and A2b. Specifically, A2a is thought to mediate most of its immunosuppressive effects. Activation of CAR-T cells can increase A2aR expression leading to paradoxical suppression of CAR-T cell therapy in preclinical mouse studies. Targeting of the A2aR through genetic or pharmacologic methods improved the efficacy of anti-HER2+ CAR-T therapy in preclinical studies, through a secondary increase in CD8+ and CD4+ T cells and cytokines [6].

Another mechanism through which CARs can be altered to survive the hypoxic and acidotic TME is through adjusting the culture/media conditions under which CAR-T cells are manufactured in order to simulate the fluctuating TME conditions. This may allow for the selection of CAR-T cells that are preadapted to the TME. Culturing with L-arginine or selecting cells with low mitochondrial membrane potential can result in the enrichment of CAR-T cells with a better metabolic fit, with the potential for improved survival and persistence [138, 139].

The preferential utilisation of glucose by cancer cells for aerobic glycolysis leaves behind a nutrient-deficient environment. Hypoxia and low glucose levels can drive T cells towards senescence or terminal differentiation [71]. CD8+ T cells that were subjected to high glycolytic activity fail to form long-lived memory T cells and fail to persist following adoptive transfer. Inhibiting hexokinase-2, with the glucose analogue 2-deoxyglucose (2DG), improves adoptive CD8+ cell effector function and promotes the formation of long-lived memory T cells in B16 melanoma mice [138]. Therefore, manipulating glycolytic metabolism during ex vivo expansion is likely to enrich CAR-T cells with improved cytolytic activity, reduced exhaustion and senescence and allow for the formation of long-lived memory cells [138].

Another method by which CAR-T cell persistence within the TME can be enhanced would be to strategically select a costimulatory domain that would thrive under certain environmental and pH conditions of a tumour's TME. As described earlier, 4-1BB was shown to have improved persistence but reduced early proliferation compared with the CD28 domain. This enhanced persistence is attributed to the ability of 4-1BB to utilise fatty acids in the production of energy, while CD28 favours glycolysis [20, 57].

Thus, several strategies are being employed to overcome the metabolic barriers of the TME for improving CAR-T cell function.

At a glance

• The TME is hostile for cell therapy- Barriers include-	• CARs to overcome the TME barriers
- trafficking barriers with chemokine gradients	- Express CC2b, CCR4; secreting CCL19
- immunosuppressive cells and cytokines	- Express switch receptors (IL-4/7) and secreting cytokines (IL-12, IL-7, IL-18)
- Stromal and vascular structures	- Express FAP, Heparanase, anti-VEGFR2
- Checkpoint inhibitory receptors and ligands	- Express anti PD-1/PD-L1 antibody, switch receptors (PD1/28)
- Metabolic hurdles	- Express Catalase, HIF1α, anti-Integrin receptors, adenosine receptors

8.3.7 Tumour Heterogeneity

Within an individual patient, a primary tumour can harbour different subpopulations with varying levels of surface tumour-associated antigens (TAAs). Inter-tumour heterogeneity is observed in primary and metastatic tumours in multiple solid organ cancers (lung, renal, colon, brain and pancreatic to name a few) as well as in CLL and ALL, with metastases generally associated with mutations [53]. Somatic mutations result in mutant peptide fragments known as neoantigens (or neo-epitopes) which can be expressed on the tumour surface [41]. CD8+ TILs respond to such neo-epitopes and are associated with improved treatment response in some breast and ovarian cancers [53]. Neoantigens are thus potentially exploitable targets when designing CARs as they are not usually found in normal tissues.

The concept of immunoediting potentially caused by CAR-T cells or the TME is an important factor to consider for therapy designs. Antigen loss and a reduction or modulation in antigen expression are ways in which tumours escape the effects of the effector cells [62, 80, 104]. Recent data from clinical trials with CD19 CAR-T cells indicate that a portion of the patients relapse with antigen escape variants. The expression of CD19 antigen is reduced due to the emergence of splice variants leaving the tumours resistant to continued CAR19 therapy [131, 136]. Similarly, it was demonstrated that targeting HER2 with CAR-T therapy in a xenograft mouse model of glioblastoma (tumours with prior high HER2 levels) induced antigen escape with the appearance of HER2-null populations of tumour cells. Co-targeting HER2 and IL-13Rα2 (using novel designs – see Sect. 8.5.1) expanded the capacity of the CAR-T cells and allowed for more enhanced anti-tumour activity in this instance [42].

Currently, "bi-cistronic" CARs targeting dual antigens, e.g. CD19 and CD20 or CD19 and CD22, are being trialled. Sequential administration or co-administration of independent CARs targeting individual antigens have also been trialled. Recently, a trivalent vector encoding three independent CARs, each targeting a separate antigen, was tested for glioblastoma [8]. The reader is also referred to Martinez and Moon's review for a comprehensive list of antigens that are being targeted in the preclinical and clinical setting [83].

It is likely that several antigens and several factors encompassing the TME will need to be targeted simultaneously for an effective response. These kinds of vectors are likely to play leading roles in the generation of CAR-T cells in the future. The section on novel designs will expand on the kinds of CAR constructs that are currently being developed.

8.4 Non-T CAR Cells for the TME

8.4.1 NK-CAR Cells

Natural killer (NK) cells are unique innate immune cells that exhibit functions analogous to those of adaptive immune cells [46, 149]. As innate cells, they possess the ability to recognise and attack viruses, compromised cells and tumours, in an HLA-independent fashion and without prior sensitisation [134]. During antigen exposure, NK cells can receive signals from APCs (such as dendritic cells), costimulatory receptors (DNAM-1) or pro-inflammatory cytokines (IL-2, IL-18 and IL-33) [46, 79, 102]. Their cytotoxicity is mediated through a variety of pathways including cytolytic perforins and granzymes, the caspase cascade as

well as antibody-dependent cytotoxicity (ADCC) [46].

NK cell function is controlled by activating and inhibitory cell surface receptors. These include killer immunoglobulin-like receptors (KIRs), NKG2D, natural cytotoxicity receptors (NKp30, NKp44 and NKp46) and the CD94/NKG2A receptor complex. KIRs have the capacity to be both activating and inhibitory and safeguard against the development of NK cell autoreactivity. NKG2D receptors recognise stressed cells and play a role in NK-mediated anti-tumour activity [110]. Checkpoint receptors are thought to serve a limited role in inactivating native NK cells; however, under pathological conditions within the TME, checkpoint receptors are upregulated. Upregulated PD-1 expression mediates NK exhaustion and functional impairment in Kaposi sarcoma [7] and Hodgkin lymphoma [147]. NK cells face similar barriers as T cells in their homing to tumour sites and within the TME. Native immature NK cells respond to CCL19 and CCL21 via their receptors CXCR3 and CCR7 in order to traffic to target tissues. Mature NK cells reach their targets via Chemerin R, CXCR1, CXCR2 and CX3CR1 in response to their cognate ligands Chemerin, IL-8 and Fractalkine [134].

The discovery that NK cells formed long-lived antigen-specific memory cells [79, 99] rendered them attractive targets for adoptive cell therapy, including CAR therapy [73, 99]. Genetic modification of NK cells to produce NK-CAR cells was founded upon the same backbone as CAR-T cells. The first successful use of NK-CAR therapy was established against tumour cells and HIV in vitro [143]. Subsequently, anti-CD19 NK-CAR cells were shown to increase NK-mediated cytotoxicity against leukaemic cells in in vitro studies [49].

HER-2 NK-CAR therapy also displayed antigen-specific potency in mouse models of glioblastoma. This was achieved through recruitment of bystander native immune cells and formation of long-lived memory cells [161]. Local therapy with a second-generation EGFR-NK-CAR therapy combined with oncolytic herpes simplex virus-1 achieved a greater cytotoxicity against breast cancer brain metastases in a murine model [14]. Multiple other in vitro and preclinical trials demonstrated efficacy of NK-redirected CAR therapy, notably using early-generation CARs including first generation, for neuroblastoma [30] and lymphoma through targeting CD20 [93], and second-generation NK-CARs targeting CSF-1 in multiple myeloma [21].

CARs targeting NKG2D have been developed, either bound to DAP10 or conjugated to costimulatory molecules. Normally, NKG2D binds to DAP10 and through the recruitment of PI3K and Grb2 activated cytotoxic NK cells [27]. An NK-CAR cell-expressing NKG2D-DAP10-CD3ζ led to enhanced NK-induced tumour necrosis against a wide range of cancers including epithelial carcinomas (colon, lung, hepatocellular and breast), sarcomas (osteosarcoma and rhabdomyosarcoma) as well as neuroblastoma [12]. Although "non-classical" CARs, they are becoming a novel mechanism of targeting tumours.

With these findings, the first phase I/II trials are currently actively recruiting in lymphoma and leukaemia, AML, solid tumours and glioblastoma (NCT 00995137, NCT 02742727, NCT 03415100 and NCT 03383978, respectively). Their targets include CD19, CD33, MUC1, NKG2D and HER2. Another clinical trial testing the safety and feasibility of novel NKG2D NK therapy is being trialled for NKG2D-ligand solid organ tumours (NCT03415100).

As NK-CAR therapy matures, additional features will be incorporated, in order to optimise its activity against the TME. It is likely that NK-CARs will compliment CAR-T cells and a combination therapy harnessing the advantageous features of both cell types will play an important role in the future of adoptive cell therapy.

8.5 Future CARs

The evolution of genetic engineering tools and CAR designs has resulted in a plethora of novel synthetic molecules that are likely to be effective against solid tumours and the TME. As mentioned previously, elimination of negative regulators like the checkpoint receptors is being realised

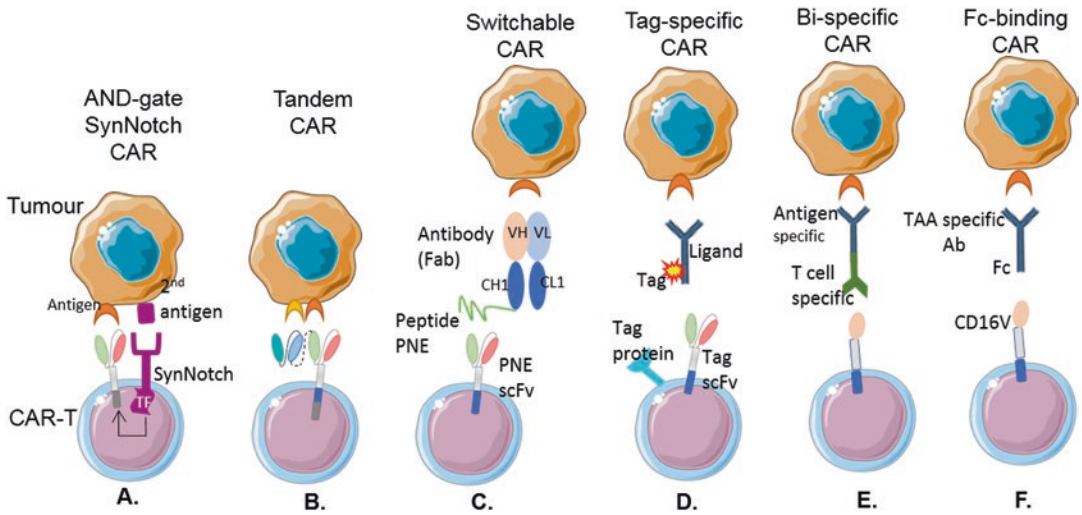

Fig. 8.7 Next-generation CAR designs. (**a**) "AND" gated CARs utilising the synthetic Notch receptor. The transcription factor domain is cleaved and activated upon binding of one antigen which in turn controls the expression of the CAR against another antigen. This increases specificity and decreases toxicity by targeting tumours that only express both antigens. (**b**) Tan CAR or tandem CARs express two separate scFv against two independent antigens but have only one downstream activating signalling domain increasing specificity and allowing for targeting more than one receptor. (**c**) The switchable CAR is a type of universal receptor CAR where the scFv is directed against peptides (peptide neo-epitopes) which are linked to antibody moieties. A single CAR-T cell can be redirected against multiple peptides depending on the antigen-antibody treatment. This design is useful for targeting antigen escape variants and can be useful to overcome tumour heterogeneity and emergence of neo-epitopes. (**d**) Tag-specific immune receptors rely on the binding to a Tag on a targeting ligand, either natural binding proteins or anti-Tag scFv-based designs. (**e**) Bispecific engaging molecules simultaneously engage the tumour cells and T cells. (**f**) Fc-binding CARs function similar to antibody-dependent cellular cytotoxicity by engaging tumour-specific antibody and an extracellular CD16 Fc-binding domain

with CRISPR technology [128]. In addition, universal CAR-T cells with TCR knockdown (to prevent GVHD) have also been created using similar genome editing tools [119, 142] and can serve as a bank of "off-the-shelf" cells.

8.5.1 Novel Designs and Multifunctional CARs

Next-generation designs allow for developing multifunctional CAR-T cells with precise targeting. Morsut et.al designed "AND-gated" CAR-T cells linked to a synthetic NOTCH (SynNotch) receptor, which ensures that CAR-T cells are only activated in the presence of two antigens to precisely eliminate the target while sparing the tissues that expressed only one of the antigens, thus minimising "off-target toxicity" [91, 123]. This system allows for control via release of the intracellular domain of the transcription factor Notch upon binding of the first antigen. It can also be used for custom sculpting responses after the initial binding signal, to enable the CAR-T cell to secrete cytokines or other therapeutic antibodies (e.g. against PD-1 or PD-L1) (Fig. 8.7).

Tandem CARs target two antigens through two scFv domains that are linked to CD3ζ through the one-transmembrane domain, allowing for conditional activation of the CAR in the presence of both antigens [64]. Proof of principle demonstrations proved effective in targeting tumours expressing both HER2 and CD19 [35] and in targeting HER2 and IL-13Rα2. In a preclinical study, targeting two antigens enhanced anti-tumour activity in a murine model of glioblastoma, compared with CARs targeting either antigen alone and without increased exhaustion of the CARs [43].

Combining titratability of antibodies with CAR-T therapy was described in novel switch CARs. Antibody-based switches were engineered by introducing peptide neo-epitopes (PNEs). The CAR is specific to the switch and a single CAR can be redirected based on providing various different switches [120]. The sCART cell design ensured that effector function was dependent on the switch for activation through selective binding only to the PNE. Switchable CAR-T cells targeting HER2 resulted in complete remission in a difficult to treat pancreatic tumour xenograft derived from a patient with metastatic pancreatic cancer with an improved safety profile [117]. This design can be useful not only to target second antigens, especially for antigen escape variants, but also to target changing components of the TME.

Versatile universal immune receptors (UIRs) can extend the functionality of CARs ([86]; Fig. 8.7). The scFv region of a conventional CAR can be substituted for an adapter region which can bind to soluble ligands (which can be tagged) targeting tumour antigens. Artificial receptors against the tags can be developed. Alternately, bispecific protein engaging molecules and those that can mimic ADCC (antigen-dependent cytotoxicity) function (using tumour-specific antibodies and Fc-binding regions) are some examples of novel designs which can be extended to improve CAR function. Next generation of CAR-T cells can hence target multiple TAAs or neoantigens in a controlled manner, with the ability to address issues of heterogeneity and antigen modulation [86].

8.6 Conclusion

The success of CAR-T cells observed for haematological malignancies has not been realised for solid tumours. While the dynamic TME poses significant challenges, research and development in synthetic immunology and genetic engineering is racing to create novel designs to overcome the hurdles. It is already possible to generate multifunctional CARs. In combination with genome editing techniques, it is likely that customised CAR-T therapy will be developed, which will be effective for sustained responses in solid organ tumours, in the near future. With the advent of transcriptomics, proteomics and epigenomics, it is likely that tumour tissue and the microenvironment can be analysed precisely for specific tumour types. Currently such preliminary data is being assembled for many cancers including colorectal cancer, ovarian cancer, breast cancer, urothelial cancers and lung cancer [25, 54, 70, 148, 163], and more data on integrative proteogenomics are likely to follow soon. This will enable a comprehensive understanding of the TME which will in turn guide evolution of CAR designs.

Lastly, novel techniques to deliver the CAR DNAs to effector cells and to the tumour sites are being developed. Exosomes derived from cells as delivery methods and intra-lesional injections of oncolytic viruses are already being tested in the context of CAR-T therapy [122]. It is envisaged that nanoparticles [135] and nanorobots [74] carrying CAR DNA constructs may act as smart bombs to accurately deliver novel therapies.

TME	CAR	Cancer model
Trafficking barrier (overcoming chemokine gradients)	CCR4-CD30	Neuroblastoma, Hodgkin lymphoma
	CCR2b-GD2	
	CCL19	
Stromal and Vascular barrier (overcoming fibroblasts and endothelium)	FAP	Ductal adenocarcinoma
	Heparanase-GD2	
	EGFR-2	Neuroblastoma
	Integrin	Renal cell carcinoma
		Pancreatic
Immuno-suppression and metabolic barrier (overcoming cytokines, checkpoint inhibition, hypoxia and nutrient depravation)	dnTGFβR-HER2, PSMA	Breast, Prostate
	IL4/7 switch-PSCA	Pancreatic
	IL-12-CAR19, MUC16	Leukemia, Ovarian
	IL-18-CEA	Colorectal
	A2aR-HER2	Melanoma
	PD1/PDL1 block-CEA, CD19, CAIX, Mesothelin	Leukemia, Lung, Renal cell carcinoma, Mesothelioma
	PD-1 switch-CAR19	Leukaemia
	Catalase-CAR-CAT-CEA/HER2	Colorectal, Breast
	HIF-CD19	Leukemia

Adapted from Gowrishankar et al (2018)

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9 Toll-Like Receptors (TLRs) in the Tumor Microenvironment (TME): A Dragon-Like Weapon in a Non-fantasy Game of Thrones

Mahmud Angrini, Aditi Varthaman, and Isabelle Cremer

Abstract

Toll-like receptors (TLRs) in the tumor microenvironment (TME) are expressed not only in innate and adaptive immune cells but also in stromal cells such as fibroblasts, endothelial cells (EC), and tumor cells. The role of TLR signaling in the TME is complex and controversial due to their wide expression within the TME. Moreover, TLR signaling may culminate in different outcomes depending on the type of tumor, the implicated TLR, the type of TLR ligands, and, most importantly, the main type of cell(s) that are targeted by TLR ligands. Understanding to what extent these complex TLR signals impact on tumor progression merits further investigation, as it can help improve existing anti-cancer treatments or unravel new ones. In most cases, TLR signaling in tumor cells and in immune cells is associated with pro-tumoral and anti-tumoral effects, respectively. A better understanding of the relationship between TLRs and the TME, especially in humans, is required to design better anti-cancer therapies, considering that most current TLR-involved treatments were disappointing in clinical trials.

In this chapter, we will discuss the impact of TLR signaling on the hallmarks of cancer, by highlighting their effects in tumor, immune, and stromal cells within the TME. Furthermore, we will discuss how the understanding of the role of TLRs can pave the way to develop new anti-cancer treatments and even predict clinical outcome and chemotherapy efficacy.

Keywords

TLRs · Tumor microenvironment · DAMPs · PAMPs · CAFs · TAMs · MDSC · TECs · TLR agonist · Cancer · Anti-tumor immune response · Chemoresistance · Angiogenesis · Metastasis · TLR polymorphisms · Immunotherapy · TLR-based treatment · Microbiota · Cancer vaccine

9.1 Introduction

In addition to cancer cells, the tumor microenvironment (TME) is composed of stromal cells, extracellular matrix, and infiltrating immune cells. Stromal cells include fibroblasts, myofibroblasts, vascular and lymphovascular endothelial cells, and stressed normal epithelial cells. These

M. Angrini · A. Varthaman · I. Cremer (✉)
Institut National de la Santé et de la Recherche Medicale (INSERM), UMRS1138, Centre de Recherche des Cordeliers, Paris, France

Sorbonne Université, Paris, France
e-mail: isabelle.cremer@crc.jussieu.fr

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_9

cells play multifaceted roles during cancer progression as, depending on local and systemic conditions, they can promote or inhibit tumor development [1].

Tumor antigens can activate the immune system eliciting an anti-tumor response which in some cases can result in tumor destruction. During early stages of tumor development, cytotoxic immune cells such as natural killer (NK) and CD8+ T cells recognize and eliminate the more immunogenic cancer cells. As the less immunogenic variants of cancer cells evolve to a clinically detectable tumor, different subsets of inflammatory cells impact on tumor fate [2]. Inflammation is considered an important hallmark of cancer; it promotes tumor development and progression and has been associated with higher tumor grades and a poor prognosis [3, 4]. NF-κB activation – which is seen in most tumor cells – plays a key role in tumor initiation, progression, metastasis, and chemoresistance by mediating the production of a large variety of pro-inflammatory cytokines, chemokines, growth factors, collagenases, and anti-apoptotic proteins [5].

As the largest family of pattern recognition receptors, Toll-like receptors (TLRs) are among the major activators of NF-κB and are involved in inflammation in the TME. In addition to the well-described roles of TLRs in innate and acquired immunity against microbial infection, and their role in regulating tissue repair and regeneration, emerging studies now implicate TLRs in inflammation-associated cancers [6].

TLRs are expressed not only in innate and adaptive immune cells but also in stromal cells such as fibroblasts, endothelial cells, and most remarkably tumor cells. Recognition of natural TLR ligands shapes the TME by mediating both pro- and anti-tumorigenic pathways. On the one hand, abnormal activation of TLRs in tumor cells can facilitate aberrant cytokine profiles associated with immune tolerance and tumor progression. On the other hand, in preclinical studies, TLR agonists incite anti-tumor responses. Nonetheless, most TLR agonists used as standalone anti-tumor drugs have yielded limited success in clinical trials [7].

In this chapter, we will describe the complex mechanisms underlying TLR responses in the TME and how TLR signaling in different cells, including tumor cells, reshapes the TME. Understanding this will facilitate the development of more effective TLR-based therapeutic strategies in a wide variety of cancers.

9.2 What Are Toll-Like Receptors?

TLRs are the largest and most well-studied family of pattern recognition receptors (PRRs). In 2011, Bruce Beutler and Jules Hoffmann were awarded the Nobel Prize in Physiology or Medicine for their discoveries of the role of TLRs in innate immunity, highlighting the importance of TLRs in physiology as well as pathology.

TLRs are type I transmembrane glycoproteins characterized by the presence of an extracellular domain (ECD), a single transmembrane helix, and an intracellular Toll-like/interleukin-1 (IL-1) receptor (TIR) domain. The ECD domain recognizes TLR ligands, while the TIR domain is responsible for downstream signaling [8].

To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively. TLR1-9 are conserved in both species; however, mouse TLR10 is not functional, and TLR11-13 have been lost from the human genome [9].

TLR1, 2, 5, 6, and 10 (extracellular TLRs) are largely localized on the cell surface, while TLR3, 7, 8, and 9 (intracellular TLRs) are localized in intracellular organelles, such as endosomal/lysosomal compartments and the endoplasmic reticulum (ER). TLR4 is unique as it is localized to both the plasma membrane and endosomal vesicles [10] (Fig. 9.1).

The adapter protein Unc-93 homolog B1 (UNC93B1) guides intracellular TLRs (nucleic acid-sensing TLRs) from the ER to their respective endosomal signaling compartments and the flagellin receptor TLR5 to the cell surface, whereas other cell surface TLRs, such as TLRs 2 or 4, function independent of UNC93B1. Loss of UNC93B1 results in near-complete loss of TLR3 and TLR7 signaling in primary splenic mouse

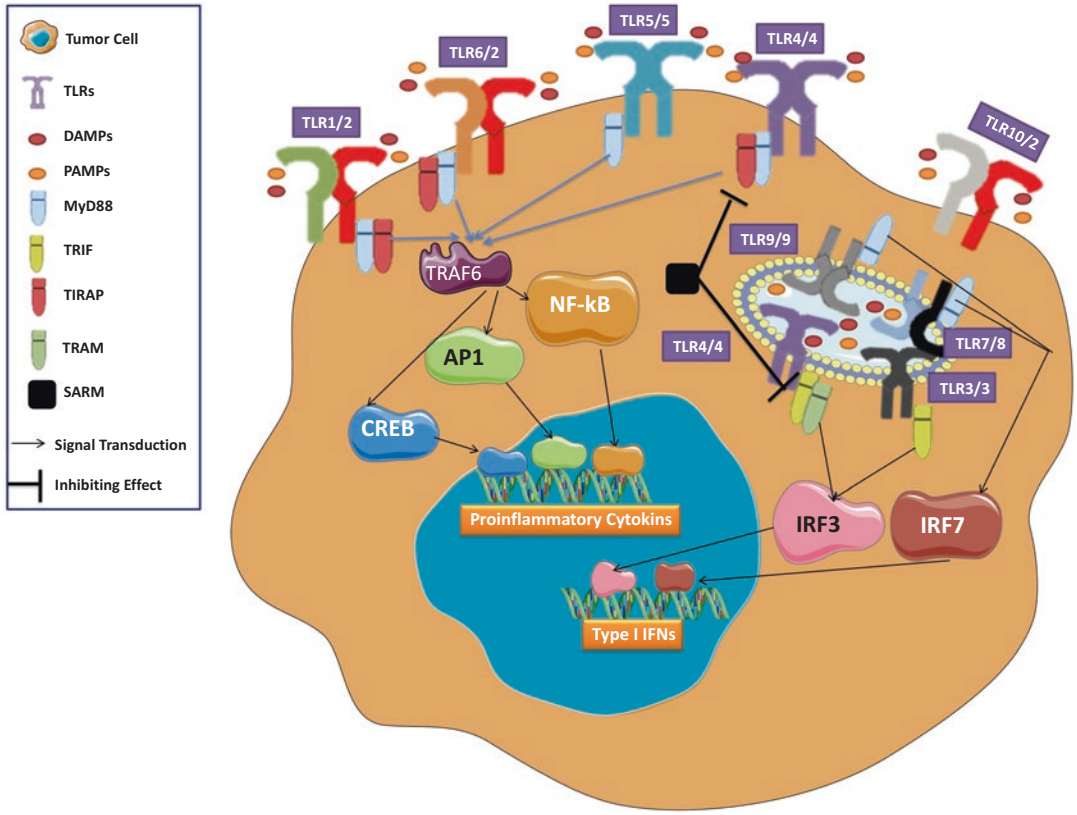

Fig. 9.1 TLR signaling pathways in tumor and immune cells. Following binding of a specific ligand, TLRs form homo- or heterodimers and recruit adaptor protein(s). This starts the signal transduction cascade (details of the cascade are not shown here, because it is out of the scope of this review). *TLRs* Toll-like receptors, *PAMPs* pathogen-associated molecular patterns, *DAMPs* damage-associated molecular patterns, *MyD88* myeloid differentiation primary response gene 88, *TIRAP* TIR domain-containing adapter protein, *TRIF* TIR domain-containing adaptor-inducing interferon IFN-β, *TRAM* TRIF-related adaptor molecule, *SARM* sterile α- and armadillo motif-containing protein

dendritic cells and macrophages [11]. Importantly, murine DCs with a non-functional mutation in Unc93b1 (3d mutation) exhibit compromised antigen cross-presentation and anti-tumor responses [12]. TLRs that reach endocytic compartments are cleaved by proteases in order to signal [13, 14].

9.2.1 Exogenous and Endogenous TLR Ligands

In addition to synthetic TLR agonists, two types of natural TLR ligands exist: exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (Table 9.1).

PAMPs represent evolutionarily conserved microbiota/pathogen-specific sugars, lipoproteins, or nucleic acids essential for the survival of entire classes of pathogens and are distinguishable from "self" moieties [15, 16]. Below are a few examples of PAMP-associated effects in cancer:

- TLR signaling is implicated in an association between the microbiota and gastric, bladder, hepatobiliary, pancreatic, lung, and colorectal cancer [17]. The microbiota and its metabolites activate TLRs and result in

Table 9.1 A list of natural TLR ligands (DAMPs and PAMPs) for each TLR [4, 5, 18, 19]

TLR	DAMPs	PAMPs
TLR1		Triacyl lipoproteins
		Soluble factors: *Neisseria*
TLR2	Heat shock proteins (HSP60, HSP70, HSP96)	Peptidoglycan
	High-mobility group protein B1 (HMGB1)	Lipoprotein/lipopeptides
	Hyaluronic acid	Lipoteichoic acid
	Pancreatic adenocarcinoma upregulated factor (PAUF)	Phenol-soluble modulin
	Monosodium urate	Heat-killed bacteria
	Versican	Porins
	Matrix metalloproteinase-2 (MMP-2)	Structural viral proteins
		Zymosan
TLR3	Self double-stranded RNA (dsRNA), mRNA	viral dsRNA
	Heat shock proteins (HSP27)	
TLR4	Heat shock proteins (HSP22, HSP60, HSP70, HSP96)	Heat shock proteins (HSP60 of chlamydia)
	Fibrinogen and fibronectin	Lipopolysaccharides (LPS)
	Heparan sulfate	RSV fusion protein
	Hyaluronic acid (hyaluronan)	MMTV envelope proteins
	HMGB1, β-defensin 2	Paclitaxel
	Surfactant-protein A	Glyco**i**nositol**p**hos**p**holipids
	Glycoprotein 96 (gp96)	Taxol: Plant product
	Peroxiredoxin	
TLR5	HMGB1	Flagellin (Gram-positive or Gram-negative bacteria)
TLR6		Lipoteichoic acid
		Diacyl and triacyl lipoproteins
		Zymosan
		Heat-liable soluble factor
		Phenol-soluble modulin
TLR7/8	Self ssRNA	Viral ssRNA
TLR9	Self DNA	Bacterial and viral unmethylated CpG DNA motifs
	mitochondrial DNA (mtDNA),	Hemozoin
	IgG-chromatin complexes	
	HMGB1	
TLR10	Unknown	HIV-1 gp41
TLR11 (not expressed in human)		Profilin-like molecule

inflammation and critical signaling pathways, which promote the malignant behavior of host cells. The microbiota can also increase susceptibility to infections that upregulate the expression of certain TLRs promoting carcinogenesis [20].

- Gut microbiota can selectively activate mucosal endothelial cells in CRC to promote specific pro-angiogenic responses in a TLR-dependent fashion [21]. Following activation by commensal microbiota-derived TLR ligands, epithelial calcineurin supports the

survival and proliferation of tumor stem cells and promotes colorectal cancer (CRC) [22].

- TLR4 and the intestinal microbiota promote hepatocellular carcinoma (HCC) by increasing proliferation and expression of the hepatomitogen epiregulin and by preventing of apoptosis [23]. Lipoteichoic acid, a component of cell walls of gut microbiota and a ligand of TLR2, increases in liver cancer and upregulates cyclooxygenase-2 (Cox-2) expression, which in turn leads to the overproduction of prostaglandins [24].
- Lung commensal microbiota promotes murine lung cancer development via γδ T cells in a mechanism that could be mediated by TLR ligands derived from the lung microbiota. TLR signaling leads to the production of IL-1β and IL-23 from alveolar macrophages and neutrophils, thereby inducing the proliferation and activation of pro-inflammatory lung-resident γδ T cells [25].

DAMPs constitute structurally diverse and evolutionarily unrelated multifunctional endogenous danger signals that communicate cell injury to the host and promote early innate and adaptive immune responses important for the mobilization of repair mechanisms. Interestingly, DAMPs are hidden from recognition by the immune system under normal physiological conditions. But, under conditions of cellular stress/tissue injury, such as cancer, these molecules can be actively produced by stressed immune cells, epithelial cells, and cancer-associated fibroblasts (CAFs). In addition, neo-antigens and tumor-associated antigens can be passively released into the TME from necrotic and apoptotic cells or from the damaged extracellular matrix to act as DAMPs [26, 27] that activate TLRs [28]. These DAMPs interact with TLRs on infiltrating inflammatory cells, fibroblasts, tumor endothelial cells (TECs), and tumor cells and play critical roles in both extrinsic and intrinsic pathways of cancer-related inflammation. Below are a few examples of DAMP-associated effects on cancer:

- HMGB1, the most abundant DAMP, is released by dying hypoxic tumor cells in the TME; binds to TLR2, TLR4, and TLR9; and contributes to tumor progression [29]. During hypoxia, HMGB1 translocates from the nucleus to the cytosol, binds to mitochondrial DNA (mtDNA) released from damaged mitochondria, and further activates TLR9 promoting the proliferation of hepatocellular carcinoma cells [30].
- The HMGB1-TLR4 interaction leads to the production of pro-inflammatory and pro-angiogenic cytokines and activation of ECs, macrophages, endothelial progenitor cells (EPC), and mesoangioblasts, all of which could contribute to vessel formation [31]. Furthermore, by inducing autophagy, HMGB1 accumulation in the TME promotes the survival, differentiation, and suppressive activity of myeloid-derived suppressor cells (MDSCs) [32], which are known to inhibit anti-tumor immunity and promote tumor progression [33].

9.2.2 TLR Signaling

Signal transduction commences with the binding of a specific ligand that leads to (a) the formation of heterodimers of two receptor chains that are structurally similar (TLR2/1, TLR2/6, TLR2/10), (b) the formation of homodimers (TLR4/4, TLR3/3, TLR5/5), and even (c) the reorientation of the TIR domains in the case of TLR7, 8, and 9 [34, 35, 36]. This interaction is followed by the recruitment of downstream signaling adapter proteins. There are five adaptor proteins containing TIR domains, which function in TLR signaling: myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adapter protein (TIRAP), TIR domain-containing adaptor-inducing interferon IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) [37] (Fig. 9.1). All TLRs recruit the canonical MyD88-dependent signaling pathway – with the exception of TLR3 and endosomally localized TLR4 which exclusively use the TRIF pathway – to induce the nuclear translocation of transcription factors, NF-κB, AP1, and CREB, leading to

the expression of pro-inflammatory cytokine genes [38, 39]. The noncanonical TRIF adaptor-dependent pathway leads to the nuclear translocation of transcription factors, IRF3 and IRF7, leading mainly to the secretion of type I IFNs [39]. In humans, SARM functions as a specific inhibitor of TRIF-dependent TLR signaling [40] (Fig. 9.1).

TLR4 is unique among TLRs in its ability to activate two distinct signaling pathways: firstly, the canonical pathway, dependent on TIRAP and MyD88, and then, upon endocytosis of TLR4, the noncanonical pathway dependent on adaptors, TRIF, and TRAM [10].

Of note, while NF-κB activation in macrophages is a common theme to all TLR ligands, the MAPK signaling pathways are more significantly enriched in gene sets induced by TLR9 and TLR1/2 agonists (CpG and Pam3CSK4), and type I interferon pathways are enriched in TLR3 and TLR4 agonists (poly(I:C) and LPS)-driven gene sets [41].

9.3 Effects of TLR Signaling in Tumor Cells

TLRs are widely expressed on tumor cells and normal epithelial cells. The expression of some TLRs in tumor cells is even higher than their normal tissue counterparts. For example, TLR4, 5, 7, 8, and 9 in non-small cell lung carcinoma (NSCLC) were markedly higher than in normal lung tissue [42].

9.3.1 TLRs and the Stage of Cancer

Expression of TLRs in tumor cells depends on the type and stage of cancer and varies from one patient to another. The expression of TLR4 is positively correlated with the degree of tumor differentiation, stage, and metastasis in lung cancer [43]. A strong expression of TLR4 is characteristic of advanced-stage (TNM III or IV) gastric cancer (GC), whereas moderate or weak staining is characteristic of early-stage (TNM I or II) GC tumors [44]. The expression of TLR9 and TLR5 is absent or weak in normal cervical squamous epithelial cells but gradually increases with the histopathological grade with invasive cervical squamous cell carcinoma (ISCC) showing a moderate to strong expression of TLR9 in 70% of cases [45, 46]. Similarly, in esophageal carcinomas, TLR9 expression is positively associated with tumor size, location, and TNM stage [47]. In oral tongue squamous cell carcinoma, cytoplasmic TLR2 and TLR4 correlate significantly with higher tumor grade, whereas high TLR5 expression associates with lower tumor grade [48].

9.3.2 Correlation with Worse Prognosis

Many studies find a correlation between TLR expression in tumors and a worse clinical outcome. TLR4 is highly expressed in gastric cancer cells and is associated with tumor aggressiveness [44]. TLR7 expression in NSCLC is markedly associated with poor clinical outcomes [49].

A meta-analysis including 2812 patients with various cancers found that higher expression levels of TLR4 or TLR7, but not TLR9, in tumor tissues predict poor survival and bad prognosis [50]. In oral tongue squamous cell carcinoma, high TLR2, 4, and 9 expression correlates with deeper tumor invasion, and high expression of TLR9 correlates with advanced tumor size [48]. Of note, breast carcinomas with high TLR3 expression in tumor cells are significantly associated with higher probability of metastasis and bad prognosis [51].

9.3.3 TLR Polymorphisms

A new controversial field investigates TLR polymorphisms in cancer. Studies show that:

- Polymorphisms in TLRs are linked to susceptibility to cancer; higher frequencies of certain polymorphisms of TLR2, TLR4, and TLR9 increase susceptibility to development and progression of colorectal cancer [52], melanoma [53], breast cancer [54], nasopharyngeal

carcinoma [55], and cervical cancer [56]. Certain TLR3 polymorphisms increase risk of cancer incidence and are associated with larger tumor size in breast [57] and rectal cancer [58].
- TLR polymorphisms may influence outcomes of therapeutic interventions; some TLR4 polymorphisms indicate resistance to chemotherapy and serve as prognosis markers of head and neck squamous cell carcinomas (HNSCC) [59].
- Certain polymorphisms of TLR2, TLR5, and TLR9 show a significantly decreased risk of gastric cancer and may play important roles in *H. pylori*-related gastric carcinogenesis [60, 61]. TLR4 single nucleotide polymorphisms may play an important protective role in the development of hepatocellular carcinoma [62].

Of note, some TLR polymorphisms are not associated with increased risk of cancer; a TLR7 variant is not involved in the susceptibility to basal cell carcinoma (BCC) [63], and TLR4 and TLR1, 6, and 10 polymorphisms are not significantly associated with the risk of prostate cancer [64, 65].

9.3.4 Mechanisms of Pro-tumoral Effects of TLRs in Tumor Cells

TLRs in tumor cells generally serve as positive regulators of cancer or, in other words, have pro-tumoral effects. Different mechanisms for these effects are suggested:

- TLR signaling upregulates the NF-κB cascade and induces anti-apoptotic proteins contributing to carcinogenesis and cancer cell proliferation. TLRs mediate the release of cytokines and chemokines by cancer cells that enhance inflammation in the TME – a hallmark of cancer [4]. NF-κB activation by TLR signaling is associated with the expansion, invasion, and tumorigenesis of cancer stem cells (CSCs). CSCs possess self-renewal and differentiation abilities, which promote tumor progression and metastasis and are responsible for treatment resistance and cancer relapse [66].
- Following TLR activation, tumor cells can undergo epithelial-to-mesenchymal transition (EMT) and epithelial-to-leucocytic transition (ELT) – by which tumors develop the ability to activate leucocytic traits otherwise inaccessible to epithelial cells – and gain the ability to (1) evade the immune system at the primary tumor site, (2) access the lymphatic system, (3) metastasize through the vasculature, and (4) avoid destruction by the immune system at the site of metastasis [67].
- TLR ligands may induce the production of immunosuppressive cytokines and molecules, including IL-10, transforming growth factor-β, IDO, and iNOS by tumor cells, which can counteract the activation of innate immunity [68, 69].
- TLRs can directly regulate cell metabolism affecting tumor and immune cell behavior and function in melanoma; prostate, head, and neck carcinoma; and breast cancer [7].

9.3.4.1 Effects of TLRs on Proliferation and Apoptosis of Tumor Cells

A primary way to enhance tumor progression is to induce cancer cell proliferation or inhibit apoptosis.

- In the majority of gastric cancer patients, elevated TLR2 expression is associated with a tumor-potentiating gene signature predicting poor patient outcome. High TLR2 expression in human and murine gastric cancers correlates with the upregulation of six anti-apoptotic genes (BCL2A1, BCL2, BIRC3, CFLAR, IER3, TNFAIP3) and the downregulation of two tumor suppressor (PDCD4, TP53INP1) genes [70]. In highly invasive breast cancer cells (MDA-MB-231), a TLR2 agonist (*Porphyromonas gingivalis* lipopolysaccharides, pg-LPS) promoted cell invasion and increased NF-kB signaling, IL-6, TGF-beta, VEGF, and MMP9 secretion, while TLR2 blockade diminished this outcome [71] and thus their invasion potential. Highly metastatic B16 melanoma releases heat shock pro-

tein 60 (hsp60) that causes persistent activation of TLR2, increasing their invasion capacity, and the release of immunosuppressive cytokines and chemokines [72].

- Furthermore, binding of lipopolysaccharide (LPS) to TLR4 on gastric cancer cells stimulates the generation of mitochondrial ROS (mROS), the activation of Akt phosphorylation, and NF-κB p65 nuclear translocation, which in turn enhances proliferation without affecting apoptosis [44]. Exposure of human melanoma cells to hyaluronan fragments induces TLR4-mediated activation of NF-kB leading to the enhanced expression of matrix metalloprotease 2 (MMP2) and interleukin (IL)-8 – factors that contribute to melanoma progression [73, 74]. LPS activation of TLR4 promotes production of the immunosuppressive cytokine TGF-beta, pro-angiogenic VEGF, and chemokine IL-8 by human lung cancer cells. TLR4 ligation also induces resistance of human lung cancer cells to TNF-alpha or TRAIL-induced apoptosis [29]. Of note, LPS stimulation increases the proliferation of glioma CD133+ cancer stem cells (CSCs) isolated from human glioma samples in a time- and dose-dependent manner [75].
- TLR3 and TLR9 are expressed in about 90% of hepatocellular carcinoma tissues. While stimulation of TLR3 with poly(I:C) activates the NF-κB pathway without affecting cell viability, stimulation of TLR9 with CpG-ODNs promotes cell proliferation and reduces cytotoxicity of the anti-cancer drug Adriamycin [76], via upregulation of apoptosis inhibitors such as survivin, Bcl-xL, XIAP, and cFLIP [77]. In esophageal carcinomas, high TLR9 expression is positively associated with tumor size, location, and TNM stage, and the TLR9 ligand, CpG ODN, significantly enhances the invasion of esophageal cancer cell line, TE10 cells. TLR9 activation also leads to NF-κB activation and enhances expression of pro-invasive matrix metalloproteinase (MMP)-2, MMP-7, and cyclooxygenase-2 (COX-2) [47].
- TLR7 and TLR8 stimulation with the agonist R848 increases proliferation of a human pancreatic cancer cell line (PANC1) by inducing the NF-κB pathway and COX-2. Furthermore, high TLR7 and TLR8 expression leads to increase in PANC1 tumor growth in Balb/c nude mice [78]. Our team previously showed that stimulation of human NSCLC cell lines with TLR7 or TLR8 agonists leads to NF-kB activation, upregulation of the anti-apoptotic protein Bcl-2, and increase of tumor cell survival [79].

TLRs in tumor cells can also directly or indirectly act as a "double-edged sword" (Fig. 9.2). Several TLR ligands, such as the TLR7 agonist imiquimod, TLR3 ligand poly(I:C), and TLR9 ligand CpG, can directly induce tumor cell apoptosis or enhance the function of tumor-infiltrating innate cells and tumor-specific T cells. Treating a human breast cancer cell line (MCF-7) with synthetic commercial agonists, poly(I:C) and imiquimod, decreases growth rate and increases sensitivity to radiotherapy [80]. However, poly(I:C) has many disadvantages in addition to the fact that, besides TLR3, it also triggers cytosolic sensors RIG-I and MDA. Its undefined chemical structure and very poor homogeneity leading to unpredictable pharmacokinetics and high toxicity in many clinical trials [81] necessitate careful interpretation of the results.

9.3.4.2 Effects of TLRs on Metastasis

Modulation of the TME and of the tumor cells themselves is required for tumor cells to metastasize to distant sites. Endogenous and exogenous ligands inducing TLR signaling are implicated during this process (Fig. 9.2).

- Versican, an extracellular matrix (ECM)-derived peptide and a DAMP, upregulated in many human tumors including lung cancer, activates TLR2/6 on MDSCs to enhance TNF-α production leading to tumor cell proliferation and metastasis [82].
- Although the chemotherapeutic effect of paclitaxel treatment is largely efficacious in controlling TLR4-negative breast cancers, paclitaxel significantly increased the incidence and burden of pulmonary and lymphatic metastasis in TLR4-positive breast tumors.

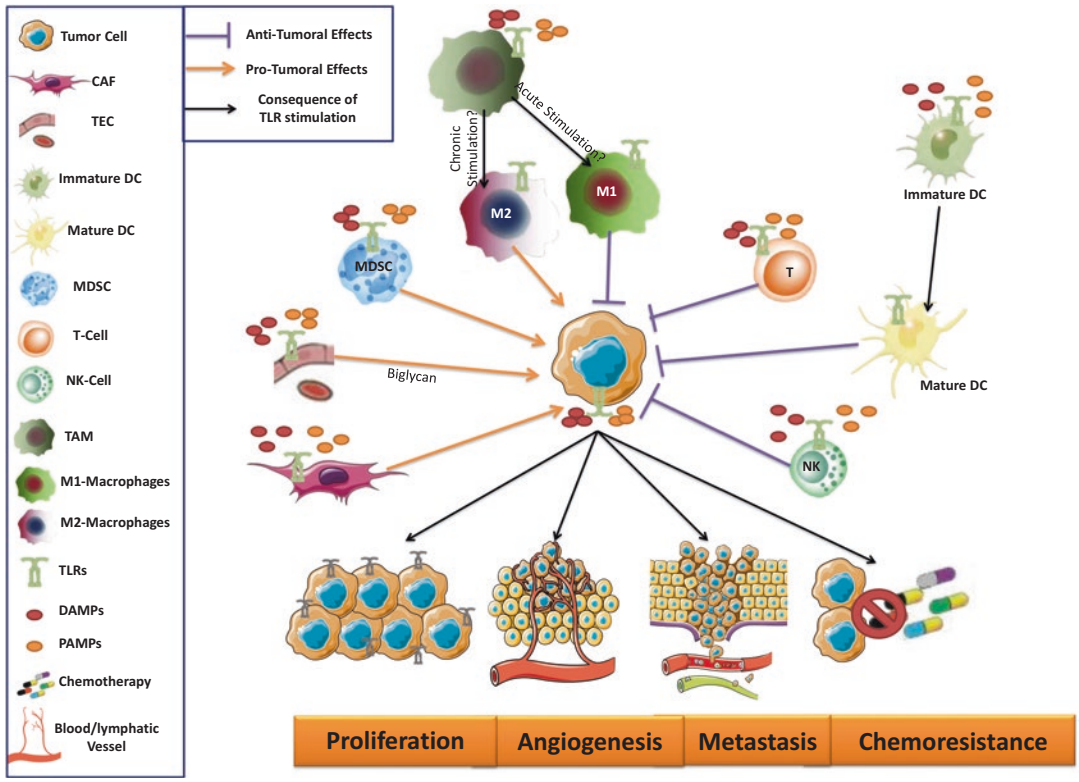

Fig. 9.2 Effects of TLR signaling on the cellular components of the TME. In tumor cells, DAMPs and PAMPs induce TLR signaling leading to enhanced proliferation, angiogenesis, metastasis, and chemoresistance. In CAFs, TECs, and MDSCs, TLR signaling also leads to pro-tumoral effects. Within immune cells, TLR signaling induces the maturation of DCs and the activation of T and NK cells resulting in anti-tumoral responses. In macrophages, TLR signaling can result in the shift of TAMs either into the anti-tumoral M1 phenotype or into the pro-tumoral M2 phenotype (possibly through acute or chronic TLR signaling activation, respectively). *CAFs* cancer-associated fibroblasts, *TECs* tumor endothelial cells, *NK cells* natural killer cells, *DCs* dendritic cells, *MDSC* myeloid-derived suppressor cells, *TAMs* tumor-associated macrophages, *TLRs* Toll-like receptors, *PAMPs* pathogen-associated molecular patterns, *DAMPs* damage-associated molecular patterns

Indeed, TLR4 activation by paclitaxel induces de novo generation of deep intratumoral lymphatic vessels that are highly permissive to invasion by malignant cells [83].

- Specific stimulation of highly expressed TLR2, TLR3, and TLR4 on human melanoma cells induces cell migration [84]. In HCC cells, LPS significantly enhances their invasive potential and induces EMT with the upregulation of vimentin, N-cadherin, Snail, and α-smooth muscle actin (α-SMA) and downregulation of epithelial markers (E-cadherin and β-catenin) [85].
- Stimulation with various TLR agonists (MALP-2 for TLR2/6, LPS for TLR4, and poly(I:C) for TLR3) induces mesenchymal characteristics and invasion potential in SK-OV-3 cells, a metastatic epithelial ovarian cancer cell line. TLR-mediated PI3K activation promotes the secretion of EMT-related cytokines in SK-OV-3 cells (TGF-β1, TNF-α, VEGF, IL-6, IL-8, and IL-10). Interestingly, TLR4-mediated galectin-1 production induces EMT in SK-OV-3 cells [86].
- TLR7 stimulation results in accelerated NSCLC growth and metastasis, accompanied by a gene expression signature linked to aggressiveness and metastatic dissemination; tumors expressing high levels of TLR7 display an EMT phenotype with high expres-

sion of vimentin and low abundance of E-cadherin [87].

9.3.4.3 Effects of TLRs on Angiogenesis

Tumor invasion and progression depends on angiogenesis. Subsets of endothelial cells with specialized functions exhibit different levels of TLR expression. TLR2 and TLR4 are ubiquitously present within the vasculature, whereas TLR7, TLR8, and TLR9 are sparse [88]. Human primary dermal and lung lymphatic endothelial cells (LECs) express TLR1–6 and TLR9, but not TLR7, TLR8, and TLR10 [89]. TLRs and their ligands contribute to the process of angiogenesis in different ways:

- Tumor endothelial cells (TECs) interact with tumor cells by secreting a small leucine-rich repeat proteoglycan known as biglycan, which acts as a DAMP binding TLR2 and TLR4 in neighboring tumor cells. Through the activation of NF-κB and ERK signaling [90], the biglycan stimulates tumor cells to metastasize, promotes gastric cancer cell migration, and induces upregulation of VEGF in neighboring TECs, resulting in enhanced capacity for tube formation, migration, motility, and proliferation in gastric and colon cancer [91–93] (Fig. 9.2).
- Peroxiredoxin-1 (Prx1), a chaperone protein and a DAMP secreted by prostate cancer cells, activates TLR4 resulting in the secretion of VEGF from tumor cells, macrophages, and ECs, thus stimulating TECs to proliferate, migrate, and differentiate [94]. LPS induces growth and lung metastasis of breast cancer cells through increased angiogenesis, vascular permeability, and tumor cell migration [95].
- In highly vascularized tumors, in both murine and human melanoma, oxidative stress, exemplified by the accumulation of lipid oxidation, ω-(2-carboxyethyl)pyrrole (CEP), is recognized by TLR2 on endothelial cells, leading to a pro-angiogenic response independent of VEGF [96].
- Activation of TLR3 by HSP27 released by tumor cells induces NF-κB activation leading to VEGF-mediated cell migration and angiogenesis [97]. In prostate cancer cell lines (PC3 cells), TLR3 activation leads to the increase of the specific 1.3 isoform of HIF-1α and nuclear accumulation of the HIF-1 complex, resulting in reduced apoptosis and secretion of functional VEGF [98].
- Some studies observe a pro-lymphangiogenic role of TLR4. TLR4 ligands LPS, HMGB1, and paclitaxel induce inflammatory lymphangiogenesis, while TLR4-deficient mice form lesser lymphatic vessels as compared to wild-type mice. TLR4 ligands induce pro-lymphatic reprogramming of human and mouse myeloid cells into myeloid/monocyte-derived lymphatic endothelial cell progenitors (M-LECP) responsible for the formation of lymphatic vessels and increased lymphatic metastasis in a model of breast cancer [99]. Indeed, LPS induces a lymphatic phenotype in endogenous macrophages in vivo, by increasing VEGFR-3 expression. These macrophage-derived lymphatic endothelial cell progenitors (M-LECPs) contribute to new lymphatic vessel formation [100].

9.3.4.4 TLRs and Resistance to Therapy

TLR ligands can induce resistance to cancer therapy, either directly by acting on tumor cells or indirectly by inhibiting the immune system (Fig. 9.2).

1. Paclitaxel is a potent anti-cancer drug commonly used for the treatment of advanced breast cancer and melanoma. However, Toll-like receptor 4 (TLR4) expression in these tumors confers resistance to paclitaxel by promoting the production of pro-inflammatory cytokines (IL-6, IL-8) and the anti-apoptotic protein XIAP [101]. LPS-induced TLR4 stimulation in ovarian cancer (OvCa) cells leads to activation of the NF-kB pathway; promotes IL-8, IL-6, and VEGF secretion; and confers resistance to paclitaxel-induced apoptosis [102].
2. In gliomas, LPS-dependent TLR4 stimulation in CD133+ cancer stem cells (CSCs) induces resistance to chemotherapy and evasion from

cytotoxic T lymphocyte-induced cytolysis [75].

3. In NSCLC cell lines, A549 and H1299, Pellino-1, a critical mediator for NF-κB activation in TLR3 and TLR4 signaling, is upregulated following the treatment with TLR agonists conferring chemoresistance to cisplatin or paclitaxel and increased cell viability. The upregulation of cIAP1 and cIAP2 by Pellino-1 reduces cisplatin-induced cleavage of caspase-3, caspase-7, and PARP accounting for the chemoresistance [103].
4. TLR7 or TLR8 stimulation induces chemoresistance to 5-fluorouracil in human pancreatic cancer cell line (PANC1) [78]. In a cohort of NSCLC patients, high expression of TLR7 on tumor cells is associated with poor clinical outcome in patients treated or not with neoadjuvant chemotherapy. TLR7 expression on tumor cells is a marker of chemoresistance to cisplatin combined with gemcitabine or vinorelbine [49].
5. Co-treatment with LPS and cisplatin induces immunosuppressive tolerogenic DCs via abundant IL-10 production, thereby skewing T helper cell differentiation toward Th2 and Tr1 cells. Together, this may provide cancer cells with an opportunity to evade the immune system [104].

9.3.5 TLRs and Tumor Cell Metabolism

In the TME, tumor cells preferably use aerobic glycolysis instead of OXPHOS (the Warburg effect) to meet their demands for growth and proliferation, especially for rapid ATP generation and biosynthesis [7]. The excess nutrient consumption and waste excretion by tumor cells, in addition to insufficient oxygen delivery in TME, result in dysfunction and loss of potential anti-tumor activity of tumor-infiltrating immune cells [105]. TLRs can directly regulate cellular metabolism by affecting tumor cell function. TLRs are also redox-sensitive receptor proteins and are implicated in the response to oxidative stress [106].

Stimulation with the TLR3-specific ligand poly(A:U) dsRNA promotes metabolic reprograming of head and neck carcinoma cells, resulting in increased tumor growth. TLR3 stimulation enhances the capacity of carcinoma cells to switch from OXPHOS to anabolic glycolysis, accompanied by the upregulation of the transcription factor HIF-1α enabling better adaptation to hypoxia and oxidative stress in the TME [107].

In patients with breast carcinoma, TLR9 regulates lipid peroxidation by trace elements (selenium, copper, zinc, magnesium, and iron) in response to oxidative stress [106].

TLR signaling not only impacts directly on tumor metabolic reprograming and tumor growth but also indirectly influences the anti-tumor immune responses in the TME by modulating cancer cell metabolites. TLR2, 3, 4, 5, 7, and 9 activation in tumor cells induces T-cell senescence in naïve/effector T cells by releasing cyclic adenosine monophosphate (cAMP) [108]. However, activation of TLR8 signaling in tumor cells downregulates cAMP, thereby reversing the immunosuppression of senescent naïve/effector T.

TLRs can modulate cancer cell metabolites by inducing indoleamine 2,3-dioxygenase (IDO) and induced nitric oxide synthase (iNOS). Intratumoral administration of the TLR7 agonist, imiquimod, significantly increases IDO and iNOS expression in tumor-draining lymph nodes, leading to the inhibition of the tumor antigen-specific Th1 response [68, 109, 110]. Inhibition of IDO or iNOS enhances the therapeutic efficacy of TLR agonists via the increase of the Th1 immune response [68, 109, 110].

9.4 Effects of TLR Signaling in Immune Cells in the TME

TLRs on immune cells play a major role as initiators of the innate immune response to defend against pathogens and are also required for an effective secondary immune response. Following pathogen recognition by TLRs, antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), are activated [111]

inducing a program of maturation that leads to the migration of APCs into lymph nodes and the release of pro-inflammatory cytokines such as TNFα, IL6, IL12 (that directs the T-cell response toward a Th1 phenotype), and IFNs, which are crucial for the activation of NK cells and cytotoxic T lymphocytes (CTLs) [112]. The idea of inciting an immune response to fight cancer dates back to more than a hundred years, when Dr. William Coley in the late nineteenth century showed that crude microbial extracts (killed *Streptococcus pneumoniae* and *Serratia marcescens*, commonly known as Coley's toxin) can promote an anti-tumor response in different types of cancers [5]. The same principle is still applied today in the form of bacillus Calmette-Guérin (BCG), which is used for the treatment of bladder cancer [113]. It is likely that these agents trigger an immune response through multiple TLRs to promote anti-tumor immunity. However, the impact of TLR signaling in the TME is more complicated and illusive today than what Coley imagined.

9.4.1 TLRs and Macrophages

During tumor progression, the TME often induces the shift of tumor-associated macrophages (TAMs) from their protective classically activated M1 phenotype to pro-tumorigenic alternatively activated M2 subtype. Within the TME, M2 macrophages are associated with tumor growth and progression by impacting on inflammation, immune regulation, angiogenesis, invasion, and metastasis. The interaction between macrophages and tumor cells results in an autocrine/paracrine loop that enhances their pro-tumorigenic properties [114]. The impact of TLR stimulation in TAMs is controversial, as different studies report different outcomes. However, it seems that the duration of TLR stimulation can explain the different outcomes (Fig. 9.2).

- On the one hand, upon LPS treatment, TAMs from ascites of ovarian cancer patients polarize toward an M1-like phenotype as demonstrated by the disappearance of membrane-bound IL-18 and upregulation of CD80 and CCR7. The LPS-activated TAMs release immunostimulatory cytokines (IL-12, soluble IL-18) that efficiently trigger the cytolytic activity and IFN-γ release of co-cultured NK cells [115]. Intratumoral administration of poly(I:C) in mice bearing colon adenocarcinoma tumors triggers an IFN-αβ-dependent (MC38) reversion of M2 macrophages to the M1 phenotype and leads to tumor regression [116].
- On the other hand, chronic TLR signaling plays a role in the conversion of macrophages from the M1 to the M2 phenotype in the TME. The DAMP HSP27 released from lung cancer cells is recognized by TLR3 in monocytes and inhibits their differentiation into DCs while skewing them toward M2 macrophages [97]. Tumor cell-derived extracellular vesicles (TEVs) can bind to TLRs in immune cells and activate them in a paracrine loop leading to the production of cytokines that increase cell proliferation and metastatic potential. TEVs carry cargo containing NSCLC tumor-associated miR-21 and miR-29a that bind to TLR7 and TLR8 in immune cells including macrophages, activating NF-κB and triggering a pro-metastatic inflammatory response [117].

Stimulation of murine macrophages (BMDM) with Pam3CSK4 (TLR1/2), poly(I:C) (TLR3), LPS (TLR4), and CpG DNA (TLR9) induces a transcriptomic profile that shows a robust activation of known inflammatory genes, including IL6, TNF, and chemokines [41]. However, re-stimulation 24 h later of the macrophages with the same doses of previous TLR agonists induces TLR tolerance shifting away from a pro-inflammatory response toward a pro-resolution and anti-inflammatory response [41].

9.4.2 TLRs and Dendritic Cells (DCs)

DCs are professional APCs that play a pivotal role in the link between innate and adaptive

immunity. Two major subtypes of DCs are recognized in mouse and human tissues: conventional/myeloid DCs (cDCs/mDCs) and plasmacytoid DCs (pDCs). cDCs encompass several subsets: cDC1 (which express CD141) and cDC2 (which express CD1c), in addition to monocyte-derived DC (Mo-DCs) [118]. While cDC subtypes produce large amounts of pro-inflammatory cytokines, including IL-12, and induce strong Th1 and CTL responses, pDCs produce large amounts of type I IFN in response to viral and bacterial stimuli [119]. Different DC subsets exhibit distinct TLR repertoires; pDCs selectively express endosomal TLR7 and TLR9 [120], cDC2 express all TLRs except TLR9 and, cDC1 highly express TLR3, TLR9, and TLR10, in addition to low expression levels of TLR1-2, TLR6, and TLR8 [121].

Within the TME, heterogenous DCs are frequently recruited by tumor-derived and stroma-derived factors. However, DCs often show impaired maturation, differentiation, and function, resulting in DC-mediated tolerance, immune suppression, and deficient anti-tumor immune responses [122]. Following long exposure with TLR3 and TLR4 ligands, bone marrow DCs (BMDCs) downregulate MHCII and co-stimulatory molecules CD40 and CD86, as well as the cytokines IL-12, TNF-alpha, and IL-6. Tumor cells induce inhibitory molecules (B7-DC, B7-H1, and CD80) on spleen DCs in vivo and on BMDCs, even in the presence of TLR ligands. This has implications for the development of DC-based cancer immune therapies using TLR ligands as adjuvants [123, 124].

TLRs appear to play an important, yet controversial, role in the development of an anti-tumor immune response.

- On the one hand, specific interaction between TLRs and their ligands (Table 9.1) results in DC maturation (Fig. 9.2), which boosts MHC-I cross-presentation [12]. In glioblastoma multiforme (GBM), intratumoral delivery of adenoviral vectors (Ad) expressing the DC-differentiation cytokine Fms-like tyrosine kinase 3 ligand (Flt3L) and thymidine kinase (TK) results in infiltration of mDCs, a clonal expansion of anti-tumor T cells, and an induction of an effective anti-GBM immune response in a TLR2-dependent manner. In fact, HMGB1, an alarmin protein released from dying tumor cells, is recognized by Toll-like receptor 2 (TLR2) and is responsible of tumor regression observed in this murine model [125]. Recognition of HMGB1 by TLR4 in DCs increases their maturation, processing, and cross-presentation of tumor antigen released from dying tumor cells during radio and chemotherapy [126] enhancing the infiltration and clonal expansion of T cells and tumor killing. HSP70, released from tumor cells, activates tumor cells through TLR4 to produce chemokines that attract DCs and T cells [127]. In addition, repeated intratumoral injections of poly(A:U), a TLR3 agonist, activate CD103+ cDC1 cells inside murine B16-OVA melanoma resulting in higher tumor-specific immunity, with an increase in infiltrating granzyme B+ CD8+ T cells and a decrease in IL-10-producing M2-like macrophages, impacting on the ability of the immune system to control tumor growth [128].
- On the other hand, studies have found pro-tumoral effects for TLR signaling in DCs in the TME. Murine and human tumors secrete TLR2 ligands, such as versican, that promote differentiation of IL-10-producing cDCs. DCs become more responsive to IL-6 and IL-10 stimulation, following TLR2 ligation, by increasing the activation of STAT3. TLR2 blockade using anti-TLR2 antibodies in vivo improves intratumor DC function and enhances anti-tumor CTL and the efficacy of immunotherapy [129]. Matrix metalloproteinase 2 (MMP-2), another DAMP, interacts with TLR2 resulting in an increase in tumor angiogenesis and dampening of the immune response. Activating TLR2 by MMP-2 upregulates an immunoregulatory receptor OX40L on DCs and leads to the secretion of inflammatory cytokines that polarizes T cells to an immunosuppressive Th2 phenotype [130]. Finally, intratumoral Mo-DCs express high

levels of TLRs (except TLR3) in metastatic breast cancer patients with circulating tumor cells (CTC), compared to CTC-negative patients [131].

9.4.3 TLRs and Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are immature and pathologically activated cells that have potent immune suppressive activity in tumors. MDSC can also support tumor progression by promoting tumor cell survival, angiogenesis, invasion, and metastasis. MDSCs are classified into two different types, as identified in mice and humans: polymorphonuclear MDSCs (PMN-MDSC) are morphologically and phenotypically similar to neutrophils, whereas monocytic MDSCs (M-MDSC) are similar to monocytes [132]. The effect of TLR stimulation in MDSCs is controversial and requires further investigation.

- TLR 7/8 agonists reverse the suppressive activity of human blood MDSC from cancer patients inducing their differentiation into tumoricidal M1-like macrophages. In contrast, agonists targeting TLR 1/2 cause M-MDSC to mature into immunosuppressive M2-like macrophages [133].
- On the other hand, our team demonstrated that TLR7 stimulation of tumor cells results in the recruitment of MDSCs to the TME, leading to accelerated tumor growth and metastasis. Depleting MDSC indicated that these cells are involved in the pro-tumoral effect of TLR7 stimulation [134] (Fig. 9.2). In addition, tumor-derived exosomes (TDEs) activate human MDSCs and trigger their suppressive function in an Hsp72/TLR2-dependent manner [135].

9.4.4 TLRs and NK Cells

In solid malignancies, tumor-associated NK cells (TA-NK cells) in peripheral blood and tumor-infiltrating NK (TI-NK) cells display phenotypes of anergy or reduced cytotoxicity [136]. Besides their well-established anti-tumor effect, NK cells may support cancer both by immunosuppression and by supporting tumor angiogenesis [137]. Considering that almost all TLRs can be expressed on human NK cells [138], many TLR agonists stimulate NK cell function directly or through accessory cells in a cytokine- or contact-dependent manner (Fig. 9.2).

Poly(I:C) through TLR3 can directly activate human NK cell lines, NK92, YTCl2, and YTS [139] promoting the secretion of IFN- γ and CXCL10 and upregulating the cytotoxic activity of NK cells against human erythromyeloblastoid leukemia and B-lymphoblastoid cell lines, which do not express the major histocompatibility complex class I (MHC-I) [139]. Poly(I:C) impairs the internalization of TLR3 and leads to the activation of NK cells within the head and neck squamous cell carcinoma (HNSCC) microenvironment [140]. In addition, TLR7/8 stimulation indirectly promotes NK cell activation and production of IFN-γ, granzyme B, and perforin exerting powerful anti-tumor immunity in melanoma and HNSCC tumors [141, 142].

9.4.5 TLR and T Cells

Certain TLRs are expressed in T lymphocytes, and their ligands can directly modulate T-cell function (Fig. 9.2). The activation of the Toll-like receptor-myeloid differentiation factor 88 (TLR-MyD88) signaling in CD8+ T cells enhances proliferation, cytotoxic function, and survival and makes them resistant to MDSC-mediated suppression [143]. TLR8 signaling reverses tumor-induced T-cell senescence by blocking cAMP production in tumor cells [144].

In human γδ T cells, TLR 3 and 7 agonists increase CD54 expression and the lysis of pancreatic adenocarcinoma cells [145].

In addition, TLR2, TLR5, and TLR8 agonists inhibit the suppressive activity of natural CD25(+)CD4(+) regulatory T cells [146].

9.5 Effects of TLR Signaling on Cancer-Associated Fibroblasts (CAFs)

Cancer-associated fibroblasts (CAFs) are the most abundant cell type within the active stroma of many cancer types [147]. CAFs trigger pro-tumorigenic signals accompanied by disruption of the normal tissue architecture creating an optimal niche for tumors to progress [148]. They induce therapy resistance, epithelial-to-mesenchymal transition (EMT), and survival or stemness-related programs and metabolically reprogram tumor cells [149]. CAFs secrete several cytokines and chemokines that are immunosuppressive (IL-6, TGF-β, IL-1β, IL-10, IDO, and PGE2), recruit inflammatory cells (CXCL1, 2, 5, 6, 9, 10, 12, CCL2, 3, 5, 7, 20, and 26), and are pro-angiogenic (VEGF, CXCL8, and FGFs) [150, 151].

Despite the fact that all ten TLRs are constitutively expressed and functional (except TLR10) in fibroblasts [152], few studies investigate the impact of TLR stimulation in CAFs on tumor progression. TLR4 in CAFs is associated with a high rate of tumor recurrence and a shortened overall survival, particularly in colorectal cancer patients [153] (Fig. 9.2).

In a cohort of luminal breast cancer patients, high TLR4 expression associates with more LC3II (a marker of autophagosomes) in CAFs and correlates with aggressive relapse rates and poor prognosis [154]. Intestinal mesenchymal cells (IMCs) and CAFs are activated by innate TLR4/MyD88-mediated signals that promote spontaneous intestinal tumorigenesis in mice. Similar TLR4/MyD88-regulated gene signatures also exist in human CAFs [155].

On the contrary, breast carcinomas with high TLR9 expression in fibroblast-like cells are associated with low probability of metastasis [156]. The polysaccharide MPSSS triggers the TLR4-NF-κB pathway and reduces the inhibitory effect of prostate CAFs on CD4+ and CD8+ T-cell proliferation [157] (Table 9.2).

Table 9.2 Summary of the effect of TLR signaling in the TME

Cell component	Effect
Tumor cells	Pro-tumoral (enhance proliferation, angiogenesis, metastasis, resistance to chemotherapy)
Macrophages	Controversial (anti-tumorigenic via M1 phenotype or pro-tumorigenic via M2 phenotype)
DCs	Mainly anti-tumoral (TLR2 signaling could be pro-tumoral)
MDSCs	Mainly pro-tumoral
NK cells	Anti-tumoral
T cells	Anti-tumoral
CAFs	Mainly pro-tumoral

9.6 TLR-Based Treatment Strategies in Cancer Therapy

Based on the importance of TLR signaling in the TME, numerous studies and clinical trials have investigated the use of TLR ligands as potential treatments for cancer. Using TLR ligands to incite a powerful humoral and cellular immune response against tumors has seemed very promising. However, most TLR ligands used as single therapies to stimulate the immune system were not successful in preclinical studies or in clinical trials. The complex nature of the TME, the fact that TLR agonists can elicit pro-tumoral effects in tumor cells, and the diverse consequences in immune and stromal cells can explain why the majority of activating TLR-based single therapies failed.

Various strategies either stimulating TLRs inciting their adjuvant effect or blocking TLR signaling have been tried with some showing promising outcomes. In addition to many preclinical studies, ten clinical trials investigating the safety and therapeutic efficacy of TLR ligands or their adjuvant impact in cancer patients have been completed. However, the majority of these studies completed between 2016 and 2019 have not been published yet.

To date, only three TLR agonists are approved by FDA for use in humans: the bacillus Calmette-

Guérin (BCG), monophosphoryl lipid A (MPL), and imiquimod. BCG is used during immunotherapy in non-invasive transitional cell carcinoma of the bladder, as an agonist for multiple TLRs: TLR2, TLR4, and TLR9. MPL (derived from the LPS of *Salmonella minnesota*) that signals through TLR2 and TLR4 is included in the formulation of Cervarix®, a vaccine against human papillomavirus-16 and human papillomavirus-18 that are strongly associated with cervical carcinoma. Imiquimod (a synthetic TLR7 agonist) is routinely used for the treatment of actinic keratosis, superficial basal cell carcinoma, and external genital warts [158, 159].

9.6.1 TLR Ligands as Stimulators of the Immune System

Some TLR ligands show a preferential stimulating effect of the immune system in some types of cancer. This could be explained by the relative abundance of the corresponding TLRs in immune cells compared to the absence/low expression or non-functionality of these receptors in tumor cells. However, further studies are required to explore the exact molecular mechanisms involved in this impact. TLR ligands in preclinical cancer models and clinical trials have shown diverse outcomes (Fig. 9.3).

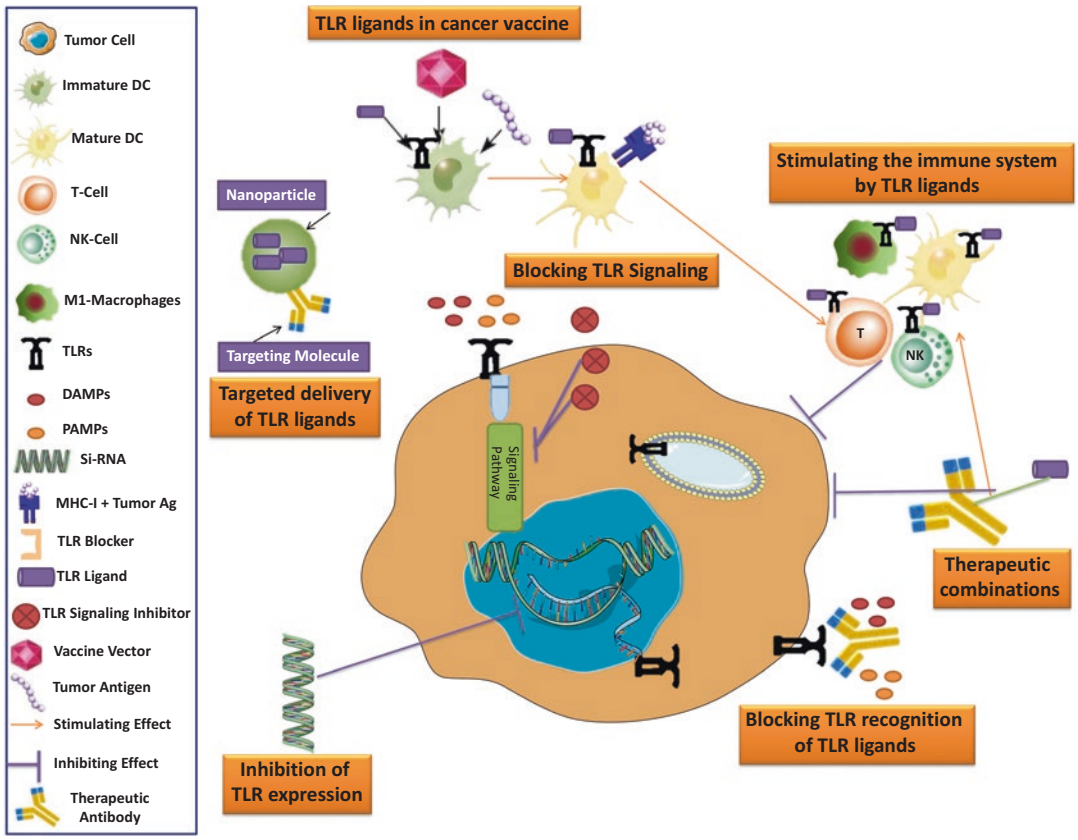

Fig. 9.3 TLR-based treatment strategies in cancer therapy. An up-to-date illustration of seven TLR-based treatment strategies used in preclinical models and clinical trials. *NK cells* natural killer cells, *DCs* dendritic cells, *TLRs* Toll-like receptors, *PAMPs* pathogen-associated molecular patterns, *DAMPs* damage-associated molecular patterns, *MHC-I* major histocompatibility complex – class 1, *Ag* antigen

- Imiquimod 5% cream tested as a therapy for high-grade squamous intraepithelial lesions and anal cancer resulted in complete or partial response in 45% and 66% of patients, following 16 and 32 weeks of consequent treatment [160]. Imiquimod treatment recruits cutaneous effector T cells from the blood; stimulates IFN-γ, granzyme, and perforin production; lowers IL-10 and TGF-β production; and inhibits tonic anti-inflammatory signals within the tumor [161].
- OM-174, a lipid A analogue (region of LPS) that is considered as a TLR2/4 agonist, reduces tumor progression and prolongs survival in mice with subcutaneous B16 melanoma. These effects are mediated by an increase of the absolute numbers and the activation of natural killer (NK) cells and cytotoxic T lymphocyte (CTL) [162]. Additionally, in a Phase I study, OM-174 shows a good safety profile for its use in patients with refractory metastatic solid tumor [163], and it is currently under development as a cancer immunotherapeutic agent (Phase Ib clinical trials). In a model of PROb colon cancer in rats, OM-174 induces complete regression in about 90% of cases. Since OM-174 is not directly toxic to tumor cells, the observed effect involves the host-mediated anti-tumor reaction [164].
- Immunomax, a plant-derived TLR4 agonist, eliminates micro-metastatic disease in the post-resection model of 4T1 mouse breast cancer. By stimulating tumoricidal NK cells, Immunomax prolonged overall survival and cured 31% of mice [165].
- SMP-105, a cell wall skeleton preparation of *M. bovis* BCG, has anti-tumoral effects in a model of subcutaneous murine NSCLC. By activating the NF-kB promoter in a TLR2-dependent manner, SMP-105 leads to a T-helper (Th) 1 type immune response and the proliferation of cytotoxic T lymphocytes (CTL) [166].
- Intratumoral injection of acGM-1.8 – a modified polysaccharide that activates TLR2 – suppresses the growth of two murine tumors, S180 sarcoma and B16 melanoma, and demonstrates higher safety than four classical TLR agonists: LPS, MPLA (agonist of TLR4), poly(I:C)(TLR3), and Pam3CSK4 (TLR1/2). The anti-tumor activity was mediated through a TLR2-mediated switch of macrophages from an M2, pro-tumor phenotype to an M1, anti-tumor phenotype [167].

9.6.2 Inhibition of TLR Expression

Small interfering RNA (siRNA) to inhibit TLR expression, mainly on tumor cells, has been tried as a strategy for cancer therapy. Even though such therapy lacks the ability to specifically target tumor cells in vivo, few studies show encouraging results.

- Silencing TLR4 in human prostate cancer cells (PC3) using small interfering RNA (siRNA) results in a dramatic reduction of tumor cell viability, migration, and invasion. In a mouse prostate cancer model, siRNA against TLR4 inhibited established tumor growth and survival [168]. Similarly, in breast cancer cells and NSCLC cells, silencing TLR4 with siRNA inhibits proliferation and survival of tumor cells and drastically decreases inflammatory cytokine secretion, such as IL-6, TNF-α, and IL-8 [43, 169].
- Knockdown of TLR2 by intratumoral injections of siRNA drastically reduces the size of human liver tumors transplanted in nude mice [170].

Although targeting TLR expression in tumor cells is beneficial, the lack of specificity and the difficulty to perform intratumoral injections for many tumor types hinder its use in humans.

9.6.3 Blocking the Recognition of TLR Ligands by TLRs

Using antibodies to block the interaction between cell surface TLRs and their ligands is an effective strategy in some cancers (Fig. 9.3).

- Activation of TLR2 with a yeast-derived ligand of TLR2, zymosan, promotes head and neck squamous cell carcinoma (HNSCC) organoid formation in an ex vivo model of tumor growth, while blockade with anti-TLR2 antibodies inhibits organoid formation. TLR2 blockade also inhibits growth of human xenografted HNSCC tumors in immunodeficient mice [171].
- In a mouse model of B16 melanoma, anti-TLR2 antibodies markedly reduce pulmonary metastases and increase the survival of mice by reversing the immunosuppressive microenvironment and restoring anti-tumor CD8+ T cells and M1 macrophages [172].
- In a model of prostate cancer, anti-TLR4 antibodies inhibit the pro-tumoral effects of LPS that include immune escape, tumor progression, and metastasis by inducing immunosuppressive TGFβ1 and the pro-angiogenic cytokine VEGF [173].
- Eritoran, which blocks TLR4, abrogates the enhanced migration of human esophageal cancer cells and colorectal cancer (CRC) and hepatic metastases in NSCLC induced by the administration of heat-inactivated *E. coli* and LPS [174–176].

9.6.4 Blocking TLR Signaling

- TAK-242 (resatorvid), a selective inhibitor of the TLR4-NF-κB signaling pathway, suppresses inflammatory mediators and inhibits growth, invasion, and metastasis of hepatocellular carcinoma (HCC) [177] (Fig. 9.3).
- Luteolin, a suppressor of the TRIF signaling pathway, reduces the volume and weight of prostate tumors in a xenograft mouse model, by targeting angiogenesis, reducing cell viability, and inducing apoptosis in cancer cells [178]. Similarly, luteolin decreases invasiveness and MMP secretion and reverses IL-6-induced epithelial-mesenchymal transition in pancreatic cancer cell lines [179].
- Inhibiting TLR7 and TLR9 signaling using a specific antagonist (IRS-954) or chloroquine reduces hepatocellular carcinoma proliferation in vitro and inhibits tumor growth in a mouse xenograft model [180].

9.6.5 TLR Ligands in Therapeutic Combinations

Different combinations of TLR agonists and immune checkpoint inhibitors have been investigated in preclinical models and clinical trials (Fig. 9.3).

- A combination of intratumoral injections of the TLR1/2 ligand, Pam3CSK4, and anti-CTLA-4 antibody enhances anti-tumor immune responses by mediating the depletion of regulatory T cells in a murine melanoma model [181].
- In murine models of melanoma (B16.F10) and colon adenocarcinoma (MC38), a combination of R848-loaded β-cyclodextrin nanoparticles (CDNPs) and anti-PD-1 synergistically results in tumor shrinkage, a stable and homogenous anti-tumor response, and protects the mice from a tumor rechallenge by triggering anti-tumor memory [182]. In colorectal cancer (CRC), a combination of Taxol and R848 results in better control of tumor growth than single treatments and other combinations [183].
- Combining imiquimod, a TLR7 agonist, with 1-methyl-d-tryptophan, an IDO inhibitor, or with an iNOS inhibitor induces a Th1 response and slows the growth of established murine tumors in both thymoma (EG7) and colon carcinoma (C26)-bearing mice, while single treatments don't have any therapeutic effects [109, 110].
- Combining IL-10 blockade with imiquimod results in long-term survival of mice with spontaneous breast tumors, while imiquimod alone doesn't show any efficacy [184].
- In a combined chemotherapy Phase II clinical trial, patients with treatment-refractory breast cancer chest wall metastases treated with a TLR7 agonist (imiquimod), a TLR4 agonist, and chemotherapy (plus albumin-bound paclitaxel) showed disease regression with an

overall response rate of 72%, and 92% of adverse events were low grade (1 and 2). However, the responses were short-lived (4–28 weeks) [185].
- A Phase II study examining the combination of an anti-CTLA-4 monoclonal antibody with the TLR9 agonist (SD-101) in patients with recurrent low-grade B-cell lymphoma showed that the combination did not constitute a promising therapeutic option [186].

9.6.6 TLR Ligands in Cancer Vaccine

TLR ligands have been extensively explored as vaccine adjuvants in preclinical and clinical studies (Fig. 9.3).

- In patients with high-risk myelodysplastic syndrome (MDS), a Phase I study investigating an NY-ESO-1 vaccine containing (PolyIC:LC), TLR3 agonist, showed the induction of an antigen-specific CD4+ and CD8+ T-cell immune response associated with a detectable population of $CD141^{Hi}$ conventional dendritic cells [187].
- A vaccine of glioma-associated antigen peptides and PolyIC:LC tested in a pilot study for pediatric recurrent low-grade gliomas was well tolerated but showed low immunological and clinical activity in 4 out of 14 kids [188].
- The safety and tolerability of a DC-based vaccine in combination with (PolyIC:LC) tested in a Phase I study in patients with locally advanced unresectable pancreatic cancer showed that, despite the good safety of the vaccine, the treatment elicited specific T-cell responses in only three of eight patients, with a median overall survival of 7.7 months [189].
- Oxaliplatin-based chemotherapy administered alone or combined with the poly(A:U), TLR3 agonist, failed to hamper the progression of murine subcutaneous melanoma (B16-OVA). However, a vaccine composed of the antigen (OVA) plus the adjuvant CpG-ODN, TLR9 agonist, given prior to oxaliplatin and poly(A:U) retarded tumor growth [190].
- Targeted delivery of tumor antigen to DCs, in addition to adjuvants (poly(I:C) and R848, TLR3 and 7/8 agonist), by encapsulating them in DC-targeting nanoparticles, enhances the maturation of DCs, the production of immune stimulatory cytokines, and the antigen-specific activation of naïve CD8+ T cells. Remarkably, such delivery reduces the serum cytokine storm and related toxicity that is associated with administration of soluble TLR ligands [191]. Using biodegradable, polymer microparticles, targeted delivery of triple adjuvant combination (TLR4/TLR7/TLR9) in murine tumor models results in increased antigen-specific antibody titer with an overall balanced Th1/Th2 response [192]. However, evaluating the anti-tumor efficacy of these vaccines in tumor models is yet to come.

9.6.7 Targeted Delivery of TLR Ligands

To avoid undesired effects of TLR ligands and to reduce acute toxicities that result from systemic diffusion of soluble TLR-based adjuvants, a new strategy that aims to deliver TLR ligands to the targeted cell type in the TME gives encouraging results (Fig. 9.3).

- Targeted delivery of a 50 bp dsRNA TLR3 agonist (Riboxxol) conjugated to anti-prostate stem cell antigen (PSCA) single chain antibody – termed "Rapid Inducer of Cellular Inflammation and Apoptosis" (RICIA) – in PSCA-positive bladder cancer xenograft models (HT1376) induces a type I interferon response and apoptosis of target cells. Although treatment in immune-deficient mice did not induce adverse effects, tumor growth was only modestly inhibited as compared to controls [193].
- Loading β-cyclodextrin nanoparticles (CDNPs) – which exhibit affinity to macrophages – with R848, an agonist of TLR7/8,

Table 9.3 Summary of TLR-based treatments of cancer in preclinical and clinical trials

TLR-based strategy	Treatment	Cancer type
Stimulation of the immune system	Imiquimod	Skin cancers (BCC and SCC)
	OM-174	Melanoma and colon cancer
	Immunomax	Breast cancer
	SMP-105	NSCLC
	acGM-1.8	Sarcoma and melanoma
Inhibition of TLR expression	siRNA for TLR4	Prostate and breast cancers
	siRNA for TLR2	Liver cancer
Blocking the recognition of TLR ligands by TLRs	Anti-TLR2 antibodies	HNSCC and melanoma
	Anti-TLR4 antibodies	Prostate, esophageal, and CRC
Blocking TLR signaling	TAK-242 (resatorvid)	HCC
	Luteolin	Prostate and pancreatic cancers
	IRS-954 or chloroquine	HCC
TLR ligands in therapeutic combinations	Pam3CSK4 + anti-CTLA-4	Melanoma
	R848 + anti-PD-1	Colon cancer
	Imiquimod + IDO/iNOS inhibitors	Thymoma and colon cancer
	Imiquimod + IL-10 blockade	Breast
	Imiquimod + paclitaxel	Refractory breast cancer
	SD-101 + anti-CTLA-4	Low-grade B-cell lymphoma
TLR ligands in cancer vaccine	NY-ESO-1 vaccine (PolyIC:LC)	MDS
	Glioma-asso. antigen (PolyIC:LC)	Pediatric gliomas
	DC-based vaccine (PolyIC:LC)	Advanced pancreatic cancer
	OVA vaccine (CpG-ODN)	Melanoma
Targeted delivery of TLR ligands	Riboxxol conjugated to anti-PSCA CDNPs + R848	HT1376 bladder cancer cells Multiple murine tumor models

alters the tumor immune microenvironment in multiple murine tumor models toward an M1 phenotype, leading to controlled tumor growth [182] (Table 9.3).

9.7 Future Perspectives

Despite advances in understanding the dual role of TLR signaling in the TME, many questions are yet to be answered. The difference between inflammation that induces tumor progression and inflammation that causes tumor regression is still unclear. Though both exogenous and endogenous TLR ligands are involved in TLR activation in tumor cells, their relative contribution to tumorigenesis remains unknown. In fact, despite the existence of natural TLR ligands, most experiments that investigate the role of TLRs in cancer use synthetic TLR ligands. While DAMPs released from dead tumor cells incite protumoral effects in tumor cells and diverse effects in immune cells, PAMPs from microbiota or exogenous pathogens (bacteria, virus, parasites) can also impact on the TME and tumor progression, especially in mucosal organs. Another intriguing question is why activation of different TLRs leads to different outcomes in the same tumor, even though most TLRs share the same signaling pathways and molecules. The answer could lie in the integration of signals of the different cells in the TME that can sense and respond to the ligands present. The outcome of activating TLR signaling in a given tumor depends on the nature of the cells that succeed to exploit it the most.

Considering that most TLR ligands have an anti-tumoral role when they activate cDCs and NK cells, and an pro-tumoral role when they activate tumor cells, looking for TLR ligands that can preferentially activate DCs and NK cells while sparing tumor cells is likely to be an effective strategy to enhance the anti-tumor response. Also, targeted delivery of TLR ligands to specific cell types using antibodies is a promising strategy to avoid off-target effects.

Tumor cells express more TLRs than their normal epithelial counterparts. In addition, some studies show that tumor cells can be more successful in sensing TLR ligands, than immune cells in the TME, considering the pro-tumoral outcome of TLR agonist treatment.

The use of synthetic TLR agonists to explore the effect of TLR signaling in the TME impedes the comprehension of the role of the TLRs. Some TLR agonists are not specific to the TLR studied activating multiple TLRs. It is possible that synthetic TLR agonists have a tropism to TLRs expressed in particular types of cells, which may not mimic the impact of natural DAMPs or PAMPs. This can explain the limited success of TLR agonists in clinical trials despite promising results in preclinical experiments.

TLR signaling can modulate the metabolic program in tumor cells, stromal cells, and immune cells. It also regulates metabolites produced by tumor cells, such as cAMP and IDO, which are potent immune suppressors, thus maintaining the tumor-suppressive microenvironment. Identification of selective TLR agonists that specifically inhibit tumor metabolism and/or regulate tumor-derived metabolites is needed for better TLR-based tumor vaccines and therapy.

Since TLR polymorphisms can play important roles in tumorigenesis and influence outcomes of therapeutic interventions, analyzing TLR expression and determining polymorphisms in patients can not only be used as prognostic marker but also opens new avenues for the use TLR ligand-based therapies in personalized medicine.

TLR-mediated reprogramming of T-cell metabolism is a novel and feasible strategy for tumor immunotherapy. Developing effective strategies targeting TLR-mediated metabolic switches to appropriately activate macrophages and DCs in the TME is a promising future therapeutic approach.

Though TLR agonists as a single agent anti-tumor drug have yielded limited success in clinical trials, combining TLR-based cancer treatment with immune checkpoint inhibitors has given encouraging results supporting growing evidence that combination drugs are the future of cancer therapy.

9.8 Conclusion

TLRs are widely expressed in tumor cells, stromal cells, and tumor-infiltrating immune cells and are involved in the regulation of tumor pathogenesis and in anti-tumor immune responses. Increasing evidence strongly indicates that TLRs can be activated in various cells within the TME and can thereby modulate the microenvironment. Like the dragon in the fantasy TV series Game of Thrones, TLR signaling is a war-changing weapon that can be exploited either by tumor cells or by immune cells, to control the TME.

A better understanding of the mechanistic effects of TLR signaling in different cells within the tumor-suppressive microenvironment will enable the development of novel strategies via TLR-mediated cancer therapies.

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10 Multiple Dynamics in Tumor Microenvironment Under Radiotherapy

Jie Huang and Jian Jian Li

Abstract

The tumor microenvironment (TME) is an evolutionally low-level and embryonically featured tissue comprising heterogenic populations of malignant and stromal cells as well as noncellular components. Under radiotherapy (RT), the major modality for the treatment of malignant diseases [1], TME shows an adaptive response in multiple aspects that affect the efficacy of RT. With the potential clinical benefits, interests in RT combined with immunotherapy (IT) are intensified with a large scale of clinical trials underway for an array of cancer types. A better understanding of the multiple molecular aspects, especially the cross talks of RT-mediated energy reprogramming and immunoregulation in the irradiated TME (ITME), will be necessary for further enhancing the benefit of RT-IT modality. Coming studies should further reveal more mechanistic insights of radiation-induced instant or permanent consequence in tumor and stromal cells. Results from these studies will help to identify critical molecular pathways including cancer stem cell repopulation, metabolic rewiring, and specific communication between radioresistant cancer cells and the infiltrated immune active lymphocytes. In this chapter, we will focus on the following aspects: radiation-repopulated cancer stem cells (CSCs), hypoxia and re-oxygenation, reprogramming metabolism, and radiation-induced immune regulation, in which we summarize the current literature to illustrate an integrated image of the ITME. We hope that the contents in this chapter will be informative for physicians and translational researchers in cancer radiotherapy or immunotherapy.

Keywords

Tumor microenvironment · Radiotherapy · Cancer stem cells · Hypoxia · Metabolism · Immune response

J. Huang
Department of Radiation Oncology, University of California Davis, Sacramento, CA, USA

J. J. Li (✉)
Department of Radiation Oncology, University of California Davis, Sacramento, CA, USA

NCI-Designated Comprehensive Cancer Center, University of California Davis, Sacramento, CA, USA
e-mail: jijli@ucdavis.edu

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8_10

10.1 Frameworks of Multiple Aspects of ITME

Artificial radiation was invented about 120 years ago and was almost immediately applied in cancer treatment [1]. Compared to other anticancer modalities such as chemotherapy, RT has demonstrated the clinical benefits of local control with relatively less systematic side effects. In the past two decades, RT efficacy has been improved with the many technological advances, including in vivo imaging and precision of tumor dose delivery. However, the overall efficacy of RT-mediated long-term cancer control remains to be further improved [2]. Accumulating new evidence demonstrates that multiple adaptive responses are induced in ITME. The mechanistic insights of such radiation-induced adaptive tumor microenvironment, especially the communication between tumor and stromal cells such as the cross talk between tumor cells and the infiltrated immune active cells, are to be further revealed [3]. On the tumor side, tumor heterogeneity has been linked to the repopulation of resistant cancer cells such as CSCs [4, 5] and adaptive tumor radioresistance.

In contrast, in the stromal side, experimental and clinical data have revealed many previously unknown features in TME, such as reprogramming cellular energy metabolism and immune active cells under radiation with a revived concept of the abscopal effect [6]. In this chapter, as illustrated in Fig. 10.1, the following fundamental issues in the TME under therapeutic ionizing radiation will be discussed: repopulation of CSCs, hypoxia in ITME, radiation-associated reprogramming cellular energy metabolism, and the potential pro- and anti-immune regulation. Further elucidating the mechanistic insights of the multiple aspects of tumor radiation response, notably, the mechanisms in charge of the communications among the cells in different categories of the ITME, as illustrated in Fig. 10.1, will be highly appreciated. These studies could be able to define dynamical alternations of intrinsic tumor gene expression conjugated with their surface immunogenicity, which will provide valuable information for inventing more effective approaches in RT and RT combined with metabolic and immune-adjusting targets.

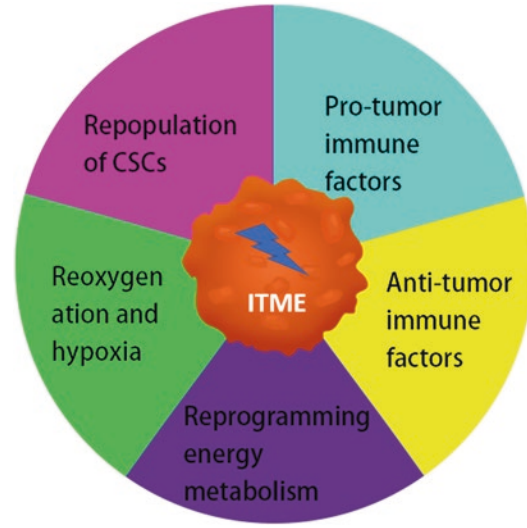

Fig. 10.1 Categorized essential adaptive rewiring TME under radiotherapy, including repopulation of CSCs, hypoxia and re-oxygenation, pro- and anti-tumor immune factors, and reprogramming energy metabolism

10.2 The Cancer Stem Cells in Irradiated TME

10.2.1 Repopulation of Cancer Stem Cells

Cancer stem cells (CSCs, also termed as stem-like cancer cells, tumor-initiating cells) are originally identified in myeloid leukemia with specific surface markers [4, 7], and then an array of solid human tumors [8–14] are linked with tumor resistance to radiotherapy and chemotherapy [15, 16]. CSCs have the capacity of self-renewal, capable of giving rise to heterogeneous lineages in the solid tumor [17, 18]. CSCs are thus loosely divided into two major subpopulations: normal and slow growth; the latter contains quiescent or dormant tumor cells [19]. Accumulating biomarkers are applied to identify and isolate CSCs revealing that CSCs can be enriched in a fraction of cancer cells that survive therapeutic radiation

[20–22], thereby being radioresistant, and may account for the failure of RT [23–25]. It has been assumed that due to unstable genomic dynamics, different subclones of CSCs with various sets of mutations and genomic alterations are present in the tumor with specific cell surface biomarker such as breast cancer with $CD44^{+}/CD24^{-/low}$ [5] as well as with enhanced aldehyde dehydrogenase (ALDH) activity [26, 27] although non-CSC tumor cells were reported to be transferred to CSCs via radiation-induced dedifferentiation [28]. CD133 + lung cancer cells were shown to be radioresistant than CD133− cells [29]. Accumulating evidence suggests that CSCs are linked with recurrence, aggressiveness, and therapy resistance, such as enhancing DNA repair capacity and forming a pro-survival microenvironment – ITME.

10.2.2 Enhanced DNA Repair Capacity in CSCs

It has long been proposed that a balance between the degree of DNA damage and activation of pro-survival signaling pathways determines the fate of an irradiated cell [30–34]. CSCs are shown to be equipped with increased capacity of DNA damage repair [35–37]. The increased capacity of CSCs for DNA repair [38] accounts for their low rate of apoptosis in response to cancer treatment. Glioma stem cells are capable of promoting radioresistance by enhancing DNA damage repair and reducing the rate of apoptosis following the repopulation of $CD133^{+}$ tumor cells after irradiation [20]. $CD133^{+}$ glioma cells are shown to survive radiation by preferentially activating DNA damage checkpoints, repairing the radiation-induced DNA damage more effectively and thus undergoing apoptosis less frequently than $CD133^{-}$ cells [20]. It may be that the transient activation of the DNA checkpoints leads to cell cycle arrest, a critical step for the initiation of DNA repair. An interesting conclusion derived from these studies is that increased activation of checkpoint proteins, with no associated change in protein expression, is detected in cancer stem cells in response to radiation-induced DNA damage. This suggests the involvement of other mechanisms in regulating the checkpoint activity and the survival of the CSCs [39]. Some irradiated cells are also able to increase their survival rate by reducing or repairing radiation-induced damage via the activation of stress-responsive signaling networks controlled by several radiation-inducible transcription factors [40–45]. For instance, interleukin family member 6 (IL-6) signaling promotes DNA repair and prevents apoptosis in CD133+ stem-like cells of lung cancer after radiation [46]. It is recently further evident that the basal expression of DNA damage sensor proteins, including ataxia-telangiectasia mutated (ATM), H2A histone family member X (H2AFX), and poly(ADP-ribose) polymerase 1 (PARP1), is increased in CSCs, indicating an increased DNA damage response in CSCs [35].

10.2.3 The Irradiated TME May Favor the Repopulation of CSCs

CSCs in an array of solid human solid tumors show radioresistant and chemoresistant properties. To improve the efficacy of current anticancer therapeutics, the cross talk between CSCs and the ITME is to be further investigated. Using a mammary chimera model in which an irradiated host is transplanted with oncogenic tumor protein 53 (Trp53) null epithelium, Nguyen et al. show that radiation enhances the development of aggressive tumors that show different molecular signatures compared to tumors arising in nonirradiated hosts, indicating that the irradiated microenvironment affects tumor phenotype [47]. ITME is also indicated to enhance the radioresistance of CSCs. To mimic ITME, Chan et al. have developed an in vitro three-dimensional (3D) NSCLC model of color-coded tumor tissue analogs (TTA) comprised of human lung adenocarcinoma cells, fibroblasts, endothelial cells, and NSCLC cancer stem cells maintained in low oxygen conditions (5% O2) to recapitulate the physiologic conditions in tumors. A single dose of 5 Gy increases the expression of cytokines and cell proliferative

factors without delaying TTA that contains CSCs [48]. Glioblastoma multiforme (GBM) cells could autocrine abundant transforming growth factor-beta (TGF-beta), a pleiotropic cytokine that promotes effective DNA damage response, promoting self-renewal and effective DNA damage response. The usage of TGF-beta inhibitors with radiation could improve therapeutic response in vitro and in vivo [49]. Tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), follicular dendritic cells (DCs), and regular T cells have been reviewed to have the capacity to regulate cancer stem cell maintenance and tumorigenesis through various cytokines, such as milk fat globule-EGF factor 8 (MFG-E8), IL6, IL17, and TGFβ1, or anchoring the ephrin type-A receptor 4 (EphA4), which in turn activates NF-κB and Src signaling pathways in CSCs [50]. The mesenchymal stem/stromal cells (MSCs) in TME play a pro-tumor function. MSCs can decrease the antitumor T cells due to MSC-released TGF-β [51, 52]. Cuiffo et al. demonstrated that the interaction between breast cancer cells and MSCs could enhance CSC function via miRNA regulation [51]. In addition, cancer-associated fibroblasts (CAFs) in non-small cell lung cancer were found to trigger the insulin-like growth factor 1 receptor (IGF1R) pathway, leading to increased stemness marker Nanog expression and a greater level of sphere formation in vitro and metastasis in vivo [53]. In hepatocellular carcinoma, CAFs are shown to secrete hepatocyte growth factor (HGF) to enhance the self-renewal capacity of tumor cells [54]. CAFs have also been shown to help maintain the stemness in gastric cancer, glioblastoma, and leukemia through secretion of TGF-β, STAT1, and NF-κB [48, 55]. The potential communication between CSCs and astrocytes in the tumor microenvironment is illustrated in breast cancer brain metastasis [56]. A recent well review on DNA repair related to CSCs has addressed on high inter- and intratumoral heterogeneity, the plasticity of CSCs, and microenvironment-stimulated tumor cell reprogramming [57]. These results demonstrate that the communication of CSCs in ITME contributes to the adaptive response of tumor microenvironment stressed with frequent genotoxic conditions.

10.2.4 Targeting CSCs in Radiotherapy

CSCs have been extensively studied for therapeutic targets [58, 59], although there is a relatively small portion of reports indicating no enhanced radioresistance in radiation-surviving tumor cells and tumors (probably enriched with CSCs) [60]. It has been reported that carbon ion radiation is more efficient to eliminate CSCs than x-ray, and the potential therapeutic synergy of radiosensitizing microRNAs and carbon ion beam has been reviewed [61], and hyperthermia combined with 6 MeV electron radiation is shown to enhance the inhibition of the clonogenicity of prostate CSCs [62]. An array of CSC targets are being tested for the elimination of CSCs based on inhibition of CSCs biomarkers and downregulation of stem cells' self-renewal genes such as Nanog, Sox-2, and Rex-1 [130]. Histone deacetylase inhibitors (HDACIs) inhibit CSC biomarkers and CSC-induced epithelial-to-mesenchymal transition (EMT) [63]. Using a radioresistant head and neck squamous cell carcinoma SQ20B cell line, UCN-01, a checkpoint kinase (Chk1) inhibitor, enhances the radiosensitivity of SQ20B-CSCs and further synergizes radiosensitivity with all-trans retinoic acid (ATRA) [64]. Honokiol, a biphenolic compound that has been clinically used in traditional Chinese medicine for treating various ailments, is shown to reduce CSC biomarkers and synergize tumor radiosensitivity [65]. Salinomycin is found to inhibit pancreatic CSCs and CD133+ persistence, and salinomycin-loaded nanomicelles showed enhanced efficiency in head and neck CSCs [66]. Graphene oxide, also a nano-compound to target CSCs, is illustrated to convert CSCs into non-CSCs via suppressing the Notch, WNT, and STAT signaling pathways [67]. Interestingly, epigallocatechin gallate (EGCG), a polyphenol ingredient of green tea, induced programmed cell death in the CD133+ and ALDH1 glioma CSCs. EGCG subdued P-glycoprotein in CSCs and consequently

enhanced its sensitivity to temozolomide [68]. In addition, some newly developed drugs such as BBI608 (inhibitor of STAT3 and β-catenin pathways), vismodegib, R04929097, and glycogen synthase kinase 3 (GSK3) inhibitors are also being tested in clinical trials [69]. Increasing interests are on the potential combination of targeting CSCs with immunotherapy [70].

Antibodies to CD44 are tested for an array of solid tumors combined with radiation to enhance tumor response [21, 71]. Additional CSC antibodies, including demcizumab (anti-Notch ligand DLL4 antibody and nanobody) [72, 73], OMP-18R5 (anti-Wnt receptor FZD monoclonal antibody), and OMP-52 M51 (anti-Notch1), have been generated and are under tests. Regulation of immune cells to target CSCs also holds great potential for generating clinical benefits. J.C. Sun et al. applied dendritic cell-based vaccines, which were treated with antigens from CD133+ hepatocellular carcinoma cells to activate specific cytotoxic lymphocytes and therefore destroy hepatocellular carcinoma CSCs. HER2-specific chimeric antigen receptor (CAR) T cells have effectively abrogated the survival of HER2 expressing glioblastoma cells, including CD133+ and CD133− carcinoma cells [74]. AC133 (an epitope of CD133)-specific CAR T cells are shown to eliminate CD133+ glioblastoma CSCs [75].

10.3 Dynamics of Hypoxia and Re-oxygenation

10.3.1 The Hypoxia and HIF-1-Mediated Gene Expression

Oxygen present on the earth surface has been linked with the biological evolution from single- to multiple-cell organisms and is an indispensable substrate to participate in the aerobic respiration of all mammalian cells that is required to maintain critical physiological activities [76]. The eukaryotes featured with their adaptive capacity in oxygen-independent energy metabolism indicate that they arose a long time before the oxygen levels reached the current level on earth [77–79]. Cells in the lowered oxygen situation, termed hypoxic condition, or hypoxia, existing in multicellular systems such as human tissue and organs as well as in the solid tumors, may reflect the ancient lifestyle of original biological units. It is thus reasonably assumed that mammalian cells with stem-like characteristics such as CSCs are evolutionally able to benefit from such hypoxic conditions.

The hypoxic region within a solid tumor is radioresistant and linked with failure of growth control and metastasis [80–82] since oxygen enhances ROS formation that increases DNA damage [83, 84]. HIF-1α, a key transcription factor, promotes the expression of many effector genes to reduce cell proliferation and survive under hypoxia, a known phenomenon of tumor dormancy. Radiation is shown to be able to re-oxygenate the hypoxic areas [85], and tumor metabolism is also linked with the hypoxic and nonhypoxic regions within the tumor as well as the surrounding stroma [86]. Such a dynamic feature of hypoxic/oxygenated status is reflected by growing evidence that radiation can cause vascular dysfunction in the tumor microenvironment, especially the application of doses delivered with less fractionated high doses, including the dosage used in stereotactic body radiotherapy (SBRT) [87–90]. Data from Oberley-Deegan's group has identified that MnTE-2-PyP (manganese (III) meso-tetrakis-(N-ethylpyridinium-2-yl)) with 5 plus charges being able to scavenge ROS is shown to reduce histone acetylation of HIF-1 effector gene PAI-1 to inhibit prostate cancer growth [91], indicating different target genes regulated by hypoxic conditions. For instance, HIF-1 is shown to regulate CD47, causing immunotolerance of breast cancer cells [92]. Future research should attempt to unearth the missing links between HIF and cancer metabolism or immune escape.

10.3.2 Hypoxia in Radiation-Induced CSC Repopulation

Hypoxia-inducible factors (HIFs) and reactive oxygen species (ROS) coordinately play a role in

CSCs [93] since CSCs favor the hypoxic condition, whereas oxygen seems to change the stem status for cells. This could be a critical mechanism underlying radiation-induced dynamics of CSCs repopulation. Hypoxia-induced biological changes are able to promote CSCs in discrete regions of the tumor [17], and HIF is shown to enhance CSCs specification and maintenance [94]. The hypoxic microenvironment is shown to be necessary for maintaining the stem-like phenotype of glioma stem cells [95]. Ovarian cancer spheroid cells with stem features contribute to therapy resistance through hypoxia-resistant metabolism [96]. Lowered ROS levels are associated with less DNA damages and metastasis of breast cancer [97]. Blazek et al. showed that $CD133^{+}$ cancer cells are enhanced by hypoxia [98]. Increased CD13 is indicated with reduced ROS and enhanced survival of liver CSCs [99]. The redox modulator NRF2 and ROS-related microRNA have been identified as potential regulators of CSCs [100–102], indicating that redox imbalance plays a critical role in the regulation of CSCs [103]. Thus, the coordinative mechanisms underlying hypoxia and ROS in CSC radioresistance are to be further investigated. In this regard, hypoxia-responsive miR-210 is found to drive the metabolic reprogramming and self-renewal activity of CSCs. In this study, HIF1A-induced expression of miR-210-3p can reduce TCA cycle activity and repress OXPHOS, suggesting a metabolic shift with increased lactate production for self-renewal capacity of CSCs [104].

10.3.3 Tumor Hypoxia and CSC Dormancy

Tumor dormancy is a recognized clinical phenomenon in which disseminated tumor cells remain occult and undetectable over a prolonged period of time [105, 106]. Such dormant cancer cells are less sensitive to clinical therapy than the cancer cells in the proliferative stage. The reasons for this may not be limited to their noncycling stage but may be associated with their unique metabolism and low oxygen microenvironment [107, 108]. Several groups have observed the existence of dormant cells in hypoxic areas of TME. These hypoxia-resistant tumor cells or hypoxia-favored cancer cells demonstrate the dormant status characterized by reduced proliferation and metabolism without significant cell death. However, the dormant state can be reversibly switched to active status with high mitochondrial respiration in an optimal cell culture condition [109, 110]. Additional experimental and clinic data also indicate that the dormant tumor cells could be enhanced by current anticancer modalities [111, 112]. Radiation is well-demonstrated to be able to modify the dormant status of a solid tumor. For instance, the quiescent neural stem cells (NSCs) can exit the dormant stage 2 days after irradiation [113]. The intrinsic molecular pathways in CSCs contribute to such therapy-mediated refactorization of cancer cells in the dormant stage. Hypoxia-induced specific factors such as leukemia inhibitory factor receptor (LIFR) are required for the dormancy in breast cancer cells metastasized in the bone marrow [114]. Conley et al. found that antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. In agreement, the population of CSCs can also be increased in breast cancer xenografts with increased tumor hypoxia by antiangiogenic agents due to HIF1-α activation, which may compromise the efficacy of antiangiogenic agents [115]. As illustrated in Fig. 10.2, the dynamics or imbalance in dormant vs. proliferative cancer cells could be a critical factor in RT-mediated CSC repopulation and lack of tumor responsiveness to RT due to the recycling of self-renewal-repopulation-dormancy. In addition, results reported by Smilowitz et al. suggested that immunotherapy efficacy can be enhanced by increased radiation dose with prolonged tumor dormancy [116]. Therefore, two opinions seem to be produced on tumor dormancy in radiotherapy. One is that RT or RT combined with immunotherapy should enhance the duration of tumor dormancy to increase disease-free survival, and the other is that treatment strategy should be applied to reduce the pool of dormant tumor cells so as to become proliferative status to increase the therapy response. However, it is highly risky to make dormant

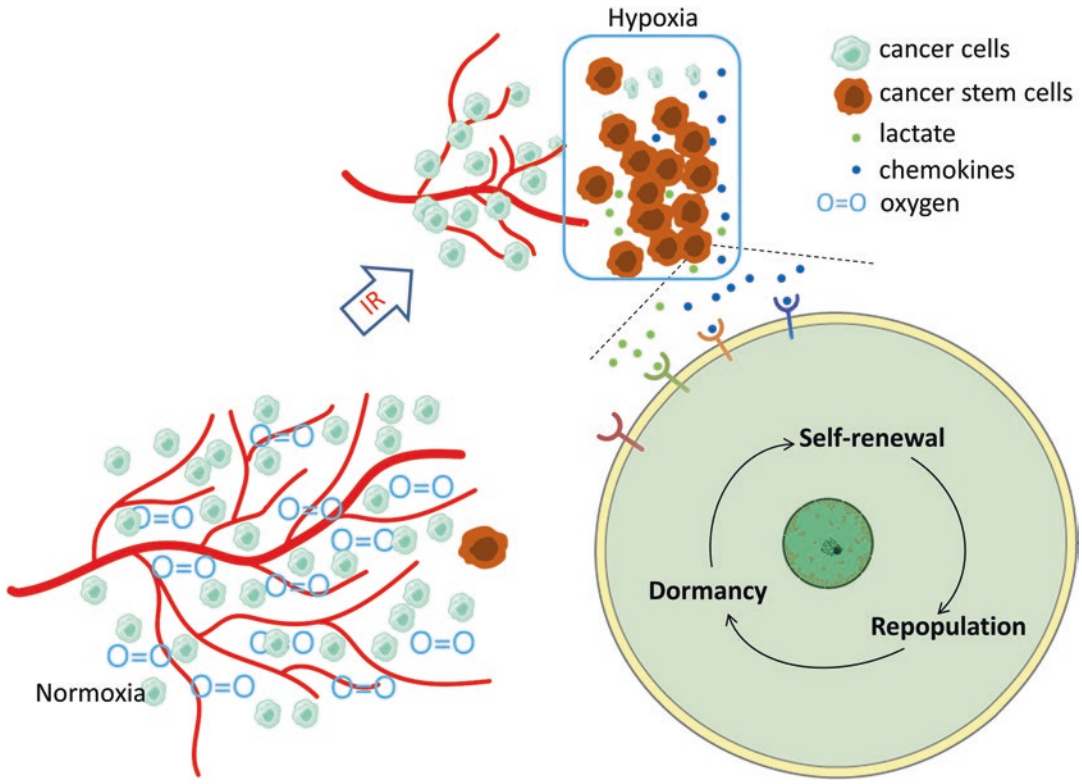

Fig. 10.2 Enhanced repopulation of CSCs due to radiation-induced hypoxia in ITME. In addition to radiation-induced re-oxygenation, which increases tumor responsiveness, radiation-induced vascular injuries and thus local hypoxia may build a hypoxic niche that favors the cycle of dormancy-self-renewal-repopulation and local tumor radioresistance

tumor cells become proliferative status because uncontrolled aggressive tumor cells lead to disease progression and failure of treatment. Thus, a conceivable strategy is to find potential molecular pathways to eliminate the dormant cancer cells at a quiescent state (by potential metabolic targets) or to reprogram or block the dormant microenvironment to limit its ability to reactivate itself [117].

10.3.4 Targeting Hypoxia for Enhancing Tumor Responsiveness

It has been long observed that tumor hypoxia is linked with radioresistance, and a great effort has been exerted to enhance tumor response under radiotherapy [85, 118, 119]. However, a precise in vivo monitoring of the hypoxic/oxygenated dynamics has been a challenge which is necessary to enhance tumor responsiveness by adjusting the radiation dose. Grimes et al. utilized avascular tumor models to mimic tumor dynamics, and spheroids exhibit a similar sigmoidal growth curve as in situ tumors [120]. Great efforts have been dedicated to the clinical hypoxia imaging to personalize radiotherapy [121–123]. White et al. described an oxygen-enhanced magnetic resonance imaging (OE-MRI) approach as a prognostic biomarker of tumor radiation response [124]. Such OE-MRI techniques were evaluated as potential noninvasive predictive biomarkers. In addition, semiquantitative blood oxygen level-dependent (BOLD) and tissue oxygen level-dependent (TOLD) contrast and quantitative

responses of relaxation rates to an oxygen breathing challenge during hypofractionated radiotherapy were applied. A recent study aiming to calculate doses required in hypoxic subareas suggests that fractionation of radiation doses is linked to the heterogeneous and dynamic tumor oxygenation [125], whereas vascular damage induced by high radiation doses may reduce the therapeutic efficacy due to enhancing the hypoxic region [89]. Many approaches have been tested to adjust tumor hypoxia, among which, the nitroimidazoles are well-studied in an array of solid tumors with limited effects in clinical trials [126, 127]. A limited therapeutic benefit for tumor radiosensitization was observed by the application of carbogen (95% O2 and 5% CO2) or misonidazole [128]. Additional approaches are aimed to reduce tumor oxygen consumption and hypoxia [129]. It is estimated that a 30% reduction of O_2 consumption can decrease the hypoxic fraction in the tumor from 37% to 11% [130]. A group of bioreductive agents has been tested in clinic trails as hypoxic cytotoxins [131]. Metformin that is able to reduce O_2 consumption and tumor hypoxia is also tested for the radiosensitization of hypoxic tumors [127, 132]. Another major approach to reduce tumor hypoxia in radiotherapy is to target the aberrant vasculature in ITME [133], which aims to prevent revascularization after therapy. Results from Dewhirst's group further demonstrate that hypoxic condition enhances the secretion of lactates that could be taken by nearby aerobic cells for mitochondrial respiration; thus blocking lactates may eliminate such lactate-fueled respiration to block tumor growth [134]. Managing the hypoxic condition in ITME, especially its correlation with reprogramming metabolism and immune regulation, remains as a critical topic in the tumor radiobiology area.

10.3.5 Pericytes in ITME

Pericytes are the multifaceted cells associated with endothelial cells with varied biological functions arranging from tissue regeneration and development of diseases [135]. There are two types of pericytes, type 1 and type 2, which can differentiate, respectively, into fat and muscle cells. The major function of pericytes is to regulate endothelial cell differentiation for angiogenesis, which plays a critical role in tumor development. As a critical component in the tumor microenvironment, pericytes contribute to the constant aggressive phenotype, metastasis, and potential immunotolerance [136]. Birbrair et al. reported that although both type 1 and type 2 pericytes can attach to blood vessels in vivo, only type 2 pericytes enhanced endothelial cells to form new vessels and can be recruited during tumor angiogenesis [137]. Although the precise mechanism of radiation-induced pericyte toxicity is to be investigated, researchers have identified that tumor blood volume was expressively reduced with significant loss of both endothelial cells and pericytes [138]. However, in the TME treated by high-dose radiation, mesenchymal stem cells (MSCs) were shown to be recruited to the irradiated site to help pericyte recovery and tumor recurrence [139, 140]. These studies further demonstrated that the migration of MSCs was guided by HIF-1α-SDF-1α/CXCR4 pathway, and as a result, MSCs can differentiate into pericytes to sustain tumor endothelial cells [139, 140].

10.4 Metabolic Reprogramming

10.4.1 Pro- and Anti-Warburg Effects in Stress-Induced Cell Metabolism

The evolutionary progression from single cells to the metazoan is paralleled with the appearance of oxidative phosphorylation from glycolysis, reflecting the flexible nature of cell energy metabolism. Cancer metabolism has been extensively studied in cancer biology and therapeutic potential [141] [141, 142]. Enhanced lactate in tumor cells with aerobic glycolysis was termed as Warburg effect, and mitochondrial deficiency was designated as a metabolic mark of

transformed cells, and cancer cells were mistakenly assumed as a result of damage of mitochondrial respiration. Although glycolysis-mediated ATP generation is not efficacious compared to oxidative phosphorylation (OXPHOS), it is believed that glycolysis-mediated factors are required for cellular function and proliferation such as pyruvate and nicotinamide adenine dinucleotide phosphate (NADPH) [143–145]. However, increasing results have challenged the Warburg effect [146–150], indicating that cancer cells remain intact structure and functional mitochondria [151]. Early studies demonstrated that forced overexpression of manganese superoxide dismutase (MnSOD) that detoxifies superoxide in the mitochondria to protect and maintain mitochondrial homeostasis enhanced the survival of breast cancer cells treated by radiation [45, 152]. On the other hand, pharmacological glycolysis inhibitors such as 2-deoxyglucose (2-DG) did not generate in vivo therapeutic benefits on tumor inhibition [153, 154]. In consistence, OXPHOS-related genes were found to be enhanced in a study with over 2000 breast cancer patients [155]. Increased mitochondrial bioenergetics was linked with CSCs [156–160] and lymphoma cells [159, 161]. An abundant amount of NADPH was detected in the mitochondria and the cytosol in cancer cells to keep the enhanced capacity of antioxidant activity and prevent the buildup of potentially detrimental ROS [162] [163]. Together, it is now generally accepted that cancers with Warburg effect still retain functional mitochondrial respiration [164]. However, the mechanisms responsible for shifting the different metabolic pathway remain to be elucidated. It is reasonable to propose that cancer cells have a pluripotent capacity of adaptive energy metabolism including OXPHOS so as to be able to adjust varied mechanisms to timely and efficiently provide the cellular fuels for the energy consumption [165, 166] and adaption of metabolic stress such as nutrient deficiency and hypoxia [167, 168]. Such plasticity in adjusting mitochondrial bioenergetics of tumor cells challenges the efficacy of cancer radiotherapy.

10.4.2 Rewiring Mitochondrial Bioenergetics

The mitochondrial antioxidant enzyme MnSOD has been shown to be necessary for maintaining mitochondrial homeostasis and in preventing cell transformation [169, 170]. Enhanced mitochondrial functions in response to genotoxic stresses such as radiation and chemotherapeutic agents are also linked with tumor resistance [160, 171–178]. The apparent "quiet" mitochondria in tumor cells thus may function as a backup energy supply to meet the increased fuel need under cytotoxic conditions such as radiation and chemotherapy. MnSOD-mediated mitochondrial protection contributes to aggressive behavior and therapy resistance [179–183]. Mitochondrial metabolism is linked with metastasis [184–186] and tumor cell invasion [187, 188], importantly, with the aggressive phenotype of triple-negative breast cancer (TNBC) [185]. Cyclin-dependent kinase 1 (CDK1) is found to relocate to the mitochondria to boost OXPHOS for cell cycle G2/M transition [189] and radiation-induced DNA repair [190]. Under therapeutic radiation, CDKs can relocate to the mitochondria to phosphorylate a cluster of OXPHOS proteins and MnSOD to boost mitochondrial metabolic functions and adaptive cellular response [191, 192]. CDK1 is able to activate sirtuin 3 (SIRT3) and p53 to enhance mitochondrial energy output and reduce mitochondrial apoptosis for cell survival [193, 194]. In addition, CDK is also indicated with Dynamin-related protein 1 (DRP1) regulation that is actively involved in mitochondrial morphological dynamics [195, 196]. Thus, blockade of mitochondrial energy reprogramming, such as CDK1 mitochondrial relocation, may inhibit the mitochondrial energy dynamics to enhance tumor control. In addition, lipogenic phenotype that is closely linked to the TCA cycle for mitochondrial biogenesis has been found to be activated in cancer cells [197, 198]. For tumor cells to proliferate, fatty acid (FA) synthesis (for membrane biogenesis) as well as glutaminolysis (for amino acid precursors) has been reported to be affected during tumorigenesis [164, 199, 200]. It is no surprise that lipid metabolism is critical for can-

cer cell survival, and lipogenic enzyme fatty acid synthase (FASN) is linked with cancer progression [197, 198]. The unsaturated fatty acids (UFAs) have been identified in cancer cells to survive under O2 deprivation [199]. However, it is to be further investigated on how such FA reprogramming is regulated in ITME, especially in the switching from glycolysis-dominated metabolism to FA-enhanced mitochondrial respiration.

10.4.3 Energy Exchange Between Tumor Cells and Stromal Cells in ITME

In the solid tumor, hypoxia-induced dynamic metabolism in tumor and stromal cells can significantly affect tumor cells' behavior in the microenvironment [201]. Cancer cells and the surrounding stromal cells, especially immune functional cells, may adjust their metabolic pathways so as to adapt to the hypoxic, acidic, and low-nutrition microenvironment. For instance, TAMs tend to be M2 polarized, featured with upregulated fatty acid β-oxidation and synthesis, whereas the cytotoxic T lymphocytes were fueled by enhanced OXPHOS with reduced glycolysis. These results illustrate that a dynamic metabolic reprogramming occurred in various cells in the tumor microenvironment. Therefore, further exploration of metabolic reprogramming in both tumor and stromal cells in ITME will provide more details of mechanistic insights regulating tumor adaptive response and metabolism-mediated immune regulation. In ITME, adjusted cellular energy metabolism may also regulate the survival of immune cells and their ability to proliferate. For instance, the NK cells need cellular energy to be functional for a prolonged period to kill the targeted malignant cells [202]. On the other hand, the adjusted tumor cells may effectively defend themselves to severely compromise the effects of NK cells, causing less effectiveness in the cell-based immunotherapies [202]. For instance, hypoxia in solid tumors can generate adenosine from the cancer-associated ectoenzymes CD39 and CD73 that enhance the immunosuppressive effects on NK cells. Such immunometabolic targets need to be further elucidated. Radiation-associated immune factors in ITME are to be discussed in detail in the following section.

10.5 Radiation Induced Anti- and Pro-tumor Immune Factors

10.5.1 The Antitumor Immune Regulation

A tumor inhibitory effect observed at a distance from the tumor site treated by local radiation therapy has long been noticed and termed as the abscopal effect [203–206]. Although the abscopal effect is rare in clinic radiotherapy, it implicates a potential fundamental mechanism by which radiation may enhance tumor priming function and systematic immune regulation in the ITME [207]. It has been proposed that RT-mediated tumor control is linked to a balance between tumor cell proliferation and T-cell-mediated killing [208]. With the increasing interest in targeted immunotherapy, a bulk of work in preclinical tumor models, supported by clinical observations, has further provided the rationale for the hypothesis that focal RT can transform cancer cells into an in situ, individualized vaccine [209]. Clinical studies are currently evaluating the synergic efficacy of RT, combined with checkpoint inhibitors [210, 211]. Characterization of radiation-induced antitumor immune factors will deepen our understanding of abscopal effects and will provide more effective immune targets in immunotherapy or immunotherapy combined with RT.

10.5.1.1 Damage-Associated Molecular Patterns (DAMPs)

In mammalian cells, high physical energy beams, including ionizing radiation, can generate so-called endogenous damage-associated molecular patterns (DAMPs) which include the high-mobility group box 1 protein (HMGB1), calreticulin (eat me signaling), and adenosine

triphosphate (ATP). Such a cluster of tumor-released molecules has been shown to cause antigen presentation to T cells, thereby activating the immune system and inducing the abscopal effects [212–214]. For instance, HMGB1 is shown to enhance the production of cytokines such as TNFα, IL-1, IL-6, and IL-8 and to mediate a pro-inflammatory effect [215] and tumor antigen presentation by interacting with toll-like receptor 4 (TLR4) on DCs. Additionally, the released DNA fragments from radiation-induced cell death are also able to stimulate the expression of the interferon genes (STING) in DCs and thus to enhance DCs' cross-priming [216]. Additionally, it is reported that merely a single dose of 0.5 Gy ionizing irradiation could recruit NOS2-expressing macrophages and T cells into the tumors [217], indicating a potential synergy on tumor control by RT combined with targeted immunotherapy mediated by DAMPs.

10.5.1.2 Radiation-Induced Neoantigen

Some specific gene mutations in tumor cells can generate immunogenic neoantigens leading to the infiltration of immune cells. Thus, the so-called tumor mutation burden (TMB) has been linked with the response to targeted immunotherapy [218]. The somatic copy-number alterations (SCNA) enhanced in tumor cells are linked with tumor immune evasion and reduced IT responsiveness [219]. Radiation and DNA-damaging chemotherapy are powerful mutagens that may alter the TMB and SCNA as well as the genomic instability. Studies by Hallahan et al. have demonstrated that radiation can be used to guide drugs to specific sites due to radiation-induced peptides that bind to irradiated tumors [220]. Cancer cells surviving these treatments often carry new mutations that are required for cancer cell survival. Such radiation "marked" cancer cells have been proposed to be an ideal target for drug delivery and imaging agents. Such peptides generated by a radiation-induced gene mutation (radiation-induced neoantigen) on the tumor cells could be the novel biomarkers and new immune targets in the radiotherapy combined with targeted immunotherapy. A cluster of such potential molecules expressed on the surface of tumor cells induced by radiation is summarized [221]. Tumor neoantigens are defined as genes with acquired mutations, and the peptides generated by proteome digestion on the neoantigen-encoded proteins bind to the major histocompatibility complex class I (MHC-I) molecules to obtain immunogenic property [222]. Such products of neoantigens must be sufficiently expressed and function typically as the key targets for T cells [223]. An analysis of an NSCLC patient who showed a well response to RT combined with ipilimumab demonstrated the expansion of CD8 T cells that could recognize a radiation-induced neoantigen encoded by the KPNA2 gene which could be enhanced to expression by radiation [211, 224]. Thus, radiation-induced neoantigens represent as a strong inducer for systemic immune surveillance. In the following, some major tumor antigen presentation pathways, a critical step for tumor immune surveillance, are derived.

Radiation-induced neoantigens can also be generated via posttranslational modifications (PTMs), proteasome splicing, and RNA splicing or from non-coding regions of the DNA, all of which are in need of a functional antigen-presenting cell system. Many of the MHC-I immunopeptidome are linked to proteasome-spliced peptides [225], and radiation is able to induce proteome where newly synthesized proteins are targeted to produce antigens [226]. The specific peptides generated via PTM which includes phosphorylation and glycosylation [227–229] can be presented by MHC to activate T cells [230]. It has been reported that such phosphorylated peptides recognized by tumor-specific T cells can be shared with different malignancies, indicating a comparable phosphorylation pattern in tumors [229, 231]. In addition, radiation-induced neoantigen can be generated by oxidation due to radiation-induced redox imbalance. Radiation-specific peptides have been identified and presented by MHC-I [232].

10.5.1.3 Radiation May Enhance Tumor Antigen Presentation

The MHC (major histocompatibility complex) is required to bind to a large scale of antigenic pep-

tides, including radiation-induced neoantigens, so as to be able to display them on the cell surface for recognition by the appropriate T cells. In human, the MHC-related genes are located in chromosome 6 containing more than 200 genes that encode many proteins in MHC. The lack of MHC in many human cancer cells is believed to contribute to the tumor evasion of immune surveillance. High-dose radiation is shown to enhance 1.5- to 10-fold MHC-I/II expression on the cell surface that can last 7–14 days postirradiation [233]. In addition, irradiated human sarcomas induce immune effectors and cancer-testis antigens with downregulation of immune suppressors [234]. Further studies are in need to identify that any of the radiation-induced neoantigens can be specifically presented by MHC.

MHC-I Presentation of the specific tumor neoantigens by antigen-presenting cells is a critical stem for tumor immunogenicity. The major histocompatibility complex class I (MHC-I) is downregulated in many human tumors which is responsible for the enhanced immune invasion in cancer cells. It is recently identified that gene methylation of MHC-I is linked to inhibited expression [235]. Paulson et al. showed that in Merkel cell carcinoma cells, radiation enhances MHC-I expression [236]. Recently, ATR inhibitor AZD6738 shown to sensitize to radiotherapy combined with fractionated radiation enhances immune cell infiltration of myeloid cells with MHC-I induction [237].

MHC-II Although radiation is shown to induce MHC-II on different cells in vitro and in vivo tumors, the details underlying the mechanism and specificity of MHC-II expression in ITME remain to be elucidated. Unlike MHC-I, MHC-II is specified to present peptides from proteins that are degraded in the endosomal pathway [238]. 2-DG, as an inhibitor of glycolysis, combined with radiation, is shown to generate synergism on tumor inhibition with enhanced mature DC functional markers CD86 and MHC-II [239]. Colon cancer mouse xenografts treated with FIR (2 x 5 Gy) show enhanced immune cell infiltration, tumor growth inhibition, and MHC-II expressing DCs [240]. MHC-II-restricted phosphopeptides are also reported as relevant targets for human CD4 T cells [241]. Peptide splicing by the proteasome is another mechanism that increases the diversity of the antigenic peptides presented to CD8 T cells [242, 243]. The peptides generated from gene mutations predicted to bind to MHC-II are found to be copious in an array of human cancers [244], indicating that MHC-II is involved in presenting a large scale of tumor peptides. Again, it is not clear what specific radiation-induced neoantigens are presented and whether such specific neoantigen could be shared by different tumors treated under radiotherapy.

CD4 T-Cell Response The so-called CD4 T-cell responses are indicated to the process by which neoantigens activate the helper function at the level of DCs and to increase the antitumor function of CD8 T cells [245]. The temporary and/or permanent enhancement of tumor mutation burden (TMB) and somatic copy-number alterations (SCNA) in ITME may be critical for achieving an efficient presentation via the endosomal pathway of antigen-presenting cells (APCs) [246, 247]. Radiation is shown to increase the antigen transfer capacity from cancer cells to the myeloid cells in ITME [248], leading to T-cell priming and immune surveillance. A neoantigen encoded in a gene upregulated by radiation is shown to be responsible for the rapid expansion of CD8 T cells in NSCLC patients [211]. It is thus assumed that radiation-induced mutanome may boost neoantigen presentation by MHC-II, enhancing the activation of CD4 T-cell responses. However, we propose that such an antitumor immune response could be severely compromised by a radiation-induced pro-tumor immune response that is to be addressed in the following Sect. 10.5.2.

10.5.2 The Pro-tumor Immune Factors in ITME

10.5.2.1 Immune Checkpoint Proteins in ITME

CTLA-4/CD80 CTLA-4 (also termed as cytotoxic T-lymphocyte-associated protein 4, CD152,

cluster of differentiation 152), a member of the immunoglobulin superfamily, plays a critical role in tumorigenesis and is responsive to radiation combined with targeted immunotherapy [249]. Although it is unclear how CTLA-4 could be included by different doses of radiation, CTLA-4 blockade is shown to synergize with RT to enhance survival in a murine glioma model [250] and in human cancers [249, 251]. The potential synergy between RT and IT with anti-CTLA-4 is recently well-summarized [251, 252], and at least two signaling pathways for irradiation-induced upregulation of CTLA-4 expression were reported, including TNF-α/CD154− NF-kB and oxidative stress response [253]. CD28 expression was increased on splenic and thymic lymphocytes after low-dose radiation (LDR), while it decreased after high-dose radiation (HDR), and CTLA-4 expression showed changes in the opposite direction. The upregulation of CTLA-4 associated with the downregulation of CD28 after HDR led to immunosuppression [254].

PD-1/PD-L1 Comparetd to CTLA-4, programmed cell death 1 (PD-1) and programmed cell death 1 ligand 1 (PD-L1) are functioning in the later phase of the immune suppression process [255] and play a key role in tumor immunotolerance. PD-L1 gene expression is indicated to be sensitive to radiation and chemotherapies, although different results are reported [256]. Their interaction leads to inhibition of secretion of TNFα, IFN-ɣ, and TGF-β, thereby favoring immunosuppression [257]. Besides, PD-1/PD-L1 is thought to have a role in the "adaptive suppression" of T-cell activation [258]. Like CTLA-4, it forces T cells to reduce their TCR expression, to become allergic to locally expressed antigens, to reprogram the methylation level of their genome, and to incline to apoptosis as well as in T-cell exhaustion [259]. Several questions urge to be solved in such combined therapies, including the dynamics of PD-1/PD-L1 expression in ITME, such as irradiated tumor cells and immune cells, and the timing to apply RT to eliminate a bulk of tumor cells that lack PD-1 expression. A dose-dependent manner of PD-L1 enhancement was reported in glioma cells treated by radiation [260]. Radiation and a number of chemo drugs induce PD-L1 expression through different mechanisms, which are likely responsible for the immune escape and acquired resistance [261, 262]. Blockade of the PD-1/PD-L1 pathway is shown to reverse tumor adaptive immune tolerance and maintains antitumor immunity [263–265]. Radiation to the tumor can induce sensitivity to PD-L1 checkpoint blockade in orthotopic models of head and neck squamous cell cancer (HNSCC) [266]. Prior irradiation resulted in significantly elevated expression of programmed cell death protein 1 (PD-1) in both CD4 + and CD8 + populations. T cells with elevated PD-1 mostly were either central memory or naïve cells. In addition, the feedback induction of PD-1 expression in activated T cells declined after radiation [267].

CD47 In addition to the above checkpoint proteins, CD47 (cluster of differentiation 47), an anti-phagocytic signal in cancer cells to evade from phagocytosis mediated by TAMs, is becoming one of the key immune targets in cancer immunotherapy [268]. CD47 interacts with signal regulatory protein-alpha (SIRPα) [269–275] on immune effector cells and generates a "don't eat me" signal to escape the immune attack [276]. Combining CD47 blockade with irradiation synergistically affected tumor growth and enhanced tumor sensitivity to irradiation in syngeneic immunocompetent mice [277]. Transcription of CD47 can be induced by IR [278], and inhibition of CD47 signaling in normal cells conferred a survival advantage to irradiated normal tissue via promoting the viability and proliferative capacity in an autophagy-dependent manner [279]. Enhanced CD47 expression in the tumor microenvironment could severely hamper tumor radiosensitivity [277, 280]. IR induces CD47 expression to make the tumor cell "invisible" for phagocytosis, enabling them to escape immune surveillance, and thus it may severely compromise the abscopal effect. Data from the author's lab support that NF-κB can co-regulate CD47 and

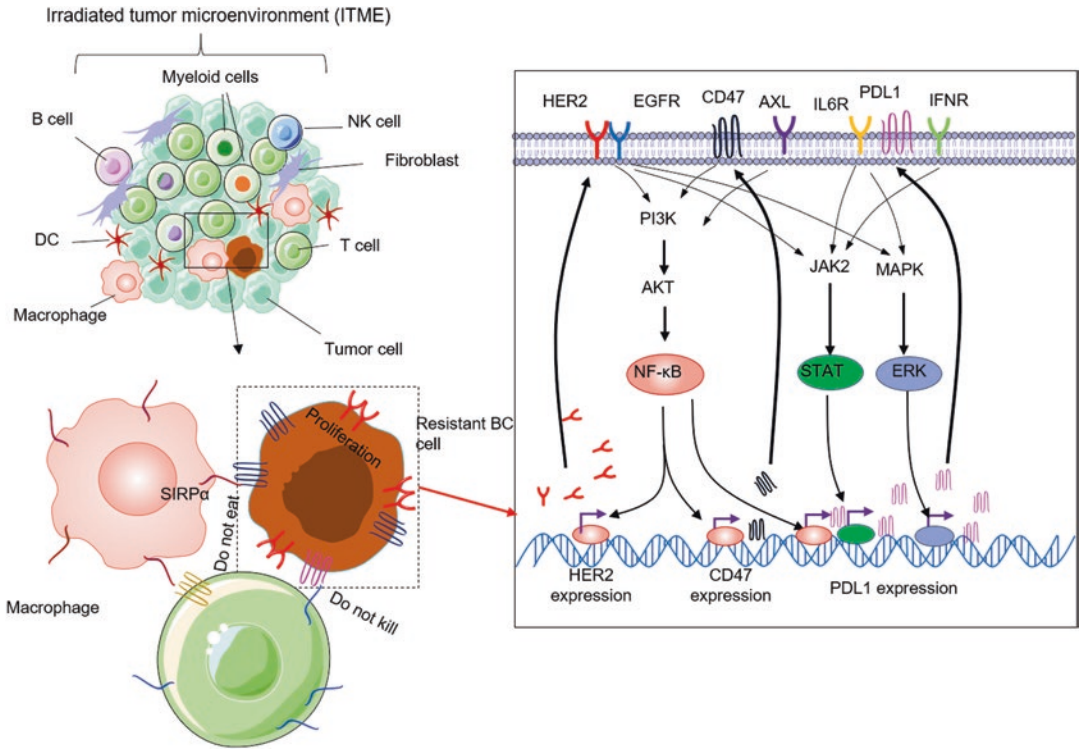

Fig. 10.3 In addition to radiation-induced tumor immunogenicity that enhances the abscopal effects, radiation also induces a pro-tumor immunotolerance via upregulating immune defending receptors such as PD-L1 and CD47 on the tumor cell surface. Together with the intrinsic radioresistant of radiation-surviving cancer cells, radiation-induced immune-suppressive factors contribute to the feature of the aggressive phenotype of radioresistant cancer

HER2. Co-expression of CD47 and HER2 is a key feature of IR-induced adaptive resistance, as illustrated in Fig. 10.3. Radiation combined with immunotherapy using anti-CD47 and anti-HER2 showed the most synergy in eliminating clonogenic cancer cells, and local tumor radiation was enhanced by the application of anti-CD47 antibody (unpublished data). These results demonstrate the complexity of tumor-acquired immunotolerance due to activation of the NF-κB-HER2-CD47 pathway, which may be a dominant feature in irradiated CSCs. Thus, dual inhibition of CD47 and HER2 may enhance the abscopal effect. A recent publication indicates that HER2 mediated STING pathway is actively involved in the immunotolerance of breast cancer, and blocking HER2-AKT1 cascade induces apoptotic cell death and STING-mediated immune surveillance [281]. To further enhance the therapeutic efficacy of immunotherapy or immunotherapy combined with RT, the cross talk between receptors charging the tumor immunotolerance and cell proliferation is to be further investigated.

10.5.2.2 Radiation-Mediated Immune Suppression in ITME

Accumulating evidence shows that RT is capable of overcoming tumor-induced immune suppression in TME [282, 283]. On the dark side, several reports have indicated that radiation may directly or indirectly suppress immune cell activity in ITME. Therefore, such radiation-associated immune suppression may severely compromise the efficacy of tumor control treated by the combined modality of RT and IT.

The Myeloid-Derived Suppressor Cells (MDSCs)

The immune-suppressive factor indoleamine-2,3-dioxygenase-1 (IDO1) that inhibits T-cell function and causes tumor immunotolerance [284] is indicated in tumor radiation response. The "rebound immune suppression" describes the immune-suppressive phenomena of TME treated by RT and other anticancer modalities [285, 286]. RT of hypofractionated doses is shown to induce the rebound immune suppression in ITME by IDO1-expressing MDSCs. IDO1-expressing MDSCs are believed to be relative radioresistant and thus able to survive and repopulate in ITME [212, 240]. The recruitment of MDSCs could also be enhanced by radiation due to radiation-induced chemokines and vasculature alterations [287]. Using murine and canine cancer models, Monjazeb et al. have provided evidence indicating that blocking IDO1-rebound immune suppression enhanced the efficacy of radio-immunotherapy [285]. IDO1 inhibitor INCB023843 combined with RT inhibited MDSC and immune suppression and sensitized LLC tumors to hypofractionated radiation [288]. Radiation and anti-PD-L1 antibody combinatorial therapy induce T-cell-mediated depletion of MDSC and tumor regression [289]. Lan et al. further demonstrated that compared to conventional fractionated radiation therapy (CFRT), the ablative hypofractionated radiation therapy (AHFRT) could enhance tumor control via reduction of MDSCs which synergized anti-PD-L1 immunotherapy [290]. Further identification of such immune-suppressive factors in ITME will help to visualize the entire immune reactions in ITME and to invent an immune-regulating cocktail to maximize the synergetic anticancer efficacy of RT combined with IT.

Repopulation of M2 MΦ

MΦ displays different functions in response to environmental alternation. Cancer-associated MΦ is believed to play a critical role in ITME [291]. The M0 *MΦ* can be polarized into M1 *MΦ and M2 MΦ* representing anti- and pro-tumor functions, respectively. All of these three *MΦ*s can reside in the same region in TME, which can dynamically change upon the environmental factor that induces MΦ polarization via IL-6/JAK/STAT3 pathway [291, 292]. Interestingly, the $CD11b^{(low)}/CD68^{+}$ MΦ is found to be specifically enriched in the hypoxic regions of TME, and the hypoxic environment in ITME seems to favor the development of M2 [293]. Leblond et al. further demonstrate that in glioblastoma, reduction of MΦ is paralleled with the enhanced number of M2 MΦ. Compared to M2 MΦ, M1 MΦ was sensitive to radiation under both in normoxia and hypoxia, indicating that radiation can repopulate M2 MΦ and M2 MΦ so as to alter tumor immunotolerance [294].

Immune-Suppressive Adenosine

The increased ATP concentration due to radiation-induced cell death is shown to function as an immune-suppressive factor [295]. Although ATP helps to recruit and activate dendritic cells (DCs) contributing to an antitumor environment, ATP can be quickly catabolized into adenosine in ITME by CD39 and CD73 on the surface of cancer cells, stromal cells, and immune cells as well. The increased adenosine concentration, in turn, induces a pro-tumor immune suppression by inhibiting DCs and CD8 T cells and increasing Treg proliferation and M2 polarization [296, 297]. Radiation also induces TGF-β expression [298, 299] resulting in adenosine formation and suppressive immune environment. In addition, TGF-β can alter the gene expression profile in M2 MΦ and increase the Th17 cells via the TGFβ-STAT3 pathway [300].

10.6 Perspective

10.6.1 Revealing the Energy Competition Between Tumor and Immune Cells

Two major aspects of the irradiated TME are the adaptive tumor energy metabolism and immune regulation, which received considerable attention over the last several years. Recent reports have revealed that tumor metabolism is tightly linked with the environment causing tumor immunotolerance. With contin-

ued advancement in both of these research disciplines, the intimate relationship between multifaceted alterations in tumor metabolism and their subsequent influence on immune regulation has become increasingly recognized as an important factor contributing to tumor growth and progression. The global metabolomic profiling that is designed to identify metabolic factors driving the aggressive phenotype of glioblastoma has identified numerous alterations in cellular metabolism that may play a contributory role in immune regulation. The metabolic race between cancer cells and immune cells is known to cause T-cell anergy and immune resistance, but the exact interaction and competition for surviving energy in the ITME are to be further elucidated.

10.6.2 Profiling Pro- and Antitumor Immune Response in ITME

The inefficiency of the immune checkpoint inhibitor can be affected by various factors in TME [218]. One of the unmet tasks is to reveal the exact different profiles of pro- and antitumor immune factors that can be instantly or permanently enhanced by radiation in TME. Since the population of the resistant cancer cells is enriched with cells expressing CSC biomarkers and responsible for the failure of treatment, efforts on immune targeting of these radioresistant cancer cells are going to continue with or without the combination of radiation. The challenge for immune targeting CSCs could be the difficulty of the delivery of efficient antibody or inhibiting agents to the local CSCs that are few and resided in a hard-to-reach area such as the hypoxic environment. Therefore, with the advantage of radiation-mediated re-structuring TME and identification of the dynamics of surface biomarkers, it should be expected that radiation-induced specific and efficient immune targets are to be invented for the combined RT and IT modality.

Acknowledgments We apologize to the authors whose publication could not be included in this chapter due to limited space. We acknowledge Dr. Ralph Weichselbaum at the University of Chicago and Dr. Yang-Xin Fu at the University of Texas Southwestern for their invaluable inputs and discussions on radiation-associated tumor immunoregulation.

Competing Interests The authors declare no competing interest exists.

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Index

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A. Birbrair (ed.), *Tumor Microenvironment*, Advances in Experimental Medicine and Biology 1263,
https://doi.org/10.1007/978-3-030-44518-8