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Alexander Birbrair *Editor*

Tumor Microenvironment

Recent Advances

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Preface

This book's initial title was *Tumor Microenvironment*. 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: Recent Advances* 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. The authors 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 the tumor microenvironment in several organs using state-of-the-art techniques. These advantages facilitated the identification of key targets and definition of the molecular basis of cancer progression within different tissues. Thus, this 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.

Karen M. Bussard and colleagues from Thomas Jefferson University discuss novel techniques to study the bone-tumor microenvironment. Ryuji Yamaguchi and Guy Perkins from the University of California San Diego compare tumor microenvironments in mice and humans. Linda A. Buss and Gabi U. Dachs from the University of Otago describe the effects of exercise on the tumor microenvironment. Georgia A. Giotopoulou and Georgios T. Stathopoulos from the University of Patras update us with what we know about the effects of inhaled tobacco smoke on the pulmonary tumor microenvironment. Bastian Zinnhardt and colleagues from the University of Münster address the importance of multimodal molecular imaging of the tumor microenvironment. Adi Karsch-Bluman and Ofra Benny from the Hebrew University of Jerusalem compile our understanding of necrosis in the tumor

microenvironment and its role in cancer recurrence. Daolin Tang and colleagues from UT Southwestern Medical Center summarize current knowledge on the multifaceted effects of autophagy on the tumor microenvironment. David H. Gutmann from Washington University School of Medicine talks about the sociobiology of brain tumors. Christian Münz from the University of Zürich focuses on the effect of γ-herpesviruses on the tumor microenvironment. Finally, Péter Bai and colleagues from the University of Debrecen give an overview of the microbiome as a component of the tumor microenvironment.

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 Mr. Murugesan Tamilselvan from Springer, who helped at every step of the execution of this project.

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1 Novel Techniques to Study the Bone-Tumor Microenvironment

Alison B. Shupp, Alexus D. Kolb, and Karen M. Bussard

Abstract

Many cancers commonly metastasize to bone. After entering the bone, cancer cells can interact with surrounding stromal cells, which ultimately influences metastasis progression. Extracellular vesicles, direct cell contact and gap junctions, and cytokines are all mechanisms of intercellular communication that have been observed to occur in the bone microenvironment. These methods of cellular crosstalk can occur between cancer cells and a variety of stromal cells, with each interaction having a different impact on cancer progression. Communication between cancer cells and bone-resident cells has previously been implicated in processes such as cancer cell trafficking and arrest in bone, cancer cell dormancy, cancer cell reactivation, and proliferation. In this chapter we review innovative techniques and model systems that can be used to study bidirectional crosstalk between cancer cells and stromal cells in the bone, with an emphasis specifically on bone-metastatic breast cancer. Investigating how metastatic cancer cells interact with, and are influenced by, the bone microenvironment is crucial to better understanding of the progression of bone metastasis.

Keywords

Breast cancer · Bone · Metastasis · Tumor microenvironment · Extracellular vesicle · Exosome · Gap junction · Cytokine · Osteoblast · Osteoclast · Crosstalk · Stroma · CD63 · Fluorescence microscopy · IL-6 · IL-8 · Vicious cycle · Bone-like scaffolds · Bioreactor

1.1 Introduction

Many cancers, including breast and prostate cancer, commonly spread to bone. Once cancer has spread to the bone, the 5-year survival rate plummets dramatically [1]. As a secondary site, bone provides a unique structural and molecular microenvironment for metastatic cancer cells. Additionally, there are many distinct characteristics of bone that may influence cancer cells in a manner not observed in metastases to other organs. However, the study of cancer metastasis to bone is complicated by physiological and structural complexity of the bone, and many models only account for certain aspects of the bone microenvironment. It is likely that a combination of techniques must be employed in order to wholly understand the events that occur in the

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bone-tumor microenvironment. In this chapter we discuss novel techniques that are being employed to study interactions between cancer cells and the bone microenvironment to better understand cancer metastasis progression in the bone.

1.2 Composition of the Bone Microenvironment

Bone is comprised of three main cell types that are responsible for maintaining structure and homeostasis. These cells are osteoblasts, osteoclasts, and osteocytes. Osteoblasts are responsible for the production of the mineralized bone matrix, or osteoid, which is composed of type I collagen, non-collagenous proteins, water, and hydroxyapatite [2]. Osteoblasts are derived from mesenchymal stem cells (MSCs) which reside in the bone marrow stroma and migrate towards the ends of bones where mature osteoblasts are found [3]. While mature osteoblasts synthesize new bone, it is the osteoclasts that degrade existing bone. Mature osteoclasts are derived from monocytes that differentiate and fuse to form large multinucleated osteoclasts [2, 4]. Osteoclasts express many enzymes that aid in the breakdown and resorption of bone [5]. To ensure that there is no net gain or loss of bone, the processes of osteoid synthesis and bone matrix resorption are tightly synchronized. The activities of osteoblasts and osteoclasts are regulated by osteocytes, which account for the vast majority of the cells in the bone. Osteocytes are derived from osteoblasts that become embedded in the osteoid as new bone matrix is synthesized. Osteocytes are capable of detecting mechanical forces and then communicating to the osteoblasts and osteoclasts to either build new bone or resorb existing bone as a result [3, 6].

1.3 Cancer Cell Metastasis to Bone

When cancer cells travel to bone, they enter via the circulation and blood vessels called venous sinusoids. These sinusoids are small blood vessels, and blood flow through them is slow, which allows for normal movement of hematopoietic and lymphoid cells in and out of the bone. However, this sluggish blood flow also enables cancer cells to easily enter and lodge in the bone [7, 8]. In the long bones, the sinusoids are located near the bone ends, which is also called the epiphysis. It has been observed that disseminated breast cancer cells preferentially travel to and colonize the epiphysis of long bones [9, 10]. The epiphysis is composed of trabecular bone, or spongy bone, which has a remarkably high rate of bone turnover [11]. Additionally, the epiphysis is the region where osteoblasts and osteoclasts can be found. Therefore, when cancer cells enter the bone via the venous sinusoids, some of the first stromal cells that the cancer cells will come into contact with are osteoblasts and osteoclasts.

There have also been reports of breast cancer cells residing in the bone marrow. The bone marrow is where hematopoietic stem cells (HSCs) can be found and is frequently referred to as the HSC niche [12]. This niche is unique in that it is capable of supporting HSC self-renewal, as well as HSC dormancy [13]. In addition to HSCs, this region also includes stromal cells such as MSCs, macrophages, and fibroblasts amongst several others. Several different cells in this niche, including MSCs, have been shown to regulate breast cancer progression and in some instances induce cancer cell dormancy, which is when cells exit the cell cycle to G_0 phase and temporarily halt proliferation [14–16].

Following cancer cell dissemination to bone, there is a probability that either the cancer cell will rapidly proliferate to form a metastatic lesion or the cancer cell may enter a dormant state. A state of cellular dormancy may be defined as proliferative arrest and in some contexts is considered an adaptive response to microenvironmental stress [17]. Over time, cancer cells that have entered a dormant state can become reawakened, through mechanisms not entirely understood, which leads to cancer proliferation and disease progression. As bone-metastatic breast cancer progresses to late stages of disease, patients commonly experience bone pain, hypercalcemia, and fractures due to osteolytic lesion formation. This is due to the phenomenon called the “vicious

cycle" of breast cancer metastasis to bone [18]. In this cycle, metastatic cancer cells produce parathyroid hormone-related protein (PTHrP), which induces osteoblasts to produce RANK-L. In turn, elevated levels of RANK-L then stimulate osteoclast formation and maturation, leading to increased bone resorption. This cycle has been well described, but it should be noted that not all metastatic lesions are osteolytic in nature. In many instances metastatic lesions are osteoblastic, and in some cases there are mixed lytic and blastic lesions [19]. Currently, there is no cure for bone-metastatic cancer. Standard of care treatments are mainly palliative in nature and aim to reduce bone degradation and pain.

1.4 Crosstalk Between Cancer Cells and Their Microenvironment as a Means to Study Cancer Progression

Stromal cells of the bone, including osteoblasts, osteoclasts, and MSCs, have been shown to interact with cancer cells. It has been shown that these cell types can communicate via direct cell contact and gap junctions, cytokines, and extracellular vesicles [15, 16, 20, 21]. While these interactions are diverse, it is highly likely that there is overlap and crosstalk amongst the different forms of cellular communication.

1.4.1 Extracellular Vesicle Communication

Extracellular vesicles (EVs) are membrane-bound structures that are released by a variety of cell types in culture and in vivo. There are several subtypes of EVs, including exosomes and microvesicles. These vesicles differ from each other based on their biogenesis, as well as size [22, 23]. Exosomes are vesicles that are derived from multivesicular bodies and contain several endosome-derived molecules. Exosomes are released into the extracellular space when a multivesicular body fuses with the plasma membrane of a cell [24]. Exosomes have been reported to be within a specific size range from approximately 30 to 150 nm [22]. In contrast to exosomes, microvesicles are produced by direct budding from a cell's plasma membrane [24]. Additionally, microvesicles have been reported to have sizes that range from 100 to 800 nm [22]. Along with exosomes and microvesicles, there are several other vesicle subtypes including apoptotic bodies and large oncosomes, as well as several vesicle subtypes that have irregular morphology such as double vesicles and membrane tubules [22, 25].

Regardless of the specific type of EV, the vesicular contents can include molecules such as proteins, RNA, and DNA from the cell of origin [23, 26, 27]. When EVs are taken up by a recipient cell, they have the potential to alter that cell's activity and function. Transfer of EVs can occur between neighboring cells, or in the body EVs can enter the circulation and thus influence the activities of cells in distant tissues. Research on EVs has grown rapidly in recent years, and a role for EVs has been implicated in nearly every area of biology. A major area of interest is the role that EVs play in cancer progression, as well as the potential of EVs to be biomarkers of disease [28–31].

1.4.2 EVs and Cancer Progression

Cancer cell-derived EVs have been reported to have a wide range of functions relating to cancer progression. The ability of EVs to circulate throughout the body has led to an accumulation of evidence demonstrating that EVs can influence cancer metastasis and prime metastatic sites. It has been reported that EVs, specifically exosomes, have a role in establishing a pre-metastatic niche. This has been shown in models of cancer metastasis to lung as well as bone [31–33]. Cancer cell-derived exosomes induce vascular leakiness at the pre-metastatic sites and can promote subsequent cancer cell colonization of the metastatic site [30–32]. In the bone specifically, breast cancer cell-derived exosomes have been shown to alter osteoclast activity, which in turn creates an osteolytic microenvironment that

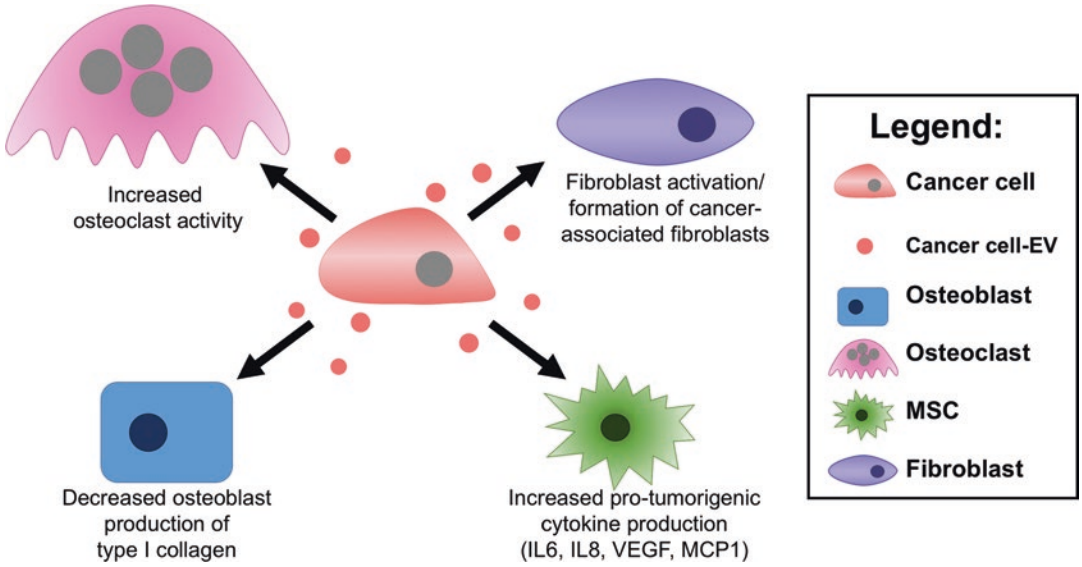

Fig. 1.1 Cancer cell-derived EVs have multiple, distinct, effects on cells in the bone microenvironment. EVs from breast cancer cells are taken up by osteoclasts, which stimulate osteolytic activity. Breast cancer-derived EVs also regulate osteoblast activity, by decreasing osteoblast production of collagen. In neuroblastoma, cancer cell-derived EVs induce MSCs to produce pro-tumorigenic cytokines such as IL6, IL8, VEGF, and MCP1. In various other cancer types, cancer cell-derived EVs have been shown to induce activation of fibroblasts into cancer-associated fibroblasts

is favorable to breast cancer growth (Fig. 1.1) [34]. In another study, it was found that breast cancer-derived EVs can regulate osteoblast activity as well and lead to decreased osteoblast type I collagen production (Fig. 1.1). This, in turn, led to the formation of a pro-tumorigenic bone microenvironment [35]. Similar findings were discovered in a model of multiple myeloma, whereby cancer cell-derived small EVs enhanced osteoclast activity while inhibiting osteoblast function [36]. In neuroblastoma it was demonstrated that cancer-derived EVs are taken up by MSCs, which results in production of pro-tumorigenic cytokines and chemokines (Fig. 1.1). Neuroblastoma commonly metastasizes to bone, and this evidence suggests that cancer cell-derived EVs are one mechanism by which neuroblastoma is able to hijack the bone microenvironment to promote cancer growth [37]. In addition to forming and modifying the metastatic site, cancer cell-derived EVs and exosomes have a broad range of functions including activation of cancer-associated fibroblasts and immune modulation (Fig. 1.1) [38–43]. Cancer cell-derived EVs undoubtedly have a large role in disease progression, but equally important seems to be the role of stromal-derived EVs in regulating cancer growth and advancement of disease.

The role of the stroma surrounding a tumor has a large impact on cancer progression. In recent years, it has been discovered that stromal cells produce EVs that influence the activity of cancer cells. Through EV transfer, stromal fibroblasts have been shown to increase growth, metastasis, and therapy resistance of breast cancer cells. These same fibroblasts were also shown to fuel an inflammatory response when stromal-derived EVs were taken up by immune cells [44]. While stromal-derived EVs have been shown to enhance cancer progression in some instances, in models of bone-metastatic cancer, EVs produced by the stromal cells in the bone have an antiproliferative effect on cancer cells. Two separate studies found that MSCs, which are found in bone marrow stroma, produce EVs that suppress proliferation of metastatic cancer cells [16, 45]. Interestingly, EVs produced by M1 macrophages, which are another cell type found in the bone

marrow stroma, played a role in reactivation of dormant cancer cells in the bone [14]. Altogether, these studies demonstrate the complexity of EV biology as it pertains to cancer progression and that EVs from different cell types exhibit different effects.

1.4.3 Techniques to Isolate Extracellular Vesicles

There are several methods that have been employed to study EV-mediated communication between cells. However, the extensive heterogeneity of vesicle subtypes complicates the study of EV functions. EVs can be isolated from many sources including cell culture media, serum, blood, and urine. Many methods to isolate EVs actually isolate a mixed population of vesicles. Techniques that are often used to isolate EVs include ultracentrifugation, filtration, size exclusion chromatography, and precipitation [46, 47]. Each of these techniques has their own benefits, but ultracentrifugation is one of the most common techniques currently being employed [46]. Because these isolation techniques capture a mixed, heterogeneous EV population, there is a need to characterize the nature of the EV samples. There are several ways to do this, and it is recommended that several methods are used in combination [48]. One way to characterize EVs is based on protein marker expression. There are a multitude of protein markers that are characteristic of small EVs, exosomes, microvesicles, etc., which can be verified via Western blotting [49]. Another means to characterize EVs is based on size or morphology. EVs less than 200 nm in size can be analyzed using nanoparticle tracking analysis, while electron microscopy can be used on EVs of any size and will give information on both EV size and morphology [50]. A final method that can be used to characterize EVs is flotation on a density gradient. The most common types of density gradients are those made with sucrose or iodixanol (also known as Optiprep). Different subsets of vesicles have been reported in the literature to migrate to specific densities, and therefore separation of vesicles in a density gradient can reveal more information about the types of vesicles present in a crude EV sample.

1.4.4 Visualization of EV Transfer

A major challenge in EV research is understanding the natural biology of EV transfer and uptake between cells. One such way to visualize EV uptake is to fluorescently label EVs. This can be done by isolating EVs and using a fluorescent cell membrane dye, such as PKH67, to label EVs. Then, cells can be cultured with the labeled EVs followed by confocal microscopy and z-stack imaging to determine if EVs were internalized by cells [51]. Another method to visualize EV transfer is to genetically engineer cells to produce fluorescent EVs. Once such way this can be accomplished is by generating stable cell lines that express a fluorescently tagged CD63 protein. CD63 is a tetraspanin protein that has been reported to be a characteristic marker for exosomes and other small EVs [49, 52]. Cells expressing fluorescently labeled CD63 can be cocultured with other cells to observe EV transfer. This has been done utilizing MDA-MB-231 human breast cancer cells that express CD63-GFP and mouse MC3T3-E1 osteoblasts that express tdTomato. Following 48 h of coculture with these two cell types, z-stack imaging was able to detect GFP-positive vesicles within tdTomato-positive osteoblasts (Shupp and Bussard, unpublished data). Additionally, CD63 fluorescently labeled EVs can be isolated from cells and utilized in various other EV-uptake experiments. It should be noted that because CD63 is a protein marker of small EVs, depending on the EV population of interest, a different EV protein marker may be better suited for certain studies.

1.4.5 Methods to Study EVs In Vivo

Studying EV transfer in between cells in culture is a challenging undertaking, but even more complex is the study of EV transfer in vivo. Several groups have attempted to study EV function

in vivo by first isolating EVs from cells in culture and then administering these EVs to mice [30, 31]. However, these experiments present several complications. For example, when injecting exogenous EVs into an animal, there is not a single established route of injection that is universally used. Some groups have utilized intravenous routes, such as retro-orbital and tail vein injections [31], while others have injected EVs directly into tumors or tissues of interest [53, 54].

When considering the bone microenvironment specifically, the route of EV administration becomes a large complication. Injecting EVs directly into the bone is a harsh procedure which can damage the bone or lead to unnecessary inflammation, especially if EVs need to be injected repeatedly. On the other hand, if EVs are injected intravenously, there is not a guarantee that the EVs will traffic to the bone. Instead, the EVs may travel to other tissues, either purposefully or as an artifact due to the injection route. Another caveat is that there are no guidelines on the concentration or number of EVs to administer and the frequency of EV administration. The rates of EV production and uptake by cells in the body are not well characterized; thus, it is currently unknown if the amount of EVs administered in vivo truly recapitulates normal biology.

Others have developed systems that use reporter cells to assess EV uptake. These systems involve genetically engineered cells that produce modified EVs. These modified EVs are then taken up by reporter cells which exhibit fluorescence signal following EV uptake [55, 56]. These techniques can be modified to visualize EV transfer in living mice. One such study utilized a reporter system in which MDA-MB-231 breast cancer cells expressed Cre recombinase. The Cre^+ breast cancer cells released EVs that contained Cre mRNA. When designated reporter cells take up these Cre^+ EVs and exhibit Cre activity, they undergo a red (tdTomato) to green (GFP) color switch. This system was used to study the ability of EV transfer to occur between cells distantly located in mice. By using the color switch reporter system, along with confocal imaging, the authors were able to observe transfer of Cre activity to reporter cells that were located in the contralateral mammary gland compared to the Cre^+ donor cells. Furthermore, by analyzing the reporter cells, it can be determined which cells and how many cells have taken up Cre^+ EVs. Additionally, functional changes, such as migratory ability and metastatic potential, in cells that have taken up EVs can be determined [55]. This EV transfer reporter system allows for the identification of cells that have taken up EVs, which is especially useful for investigating EV-mediated crosstalk between cell types, such as with cancer cells and their surrounding microenvironment. These visualization systems are extremely valuable, but as with any in vivo experiment, the systems are not perfect. For example, other mechanisms of cellular communication could also lead to Cre mRNA transfer, such as gap junction intercellular communication, which cannot be overlooked if an experiment is designed to investigate local EV transfer. These reporter systems also do not necessarily control for the specific subset of EV that is produced, and thus characterization of the Cre^+ EVs that are produced by the donor cells needs to be done before a functional effect can be attributed to an EV population.

1.4.6 Cell Contact and Gap Junction Communication

Direct cell contact is yet another way by which cells within a tissue can communicate with one another. Cell-to-cell contact is commonly disrupted in neoplastic cells, and many cell adhesion molecules have been implicated in cancer metastasis and progression. Specifically, within the bone microenvironment it has been shown that direct cell contact with osteoblasts can increase the survival and proliferation of metastatic breast cancer cells (Fig. 1.2). One such interaction between osteoblasts and breast cancer cells was via Jagged1 and Notch (Fig. 1.2). It was found that Jagged1 on breast cancer cells interacts with the Notch ligand on osteoblasts to promote metastasis progression [57]. Additionally, in response to chemotherapy, osteoblasts can upregulate their expression of Jagged1, which feeds

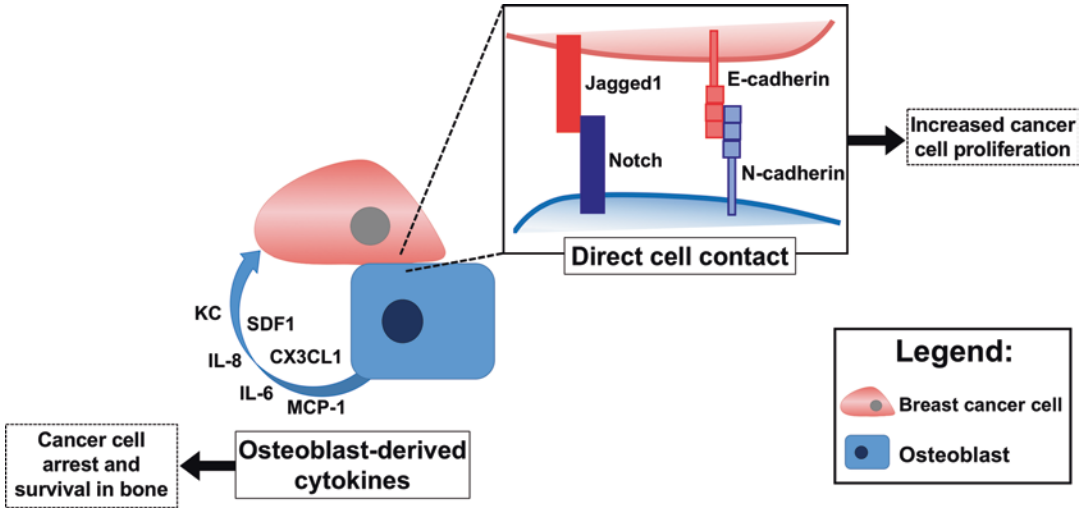

Fig. 1.2 Intercellular communication via cytokines and direct cell contact between breast cancer cells and osteoblasts. Osteoblasts produce chemokines (SDF1 and CX3CL1) that promote arrest of breast cancer cells in the bone. In early stages of disease when osteoblasts first encounter breast cancer cells, the osteoblasts also produce cytokines (IL-6, IL-8, KC, MCP-1) that act as maintenance factors to disseminated cancer cells and promote cancer cell survival in the bone microenvironment. Direct cell contact between osteoblasts and breast cancer cells is another method of intercellular communication that occurs in the bone-tumor microenvironment. Jagged1 on breast cancer cells interacts with the Notch ligand on osteoblasts to promote progression of bone-metastatic breast cancer. Adherens junctions involving E-cadherin on cancer cells and N-cadherin on osteoblasts led to increased tumor proliferation

back to the cancer cells to activate Notch signaling and promote chemoresistance of bone-metastatic breast cancer [58]. Another documented interaction between breast cancer cells and osteoblasts occurs via heterotypic adherens junctions (Fig. 1.2). One study found that when E-cadherin on breast cancer cells interacted with N-cadherin on osteoblasts, cancer cell proliferation increased [20]. Both of these studies found that *osteoblasts* were able to enhance cancer cell proliferation through two distinct mechanisms. However, there is a large body of evidence that actually implicate the *osteoclast* as being responsible for increasing cancer progression and even "reawakening" dormant cancer cells in the bone. One example by which direct contact with osteoclasts induces breast cancer proliferation is through vascular cell adhesion molecule 1 (VCAM-1) on the cancer cell surface. In the bone microenvironment, breast cancer cells can recruit and engage with osteoclasts via VCAM-1, which promotes progression of cancer to overt metastases [59]. Furthermore, in multiple myeloma, osteoclasts were also found to promote cancer progression by reawakening dormant multiple myeloma cells. This effect was a result of osteoclast activity and active bone resorption [60].

Another means of cellular communication through direct cell contact is via gap junctions. Gap junctions are channels that connect the cytoplasm of two cells and are made of connexin proteins that span the cell membrane. Six connexins (Cx) oligomerize to form a unit called a connexon. Each gap junction is composed of two connexons, one connexon from each cell. Gap junctions allow for the transfer of small molecules, typically less than 1 kilodalton (kDa), between cells. These molecules can include ions, inositol phosphates, and nucleotides such as small RNAs [61]. Within the bone, Cx43 is the most prevalent connexin, but Cx45 and Cx46 have also been reported to be present [61–63]. In normal breast tissue, Cx43 is also the most prevalent connexin; however, there are others that are also expressed [64]. Interestingly, loss of Cx43 is associated with primary breast cancer compared

to normal breast tissue, and higher Cx43 expression levels in breast tumors are predictive of better patient survival [65, 66]. Additionally, loss of Cx43 in breast cancer cells has been shown to increase cancer cell metastasis [67, 68]. Once breast cancer cells travel to the bone, the role of gap junction-mediated communication becomes more complicated. In one study, it was observed that gap junctions between breast cancer cells and bone marrow stromal cells resulted in decreased cancer cell proliferation [15]. In a separate study, it was determined that gap junctions formed between metastatic breast cancer cells and osteoblasts, which ultimately promoted metastasis progression [69]. It is evident that the metastatic niche has a considerable influence on the fate of cancer cells, and gap junctions are just one way in which the stroma can influence cancer progression.

1.4.7 Methods to Study Gap Junction-Mediated Communication

Traditionally, studies of cell-to-cell communication via gap junctions are carried out by measuring transfer of dyes or small fluorescent molecules between cells. These methods allow for the study of gap junction normal physiology as well as the function of gap junctions in different disease states or in response to chemical or drug exposure. Additionally, these techniques can be used to study how the permeability of gap junctions is altered by different connexin isoforms or genetic mutations.

Dyes and molecules that can be used in gap junction coupling experiments need to be small enough (<1 kDa) to traverse gap junction channels, and they must also be membrane impermeable so that they do not leak out by crossing the cell membrane. One technique to investigate dye transfer across gap junctions is through microinjection of tracer dyes. This technique involves a micropipette injection of membrane-impermeable dye into a single cell. If neighboring cells are coupled via gap junctions, the dye will diffuse to adjacent cells, which can be visualized with standard fluorescent microscopy [70]. There are several other techniques that can be used to introduce dyes into donor cells, such as electroporation, or scrape loading, in which adherent cells are scratched, in a method similar to a wound healing assay, in order to introduce the tracer dye to the cell cytoplasm [71–73]. A similar but distinct technique, called the parachute assay, also utilizes diffusion of gap junction-permeable dyes. In a parachute assay, one population of cells is labeled with calcein, or other dyes that can pass through gap junctions, but do not otherwise leak through a cell membrane. A separate population of cells is then labeled with a dye that is gap junction impermeable, such as Di-I. Then the two populations of cells are plated in a culture dish, and transfer of dye from donor cells to recipient Di-I labeled cells can be visualized. In this method, gap junctions must first form between adjacent cells in order to assess subsequent gap junction-mediated communication [74].

A different technique that still exploits diffusion of fluorescent molecules to visualize gap junction communication is called gap-FRAP (fluorescence recovery after photobleaching). In this method, a fluorescent tracer dye, such as calcein acetoxymethyl ester, is taken up by live cells in culture. After the dye is internalized, the cells are washed and the media is replaced to prevent further dye loading. Then the fluorescence of one cell is photobleached by a high-powered laser, such as on a confocal microscope. If neighboring cells are coupled via gap junctions, the fluorescent dye will diffuse into the photobleached cell from adjacent non-photobleached cells. Compared to other dye transfer methods, gap-FRAP has the advantage of generating better-quality quantitative data on gap junction coupling and kinetics, whereas these types of quantitative data are not traditionally obtained in other dye loading experiments [73, 75].

Several efforts have been made to identify inhibitors of gap junction communication to either prevent gap junction formation or inhibit channel permeability. One well-studied gap junction inhibitor is carbenoxolone, which is a derivative of glycyrrhetinic acid [76]. In addition to antagonizing gap junction communication, car-

benoxolone was also found to increase cellular expression of Cx43, which may be part of a feedback mechanism in response to gap junction inhibition [77]. Another compound that was found to antagonize gap junction intercellular communication is meclofenamic acid [76, 78, 79]. Meclofenamic acid was found to be a more effective gap junction antagonist than carbenoxolone. Additionally, the effects of meclofenamic acid on gap junction coupling was easily reversed by removal of the compound [79]. Long-chain alkanols, such as the compound octanol, have also been used to inhibit gap junction-mediated communication, specifically through interfering with gating of gap junction channels [15, 80]. A major caveat of using such compounds to inhibit cell communication via gap junctions is that the compounds do not necessarily have a specificity or selectivity for any particular connexin isoform. Instead, these compounds were previously used to broadly inhibit gap junction intercellular communication [76, 81].

1.4.8 Cytokines and Secreted Factors

The bone is a metabolically active tissue that has a plethora of cytokines and other secreted proteins. During bone resorption, various growth factors and cytokines are released from the bone matrix. Additionally, the cells within bone produce a variety of soluble factors themselves. These secreted factors have been reported to have a multitude of effects on cancer progression in the bone, depending on the specific metastatic niche as well as the stage of disease. A well-characterized cytokine interaction is the SDF-1/CXCR4 chemoattractant axis. It was demonstrated that osteoblasts produce SDF-1 which may promote cancer cell dissemination to bone (Fig. 1.2). Additionally, inhibiting CXCR4 on cancer cells with a neutralizing antibody was found to suppress bone metastasis [82]. Another chemokine that plays a significant role in the arrest of circulating cancer cells in the bone is fractalkine (CX3CL1). Mature osteoblasts and bone marrow endothelial cells produce fractalkine (Fig. 1.2), and both breast cancer and prostate cancer cells express the corresponding receptor, CX3CR1 [83–85]. Furthermore, in fractalkine-null mice, breast cancer dissemination to bone is significantly impaired, suggesting that fractalkine is crucial for initial cancer cell trafficking and arrest in the bone.

While there are several soluble factors that play a role in the attraction of cancer cells to the bone, there are many additional factors that are important for cancer cell maintenance and proliferation in the bone microenvironment. There are several cytokines that are produced by osteoblasts following interaction with cancer cells. It was observed that osteoblast production of IL-6, IL-8, KC, and MCP-1 greatly increases after short-term interaction with metastatic breast cancer cells. It is believed that these cytokines are involved with cancer cell survival and angiogenesis [7, 10, 86].

Interestingly, however, after prolonged exposure to metastatic breast cancer cells, osteoblasts are altered, or "educated," to produce different soluble factors compared to naïve osteoblasts [21, 87]. This unique subpopulation of osteoblasts that have been "educated" by the presence of cancer cells produce factors that modulate cancer cell growth and proliferation. Two of these factors are NOV (CCN3) and decorin. It was observed that conditioned media from "educated" osteoblasts led to decreases in breast cancer cell proliferation, and this effect was mediated through alterations in levels of NOV and decorin. Furthermore, when "educated" osteoblasts were present in the bone microenvironment in vivo, there was a decrease in inflammatory cytokines, such as IL-6. Other mechanisms have been reported by which osteoblast-derived factors exhibit an anticancer effect. One such mechanism that has been reported to inhibit proliferation in prostate cancer was an interaction between AXL on the cancer cell surface and GAS6 (growth arrest-specific 6) produced by osteoblasts. This interaction suppresses cancer growth and leads to cell cycle arrest of prostate cancer cells [88, 89]. Together, these studies suggest that osteoblasts may play differing roles during early cancer cell dissemination to bone and late-stage

metastatic disease. It is not yet known what causes a switch between these two stages and allows for metastatic outgrowth.

1.4.9 Methods to Study Secreted Factors

Current innovative techniques to study cytokines and soluble proteins involve multiplex arrays and proteomic approaches. One particular technique, the reverse phase protein array (RPPA), is a high-throughput proteomic technology that identifies and quantifies proteins. RPPA is an antibody-based technique that can be used to identify protein targets or protein changes in response to a given treatment. Moreover, RPPA can analyze the expression of approximately 400 different proteins while also providing information on phosphorylated proteins in addition to total protein levels. Samples for RPPA analysis can be derived from total cell or tissue lysates [90, 91].

Another array that can quantify cytokine levels is a multiplex ELISA (enzyme-linked immunosorbent assay)-based array. This technology comes in different formats of targeted pathway or custom designed arrays and oftentimes can identify up to 1000 protein targets. Moreover, these arrays can be done on various starting materials such as cell culture media, serum, plasma, and other body fluids. Both of these technologies are extremely valuable for identifying protein biomarkers in human samples.

1.5 In Vivo Models to Study Bone-Metastatic Breast Cancer

There are multiple experimental models that are used to look at specific aspects of metastatic disease; however, each of these models has limitations. Some models are designed to investigate a certain stage of metastasis, while others are designed to look at specific changes in the bone microenvironment. The choice of murine model and/or cell lines to use is entirely dependent on the experimental question, and often a combination of models is the best choice.

1.5.1 Mouse Models of Metastasis to Bone

Many mouse models of cancer involve cancer cell lines which are inoculated into mice. These types of models can be categorized into two groups: syngeneic models and xenograft models. Syngeneic models traditionally refer to cell lines that are derived from the same genetic background as the animal being used. An example of this would be murine cancer cell lines that are injected into a mouse [92]. Syngeneic models are useful because immunocompetent animals can be used, which allows for the study of how the immune system impacts disease. Additionally, because the host animal and the cell lines are from the same species, these models may be valuable for studying interactions between injected cells and endogenous host cells, such as within a tumor microenvironment [92]. On the other hand, human xenograft models involve implantation of human cancer cell lines or tissues into a host animal, such as a mouse [92]. In xenograft models, the host animal must be immunocompromised to allow for the human cells to grow [92, 93]. While human xenograft models do not account for immune-mediated effects, they do have their own benefits. Human cancer cells and tumors have genetic diversity and mutations that may not be observed in certain mouse cell lines [94]. The use of human cancer cells in a xenograft model also allows for the discrimination of human cancer cells from mouse host cells, which is crucial for determining spatial interactions in the tumor microenvironment.

One specific mouse model to study cancer metastasis is an orthotopic model. This model involves transplanting cancer cells or a fragment of a tumor into the same anatomic location from which the cancer was derived [92]. For example, this would include breast cancer cells being injected into the mammary fat pad of a mouse. Often, growth of the primary tumor may be more

rapid than the formation of metastases. To prevent animal morbidity due to primary tumor growth, surgery is often done to remove the primary tumor and metastasis formation is subsequently monitored [92]. Additionally, metastases to other organs may also impact animal morbidity [95]. For example, metastases to lung may shorten the life span of a mouse thereby limiting the time frame that bone metastases may occur. Therefore, depending on the metastatic site of interest, cancer cells with organ tropism may be useful [95, 96]. With regard to bone metastases, several bone-tropic breast cancer cell lines have been developed that preferentially home to the femurs and tibiae of mice [97, 98]. Orthotopic metastasis models are beneficial for looking at multiple aspects of the metastatic cascade and can potentially be used to study cancer dormancy by periodically monitoring for disseminated cells or micrometastatic lesions at secondary sites.

One experimental metastasis model that is often used to specifically study bone metastasis is intracardiac injection of cancer cells. The intracardiac injection route results in cancer cells that travel to the bone and bypasses the lung circulation, which decreases the development of high tumor burden in the lungs. Another model of bone metastasis utilizes intratibial injections of cancer cells. This model is useful for studying interactions between the tumor cells and the bone microenvironment, specifically if there is interest in using genetic modifications of the host or cancer cells. This model also allows for modulation of the bone microenvironment by co-injecting cancer cells along with MSCs, osteoblasts, osteoclasts, or bone cells. Both intracardiac and intratibial injections bypass the early stages in the metastatic cascade and are useful for studying the later stages of metastasis [95, 96].

1.5.2 Analysis of Disseminated Cancer Cells in Bone

After cancer cells disseminate to bone, there are several experimental assays that can be done to identify cancer cells in the bone microenvironment and characterize interactions between cancer cells and the surrounding stroma. A straightforward approach to visualize cancer cells in the bone is to harvest the bones and use immunofluorescence microscopy on the bone sections. This technique works well if the cancer cells are already fluorescently labeled. Alternatively, antibodies against cytokeratins or EpCAM (epithelial cell adhesion molecule) have previously been used to identify cancer cells of epithelial origin within the bone marrow [99, 100].

There is also a large interest in analyzing genetic alterations and gene expression profiles of single disseminated cancer cells that may reveal differences between disseminated cells and the primary tumor as well as insights into cancer cell evolution. To perform such genetic analyses on single disseminated cancer cells, these cells must first be isolated from the microenvironment. Traditionally, this is done by flushing the bone cavities to collect cells located inside the bone. Alternatively, this can also be done using laser capture microdissection, which cuts away and excises unwanted cells to isolate histologically pure populations of cells [101, 102]. Further sorting of cells can be done to purify the cancer cells from stromal cells. Sorting of cancer cells, such as with fluorescence-activated cell sorting (FACS), is typically done by using antibodies against cytokeratins or EpCAM and sorting for positively marked cells. Once disseminated cancer cells are sorted, transcriptomic and genomic analyses may be carried out [103, 104].

Using PCR-based genomic analyses, Schardt et al. were able to determine a sequence of genetic alterations that occur during breast cancer metastasis to bone [104]. Additionally, using a similar approach, it was found that disseminated cancer cells in the bone marrow harbor less genomic aberrations than cancer cells present in the primary tumor. This ultimately brought forth the idea that dissemination of cancer cells is an early event in cancer progression [105]. Another technique, single-cell sequencing, has also been done on cancer cells isolated from the bone marrow. Single-cell RNA-seq was recently been done on multiple myeloma cells that were iso-

lated from bone marrow aspirates [106]. Single-cell RNA-seq is expected to become more prevalent in studies of metastasis and the tumor microenvironment in order to assess tumor heterogeneity and cancer-related gene signatures.

1.6 In Vitro Models of the Bone Microenvironment

There is a lack of model systems to study cancer metastasis to bone; however, the use of three-dimensional (3D) models, such as scaffolds, hydrogels, and bioreactors, is becoming a popular tool to study interactions between cancer cells and their microenvironment [107–109]. The unique biophysical properties and functions of these systems better replicate biological tissue microenvironments in vitro compared to standard 2D cell culture. Seminal work by Bissell and colleagues demonstrated that normal human breast epithelial cells behaved like tumor cells when in 2D culture compared to 3D tissue culture [110]. It was also found that normal breast epithelial cells need interactions with the basement membrane present in 3D cultures in order to maintain and display normal breast differentiation [110]. Therefore, in many instances 2D model systems are insufficient to model specific characteristics of disease because they lack the key structural features of 3D model systems [107, 111].

1.6.1 Hydrogels and 3D Models

One important area to consider when designing 3D models is the structure, including pore size, and the type of material used to develop the 3D mimetic system [108, 111, 112]. For example, pore size can affect cell attachment, invasion, migration, and angiogenesis [108]. In the context of bone metastasis, angiogenesis, invasion, and migration are all important components of tumor dissemination and growth [108]. One also has to consider the composition of trabecular bone and cortical bone and how these two bone types are different, for example, trabecular bone is very porous and well vascularized compared to cortical bone, which is a hard-packed, dense structure [108, 113]. Therefore, pore size is an important biophysical property and needs to be controlled when developing 3D mimics of a bone-tumor microenvironment. In addition, the material of the 3D system is important [111, 112]. To develop the most realistic 3D model, it is first necessary to understand how the cells respond to the environment. For example, hydrogels are a special type of scaffold made of water-swollen networks of polymers [111]. Hydrogels come in two forms: natural or synthetic [111]. Natural hydrogels are those made from natural materials, such as rat tail collagen, whereas synthetic hydrogels are those made from man-made materials, such as polyacrylamide [112]. To develop a hydrogel to study tumor growth in the trabecular bone, it would be best to use a natural hydrogel consisting of collagen because this structure would accurately portray the composition and biophysical properties of trabecular bone [109]. It is hard to replicate the rigid, mineralized extracellular matrix of bone [108]; therefore, it is important to select the material that will most closely replicate the bone matrix.

1.6.2 Bone-Like Scaffolds

In some instances, materials such as hydrogels cannot recapitulate the high mechanical properties needed to model the rigid extracellular matrix of bone. Therefore, other biocompatible materials have been used to model a stiff bone microenvironment. These materials can be 3D printed into various scaffold structures onto which cells of interest are subsequently seeded. These biomimetic scaffolds can be made of a synthetic polymer, such as poly-(ϵ-caprolactone) (PCL), that is printed into a desired structure using a 3D bioprinter for subsequent in vitro experiments (Fig. 1.3a). PCL is an FDA-approved biocompatible polymer that has gained attraction as a 3D bone model to use in the laboratory [114, 115]. We have successfully cultured GFP-labeled MDA-MB-231 breast cancer cells on PCL scaffolds and were able to implant these scaffolds into mice (Fig. 1.3). Following scaffold implanta-

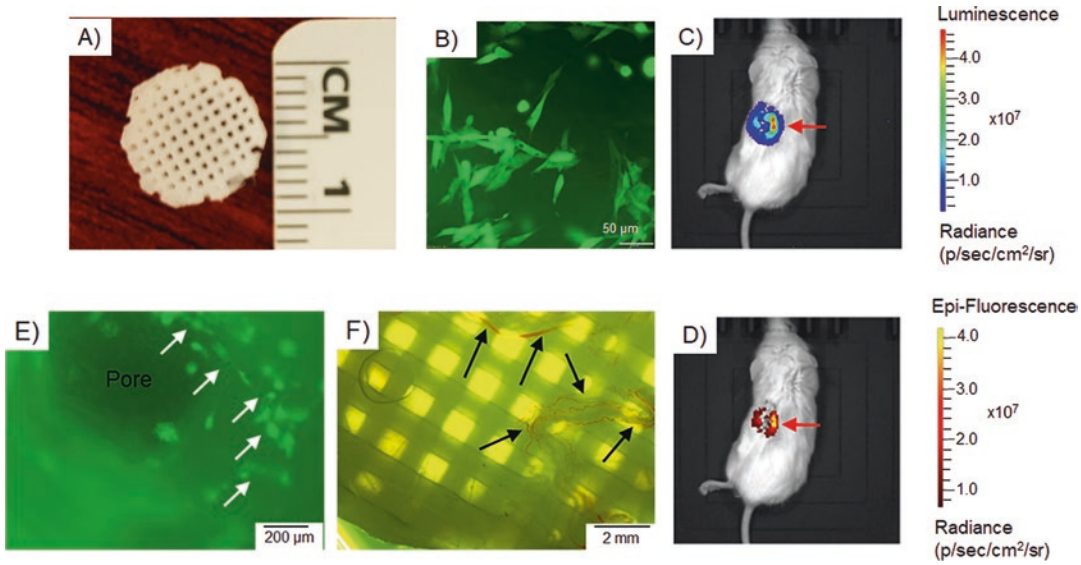

Fig. 1.3 Bone-like biomimetic scaffolds to model the tumor microenvironment. Human MDA-MB-231luc-GFP breast cancer cells were mixed with Matrigel and seeded into a 3D bioprinted scaffold. (**a**) Unseeded bioprinted scaffold. (**b**) luc-GFP breast cancer cells on seeded scaffold 2 days post seeding, but pre-mouse implantation. (**c**) Bioluminescent image of seeded scaffold implanted into mouse flank 7 days postimplantation; red arrow indicates seeded scaffold. (**d**) Fluorescent image of seeded scaffold implanted into mouse flank 7 days postimplantation; red arrow indicates seeded scaffold. (**e**) luc-GFP breast cancer cells (white arrows) in retrieved scaffold 7 days postimplantation. (**f**) Blood vessel formation (black arrows) in retrieved scaffold 7 days postimplantation

tion, we were able to subsequently measure tumor growth using IVIS in vivo bioluminescent and fluorescent imaging (Fig. 1.3c, d). Additionally, after removal of scaffolds from mice, we observed blood vessels that had infiltrated the scaffold, indicating that the breast cancer cells on the scaffolds are able to establish a vascular network (Fig. 1.3f). Patricio et al. have shown that blending polylactic acid (PLA) polymer with PCL (PCL/PLA) enhances the biomechanical properties of biomimetic bone-like scaffolds [116]. Furthermore, osteosarcoma cells were seeded and were able to grow properly on the scaffolds while mimicking normal architecture. This indicates that PCL and PCL/PLA scaffolds are a suitable model to represent a 3D bone-like microenvironment [116]. Because the scaffolds are made of biocompatible materials, they are able to be implanted into mice and used to study the bone microenvironment in vivo [107, 117]. Bone-like scaffolds are useful for in vivo studies because they allow for manipulation of the microenvironment and the cell types that are present on the scaffold.

1.6.3 Bioreactors

Another tool that can be used to study the bone-tumor microenvironment in vitro is a bioreactor [118–120]. There are currently limited models to study the bone-tumor microenvironment in vitro, specifically with regards to bone remodeling [119]. Dhurjati et al. developed a 3D, in vitro culture system, termed a bioreactor, which can replicate the mineralized, collagenous tissue of bone [118]. Both murine and human osteoblast cell lines (MC3T3-E1 or hFOB, respectively) successfully grew in the long-term culture system for up to 120 days. After only 15 days in culture, cells displayed characteristics comparable to normal osteoblasts in vivo [118]. This bioreactor can

be seeded with osteoblasts as well as other bone cells or even cancer cells. Krishnan et al. utilized a bioreactor to mimic early colonization of breast cancer cells in the bone [119]. They were interested in whether bone remodeling by osteoclasts could be replicated in vitro in a bioreactor and how breast cancer cells are involved in bone remodeling during early stages of cancer cell colonization. They were able to show that murine osteoblasts and osteoclasts grew together and mimicked normal bone development over time in culture. Secondly, when human breast cancer cells were added to the co-culture bioreactor systems, this recapitulated the "vicious cycle" of bone degradation in vitro. They observed a decrease in matrix thickness, an increase in osteoclast bone resorption, and cancer cell proliferation, suggesting that this model can mimic the events that occur during breast cancer cell colonization of bone [119].

1.7 Conclusions and Future Directions of the Field

In the bone-tumor microenvironment, there are several ways in which the cells of the bone can influence metastasis progression, as well as ways in which the cancer cells can influence the surrounding stroma and bone homeostasis. It has been shown that osteoblasts, osteoclasts, MSCs, and other stromal cells can all influence cancer progression through various mechanisms of intercellular communication. Examples of such communication include EV transfer, direct cell contact and gap junctions, and cytokines and soluble factors. A large area of research that is likely to continue to rapidly expand is the study of EVs. EVs are a major area of interest in the field of cancer biology, due to their ability to mediate communication both locally and systemically, as well as their potential to serve as disease biomarkers. As knowledge of EVs continues to grow, there will likely be increased investigation of the role of EVs in vivo pertaining to both normal biological function and disease states.

The study of bone-metastatic cancer presents several challenges that are associated with a structurally complex tissue such as the bone. Important considerations to make when studying the bone-tumor microenvironment can range from the medium on which cells are cultured in vitro to the choice in vivo mouse model. While there are many model systems and techniques available, each model has its own specific limitations. Therefore, models of bone-metastatic cancer should be carefully selected to answer a specific experimental question, and often a combination of models will be the best option.

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2 An Emerging Model for Cancer Development from a Tumor Microenvironment Perspective in Mice and Humans

Ryuji Yamaguchi and Guy Perkins

Abstract

In the past, cancer development was studied in terms of genetic mutations acquired in cancer cells at each stage of the development. We present an emerging model for cancer development in which the tumor microenvironment (TME) plays an integral part. In this model, the tumor development is initiated by a slowly growing nearly homogeneous colony of cancer cells that can evade detection by the cell's innate mechanism of immunity such as natural killer (NK) cells (first stage; colonization). Subsequently, the colony develops into a tumor filled with lymphocytes and stromal cells, releasing pro-inflammatory cytokines, growth factors, and chemokines (second stage; lymphocyte infiltration). Cancer progression proceeds to a well-vesiculated silent tumor releasing no inflammatory signal, being nearly devoid of lymphocytes (third stage; silenced). Eventually some cancer cells within a tumor undertake epithelial-to-mesenchymal transition (EMT), which leads to cancer metastasis (fourth stage; EMT). If a circulating metastasized cancer cell finds a niche in a new tissue and evades detection by NK cells, it can establish a new colony in which very few stromal cells are present (fifth stage; metastasis), which is much like a colony at the first stage of development. At every stage, cancer cells influence their own TME, and in turn, the TME influences the cancer cells contained within, either by direct interaction between cancer cells and stromal cells or through exchange of cytokines. In this article, we examine clinical findings and animal experiments pertaining to this paradigm-shifting model and consider if, indeed, some aspects of cancer development are governed solely by the TME.

Keywords

Tumor microenvironment · Epithelial-to-mesenchymal transition (EMT) · Metastasis · Natural killer cell (NK) · Cancer development · Malignant pleural mesothelioma (MPN) · Lymphocytic infiltration · Ductal carcinoma in situ (DCIS) · Cancer associated fibroblast (CAF) · Tumor associated macrophage (TAM) · Myeloid-derived suppressor cell (MDSC) · Cytokines · Chemokines · Pleural effusion

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2.1 Introduction

In this chapter, we discuss an emerging model for cancer development from the perspective of the role the tumor microenvironment (TME) plays. In the first section, we examine how the model fits the relevant clinical data. Inevitably though, these data are like snapshots of cancer at discrete points and thus represent partial views of a dynamic process. In the second section, we examine mouse cancer models in which cancer development takes place reliably and reproducibly and therefore allow predictions for the TME's influence. In the third section, we discuss the implications of the cancer developmental model and testable hypotheses. In the last section, we examine malignant pleural mesothelioma in detail, comparing genetic, transcriptional, and TME perspectives, and assemble a comprehensive model of its development.

2.2 TME Categories and Cancer Developmental Stages

Cancer inevitably starts with DNA mutation. The mutation could be an error during DNA replication in a cycling cell, or it could be massive DNA damage caused by a catastrophic event such as exposure to radiation or inhalation of asbestos. By the time most biopsies are performed to test for cancer, a cancerous tumor usually contains more than 30 mutations in the known cancer-causing oncogenes [1]. But the fact that a cell may have 30 mutations in cancer-related genes alone does not mean that this cell will cause cancer growth when it is grafted onto a syngeneic individual. In fact, when a murine cell with multiple genetic mutations that had also acquired immortality is grafted onto a syngeneic host, it is often eliminated by either the innate or the adaptive mechanism of the immune system [2], suggesting that to initiate carcinogenesis, a cancer cell must acquire a way to manipulate the immune system and start building its own microenvironment. Thus, in order to predict how a cancer responds to a particular immunotherapy, knowing its tumor microenvironment is paramount.

We present a schema to categorize tumors by their TMEs. The four TME categories are: (a) small cluster of homogeneous cancer cells, (b) tumor with lymphocyte infiltration, (c) tumor with complex stromal and cancer cell architecture but without lymphocytic infiltration or release of cancer cells to blood circulation, and (d) tumor in which some cancer cells are undertaking epithelial-to-mesenchymal transition (EMT) and some cancer cells are being released into the blood circulation. (b)–(d) are well-characterized tumors found in many cancer types, including breast, lung, stomach, and liver.

(a) A small cluster of homogeneous cancer cells. Examples of a small cluster of homogeneous cancer cells have been found in breast duct cancer (ductal carcinoma in situ, DCIS) [3] and some melanoma biopsies [4]. These tumors evaded immune surveillance. Possibilities are that either the cancer is at a very early stage of development, such as DCIS, or the cancer is a newly metastasized colony. This type of tumor is difficult to find partly because of its small size and has not appeared often in the literature.

(b) A tumor with lymphocyte infiltration. This class of tumor releases cytokines, such as TNF-α, TGF-β, chemokines, and pro-inflammatory cytokines such as IL-10 and IL-4, from lymphocytes and directly engages with cancer cells, recruiting lymphocytes, monocytes, neutrophils, and other blood cells into the tumor. During the process, nearby macrophages and fibroblasts are converted into tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs). If PD-1 and CTLA-4 are in the tumor, cancer cells can be targeted by immune checkpoint therapies. In many tumors, granulocyte-macrophage colony-stimulating factor (GM-CSF) is released, recruiting Gr1+CD11b+myeloid cells and turning them into a species of myeloid-derived suppressor cell (MDSC). In other tumors, IL-10 and IL-4 may recruit Gr1+Mac1+myeloid cells into a tumor, turning them into another species of MDSC.

(c) A tumor without infiltrating lymphocytes. In this class, CAFs encapsulate the tumor with extracellular matrices (ECMs). Even so, the tumor remains well vesiculated. It is filled with many stromal cells including TAMs, CAFs, and MDSCs and does not release cancer cells into the blood circulation. There are a number of ways that lymphocyte infiltration can be blocked. For example, chemokines that attract lymphocytes may be nitrated within the tumor and inactivated, or infiltrating lymphocytes are made to undergo apoptosis by expression of FasL in endothelial cells and many other ways reviewed in the article by Joyce and Fearon [5]. Unless combined with other types of therapies, this class of tumor is expected to be resistant to all immune therapies.

(d) A subgroup of cancer cells in the tumor undertaking epithelial-mesenchymal transition [6, 7]. This transition downregulates genes such as E-cadherin, β-catenin, and cytokeratins 5/6 and upregulates genes such as E-cadherin, vimentin, α-smooth muscle actin, Snail, Slug, Twist, ZEB1 and 2, S100A4, and MMP2 and 3. Subsequently, some cancer cells become metastasized, activating mobility-enhancing genes, such as S100 calcium-binding protein P (S100 CBP P), and are then released into the blood circulation. Released cancer cells are often chaperoned with platelets and other stromal cells [8].

All four classes of tumors are found in clinical samples of metastatic melanoma biopsies, and the phylogenic tree based on their transcriptomes suggests a natural progression of cancer from (b) to (c) to (d) and finally to (a) [4] (Fig. 2.1). This progression is not an unreasonable assumption. For example, when tumors in (c) are considered, in order to attain the very complex cytoarchitecture of tumors having MDSCs, signals from cancer cells as well as stromal cells are required to recruit and convert neutrophils and monocytes into MDSCs [10]. Only then can lymphocyte infiltration be blocked. Thus, tumor (c) must have come through (b). Further, a cancer cell is stimulated to undertake EMT by cytokines and growth factors present in a hypoxic environment, the environment proposed for (c). Thus, tumor (c) precedes tumor (d).

What does a newly translocated metastatic tumor in the body look like? Joan Massague's group isolated cells from human cancer cell lines that when grafted onto a new host form a small, slow-growing colony that can evade NK surveillance [11]. Therefore, it seems likely that the TME of a newly metastasized cancer would fall into category (a).

By associating (a) with Stage 1 and 5 and (b)–(d) with Stages 2–4, we modeled cancer development as described in Fig. 2.2.

2.3 TME and Cancer Developmental in Mice

We presented above a model that fits the clinical observations. However, it is a model based on an inductive argument. To make the argument more deductive, we explore whether the model fits observations of murine cancer progression. Furthermore, will the model shed light on the questions, "Could some facet of cancer development be stopped or reversed by chemical manipulation of the TME, or will a particular immunotherapy alter the TME?"

Inducing Stage 2 to Stage 3 cancer progression is straightforward in murine models. Grafting B16F10 melanoma onto wild-type mice [12] or grafting mouse fibrosarcoma (CSA1M) cells into wild-type mice [13] causes the development of melanoma and fibrosarcoma, respectively, and the tumors in these mice progressed sequentially through Stage 5/Stage 1, Stage 2, and Stage 3. It was observed that in B16F10, CSA1M, and RMS grafts, secreted IL-10 and IL-4 recruited myeloid cells into the tumor, turning the cells into MDSCs and converted Stage 2 cancer to Stage 3 cancer. In all cases, it took only a few weeks to progress from Stage 2 cancer to Stage 3 cancer.

In a mouse model for pancreatic cancer expressing $Kras^{G12D}$ and $Trp53^{R172H}$ in the pancreas [14–16], pancreatic ductal adenocarcinoma

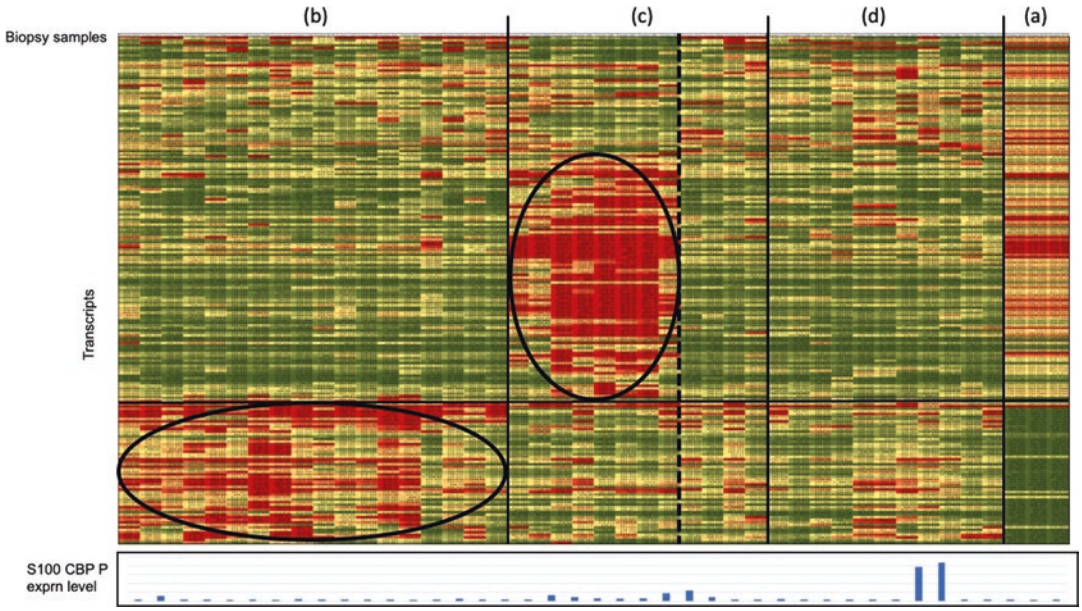

Fig. 2.1 Unsupervised hierarchical analysis of melanoma biopsies. Harlin and colleagues at the University of Chicago took 44 metastatic melanoma biopsies and bulk analyzed their RNA contents using Affymetrix GeneChips. The data were put through unsupervised cluster analysis [9]. Their data were reanalyzed to create a heatmap (upper panel, red square for high expression and green for low expression). The 44 samples on the horizontal axis were grouped into b, c, d, and a classes [4]. Transcription levels of 201 transcripts are displayed on the vertical axis. The horizontal ellipse represents lymphocyte-specific transcripts, such as TCRα, β, and γ. The vertical ellipse represents transcripts of genes important for survival of dermal layers, such as keratins, desmoplakin, gap junctions, and aquaporines [4], illustrating differences in the contents of TMEs. Note that transcripts of class (a) tumors are almost exclusively those of melanoma cancer cells. The transcription level of S100 calcium-binding protein P, known to enhance cell motility and invasion, is bar-graphed on the lower panel, suggesting the presence of metastatic cells. For a more detailed description of tumor groups, see the main text

(PDAC) tumors developed that released GM-CSF, thus recruiting myeloid cells and turning them into MDSCs. The same progression to MDSCs occurred in a mouse mammary tumor virus-driven polyomavirus middle T antigen (MMTV-PyMT) transgenic mouse, a model mouse for breast cancer [17], and in a prostate-specific PTEN deletion [18] prostate cancer model mouse. In all three cases, Stage 2 cancer was converted to Stage 3 cancer.

Mesotheliomas are described by three histology types. Cell lines generated from asbestos-induced murine mesotheliomas fall into three groups having distinct histological characteristics, much like biopsy samples from human mesotheliomas [19]. The three histology types are: epithelioid, sarcomatoid, and biphasic (mixture of epithelioid and sarcomatoid). Analysis of their cDNA expression profiles showed that epithelioids expressed E-cadherin, β-catenin, and cytokeratins 5/6, whereas sarcomatoids expressed N-cadherin, vimentin, a-smooth muscle actin, and metalloproteases MMP2 and 9, and biphasic expressed intermediate protein levels of epithelioid and sarcomatoid expression profiles, suggesting that EMT transition took place from epithelioid to sarcomatoid and biphasic in between [20]. In humans, the sarcomatoid mesothelioma associates with poor survival, whereas the epithelioid mesothelioma fairs better and biphasic in between [20, 21]. Asbestos inhalation can induce mesotheliomas. Mice with asbestos-driven mesotheliomas developed Stage 4 cancer that is dependent on their TME [22].

The brain microenvironment can support early metastatic growth in mouse. When Schwartz and

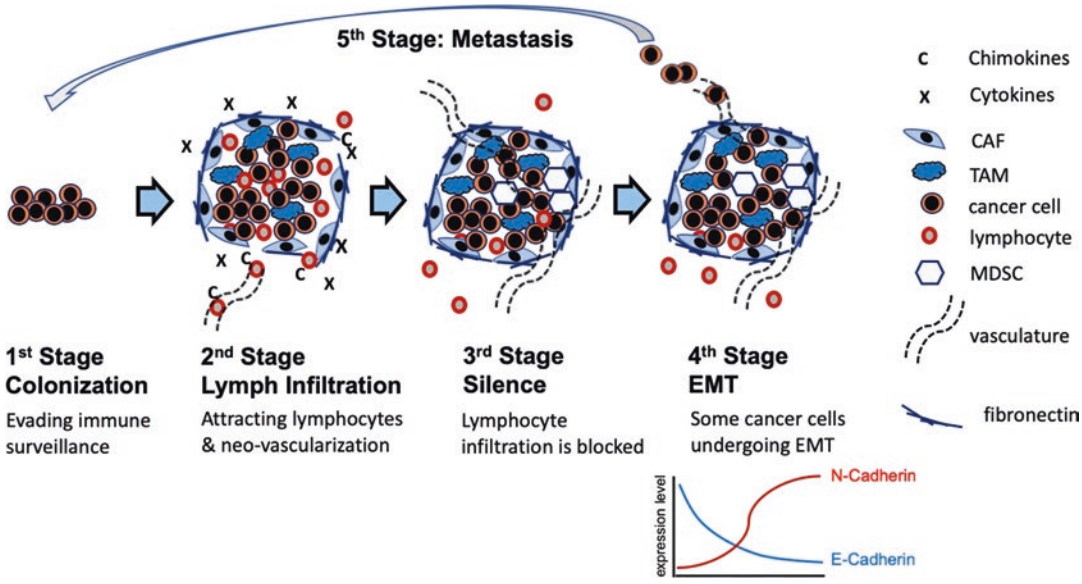

Fig. 2.2 Cancer developmental stages and TME. Four cancer developmental stages and their respective TME contents are drawn. In Stage 4, expression levels of N- and E-cadherins change as some cancer cells undergo epithelial-mesenchymal transition

colleagues subcutaneously injected Ret-melanoma sorted (RMS) cells into wild-type mice, they found that not only did the tumors form at the site of injection, but also metastasized to the brain [23]. Interestingly, astrocytes facilitated initial growth of melanoma cells. Further, astrogliosis that was naturally instigated as a brain tissue damage response was hijacked by tumor cells to support brain metastatic growth. Unfortunately, they did not determine at what stage of the tumor development these melanoma cells metastasized to the brain.

2.4 Implications and Testable Hypotheses

The obvious implication of the TME-based model of cancer development is that when cancer reaches a certain stage of development, it passes beyond the reach of certain immunotherapies. For example, immune checkpoint therapies such as PD-1 antibody and CTLA-4 antibody therapies are effective only when the targeted proteins are found in a tumor (Stage 2). Furthermore, if lymphocytes cannot infiltrate the tumor, then almost any immunotherapy would be ineffective (Stages 3 and Stage 4 tumors). This includes chimeric receptor antigen therapies (CAR-T therapies) and vaccine therapies.

An important testable question is whether the onset of Stage 3 could be delayed or even a Stage 3 to Stage 2 reversal, thus prolonging the period during which immunotherapies are effective. In certain mouse cancer models, the answer is yes. Inhibition of colony-stimulating factor 1 receptor (CSF1R) [24, 25] or granulocyte-macrophage colony-stimulating factor (GM CSF) [14, 15] enhanced CD8 T cell tumor infiltration, prolonging Stage 2 cancer, and improved the response to checkpoint immunotherapies in breast, cervical, and pancreatic cancer model mice.

An effective therapy to alter almost all categories of TME in many types of cancer is localized radiation treatment. This treatment destroys cells in the focused area indiscriminately, makes the immediate vicinity inflammatory and immunogenic, and produces antigen-presenting dead cancer cells. Thus, there is a great deal of interest in combining localized radiation with immunother-

apies [26, 27]. However, one of the most obvious drawbacks is the formation of scars (fibrosis) after the radiation that limits vascular permeability of tumors and thus limits tumor-infiltrating lymphocytes [26]. CAFs are also known to be activated by radiation that secrete integrins that help tumors to anchor to the ECM; this anchoring caused breast cancer cell proliferation in one study [28].

Perhaps a better approach is to test the hypothesis that judiciously combining immunotherapies with chemotherapies will induce apoptosis only in targeted cancer cells. In this hypothesis, a chemotherapy would create apoptotic cells inside the tumor that would trigger activation of macrophages for phagocytosis and attract lymphocytes. Indeed, when BRAF inhibitor alone or with MEK inhibitor was applied to patients with metastatic melanoma, an increase in infiltrating lymphocytes and melanoma antigens and a decrease in immunosuppressive cytokines (IL-6 and IL-8) were observed. An increase in PD-1 expression was also observed in melanoma cells grafted onto syngeneic mice [29] and in melanoma patients [30]. However, a large number of patients experienced high-grade toxicities in clinical trials [31].

In perspective, there seems to be aspects of cancer development that are independent of cell types and DNA mutations. The hypothesis is that the aspects that are governed solely by the TME can be discovered. Therefore, a productive approach would be to study a particular solid cancer and learn when and how a tumor recruits lymphocytes, fibroblasts, and macrophages to build a Stage 2 environment and when and how the environment is changed into Stage 3 and Stage 4 and then test how chemotherapeutics and immunotherapies would affect these environments. It is anticipated that most of the knowledge gained would be applicable to essentially any other solid cancer.

In the next section, we look at one cancer, malignant pleural mesothelioma (MPM), in detail, comparing genetic, transcriptional, and TME perspectives and assemble a comprehensive model of its development.

2.5 Malignant Pleural Mesothelioma (MPM)

Asbestos inhalation is the major cause of MPM. Asbestos may be one of six naturally occurring silicate minerals (crocidolite, amosite, anthophyllite, tremolite, actinolite and chrysotile), all forming long and thin crystals. Most industrial asbestos fibers are 0.1–40 μm in length and, when inhaled, lodge in a narrow pleural space between mesothelial cell linings (visceral pleura facing the lung and parietal pleura facing the chest wall or the diaphragm) surrounding the lung. In mice and humans, the upper part of the pleural space may be as narrow as 10–20 μm in width. The lower part of the pleural space may be much wider, partly due to the weight of the fluid, called "pleural effusion," that accumulates in the pleural space. In the diseased lung, the pleural effusion accumulates above the diaphragm, voluminous enough to show in a chest X-ray, a process called "water in the lung." Asbestos fibers less than 5 μm in length are cleared from the lung more efficiently than longer asbestos fibers [32]. Long asbestos fibers can be found in patients' lungs and pleural space decades after the exposure [33]. This is partly because macrophages do not phagocytize long asbestos fibers and also because long fibers are physically lodged in the narrow pleural space. However, a mesothelial cell may partially phagocytize asbestos fibers much longer than itself. For example, in one in vitro experiment, mesothelial cells expressing a fluorescent protein were exposed to 20-μm-long vitronectin-coated asbestos fibers, and when the cells were lysed, the fibers were still intact with about half the length coated with the fluorescent protein, suggesting that about half the fiber had been inside the cell [34]. Since mesothelial cells secrete vitronectin, it is reasonable to assume that asbestos fibers in the pleural space are coated with vitronectin, which enhances internalization by mesothelial cells. When mesothelial cells phagocytize these asbestos fibers, it causes massive DNA damage to the cells, inducing apoptosis in most of the cells [35]. It is a reasonable

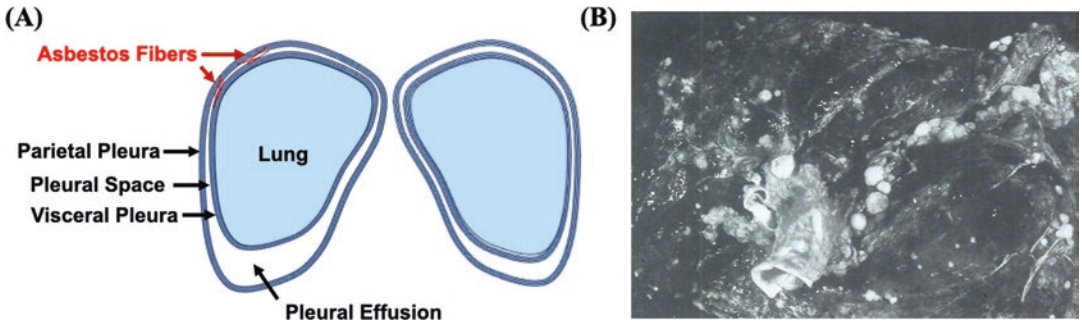

Fig. 2.3 Malignant pleural mesothelioma. (**a**) Asbestos fibers are found in mouse pleural space within hours of exposure (G.M. Davis, Br J Exp Path (1974) 55, 64–70). (**b**) Early diffuse malignant pleural mesothelioma may be seen as nodules on visceral pleura surface (Pathology for the Surgeon, Ed. by Banks & Kraybill, W.B. Saunders Company, 1996)

assumption that some mesothelial cells that phagocytize asbestos fibers in the pleural space survive with massive DNA damage and become cancer cells (Fig. 2.3). Of course, to form a cancer colony, cells still need to evade surveillance by immune cells. It is possible that evasion is easier when cancer cells are confined to a very narrow space that would impede access for immune cells. In support of this possibility, in very rare cases, asbestos-induced mesotheliomas were found in tunica vaginalis [36], a very narrow confined space.

How may tumor suppressor genes affect mesotheliomas? To survive in the face of massive DNA damage, disabling p53 would be an advantage [37], but the loss of p53 function is not a prerequisite for survival. Disabling other tumor suppressor genes such as BAP1, CDKN2B, CDKN2A, and NF2 provides pro-survival advantage as demonstrated for many mesotheliomas [38]. The most frequently mutated genes, BAP1 and CDKN2A, in mesothelioma cells were shown to be reasonably specific to MPM, but they cannot be markers for all mesotheliomas [38–40]. In other words, there is no single suppressor gene preventing variously mutated mesothelial cells from becoming cancerous. Furthermore, massive parallel sequencing of MPM genes showed that there were four pathways found mutated: p53/DNA repair, cell cycle, mitogen-activated protein kinase, and phosphoinositide 3-kinase (PI3K)/AKT pathways [41]. Compromising any of the four pathways could drive carcinogenesis, but there has not been found a single oncogenic mutation common to all mesotheliomas. This was a complicating issue when we tried to diagnose mesotheliomas, or tried to identify a target gene to treat mesotheliomas. In fact, it is rare for cancer to have one oncogene responsible for generation of a cancer type (called oncogenic addiction); exceptions are BRAF for melanoma and Ras for pancreatic cancer. And even then, every melanoma inevitably develops resistance to a specific inhibitor of BRAF in less than a year [42]. Further, most cancers have heterogenetic cancer cells in a single tumor and would not be addicted to just one oncogene [42]. Mesothelioma may be even more difficult to treat because asbestos-induced mesothelioma may originate from multiple locations, and at each location, a different set of DNA mutations may be driving carcinogenesis.

What is new with mesothelioma diagnosis? With advances in nanoString technology, there is less need to identify cancer cells by genetic mutation. For example, MPM can be distinguished from mesothelial cell hyperplasia by gene expression pattern (transcriptome analysis). By analyzing the transcriptome of cells in the pleural effusion, AI programs distinguished mesotheliomas from mesothelial cell hyperplasia with remarkable accuracy [43, 44]. Mesothelioma diagnosis can also be done by examining the gross chromosome structure in the nucleus of cells released into the pleural effusion [45], using a recently developed technology called transport-

based morphometry [46]. Apparently, damage to chromosome structures in mesotheliomas is so extensive that a computer-aided imaging analysis can distinguish a mesothelioma nucleus from a mesothelial cell hyperplasia nucleus with very high accuracy. In perspective, trying to characterize a mesothelioma based on its cancer genome by identifying a target gene(s) and devising a strategy for treatment is a formidable task.

What are the possibilities for combining immunotherapy with chemotherapy to treat MPM? Systemic chemotherapy is a standard component in the treatment for MPM [47]. Even with treatment, 1-year and 5-year survival rates are 33% and 5%, respectively [48]. The median survival times after initial diagnosis for MPM are between 7 and 15 months depending on the statistics taken [49]. In recent years, immunotherapies with or without chemotherapeutics have been tried with limited success [47]. One reason for the limited success is the heterogeneity in immune cell contents in MPM [50]. For example, the immune checkpoint PD-1 is not expressed in all tumors. Also, if MPM is already in Stage 3, then checkpoint blockade, adoptive cell therapy, or chimeric antigen receptor T (CAR-T) therapy (using anti-MSLN antibody) will all be ineffective. Therefore, there is an urgent need to develop an effective way to reverse a Stage 3 tumor to a Stage 2 tumor. Thus, the problems facing MPM therapies are a reflection of the troubles facing cancer therapies generally. MPM, however, has one advantage for therapy development; mouse models for MPM are already available. With trial and error on mouse MPM, it seems possible to devise an effective therapy scheme readily translatable to clinical settings.

Lastly, we describe an effort to develop a dual inhibitor to induce apoptosis in all MPM.

Varin and colleagues' treated mesothelioma cells that are highly refractory to conventional chemotherapy, by knocking down two genes, Mcl-1 and Bcl-xL, inducing apoptosis in all the treated cells [51]. Knocking down just one of the genes did not induce apoptosis because most human cells are protected from spontaneously induced apoptosis by having two anti-apoptotic proteins—Mcl-1 and Bcl-2 (including Bcl-2's homologues). Most cells express Mcl-1 and either Bcl-xL or Bcl-2. For example, megakaryocytes express Mcl-1 and Bcl-xL [52]. When both proteins are inactivated, apoptosis is induced in any cell, cancerous or otherwise [53]. The dual inhibition of Mcl-1 and Bcl-xL would be very toxic to most MPM because 34 out of 35 known MPM expressed the Mcl-1 and Bcl-xL combination [54]. This dual inhibition also induces apoptosis in those cells that do not express Bcl-2 such as liver and megakaryocytes. However, there is a way to limit Mcl-1 inhibition. Mcl-1 is inhibited by the treatment of 2-deoxyglucose and beta-cyclodextrin [55]. 2-Deoxyglucose is taken up by cells with elevated glucose uptake. A pan-Bcl-2 inhibitor such as ABT-263 can inhibit all Bcl-2 family proteins. However, it cannot cross the blood-brain barrier (BBB). Thus, a triple combination of 2-deoxyglucose, beta cyclodextrin, and ABT-263 may effectively kill cancer cells outside the brain, but with the drawback of also killing megakaryocytes [53]. It is known that MPM is highly glycolytic, overexpressing the glucose transporter Glut-1. Thus, the triple combination therapy would also induce apoptosis in most MPM (those that do not express p-glycoprotein, which excludes ABT-263) while leaving hepatocytes unaffected because hepatocytes are not highly glycolytic. If the challenge of not exposing megakaryocytes to all three agents during the treatment can be met, the triple combination therapy could eliminate MPM with few side effects.

In summary, even though the incidence of MPM is expected to decline, MPM may still be an excellent model cancer to study because in many ways, difficulties in treating MPM share many of the same problems facing the wider spectrum of cancer therapies. Progress in treating MPM is thus expected to translate to progress in treating cancers generally.

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Effects of Exercise on the Tumour Microenvironment

3

Linda A. Buss and Gabi U. Dachs

Abstract

Epidemiological evidence suggests that exercise improves survival in cancer patients. However, much is still unknown regarding the mechanisms of this positive survival effect and there are indications that exercise may not be universally beneficial for cancer patients. The key to understanding in which situations exercise is beneficial may lie in understanding its influence on the tumour microenvironment (TME)—and conversely, the influence of the tumour on physical functioning. The TME consists of a vast multitude of different cell types, mechanical and chemical stressors and humoral factors. The interplay of these different components greatly influences tumour cell characteristics and, subsequently, tumour growth rate and aggression. Exercise exerts whole-body physiological effects and can directly and indirectly affect the TME. In this chapter, we first discuss the possible role of exercise capacity ('fitness') and exercise adaptability on tumour responsiveness to exercise. We summarise how exercise affects aspects of the TME such as tumour perfusion, vascularity, hypoxia (reduced oxygenation) and immunity. Additionally, we discuss the role of myokines and other circulating factors in eliciting these changes in the TME. Finally, we highlight unanswered questions and key areas for future research in exercise oncology and the TME.

Keywords

Exercise · Physical activity · Cancer · Tumour microenvironment · Hypoxia · Anti-tumour immunity · Tumour perfusion · Myokines · Exercise capacity · Vascularity · Exercise adaptation · Immunology · Preclinical · Metastasis · Physiology

3.1 Introduction

Gone are the days in which cancer patients are treated to the adage 'rest is best'. A wealth of epidemiological studies over the past two decades have provided evidence that physical activity or exercise reduces the risk of developing a range of different cancers (such as breast, colorectal and lung) and is even associated with improved survival outcomes in breast and colorectal cancer patients [1, 2]. Furthermore, exercise exhibits a remarkable safety profile compared with cancer therapeutics—it is not associated with any toxicities of its own and may even reduce the rate or severity of treatment-associated adverse events [3]. In addition to survival benefits, exercise has been shown to improve cognitive and physical

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functioning, reduce anxious and depressive symptoms and improve health-related quality of life in cancer patients (reviewed in [3]).

However, there are indications that exercise is not universally beneficial for all cancer patients. Occasional preclinical studies have suggested that, in some situations, exercise may not affect tumour growth rate at all—or even increase tumour growth rate [4, 5]. It is unclear what the determinants of an exercise-responsive versus non-responsive tumour are, or how to identify which patients will benefit from exercise. The key to this may lie in how exercise affects the tumour microenvironment (TME).

The TME is the local cellular, physical, chemical and humoral environment that tumour cells inhabit. More specifically, it includes a range of cell types other than cancer cells, such as endothelial cells, immune cells, fibroblasts and adipocytes. Physical, chemical and humoral factors include the stiffness of the extracellular matrix, pH, oxygen tension, cytokines and growth factors. It is now well-known that tumour growth characteristics and metastatic capacity are in large part defined by the make-up of the TME. Therefore, understanding the effects of exercise on the TME is integral to understanding how exercise exerts its beneficial effects (or lack thereof).

This chapter will summarise and synthesise available preclinical (and clinical, where available) data on the effects of exercise on the TME. We will describe the factors affecting individual exercise capacity and exercise adaptability and briefly summarise preclinical and epidemiological evidence on how exercise affects tumour growth/patient survival. The main focus of this chapter will be to discuss the finer details of how exercise affects the TME, including the role of myokines, tumour vasculature/perfusion and hypoxia and immunity. Finally, we will suggest future avenues of investigation for the field of exercise oncology.

3.2 Exercise Capacity and Exercise Adaptability

3.2.1 Exercise Capacity

Exercise capacity is defined as the maximum amount of physical exertion that an individual can sustain and is colloquially referred to as ‘fitness’ [6]. The major variable affecting exercise capacity is, of course, the intensity and duration of exercise performed by an individual on a regular basis. However, other factors can play an important role, including nutrition, age, gender and genetics.

There is a clear heritable component to exercise capacity. Rodents can be selectively bred for high inherent exercise capacity; these animals can run for longer and at a higher speed (in an untrained state) than their low inherent exercise capacity counterparts [7]. In addition, different inbred mouse strains have significantly different exercise capacities in the untrained state [8]. In humans, studies have suggested that there is a large heritable component to exercise capacity (reviewed in [9]).

To our knowledge, there is just one study that has investigated the effect of inherent exercise capacity on cancer risk. Rats were selectively bred for high or low inherent exercise capacity and exposed to the carcinogen 1-methyl-1-nitrosurea (MNU) [10]. Rats with high inherent exercise capacity had lower tumour incidence (fewer rats with any breast malignancy), and those that did develop tumours had fewer tumours than rats with low inherent exercise capacity [10]. This suggests that there may be a large heritable component to the protective effect of exercise on cancer risk.

3.2.2 Exercise Adaptability

There also appears to be a heritable component to exercise adaptability, that is, the ability of an

individual to effect the physiological changes required to improve exercise capacity in response to exercise. These include muscular adaptations (increased capillary density and mitochondrial expansion) and improved pulmonary and cardiovascular capacity [11]. In mice, different inbred strains exhibit significantly different changes in exercise capacity following the same training protocol [8]. Similarly, in humans, training-induced increases in maximal oxygen uptake (VO_2 max) vary significantly more between families than within families [12]. It has been suggested that the heritability of exercise adaptability may be as much as 50% (reviewed in [9]).

To our knowledge, there are no published studies specifically investigating the role of exercise adaptability on risk of cancer. However, higher VO_2 max was associated with improved survival in metastatic breast cancer patients and non-small cell lung cancer patients [13, 14]. We found a single clinical study in which exercise adaptations in the skeletal muscle of cancer patients were compared with healthy controls [15]. In that study, the authors found that a greater number of healthy individuals had an increase in muscle fibre cross-sectional area with exercise training compared with cancer patients [15]. In addition, healthy individuals had an increase in muscle capillarisation and quadriceps strength while cancer patients did not [15]. Although this study is limited by small numbers (n = 12–16 per group), it provides preliminary evidence that cancer patients may not adapt to exercise to the same degree as healthy individuals. Furthermore, comparison of a systematic review on improvements of VO_2 max in cancer patients with data in healthy subjects suggests that the magnitude of improvement in VO_2 max is lower in cancer patients despite following similar exercise programs to the healthy subjects [16].

It is largely unknown what role exercise adaptability and exercise capacity play in cancer patients' response to exercise and, specifically, in exercise-induced changes in the TME. There are two possibilities: (1) the exercise adaptation response of the tumour is just as or more variable than the muscular/cardiopulmonary response or (2) the tumour response is less variable as tumour tissue is not directly involved in determining exercise capacity. In addition, the anatomical location of the tumour may be important. We speculate that tumours located in exercise-involved tissues (such as lung) may respond more strongly to exercise.

The potential role of (inherited) exercise capacity and exercise adaptability has thus far been largely unacknowledged in exercise oncology research. Given that higher exercise capacity is associated with lower tumour incidence in rodents [10] and improved survival in cancer patients [13], inherited exercise adaptability and exercise capacity may play a significant role in whether or not a particular cancer patient will benefit from exercise. In addition, impairments in exercise adaptability due to tumour or treatment burden may limit the effectiveness of exercise.

3.3 Effect of Exercise on Tumour Growth/Patient Survival

In this section we will give only a brief overview regarding the effect of exercise on tumour growth/progression and patient survival in order to retain the main focus on the effects of exercise on the TME. We refer the interested reader to the many comprehensive reviews on the effect of exercise on cancer patient survival [2, 3, 17, 18] and the effect of exercise as a sole intervention in preclinical studies [3, 19–22].

3.3.1 Clinical Studies

The majority of clinical studies investigating the effect of exercise or physical activity on cancer patient survival have been observational studies. There is evidence that meeting the World Health Organization (WHO) guidelines of 150 min of moderate-intensity or 75 min of vigorous-intensity exercise per week confers a significant survival benefit for cancer patients and for some cancer types, such as colorectal cancer, a dose-response relationship between exercise volume and survival has been reported [2]. Current evidence supports a 40–50% reduction in all-cause

mortality for breast, colorectal and prostate cancer survivors engaging in high levels of physical activity; other cancers have not yet been sufficiently studied in this context [18]. However, there is a large possibility of reverse causation for the relationship between cancer survival and physical activity level (patients who are less well may exercise less, rather than high activity levels causing an improved outcome) [18], which emphasises the importance of conducting randomised controlled intervention trials to fully investigate the role of exercise in improving patient survival. Two large intervention studies (the CHALLENGE trial and the INTERVAL trial) are ongoing to address this and will shed more light on whether a targeted exercise intervention can improve survival [23, 24].

3.3.2 Preclinical Studies

The effect of exercise on tumour growth in preclinical studies is less clear than in epidemiological studies. This is likely to be largely due to heterogeneity in study design. Key factors that influence outcomes of preclinical studies are the rodent strain used (as discussed above, different strains have different inherent exercise capacity and adaptability), immunocompetency of the rodent strain, timing of exercise initiation (pre- vs post-'diagnosis'), tumour type and anatomical location, exercise modality (forced, swimming or treadmill; voluntary, wheel running), tumour burden and study endpoint (predetermined time after tumour initiation or ethically determined by tumour size). Very few studies have more than a few of these factors in common, making comparisons difficult. Comprehensive discussion of these parameters is beyond the scope of this chapter (refer to [20] for comparison of the effects of some of these parameters on tumour growth), but we will briefly discuss the role of pre- versus post-implantation (mimicking pre- vs post-diagnosis) exercise on tumour growth.

In many preclinical studies, post-implantation exercise either only marginally affects tumour growth rate [25–28] or does not affect tumour growth rate at all [29–33]. In agreement with this, only a 'small to moderate' effect size of exercise on final tumour size was reported in a recent systematic review [20]. Of those studies included in the analysis that did find a statistically significant difference in final tumour size, 4/8 had a 'probably high' risk of bias and one even showed an increased tumour size with exercise. Together with the observation that many studies find a statistically but not clinically significant result, this suggests that exercise as a sole intervention (monotherapy) is minimally effective at slowing primary tumour growth rate.

The documented effect of pre-implantation exercise is more consistent than that of post-implantation exercise. In the above-mentioned systematic review [20], studies in which exercise was performed both pre- and post-implant had a larger effect size for exercise to reduce tumour growth than studies in which exercise was performed only after tumour implant [20]. In addition, Pedersen et al. found that growth rate of B16-F10 melanoma was slowed with pre-implantation or pre-and post-implantation exercise, but not post-implantation exercise only [30]. Similarly, a number of studies using carcinogen-induced models (which typically start exercise after carcinogen administration but before tumours become detectable) have found a reduction in malignant tumour incidence and/or overall tumour burden (by number of tumours per animal or combined weight of tumours) [30, 34]. This suggests that while exercise monotherapy may not be very effective (as discussed above), exercise preconditioning may be important both for prevention and slower growth of cancer once it has arisen. In a clinical setting, this may also translate to reduced rates of recurrence after tumour control following treatment, although this remains speculative.

It seems clear that exercise can reduce tumour growth rate, but likely only to a significant extent in a pre-implantation setting or possibly in combination with cancer therapies (discussed in Sect. 3.7). However, some studies have found differences in the exercise responsiveness of different

tumours, using the same exercise protocol, with some tumours (of the same subtype) exhibiting either no change in growth rate or even an increased growth rate with exercise [4, 5]. This is important, as it indicates that exercise treatment is far from a one-size-fits-all approach and some patients may not benefit from exercise.

3.3.3 The Effect of Exercise on Metastasis

Metastasis is the process whereby tumour cells migrate from the site of the primary tumour to establish secondary tumours in different tissues. For many cancer types, it is the emergence of secondary tumours in vital tissues which ultimately causes death.

Before cancer cells can seed in secondary sites, they must survive transport through the circulation. Regmi et al. have shown that high shear stresses (such as those present in the vasculature during intense exercise) can kill circulating tumour cells using an in vitro microfluidic system [35]. However, most of the time points used were not clinically relevant. Cells were circulated under high shear stress for up to 18 h—this mimics the scenario of vigorous exercise for 18 h, which is highly unrealistic for the vast majority of the healthy population, let alone cancer patients. In one experiment, increased lactate dehydrogenase (LDH) release (a proxy for necrotic cell death) was seen after 1 h of circulating under high shear stress, which represents a more achievable length of exercise time. Much longer than this is not realistically achievable in a clinical setting. It would be prudent to repeat these experiments with shorter time points that more accurately mimic the exercise behaviour of the average population (and specifically, cancer patients).

As with preclinical data examining the effect of exercise on primary tumour growth, it is unclear how exercise affects metastasis due to varying results. However, there are several studies describing a reduced number or mass of metastases with both spontaneous [32, 36, 37] and experimental metastasis [25, 30, 38–40], with fewer studies reporting no change [39, 41] or an increase in the number/mass of metastases [25, 42], suggesting that in many situations, exercise can inhibit metastatic tumour formation. Stress may play a role in how exercise affects metastasis. Zhang et al. found that swimming for 8 min/day (which mice performed without added encouragement) reduced the relative size of experimental lung metastases, whereas when mice were forced to swim for 16 or 32 min/day, the relative size of metastases was increased [25]. More mechanistic studies are required to help delineate how exercise affects different aspects of the metastatic cascade.

3.4 Myokines and Other Circulating Factors

During exercise, muscle tissue releases a vast array of factors into the circulation, collectively termed ‘myokines’ (from ‘muscle-derived cytokines’). These myokines have known effects on peripheral tissues, such as skeletal muscle remodelling in response to exercise and improvements in cognitive function (reviewed in [43]). It is thought that the action of myokines (and other factors) is directly (affecting cancer cells) and/or indirectly (affecting other cells of the TME) responsible for many of the changes seen in the TME with exercise.

A number of in vitro studies have found that post-exercise serum (serum harvested from humans or animals following an acute exercise bout) can directly inhibit cancer cell proliferation, viability or survival when supplemented into the cell culture media [44–48]. This has been attributed to a few different myokines, including secreted protein acidic and rich in cysteine (SPARC) [49], irisin [50] and oncostatin M [47].

Direct in vivo data indicating the effect of select myokines on tumour growth is still largely lacking. However, Aoi et al. found that the protective effect of exercise against azoxymethane-induced colon tumourigenesis was nullified in SPARC knockout mice, and

SPARC was able to induce colon cancer cell apoptosis in vitro [49]. This suggests that the myokine SPARC plays an important role in the protective effect of exercise on cancer development and highlights the value of further investigating the effects of exercise-induced myokines on tumour growth.

Research by Hojman and colleagues indicates that exercise-induced catecholamines (epinephrine and norepinephrine) have both an indirect and a direct effect resulting in the reduction of tumour growth rate [30, 45]. Human breast tumour cells preconditioned with exercise serum were less able to form xenograft tumours in mice, and this effect was completely abolished when the beta-blocker propranolol was also added to the pretreatment [45]. In addition, daily injections of epinephrine or norepinephrine were able to significantly slow MCF-7 and MDA-MB-231 xenograft growth rate [45]. Indirectly, catecholamines (and IL-6) are essential for the exercise-induced mobilisation of natural killer (NK) cells to the tumour site, which are themselves essential for the exercise-induced delay in tumour growth seen in this study [30].

A third factor that has been linked to an exercise-associated delay in tumour progression is dopamine. Zhang et al. found that moderate swimming exercise reduced the tumour weight of subcutaneous and pulmonary hepatomas in mice, and this was mirrored by an increase in dopamine levels in the prefrontal cortex, serum and tumour tissue [25]. Moreover, dopamine treatment was able to reduce tumour weight to the same extent as swimming, and a dopamine receptor 2 antagonist (domperidone) abolished the tumour growth inhibitory effect of both dopamine and swimming exercise [25].

Although there is still a scarcity of data investigating the effects of exercise factors on tumour growth and the tumour microenvironment, those studies that have been done indicate that exercise-induced systemic factors may mediate the effects of exercise on the tumour microenvironment.

3.5 Effect of Exercise on Tumour Vascularity, Hypoxia and Perfusion

3.5.1 Effect of Exercise on Normal Vasculature

Acute exercise modulates blood flow to different organ systems, with some receiving increased (such as skeletal muscle) and some receiving decreased (such as skin) blood flow during exercise [51]. This enables the body to cope with the stress of acute exercise by providing those tissues directly involved in exercise with more oxygen and nutrients. Meanwhile, chronic exercise can induce vascular remodelling [52]. Skeletal muscle is the tissue most affected by these changes, but many tissue types are affected to some degree, including the brain, heart and bone [53–55].

It has been shown in vitro and in vivo that exercise can directly affect endothelial cell behaviour. Schadler et al. transplanted Matrigel plugs (an artificial matrix containing gelatine and basement proteins) containing primary mouse endothelial cells into mice and found that those implanted into exercising mice were better perfused and showed elongated vessels compared with those implanted into non-exercising mice [31]. In addition, endothelial cells exposed to exercise-conditioned serum in a microfluidic system showed reduced sprouting (i.e. less angiogenesis), as did those exposed to high shear stress (mimicking that present during exercise) [31]. This seems counterintuitive, but the authors argue that this reflects increased vascular maturity which is ultimately conducive to more stable vascular networks. Interestingly, exercise may also reduce age-associated venous endothelial cell senescence in humans [56].

Given that exercise can affect endothelial cell behaviour and induce vascular remodelling in a variety of normal tissues, it is possible that tumour vasculature may also be affected by exercise. A number of preclinical studies have investigated how exercise affects tumour hypoxia, perfusion and vascularity in various tumour types and locations (Fig. 3.1).

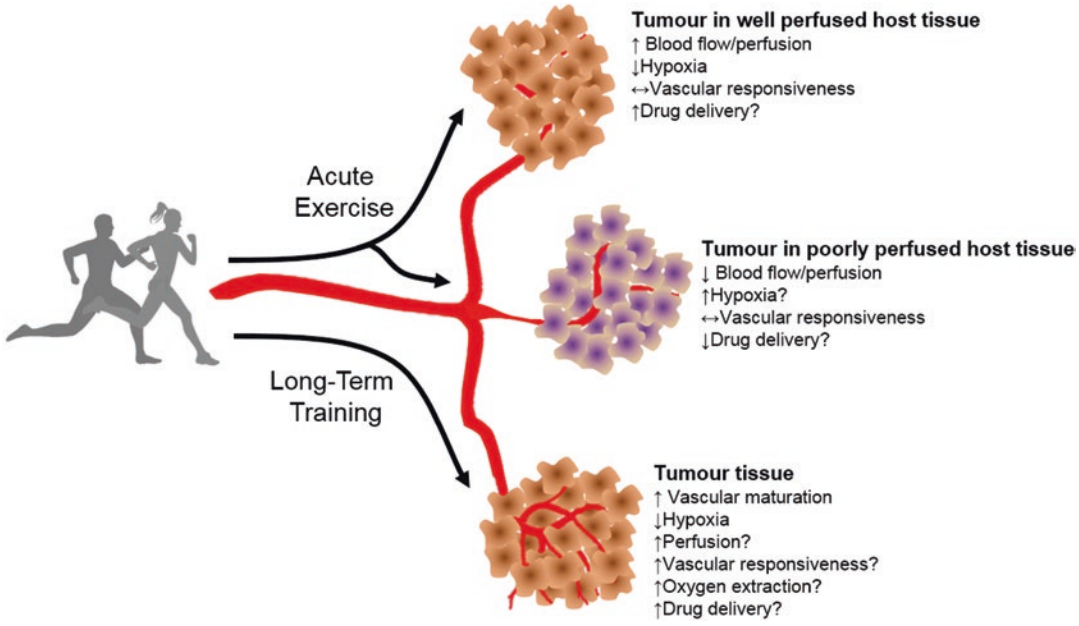

Fig. 3.1 Effects of acute and chronic exercise on tumour vascular characteristics and hypoxia. Acute exercise increases blood flow to tumours located in tissues that receive constant or increased blood flow during exercise while decreasing blood flow to tumours located in tissues that are poorly perfused during exercise. This may alter levels of hypoxia and affect drug delivery in the indicated directions. Vascular contractility and dilatory responsiveness are impaired in tumour vessels and this impairment is unchanged by acute exercise. On the other hand, long-term training (chronic exercise) may improve vascular maturation by improving responsiveness and pericyte coverage, which may improve perfusion, oxygen extraction and drug delivery and reduce hypoxia. Areas in need of further research are indicated with?

3.5.2 Effect of Exercise on Tumour Hypoxia

Just like any other tissue, tumours require blood flow delivering oxygen and nutrients in order to survive. However, tumours are not evenly perfused and are characterised by disorganised, dysfunctional vasculature and regions of hypoxia [57]. This leads to the activation of hypoxia factors such as the hypoxia-inducible factors (HIFs), which stimulate the transcription of a large array of genes designed to help the cell adapt to low oxygen conditions. These include genes central to angiogenesis, cell metabolism and metastasis [58]. In normal tissue, this results in improved vascular coverage and subsequent improved perfusion and alleviation of hypoxia. However, in tumours, the hypoxic response does not improve perfusion as the new blood vessels formed are often immature and may be leaky or lack proper haemodynamic control [59, 60]. This perpetuates tumour hypoxia rather than alleviating it.

In hyperlipidaemic *ApoE*$^{-/-}$ mice bearing orthotopic breast tumours, we found that mice with high levels of cytochrome c oxidase subunit 4 (COX-IV, a marker of mitochondrial content and proxy for training status) in the quadriceps femoris muscle exhibited greatly reduced tumour hypoxia, although there was no change in tumour perfusion or CD31^{+} vessel density [37]. A second study found that hypoxia in orthotopic prostate tumours was reduced *during* exercise [61], and this was also evident in tumours from trained rats [33]. Furthermore, Betof et al. observed that tumour hypoxia was decreased in orthotopic 4T1 breast tumours from exercising mice [28].

Levels of the hypoxic response protein HIF-1α were decreased with exercise in an orthotopic breast cancer model [4]. In contrast, HIF-1α levels were increased with exercise in human-derived breast xenografts in athymic mice [62]

and in orthotopic breast and prostate cancer in immunocompetent mice [4, 32]. In subcutaneous Ewing sarcoma xenografts, *HIF-1*α and carbonic anhydrase IX (*CA-IX*, a HIF-1 target gene) mRNA were decreased with exercise in one of two tumour cell lines used [63]. It is unclear why this disparity exists between measured hypoxia and levels of HIF-1α, given that HIF-1α protein stability is strongly dependent on oxygen levels. However, it can partially be regulated independently of hypoxia, which may explain the above-described results [58].

To our knowledge, hypoxia in tumours from mice starting exercise prior to tumour cell inoculation has not yet been investigated nor has tumour hypoxia in exercising cancer patients. It is also unknown whether the reduction in tumour hypoxia occurs in tumour types other than breast and prostate cancer.

3.5.3 Tumour Perfusion and Vessel Density

Initial anticancer strategies targeting tumour blood vessels focussed on inhibiting angiogenesis, as tumour cells will die if completely deprived of oxygen and nutrients. However, as with all cancer therapies, many tumours develop resistance to anti-angiogenic agents. A more recent strategy is vascular normalisation [59], which aims to promote the normal development of tumour vessels to form a functional, evenly perfused network which more closely resembles that of normal tissue. This would reduce hypoxia, thereby reducing metastatic potential and enhancing radiosensitivity. In addition, drug delivery throughout the tumour would be improved.

The effect of exercise on tumour vascularity and perfusion remains unclear. Studies by various groups have demonstrated an increase in perfusion homogeneity, the level of perfusion and/or vessel density or *Cd31* mRNA levels (breast, prostate and pancreatic cancer) [28, 31, 32, 64, 65], but others have found no change in the mean level of perfusion [15, 19] or vessel density in breast and/or prostate cancer [66]. Further studies even found reduced numbers of blood vessels in breast tumours or lymphomas from exercising mice [67, 68].

McCullough et al. observed increased blood flow to orthotopic prostate tumours during acute exercise (but not with exercise training) which was associated with a reduction in tumour hypoxia, which suggests that levels of tumour perfusion may change with acute exercise but this is not necessarily maintained after exercise cessation [61].

A potential reason for the differing results observed in different studies may be the method used to detect tumour perfusion. We and McCullough et al. used IV injection of Hoechst 33342 prior to euthanasia to label perfused blood vessels [33, 37], while other studies used MRI to generate a perfusion map of the entire tumour while the animal was still alive [28, 32]. MRI is likely a more representative method of whole-tumour perfusion, as Hoechst injection and subsequent imaging of thin tissue sections presents only a snapshot of tumour perfusion in time and space, which fails to take into account the dynamic nature of tumour blood flow. Nevertheless, this method has been able to detect differences in perfusion in the past [62] and has been validated for this purpose [69].

A further important consideration is the impact of anatomical location of the tumour on blood flow responses to exercise. Garcia et al. elegantly demonstrated that blood flow during exercise is increased to orthotopic prostate tumours in rats, but decreased to subcutaneous tumours of the same type [51]. They further measured blood flow to different organs during exercise, including the bladder, prostate (location of the orthotopic tumour), soleus muscle, kidneys, skin, subcutaneous adipose (location of the ectopic tumour) and visceral adipose tissue. Blood flow to the bladder and prostate was unchanged, but increased to the soleus muscle and decreased to the kidneys, skin, subcutaneous and visceral adipose tissue [51]. This suggests that host tissue haemodynamics in response to exercise also play a role in regulating blood flow to the tumour. As such, tumours located in tissues that become less well perfused during exercise may not benefit from the increased perfusion seen in prostate

tumours and may even become more poorly perfused during exercise, which could exacerbate tumour hypoxia. This has yet to be further investigated in different tumour models.

Tissue blood flow is largely regulated by vascular contractility and myogenic tone. Due to their poor maturation (lack of smooth muscle cells and innervation), tumour vessels have poor contractile and dilatory responsiveness, which limits their ability to regulate blood flow [51, 61, 70]. Contractile responsiveness to norepinephrine does not change with exercise training, indicating that tumour vessel response to both acute and chronic exercise is impaired compared with normal tissue [51, 61]. Due to this, tumour blood flow cannot be regulated to provide optimal conditions for oxygen extraction. Optimal oxygen extraction relies on complex haemodynamics—parameters such as capillary transit time, microvascular pressure and apparent blood viscosity all affect how much oxygen can be extracted from the bloodstream [57, 71]. Thus, optimal tissue oxygenation relies on more than just the presence of perfused vessels. As mentioned above, acute exercise does not alter tumour vessel contractility, but it is unknown whether long-term exercise could improve vascular maturation to a point where vessel contractile and dilatory responses are restored, thus improving tissue perfusion and oxygen extraction.

Part of the therapeutic appeal of vascular normalisation is the enhanced delivery of anticancer agents to the tumour. Two preclinical studies have investigated how exercise affects chemotherapy delivery to the tumour [31, 63]. Schadler et al. found that although exercise alone did not reduce tumour growth rate of subcutaneous, pancreatic PDAC or B16-F10 melanoma, chemotherapy in combination with exercise significantly slowed tumour growth rate over and above the effect of chemotherapy alone [31]. Immunofluorescence analysis revealed that there was increased expression of the DNA damage marker γH2AX in PDAC tumours from mice receiving both chemotherapy and exercise compared with those only receiving chemotherapy and higher levels of doxorubicin fluorescence in B16-F10 tumours from exercised mice receiving chemotherapy compared with those receiving chemotherapy only. This was only the case in tumours from trained mice; one acute exercise session was insufficient to enhance doxorubicin delivery to the tumour [31]. Furthermore, the authors demonstrated that pharmacologically increasing tumour blood velocity by the use of an antihypertensive agent (prazosin) also enhanced the growth inhibitory effect of gemcitabine on PDAC tumours [31]. Similarly, Morrel et al. found that exercise improved doxorubicin delivery to subcutaneous Ewing sarcomas, and this was associated with a further reduced tumour growth rate compared with exercise or doxorubicin alone (although exercise alone also had a strong growth inhibitory effect) [63]. These data suggest that exercise can induce vascular changes leading to improved tumour blood flow even in tumours that are located in tissue that does not receive enhanced blood flow during acute exercise (i.e. subcutaneous adipose tissue), although this is yet to be corroborated by other groups.

3.5.4 Markers of Angiogenesis and Vascular Maturation

Further to the above-described effects of exercise on tumour hypoxia, vascularity and perfusion, a few studies have investigated markers of angiogenesis and vascular maturation in tumours following exercise.

Betof et al. found that voluntary wheel running not only increased $CD31^+$ vessel density, but also enhanced pericyte coverage (a marker of vascular maturation) in orthotopic 4T1 breast tumours [28]. In addition, pericyte coverage was increased by exercise in two Ewing sarcoma models [63]. Conversely, Schadler et al. found that the α-smooth muscle actin (α-SMA, a pericyte marker) to CD31 ratio did not change with exercise in subcutaneous B16-F10 tumours [31]. This discrepancy may simply be due to differing tumour models, but future studies should aim to clarify this.

A few studies have investigated tumour levels of the angiogenic factor vascular endothelial growth factor (VEGF, a HIF-1 target) in mam-

mary tumours. One study observed increased mRNA levels of *Vegfa* [28]. In agreement with this, another group found increased VEGFA protein expression in mammary tumours from exercised rats [64]. In contrast, two other studies found that VEGF protein expression was reduced in tumours from mice exercising after tumour implant [27, 67].

3.5.5 Conclusions on Tumour Vascularity, Hypoxia and Perfusion

It seems clear that acute exercise can regulate tumour blood flow, either increasing or decreasing blood flow depending on tumour location [51]. However, it remains unclear whether or how long this persists after exercise cessation and whether chronic exercise can remodel the TME in such a way as to normalise the vasculature to improve perfusion and oxygen extraction even at rest. These questions are central to future work in this area, as a thorough understanding of tumour blood flow and perfusion dynamics (with respect to acute and chronic exercise) is required to inform relevant intervention trials and subsequent clinical practice to achieve the greatest benefit from exercise together with standard cancer therapies.

3.6 Effect of Exercise on the Immune Microenvironment

3.6.1 Effect of Exercise on Immunity in Healthy Individuals

Acute exercise causes a rapid rise in the number of circulating immune cells; this includes an increase in numbers of all major subclasses (lymphocytes, monocytes and granulocytes) [72]. Lymphocytes, in particular NK cells, are among those that respond most strongly to acute exercise [73]. Following exercise cessation, lymphocyte counts in the blood rapidly decrease, falling below pre-exercise levels by 1 h post-exercise [72]. This was previously thought to be due to lymphocyte apoptosis and attributed to an immunosuppressive effect of exercise, but based on evidence that lymphocyte apoptosis post-exercise only accounts for a small fraction of the observed lymphocytopenia, it seems more likely that the bulk of this is due to egress into peripheral tissues and may present a mechanism for heightened immune surveillance of tissues post-exercise [74]. Direct evidence for this is still lacking, but is supported by evidence that leukocyte subtypes that are preferentially mobilised by exercise tend to be cytotoxic subtypes and express markers associated with extravasation and tissue migration (such as integrins and chemokine receptors) [75–77].

Regular moderate-intensity exercise has been linked with enhanced overall immunity, such as improved NK cell cytotoxic activity, increased lymphocyte proliferation, reduced T cell senescence and enhanced vaccine responses [78–80]. There is some controversy regarding the effect of intensive exercise on immunity, with the open window hypothesis stating that intense exercise is followed by a transient state of immune depression, which becomes chronic if regular intense exercise is performed [81]. This has recently been challenged by Campbell and Turner, who argue that the evidence supposedly supporting the open window hypothesis (increased frequency of upper respiratory tract infections, a fall in salivary IgA and lymphocytopenia following intense, acute exercise such as a marathon) has been largely misinterpreted [82]. They argue that the supposed increase in incidence of upper respiratory tract infections is either due to symptoms of an infection but no actual infection (rather caused by airway irritation due to increased ventilation or non-specific inflammation) or an actual infection caused by factors not directly related to intense exercise such as increased exposure to pathogens due to a large accumulation of people. As discussed above, acute lymphocytopenia following exercise is now thought to be due to lymphocyte egress into peripheral tissues.

3.6.2 Effect of Exercise on Immunity in the TME

3.6.2.1 Peripheral Immunity in Cancer Survivors

Changes in circulating levels of immune cells with exercise may provide an indication of whole-body immunity, including effects on the tumour, in cancer patients. In some patients, the acute exercise-induced increase in circulating immune cells is attenuated or even abolished [83, 84]. Lymphocytes seem to be most strongly affected by this, with two studies showing a nullified or attenuated lymphocytosis but intact neutrophil [84], granulocyte and monocyte response with acute exercise [83]. Another study has found an increase in both lymphocytes and granulocytes immediately following acute exercise in chronic myeloid leukaemia patients [85]. However, other studies were in patients with solid tumours [83, 84], which may impact systemic immune responses differently. These results suggest that either tumour burden or treatment may negatively affect immune cell mobilisation in response to exercise, which may reduce immune surveillance of peripheral tissues.

Chronic exercise does not alter numbers of circulating immune cells in most studies [84, 86–90]. However, occasionally some studies have found an increase in various immune cell types with chronic exercise, including granulocytes, leukocytes, lymphocytes and neutrophils (systematically reviewed in [89]). Others have reported a decrease in lymphocytes or monocytes [89]. In addition, exercise training was unable to prevent the chemotherapy-associated decline in immune cell numbers [90]. Taken together, this suggests that exercise training does not alter numbers of circulating immune cells in cancer patients and other factors may be responsible for the observed increases or decreases in certain components in some studies.

3.6.2.2 Ex Vivo Immunity

Preclinical exercise studies reporting on ex vivo or intratumoural immunity are summarised in Fig. 3.2.

The effect of exercise on immune cell function is difficult to measure in vivo. However, a number of studies have isolated immune cells from either the spleen, tumour or peritoneum of exercising and non-exercising animals and compared their cytotoxic capacity, phagocytic capacity or cytokine production in vitro.

The first studies investigating ex vivo immune function against tumour targets were conducted by MacNeil and Hoffman-Goetz in the early 1990s. Splenic NK cells isolated from healthy mice immediately following an acute exercise session had higher activity when stimulated with IL-2 than those from non-exercised mice [91]. In addition, splenic NK cells isolated from tumour-bearing mice performing chronic exercise beginning prior to tumour implant exhibited increased activity against tumour targets [92–95]. In contrast, Pedersen et al. found no change in the cytotoxic activity of splenic NK cells isolated from trained compared with non-exercised mice bearing B16-F10 melanoma [30]. This discrepancy may be due to the activation status of the NK cells. In a few of the above-mentioned early studies, the authors showed that only IL-2 activated but not unactivated NK cells from exercised mice had increased cytotoxicity against tumour targets [91, 93]. In addition, unactivated NK cells are poorly effective against lysis-resistant tumour cell lines, but are able to achieve up to ~60% lysis when pre-stimulated with IL-2 and IL-12 [96]. This suggests that the in vivo antitumour activity of NK cells may be dependent on the intratumoural milieu. In support of this, exercise prior to tumour implant causes significantly slower growth of B16-F10 tumours, and these tumours show higher mRNA expression of IL-2 and other NK-cell activating factors [30].

Macrophage phagocytosis and phenotype has also been reported to change with exercise in tumour-bearing rodents. Peritoneal macrophages from exercised mice produce more IFN-γ, IL-12, TNF-α and IL-4 than those from non-exercised mice and less of the immunosuppressive cytokines TGF-β and IL-10, suggesting a polarisation towards an antitumour M1 phenotype [97]. Furthermore, peritoneal macrophages from exer-

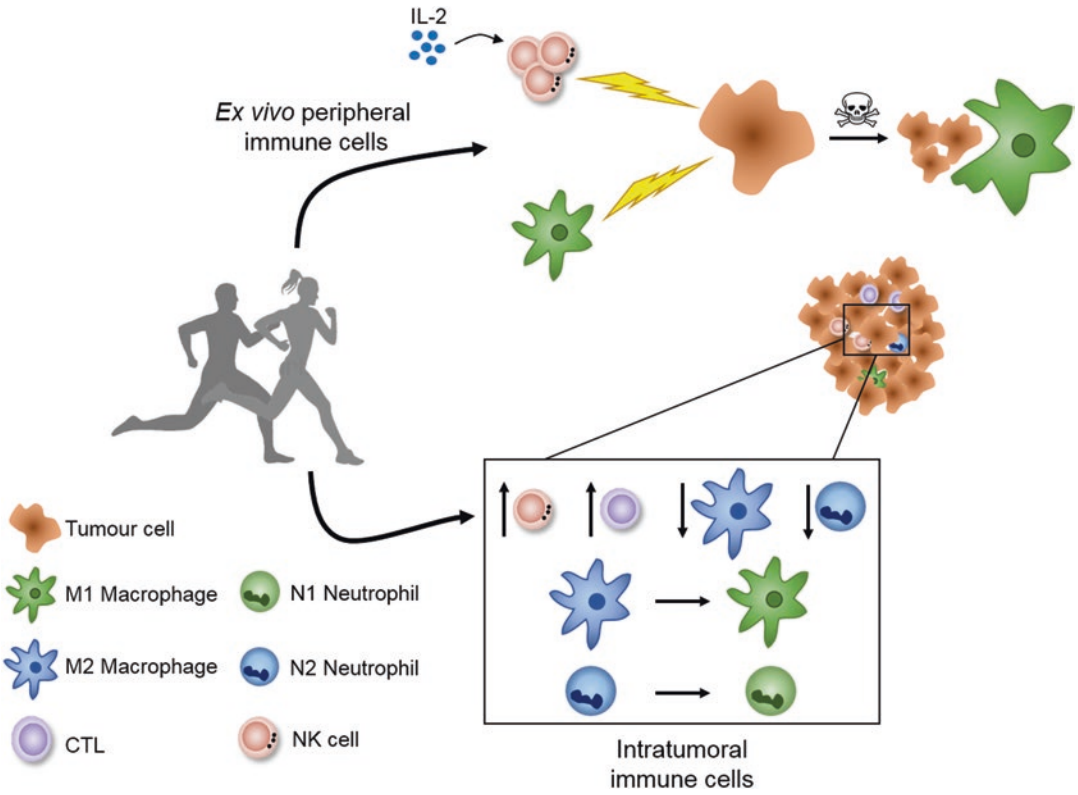

Fig. 3.2 Effects of exercise on peripheral immunity and the immune TME. Exercise increases cytotoxicity of peripheral, IL-2 activated NK cells and macrophages against tumour targets and enhances phagocytic activity of macrophages ex vivo. In addition, exercise may increase recruitment of cytotoxic lymphocytes (NK cells and CTLs) to the tumour site while decreasing number and/or changing phenotype of myeloid cells such as neutrophils and macrophages to an anti- (M1/N1) rather than pro-tumour (M2/N2) state. *CTL* cytotoxic T lymphocyte, *NK cell* natural killer cell, *IL-2* interleukin 2

cised, tumour-bearing rats are more phagocytic than those from non-exercising rats [98] and macrophages isolated from healthy, trained mice are able to induce higher cytolysis of tumour targets compared with those from non-exercised mice [99]. Finally, phagocytes isolated from subcutaneous breast tumours in moderately exercised mice have higher phagocytic activity against *Staphylococcus aureus* than those from non-exercised (or exhaustively exercised) mice [100].

In humans, it has been found that both acute [101, 102] and chronic [80, 103] exercise can improve cytotoxic activity of peripheral blood NK cells from healthy individuals against tumour targets, although one study found no change in NK cell cytotoxicity following exercise training [104] and another found decreased activity [105]. In cancer patients, chronic exercise has also been shown to increase NK cell cytotoxicity ex vivo [89, 106, 107]. In addition, ex vivo lymphocyte proliferation and phagocytic activity of monocytes is increased in post-exercise training, while neutrophil oxidative burst is unchanged [89].

Taken together, ex vivo immune functionality data from both human and animal studies suggest that exercise can improve antitumour cytotoxicity. In future, studies should aim to corroborate this in vivo if possible. In addition, only one study has utilised immune cells isolated from the tumour itself rather than from the spleen or peripheral blood [100]. As the phenotype of immune cells in the TME may differ from peripheral immune cells due to crosstalk with tumour

cells, future studies should investigate the functionality of phagocytes and lymphocytes isolated directly from the tumour.

3.6.2.3 Intratumoural Immunity

Given the hypothesis that the transient lymphocytopenia following exercise is due to cytotoxic lymphocyte egress and surveillance of peripheral tissues, it follows that exercise may also redistribute these cells to the tumour. This is indirectly supported by work showing that NK cell and T cell numbers are increased in subcutaneous B16-F10 tumours following 6 weeks of exercise training [30] and that *Cd8* gene expression is increased in mucosal scrapings from exercised compared with non-exercised $Apc^{Min/+}$ mice [108]. In addition, Zielinski et al. found increased intratumoural lymphocyte density in subcutaneous EL-4 tumours following exhaustive exercise training compared with non-exercise mice [68]. Conversely, we observed no difference in T cell numbers in EO771 tumours between exercising and non-exercising $ApoE^{-/-}$ mice [37], and Bianco et al. found no change in numbers of tumour-infiltrating T cells into 4T1 tumours with post-implant exercise [109]. This may be due to the length of exercise, timing of exercise initiation or tumour model used. Pedersen et al. began exercise 4 weeks prior to tumour implant, whereas we and Bianco et al. started exercise at tumour implant [30, 37, 109]. Although Zielinski et al. also had a short exercise period of approximately 2 weeks, they used a tumour model which spontaneously regresses, indicating that this tumour cell line induces a strong antitumour immune response in vivo, which is enhanced by exercise [68]. Just one study has investigated B cell numbers and found that they were unchanged in the tumour with chronic exercise [30].

Whether or not absolute numbers of lymphocytes within the tumour change may be less important than the phenotype and cytotoxic functionality of those that are present. As described in the previous section, ex vivo data indicate that exercise may improve NK cell cytotoxicity. Data on T cells is much scarcer. Some studies suggest that exercise reduces T_{reg} cell recruitment to the tumour (inferred from lower levels of the T_{reg} cell recruiting cytokine CCL22 or lower mRNA expression of Foxp3 [108, 110]), but others have found no change in the proportion of intratumoural T_{reg} cells [37, 109] or even an increase in intratumoural Foxp3 mRNA (alongside increased expression of inflammatory/cytotoxic cell markers) following exercise training [30]. Ex vivo functionality assays investigating the effect of exercise on intratumoural T cells have not yet been conducted.

The tumour microenvironment promotes an immunosuppressive phenotype of infiltrating immune cells, causing them to aid rather than inhibit tumour growth both by the inhibition of cytotoxic immune cells and by secreting factors that aid tumour growth such as VEGF [111]. Myeloid cells seem to be particularly susceptible to this reprogramming and often take on an immunosuppressive phenotype within the TME (e.g. M2 macrophages) [112]. Two studies have found reduced neutrophil infiltration into tumours with exercise [68, 113], and two have found reduced macrophage density [68, 114]. Additionally, gene expression of general macrophage markers (F4/80) and M2-specific markers (CD206, arginase) was reduced in mucosal scrapings from exercised compared with non-exercised mice [108]. Together with the above-described ex vivo data, this suggests that exercise reconditions the TME to reduce recruitment of and/or repolarise myeloid cells such as neutrophils and macrophages toward a more antitumour phenotype.

Comprehensive analysis of the types and subtypes of immune cells within the tumour microenvironment following exercise is still lacking. Current preliminary evidence suggests that exercise may repolarise immune cells to an antitumour phenotype and/or increase numbers of antitumour immune cells such as NK cells and $CD8^+$ T cells, but this requires confirmation via flow cytometry, multiplex immunohistochemistry and functional assays, as well as investigation of possible differences between tumour type and exercise protocol.

3.6.2.4 Interplay of Immunity with Hypoxia and Angiogenesis

Hypoxia inhibits antitumour immunity by inhibiting lytic functions of cytotoxic T cells (CTL) and NK cells and promoting an immunosuppressive phenotype in both lymphoid and myeloid cells (reviewed in [115]). In addition, tumour vasculature is prohibitive to T cell entry in that it downregulates adhesion molecules required for extravasation and upregulates inhibitory and apoptotic ligands [116]. Conversely, T_{reg} cells and M2 macrophages can promote angiogenesis, while Type 1 T helper (T_H1) cells can promote intratumoural vessel normalisation [117–119]. Thus, the influence of hypoxia on immune cells and their influence on tumour vasculature (and vice versa) are integral to the overall tumour phenotype.

To our knowledge, just one study has directly investigated T cell subsets, hypoxia and perfusion in the same tumours, although this was in hyperlipidaemic *ApoE*$^{-/-}$ and not wild-type mice [37]. In this study, we found no change in the fraction of intratumoural $CD8^+$ or $Foxp3^+$ T cells, or perfusion, with post-implant exercise, but found a decrease in hypoxia in EO771 tumours from trained mice (using muscular COX-IV expression as a proxy for 'fitness'). In order to better investigate how these aspects of the tumour microenvironment interact and change with exercise, an exercise protocol known to elicit tumour microenvironmental changes (such as a few weeks of pre-implant exercise continuing post-implant) could be used and the tumours analysed for hypoxia, perfusion, vascularity and immune cell composition.

3.6.3 Conclusions on the Immune Microenvironment

In cancer patients, acute exercise-induced lymphocytosis may be partially suppressed [83, 84] and chronic exercise may not be able to protect against chemotherapy-induced lymphopenia [90]. However, preclinical and clinical functional data indicate that exercise improves peripheral NK cell cytotoxicity (from both healthy individuals and cancer patients) [89, 91, 93] and possible repolarisation of macrophages towards an antitumour M1 phenotype [120]. It remains unclear whether these improvements in functionality of peripheral immune cells are translated to improved antitumour immunity within the TME, but they are a promising indication that exercise could improve immune responses in cancer patients.

3.7 Future Directions

As mentioned at the beginning of the chapter, the role of an individual's ability to perform the physiological adaptations required for improvements in exercise capacity (exercise adaptability), and indeed inherent exercise capacity itself, has thus far been largely neglected in exercise oncology. Importantly, there is large inter-individual variation in these factors in both rodents and humans, determined by both inherited factors and activity levels [7–9]. In simple terms, this means that two individuals undergoing exactly the same exercise program will (a) not adapt to exercise to the same degree (chronic response) or (b) feel the same level of exertion (acute response). It is unclear what effect (if any) this might have on how exercise affects tumour characteristics, but is an important avenue of investigation if we are to fully understand how exercise effects physiological change in the TME—the key question being whether improvements in exercise capacity/muscular adaptations are required for beneficial effects of exercise on the TME. Parameters such as VO_2 max, muscle protein synthesis/degradation and skeletal muscle mitochondrial content/function can be used to ascertain exercise capacity and exercise adaptability.

The vast majority of preclinical studies in exercise oncology to date have utilised exercise as a sole intervention (monotherapy). While these

provide valuable insight into exercise-induced systemic and local changes affecting overall tumour growth characteristics, they do not accurately reflect the clinical situation in which a person diagnosed with cancer will almost always receive some form of treatment. A small number of studies have investigated the effect of exercise in combination with other cancer therapies (chemotherapy and hormone therapy) and found a reduction in tumour growth above and beyond that of the cancer treatment alone and sometimes in the complete absence of an exercise-only effect [28, 31, 67, 121, 122]. In addition to growth inhibitory effects, it is likely that the combination of exercise with other therapies will alter the TME distinct from the alterations caused by either therapy or exercise alone. Therefore, it is essential that future work combines exercise with cancer therapies such as chemotherapy, immunotherapy and surgical resection and measures not only the effect on tumour growth rate or survival but also the components of the TME outlined in this chapter.

It is well established that exercise can improve ex vivo antitumour toxicity of select immune cell types (particularly NK cells) isolated from both healthy and tumour-bearing mice [91–95, 98–100] or humans [80, 89, 101, 103, 106, 107]. However, with one exception, these studies have been conducted using immune cells isolated from either peripheral blood or the spleen. While these may give an indication of the individual's general immune functioning, they do not account for effects that the TME might be having on immune cell phenotype. Future studies should aim to determine whether immune cells isolated from the tumour itself display the same enhanced cytotoxic capabilities as those isolated from the spleen or peripheral blood. Ideally, this would even be conducted in vivo, but this is limited by current technology.

With a few exceptions [31, 51, 63], investigation of the effects of exercise on the vascular TME have largely been limited to basic assessment of vessel number (overall, perfused or associated with pericytes) and hypoxic area. This gives a basic idea of functionality, but more comprehensive research into the effect of chronic exercise on tumour vessel haemodynamics would provide a more complete picture of whether exercise can induce similar vascular adaptations in tumour tissue as in skeletal muscle. In addition, how exercise affects the interaction between immune cells and tumour vessels has not yet been fully investigated.

3.8 Concluding Remarks

Exercise oncology is a hugely complex field and requires the collaboration of clinical oncologists, preclinical cancer researchers, immunologists and exercise physiologists (to name a few) for a thorough understanding of exercise and tumour physiology. The current state of knowledge supports a beneficial role of exercise in cancer prevention and survival in some cancer types, but comprehensive mechanistic data remain elusive and robust predictors of tumour response to exercise are non-existent. Delineating the effects of exercise on the TME (and of the tumour on the body, Fig. 3.3) may be the key to unravelling how and in which situations exercise exerts a tumour growth inhibitory effect.

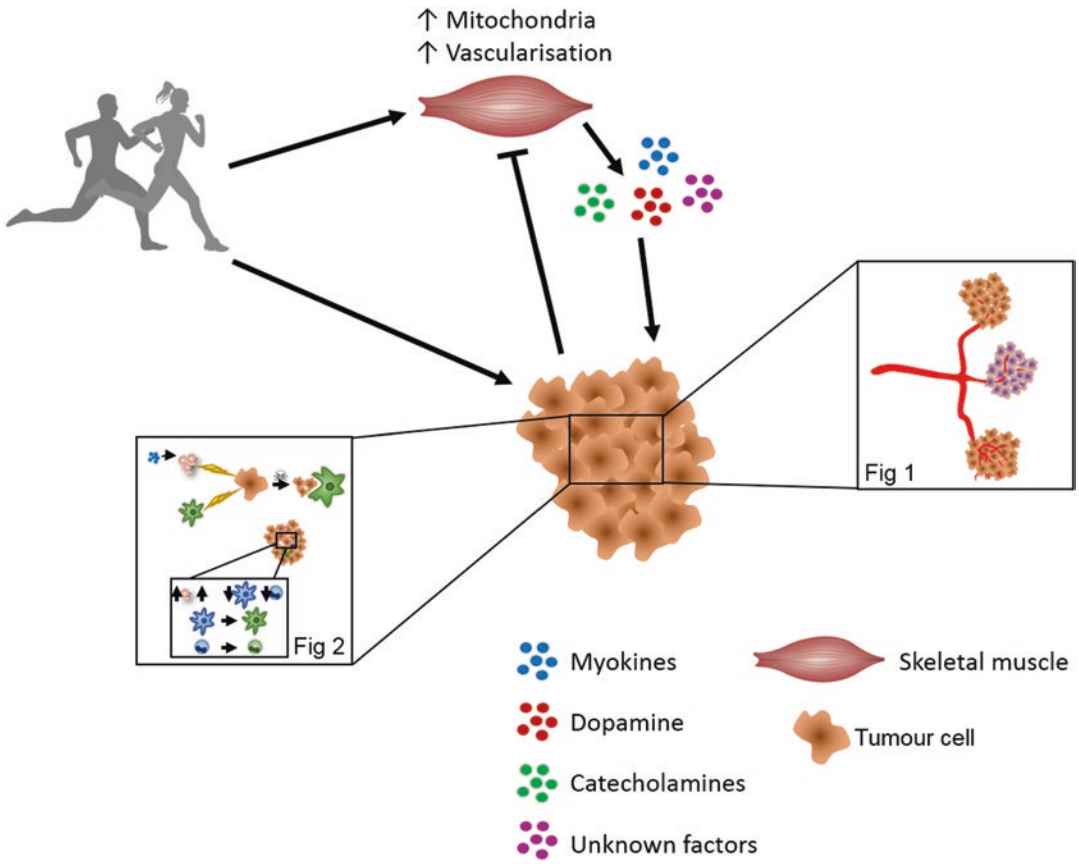

Fig. 3.3 Summary of exercise effects on the TME. Chronic exercise induces skeletal muscle remodelling to improve exercise capacity, including an increase in mitochondrial content and function, and improved vascularisation. However, this response may be blunted in cancer patients and varies strongly between individuals. It is unknown whether this variation in exercise adaptability affects the degree to which exercise can alter the TME. Myokines, dopamine, catecholamines and further unknown factors are released into the circulation with exercise and exert effects on the TME. Effects on vascularisation/hypoxia/perfusion and immunity are summarised in Figs. 3.1 and 3.2, respectively

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4 Effects of Inhaled Tobacco Smoke on the Pulmonary Tumor Microenvironment

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Abstract

Tobacco smoke is a multicomponent mixture of chemical, organic, and inorganic compounds, as well as additive substances and radioactive materials. Many studies have proved the carcinogenicity of various of these compounds through the induction of DNA adducts, mutational potential, epigenetic changes, gene fusions, and chromosomal events. The tumor microenvironment plays an important role in malignant tumor formation and progression through the regulation of expression of key molecules which mediate the recruitment of immune cells to the tumor site and subsequently regulate tumor growth and metastasis. In this chapter, we discuss the effects of inhaled tobacco smoke in the tumor microenvironment of the respiratory tract. The mechanisms underlying these effects as well as their link with tumor progression are analyzed.

Keywords

Tobacco smoke · Carcinogenicity · Lung cancer · Tumor microenvironment · Tumor-associated macrophages · Cancer-associated fibroblasts · Extracellular matrix · Epithelial-mesenchymal transition · Angiogenesis · Metabolism · Acute inflammation · Chronic inflammation · Epigenetics · Tumor growth · Metastasis

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4.1 Tobacco Smoke

4.1.1 Composition of Tobacco Smoke

4.1.1.1 Nicotine

Nicotine, composing 0.2–0.6% of the particulate phase of tobacco smoke, is the main addictive compound of tobacco smoke and, while it is a weak carcinogen, is responsible for tobacco addiction and continued smoking. Nicotine exerts its addictive functions by its interaction with neuronal nicotinergic acetylcholine receptors in the brain [1]. As soon as it is inhaled, smoke reaches the airways and alveoli, and nicotine is absorbed by the lungs. Pulmonary absorption of nicotine is mediated by the alkaline pH of cigarettes, which converts nicotine to its nonionized form. Following absorption, nicotine enters the bloodstream and is distributed to the various bodily organs. Nicotine is metabolized in the liver by the

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enzymes cytochrome P450 2A6 (CYP2A6), uridine diphosphate glucuronosyltransferase (UGT), and flavin-containing monooxygenase (FMO) to a number of metabolites, the most important of which is cotinine. Cotinine is the most well-known biomarker for detecting nicotine levels, measured in blood, saliva, urine, hair, and nails [2].

4.1.1.2 Chemical Carcinogens

Polycyclic Aromatic Hydrocarbons (PAH)

PAH have been linked with the induction of tumors in the skin and lungs [3, 4]. The members of this compound family that are proven to be carcinogenic are benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, dibenzo[*a,i*]pyrene, indeno[1,2,3-*cd*]pyrene, dibenz[*a,h*]anthracene, and 5-methylchrysene [5]. Smokers present higher metabolic activation of dibenzo[*a,i*]pyrene (BαP), mediated by aryl hydrocarbon hydroxylase (AHH) activity [6, 7], which is connected with higher cancer risk [8]. Furthermore, PAH are responsible for the induction of DNA adduct formation in the *TP53* gene [9].

Nitrosamines

N-Nitrosodimethylamine was found in 1956 to induce liver tumors in rats [10]. Since then, increasing interest on the carcinogenic potential of nitrosamines aroused. Metabolism of nicotine produces nitrosamines, with *N′*-nitrosonornicotine(NNN),4-(methylnitrosamino) -1-(3-pyridyl)-1-butanone (NNK), and 4-(methylnitrosamino)-1-(3-pyridyl)butanal (NNAL) being the most carcinogenic [11, 12], mainly causing adenomas and adenocarcinomas [13].

Butadiene

Exposure of mice to inhalation of 1,3-butadiene induced alveolar and bronchiolar carcinomas, as well as lymphoma and forestomach papilloma [14]. Butadiene is metabolized to carcinogenic epoxybutene, diepoxides, and diol epoxide.

Ethyl Carbamate (Urethane)

Urethane, also known as ethyl carbamate, or carbamic acid ethyl ester, is an ester of carbamic acid. Many studies in experimental animals support the carcinogenic role of urethane in various tissues and through different routes of administration. Urethane-induced tumors of the lung (adenocarcinomas and squamous cell carcinomas), as well as of the liver (hepatocellular carcinomas), and blood vessels (hemangiomas or hemangiosarcomas of the liver, spleen, uterus, or unspecified site) have been observed in many studies [15–18]. Since then, urethane has been used for induction of tumors in mice models [19–21].

4.1.1.3 Radioactive Materials

Except for chemicals, tobacco smoke also contains radioactive elements, including uranium and thorium isotopes (^{234}U, ^{238}U, ^{228}Th, ^{230}Th, ^{232}Th), as well as products of their decay (e.g. ^{226}Ra, ^{210}Pb, ^{210}Po) [22, 23]. Radioactive materials enter the tobacco plant through the soil and phosphate fertilizers, or through direct deposition of airborne ^{222}Rn products. Smoking results in their absorption by the respiratory system and the subsequent increased risk for lung cancer [24, 25].

4.1.1.4 Reactive Oxygen Species (ROS)

ROS are a family of oxygen-derived small molecules that contain oxygen radicals such as superoxide (O_2), hydroxyl (OH), peroxyl (RO_2), and alkoxyl (RO), as well as non-radicals such as hypochlorous acid (HOCl), ozone (O_3), and hydrogen peroxide (H_2O_2). ROS play key roles in homeostasis and intracellular signaling. However, the disruption of the balance between antioxidant defense mechanisms and ROS production leads to DNA damage, mediates oxidative stress, and is implicated in cancer progression. ROS are directly synthesized by the enzymes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (MPO). ROS are produced endogenously as a product of cellular respiration, although there are also exogenous factors driving their production, such as ionizing radiation and tobacco smoking [26]. ROS dam-

age airway epithelial cells through lipid peroxidation of the cell membrane, activation of oxidative-sensitive cellular pathways, and DNA damage [27].

4.1.1.5 Tobacco Additives

The word additive is used for compounds "...the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristic of any tobacco product …" [28]. Tobacco additives are used in order to reduce its alkaloid bitterness resulting in easier nicotine delivery to the user. Levulinic acid decreases the sensitivity of the upper respiratory tract, resulting in deeper inhalation in the respiratory system, while at the same time it mediates the binding of nicotine to neurons [29]. Pyrazines enhance product appeal, mediate easier initiation of smoking, and promote relapse [30]. Menthol increases the smoothness of the smoke and subsequently enhances deeper inhaling due to its cooling effect. Therefore, tobacco additives increase the attractiveness and addictiveness of tobacco increasing smokers' exposure to toxic compounds contained in smoke and resulting to health risks. However, there are no sufficient studies regarding the toxicity of the additives alone, since tobacco smoke is a multicomponent mixture, with the different compounds interacting with each other [31].

4.1.1.6 Other

Tobacco smoke contains inorganic compounds—metals, such as arsenic, cadmium, chromium, and nickel, all of them related to high risk of different types of cancer [32]. Other agents contained in tobacco smoke and also related to increased risk for lung cancer are isoprene, benzene, acetaldehyde, and formaldehyde [5].

4.1.2 Carcinogenicity of Tobacco Smoke

4.1.2.1 Epidemiologic Evidence

Tobacco smoke constitutes the largest exposure of humans to chemical carcinogens. It causes one out of five cancer-related deaths in the world and 1.4 million deaths per year. The largest effect of tobacco smoke is on lung cancer, constituting the cause for 80% and 50% of global lung cancer deaths for men and women, respectively [33]. However, tobacco smoke has also been linked with a variety of cancers other than lung cancer types, such as cancers of the oral cavity, pharynx, larynx, esophagus, pancreas, bladder, stomach, liver, kidney, ureter, cervix, and nasal cavity, as well as myeloid leukemia [32, 34].

4.1.2.2 Molecular Evidence

The Cancer Genome Atlas (TCGA) project aims to collect and analyze human tissues in order to generate comprehensive multidimensional maps of the key genomic changes in 33 types of cancer [35]. Lung cancer is a dominant malignancy, resulting in the largest number of cancer-related deaths worldwide [36] and lung adenocarcinoma (LADC) is its most frequent histologic subtype [37, 38]. LADC is mainly caused by environmental exposures such as tobacco smoke (TS) and high-energy transfer irradiation (IR) [39–42]. TS is the predominant cause of lung cancer [43]; however, there is a worldwide increase in the number of lung cancers in nonsmokers [44, 45]. Molecular profiling of lung cancers has revealed a heterogeneous disease that harbors thousands of mutations per cancer genome, including single nucleotide variants (SNV), copy number alterations (CNA), dysregulation of alternative splicing (exon skipping, EXS), balanced inversions resulting in gene fusions, and major chromosomal events like kataegis and chromothripsis [35, 46, 47]. LADC mutations lead to activation of proto-oncogenes such as *KRAS*, *EGFR*, and *PIK3CA* and inactivation of tumor suppressors such as *TP53*, *STK11*, and *PTEN* [48]. Interestingly, the genomic profiles of LADC differs between smokers and nonsmokers, with smokers displaying higher mutation burdens [35].

4.1.2.3 Experimental Evidence

The carcinogenicity of some compounds of tobacco smoke has been proven in vivo. Using single-hit models, LADC development was achieved in carcinogen-sensitive *FVB* mice

6–9 months posttreatment with intraperitoneal injection of urethane and diethylnitrosamine [21, 49]. Moreover, metabolically activated 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N′*-nitrosonornicotine (NNN) are implicated in carcinogenesis by inducing mutations which result in the formation of DNA adducts, promoting tumor growth, cancer cell survival, and migration [50]. In vivo studies have shown that NNN causes esophageal and nasal tumors in rats and respiratory tract tumors in mice and hamsters [51–53]. Furthermore, Westcott et al. showed that the mutational signatures of LADC differ according to the causative chemical: genome, exome, and transcriptome sequencing of genetic- and chemical-induced *KRAS*-driven murine LADC revealed that the chemical carcinogens urethane and *N*-nitroso-*N*-methylurea (MNU) caused humanlike SNV and distinct *KRAS* mutations (Q61R for urethane and G12C for MNU) [54].

4.1.2.4 Signatures of Tobacco Smoke

Alexandrov et al. defined mutational signatures in the trinucleotide context (i.e., the bases immediately 5′ and 3′ to each mutated base) and correlated these with clinical exposure data across more than 20 cancer types and 10,000 patients, identifying the smoking signature 4 (C>A transversion) [34, 55]. Lung tumors of smokers and nonsmokers do not only display distinct mutational signatures and gene expression profiles [34, 55], but also different inflammatory signatures [56]. In comparison with never-smokers, the tumor microenvironment of smokers includes fewer resting mast cells and CD4+ memory T cells, both linked with favorable survival [56]. Furthermore, tobacco smoking induces pro-inflammatory changes in the tumor microenvironment of squamous cell lung carcinomas, as determined by interferon-c signaling, cytosolic activity, and immune infiltration [57]. These data are in line with clinical studies that show that smokers with LADC have a higher response to immune checkpoint inhibitors [58]. Moreover, lung tumors of smokers and nonsmokers exhibit distinct DNA methylation profiles [59, 60].

4.2 The Tumor Microenvironment

4.2.1 The Role of the Microenvironment in Tumor Formation and Progression

In addition to the molecular heterogeneity of tumor cells, there is also cellular heterogeneity of the tumor microenvironment with which tumor cells interact [61, 62]. While tumor initiation is mediated by mutations in oncogenic driver genes, tumor progression is rather affected by interactions between cancer cells and their microenvironment. Oncogenic changes of tumor cells establish complex inflammatory signaling networks through suppression of homeostatic chemokines and de novo production of cytokines, chemokines, and their receptors by both cancer and stromal cells [63–66]. This complex network results in the migration and infiltration of various cellular populations, including tumor-associated macrophages (TAMs), mast cells, lymphocytes, and other cells to the stoma in response to chemokine gradients created by stromal and malignant cells of a tumor, which results in the establishment of an inflammatory microenvironment [67].

4.2.1.1 Tumor-Associated Macrophages (TAMs)

TAMs are the most abundant inflammatory cell type in tumors, represent a crucial component of the tumor microenvironment, and have a key role in cancer progression as indicated by several studies which describe a slower tumor growth after the depletion of macrophages, as well as by the association of TAM with poor disease outcome [68, 69]. The expression of growth factors such as colony stimulating factor (CSF)-1 and chemokines in cancers results in the recruitment of circulating monocytes which differentiate to macrophages. In addition to their physiological roles in immune response, phagocytosis, antigen-presentation, and pathogen killing, macrophages are implicated in tumor promotion via immunoediting [64, 70], although there is also evi-

dence of their antitumor functions, dependent on the cytokine microenvironment of the tumor [70]. Macrophages enable angiogenesis through secretion of proangiogenic mediators like vascular endothelial growth factor (VEGF) and angiopoietins (ANG)-1 and ANG-2 and mediate invasion and metastasis by producing growth factors and matrix metalloproteases (MMP). In order for TAM to acquire protumorigenic functions, they polarize from a pro-inflammatory (M1) to an "alternatively activated" anti-inflammatory (M2) phenotype.

4.2.1.2 T Lymphocytes

T cell populations infiltrate tumors and play key roles in the establishment of an inflammatory microenvironment which favors cancer progression. CD8 memory T cells are antigen-presenting cells with tumor suppressor activity and are related with good prognosis in human tumors [71]. The interplay between CD8 and CD4 T cells is important for tumor immunity. CD4 T helper 1 (Th1) cells enable recruitment and proliferation of CD8 T cells through an interferon (IFN)-γ- and IL-2-dependent mechanism [72]. CD4 cells' presence in the tumor microenvironment has also been linked with good prognosis [71]. Th2 CD4 cells have ambiguous roles in tumor progression, as Fridman et al. reported that they promote tumor growth [71], although other studies link them with favorable outcome in breast cancer patients [160, 161]. T regulatory cells (Treg) function as immune suppressors, which, through the secretion of IL-10 and transforming growth factor (TGF)-β, prevent the clearance of cancer cells by the immune system [73, 74].

4.2.1.3 B Lymphocytes

B lymphocytes are recruited to tumor sites in response to T helper cell-secreted C-X-C-motif chemokine ligand (CXCL) 13 [75]. Tumor-infiltrating B cells activate nuclear factor (NF)-κB canonical and noncanonical pathways through the secretion of lymphotoxin, mediating tumor growth and cell proliferation, as well as angiogenesis [76–78]. Furthermore, B cells promote metastasis by inducing increased expression of IL-8 [79].

4.2.1.4 Cancer-Associated Fibroblasts (CAFs)

CAFs are an important cell population within the tumor microenvironment that promotes cancer progression and invasion [80, 81]. As a component of the stroma, fibroblasts are responsible for the production of collagens and fibronectin and the subsequent synthesis of the extracellular matrix (ECM) [82] and the basement membrane [83]. During carcinogenesis, normal stromal fibroblasts undergo several changes including their morphological characteristics, their expression of cell surface markers [81], and their metabolism via the reverse Warburg effect [84]. The causes for transformation of fibroblasts to CAF are unknown, but mutations appear to occur in these cells, too, such as inactivation of *TP53* and *PTEN* [85] and loss of heterozygosity (LOH) [86]. Furthermore, CAF production can be induced by epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT) [81]. CAFs have been associated with enhanced tumor growth [87, 88], cell migration and invasion [89], and a pro-inflammatory microenvironment that facilitates metastasis [90–92].

4.2.1.5 The ECM of the Tumor Microenvironment

The ECM is a complex network of macromolecules with different physical and biochemical properties, and its deregulation is one of the hallmarks of cancer [93]. The deposition of different collagens is increased during tumor formation and progression [94]. Furthermore, breast cancer ECM appears to be stiffer than normal breast ECM, mediating tumor cell invasion and progression via a lysyl oxidase (LOX)-dependent mechanism [95]. ECM changes potentiate the deregulation of cellular behavior and enable malignant transformation [96]. Moreover, tumor ECM has a key role in angiogenesis, as many ECM compounds interact with VEGF regulating the formation of new vascular branchings [97]. Tumor cells, TAM, and CAF secrete MMP that remodel the ECM of tumors [61] and mediate angiogenesis [96]. ECM can also mediate the differentiation and maturation of immune cells and

the promotion of an inflammatory tumor microenvironment [96].

4.3 Impact of Tobacco Smoke on the Tumor Microenvironment

4.3.1 Acute Effects of Continued Smoking

4.3.1.1 Angiogenesis

Exposure to tobacco smoke has been linked to the formation of new vessels (neoangiogenesis) [98], with nicotine being the most well-studied compound responsible for this [99]. Angiogenic dysplasia lesions were more frequent in the bronchi of smokers compared with nonsmokers and were related to higher risk for lung cancer [100]. Furthermore, exposure to environmental tobacco smoke induced tumor growth and enhanced vessel density in a murine model of lung cancer and stimulated circulating endothelial cell precursors [101], in accord with data that demonstrate that tobacco smoke exposure of murine lung tissues increases angiogenesis and circulating leukocytes [102]. When Lewis lung cancer cells were injected in mice, systemic nicotine administration enhanced tumor growth by increasing capillary density [103]. The mechanism of tobacco smoke-mediated angiogenesis includes stimulation of endothelial nicotinergic acetylcholine receptors (nAchR) of the α7 homodimeric type by nicotine with subsequent interactions between nAchR and angiogenic growth factor receptors [104].

4.3.1.2 Tobacco-Triggered EMT

During carcinogenesis, polarized epithelial cells undergo EMT and acquire a mesenchymal phenotype. EMT has been linked with molecular, biochemical, and morphological cellular changes that lead to detachment from the basolateral membrane, loss of cell adhesion, cytoskeletal reorganization, changes in the interaction with the ECM, and angiogenesis. Cells that undergo EMT acquire higher migration capacity and invasion potential, both required for conversion of benign cells to invasive cancer cells [105]. Furthermore, EMT can give birth to CAF in the tumor microenvironment that, in turn, contribute to cancer progression [81]. Tobacco smoke has been linked with EMT: MCF7 breast cancer cells acquired mesenchymal phenotypes upon long-term aqueous tobacco smoke exposure in vitro, which enhanced their potential for growth, migration, and invasion, as well as their metastatic potential in vivo [106]. Endobronchial biopsies of COPD patients revealed that smokers had a hyperfragmented basement membrane with increased expression of MMP9, the fibroblast protein S100A4, and the mesenchymal marker vimentin compared to nonsmokers [107]. Tobacco smoke induces the expression of mesenchymal markers α-smooth muscle actin (α-SMA), vimentin, and type I collagen in human bronchial epithelial cells (HBEC) derived from nonsmokers [108]. These data together indicate that tobacco smoke contains a variety of active compounds that trigger EMT via different signaling pathways.

ROS and EMT

Milara et al. reported that tobacco smoke-induced EMT is mediated by ROS [108]. Increased ROS production results in NF-κB activation [109], as well as Rac1-mediated MMP3 expression [110]. The subsequent Rac1/MMP3-mediated binding of NF-κB subunits p65 and cRel to the Snail promoter, a key transcription factor for EMT [111], which inhibits the expression of epithelial junction proteins while inducing the expression of cytoskeleton proteins [112]. Another mechanism which underlies the potential of ROS to induce EMT includes activation of tumor necrosis factor (TNF) converting enzyme (TACE) [113] which subsequently activates epidermal growth factor receptor (EGFR) signaling via the Ras/Raf/MAPK, PI3K/Akt, and Src pathways, thereby enhancing cell proliferation and migration [114]. Src signaling, a key for EMT, is directly activated by ROS family members peroxynitrite and H_2O_2 resulting in increased expression of mesenchymal proteins, cytoskeletal reorganization, and disruption of cell matrix adhesion [115]. Along other lines, tobacco

smoke-induced ROS decreased Na,K-ATPase activity and NaK-α1 levels, resulting in disruption of tight junctions, alterations in cell polarity, and early EMT [116]. In conclusion, ROS mediate EMT by increasing cellular invasion potential into the ECM, by mediating ECM remodeling, by decreasing cellular adhesion, and by increasing cell motility (Fig. 4.1) [117].

Nicotine in EMT

The role of nicotine in inducing tumor growth and metastasis has been described in mouse models of LADC in vivo [118]. Nicotine mediates EMT through nAchR-dependent and nAchR-independent mechanisms [119]. nAchR-independent nicotine-induced EMT primarily rests on activation of TGFβ signaling [108, 120–123], which results in disassembly of epithelial tight junctions, cytoskeletal changes, downregulation of E-cadherin, and nuclear translocation of β-Catenin. Wnt signaling is also activated by nicotine and promotes EMT [124]. Moreover, Wnt enhances expression of Snail, a key to EMT [125]. nAchR-independent nicotine-induced EMT is also mediated by periostin, which is upregulated by nicotine, subsequently increasing Snail expression, cell proliferation, and invasion [119, 126]. Nicotine also mediates EMT via nAchR binding with subsequent recruitment of β-arrestin and Src and activation of MAPK [119, 127, 128]. In addition, nicotine increases mucin MUC4 production in pancreatic cancer through

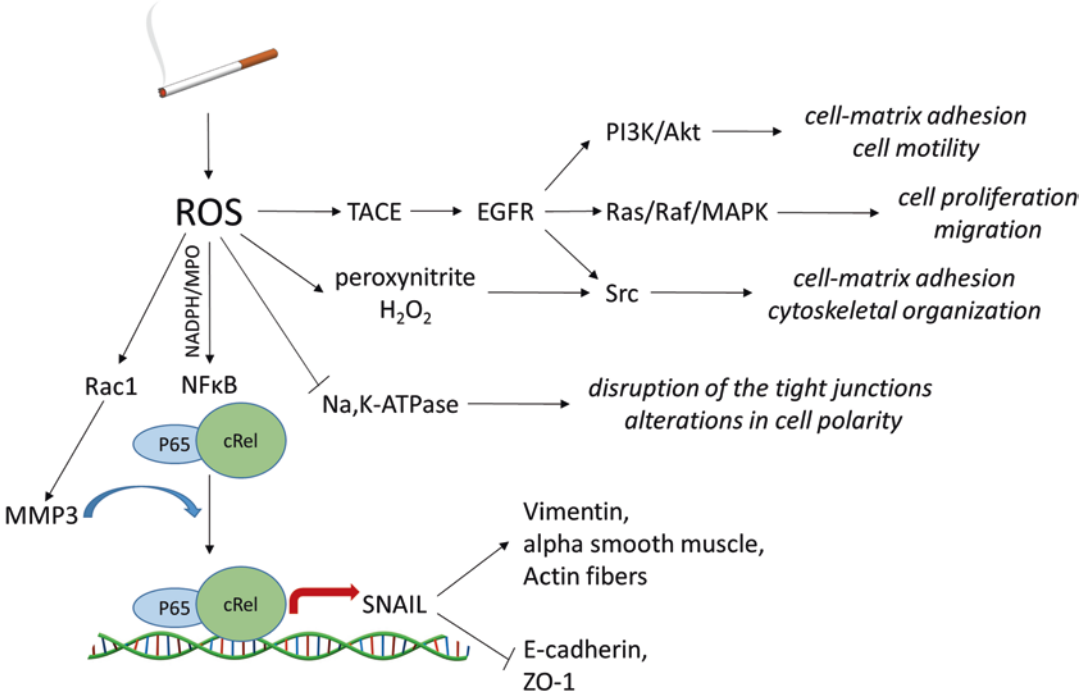

Fig. 4.1 ROS mediate tobacco smoke-induced EMT. The molecular mechanisms implicated in the induction of EMT driven by ROS include the activation of NF-κB signaling pathway, which in combination with the Rac1-mediated MMP3 expression regulates the transcriptional activation of Snail, resulting in the inhibition of the expression of epithelial junction proteins and the induction of the expression of cytoskeleton proteins. The cascade of TACE activation, EGFR signaling, Ras/Raf/MAPK, PI3K/Akt, and Src pathways regulate cell-matrix adhesion and enhance cell motility. ROS- mediated inhibition of Na,K-ATPase activity further contributes in EMT

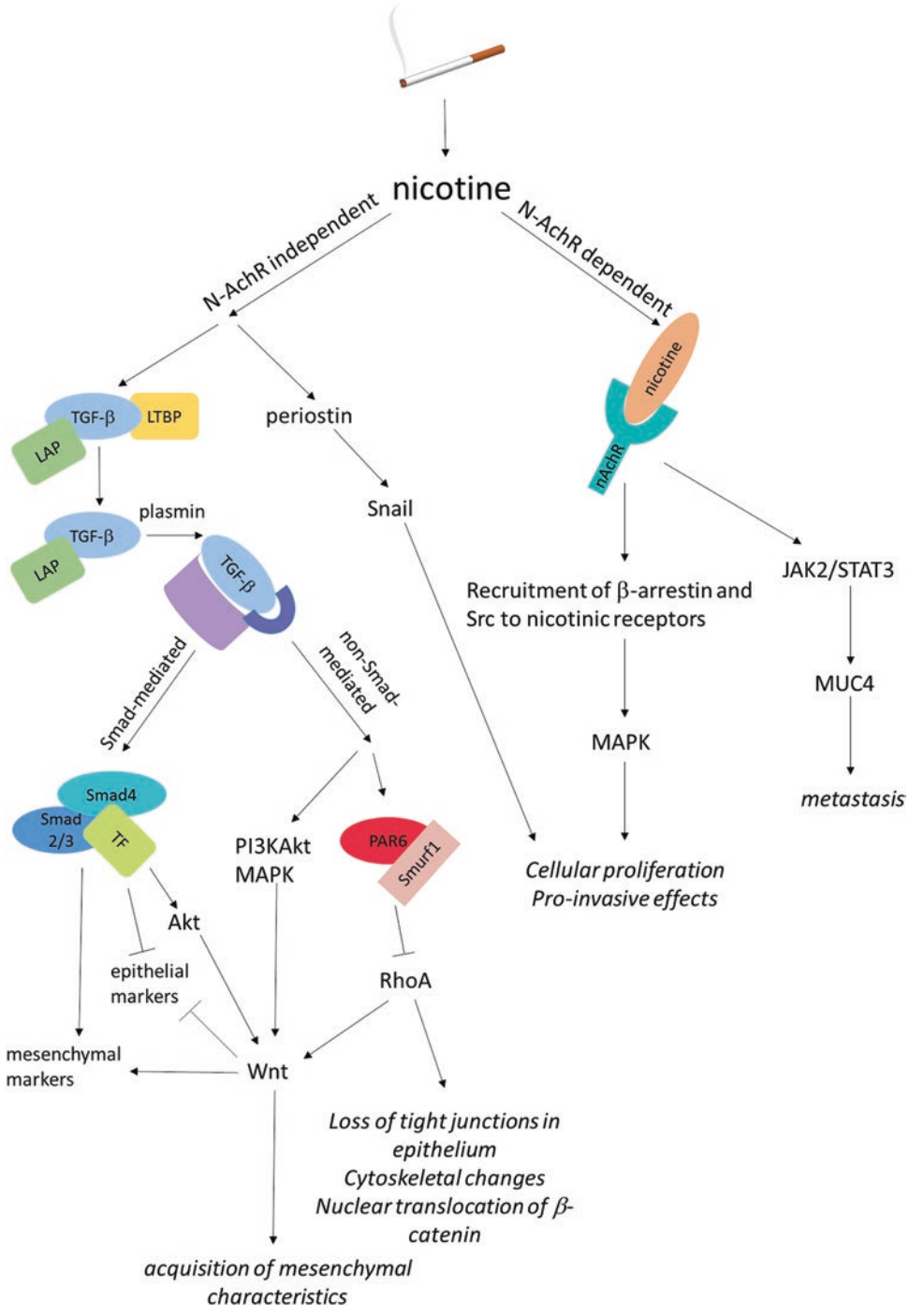

Fig. 4.2 Nicotine mediates tobacco smoke-induced EMT. The nA-chR-independent molecular mechanisms of nicotine-mediated EMT include TGFβ signaling, Wnt signaling and periostin-mediated Snail activation. The nAchR-dependent mechanisms include the recruit-ment of β-arrestin and Src to nicotinic receptors, the activation of MAPK cascade as well as the activation of activation of α7nAchR/JAK2/STAT3 signaling. The subsequent alterations in epithelial tight junctions, cytoskeletal changes, enhancement of cell motility and invasion enhance EMT

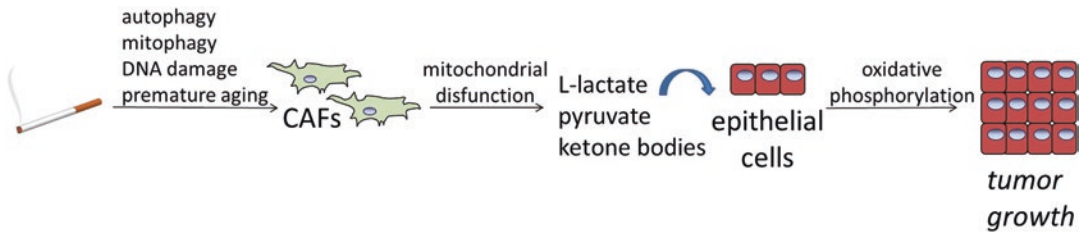

Fig. 4.3 Tobacco smoke-induces metabolic alterations through two-compartment tumor metabolism. Autophagy, mitophagy, DNA damage and premature aging convert immortalized human stromal fibroblasts in CAFs which undergo myofibroblast differentiation and mitochondrial dysfunction, resulting in secretion of high-energy mitochondrial fuels. The epithelial cancer cells use these metabolites enhancing tumor growth

activation of α7nAchR/JAK2/STAT3 signaling, thereby inducing metastasis (Fig. 4.2) [129].

PAH in EMT

PAH are organic compounds which consist of two or more fused aromatic rings. BαP, a compound of tobacco smoke that belongs to the PAH family, is connected with increased expression of EMT-related genes such as fibronectin, TWIST, and TGF-β2 [130]. Furthermore, PAH mediate activation of arylhydrocarbon receptors (AhR), which subsequently activate the transcription factor Slug, thereby enhancing EMT [131]. AhR-induced c-Jun N-terminal kinase (JNK) activation results in cytoskeletal remodeling and increased cellular migration [132].

4.3.1.3 Tobacco-Induced Metabolic Alterations

Tobacco smoke metabolically mediates cancer progression via autophagy and premature aging in the tumor microenvironment [133]. Tobacco smoke induces autophagy, mitophagy, DNA damage, and premature aging of immortalized human stromal fibroblasts, resulting in the production of CAF that mediate tumor growth [80, 133, 134]. CAFs undergo myofibroblast differentiation and mitochondrial dysfunction, resulting in secretion of high-energy mitochondrial fuels, such as L-lactate, pyruvate, and ketone bodies. These metabolites are subsequently used by epithelial cancer cells, thereby enhancing ATP generation via oxidative phosphorylation and promotion of tumor growth. This energy shuttling has been coined two-compartment tumor metabolism [134]. Tobacco smoke can also induce the reverse Warburg effect [84], by accelerating aging in the host microenvironment, which through a paracrine mechanism leads to cancer promotion (Fig. 4.3).

4.3.1.4 Tobacco Smoke-Induced Acute Inflammation of the Tumor Microenvironment

Acute effects of tobacco smoke have been studied in both in vitro and in vivo systems. In all models, neutrophils were found to be recruited immediately after acute smoke exposure, followed by alveolar macrophages. Eosinophils also increase in response to acute smoke exposure. Fibroblasts are implicated in the respiratory inflammatory signature induced by acute smoke exposure, through their inhibition and subsequent abnormalities in the repair mechanisms of the lung [135]. Except for the regulation of the recruitment of immune cells, tobacco smoke acute effects on inflammatory processes are also mediated via regulation of expression of various inflammatory mediators, such as neutrophil elastase, leukotrienes, and IL-6 [135].

4.3.2 Perpetual Impact of Past Smoke Exposure

4.3.2.1 Tobacco Smoke-Induced Chronic Inflammation

Chronic inflammation is the result of the failure of inflammatory cells to eliminate pathogens and

it involves both the adaptive and innate immune systems. The lungs are continuously exposed to environmental agents that can cause injury and have been strongly linked to chronic obstructive pulmonary disease (COPD) and lung cancer [136–138]. Tobacco smoke contains many components with immunomodulatory function, such as nicotine, ROS, nitrogen oxide, acrolein, carbon monoxide, and toxins [139, 140]. These components induce inflammatory mediator release (IL-8 and TNF-α) and chemokine secretion by airway epithelial cells [141, 142], through induction of epithelial intracellular cascades, such as Ras [143], MAPK, NF-κB, STAT, AP/1, and ERK [144–146]. These result in regulation of the inflammatory cell cycle, but also altered regulation of cell death [146], culminating tobacco smoke-induced airway inflammation. Another mechanism that has been suggested to mediate tobacco smoke-induced inflammation of the airways involves thymic stromal lymphopoietin (TSLP) secreted by both epithelial [147] and airway smooth muscle cells [148], which induces dendritic cell activation resulting in Th2 polarization [149] and subsequent allergic airway inflammation. Except for the induction of pro-inflammatory responses, tobacco smoke also diminishes the responsiveness to infections, with both mechanisms synergistically leading to chronic inflammation. Tobacco smoke downregulates the expression of the endogenous secreted antimicrobial peptide human beta defensin-2 compromising immune responses [150]. Furthermore, tobacco smoke suppresses the phagocytic function of alveolar macrophages [151], as well as the functions of circulating NK cells by downregulating IFN-γ and TNF-α in smokers [152]. Moreover, tobacco smoke induces mucus hypersecretion, resulting in diminished clearance of infections [153]. In conclusion, tobacco smoke triggers airway inflammation and impairs defense against infections and pathogens, all together leading to chronic inflammation (Fig. 4.4).

4.3.2.2 Epigenetic Changes

Smokers and nonsmokers show distinct profiles of DNA methylation [59, 60]. In vitro studies demonstrated that exposure of respiratory epithelial cells to tobacco smoke induces epigenetic changes [154]. Vaz et al. exposed HBEC cells to tobacco smoke for 10–15 months and observed changes in colony formation potential, EMT properties, MEK, RAS, EGFR, and WNT signaling and malignant phenotype after induction of $KRAS^{V12}$ mutations. However, whole exome sequencing did not reveal any driver mutations underlying the effects of tobacco smoke exposure. Changes in the DNA methylation pattern of the cells exposed to tobacco smoke were observed in genes which are frequently methylated in lung adenocarcinoma and squamous cell carcinoma, such as SFRP2, SFRP5 and WIF1, implicated in WNT signaling; MSX1, mediating the p53 function; and BMP3, WIF1 and GATA4, important for the RAS/MAPK signaling cascade. [155]. The mechanism underlying the effects of tobacco smoke on DNA methylation pattern might include AhR, which is a transcription factor mediating downstream histone modification related to risk of cancer [156, 157]. Thus tobacco smoke causes epigenetic changes, driven by mutations such as single $KRAS^{V12}$ mutation, which synergistically lead to oncogenic transformation of respiratory epithelial cells [158].

4.4 Future Trends and Directions

The pattern of accumulation of mutations inflicted by tobacco smoke during oncogenesis, the cell types of origin of lung adenocarcinoma, and the molecular mechanisms implicated during the progress of the disease have not been completely determined [35, 54, 159]. Understanding the cellular and molecular base of different causative factor-induced LADC through physiologically relevant mouse models of environmentally induced LADC, high-throughput sequencing, and carefully phenotyped and molecularly characterized human cohorts could lead to the discovery of new therapeutic targets, contribute to personalized medicine, and help for integration of exposure/molecular data into mechanistic risk prediction models.

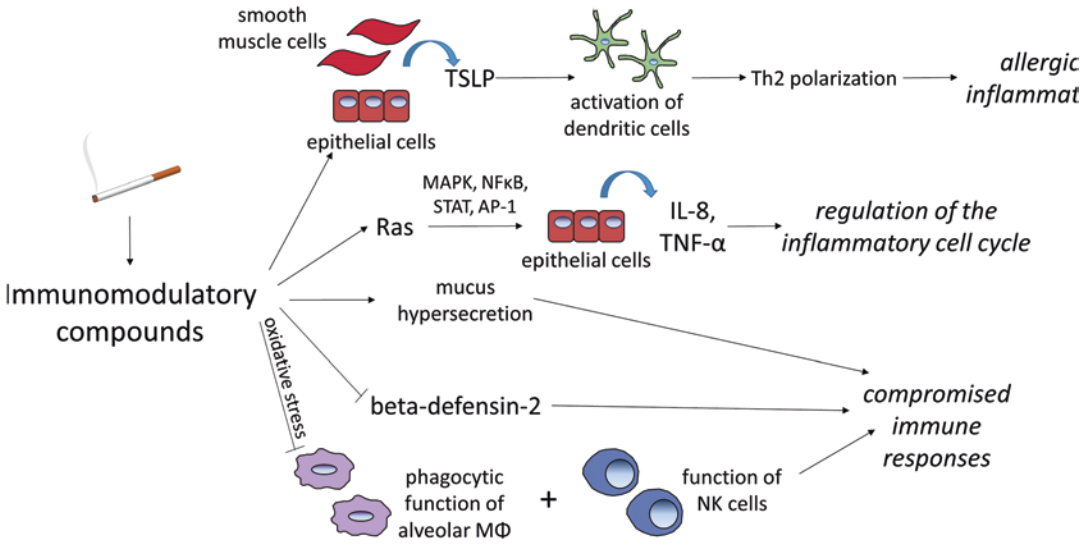

Fig. 4.4 Tobacco smoke favors chronic inflammation. The immunomodulatory compounds of tobacco smoke induce the secretion of TSLP from the epithelial and airway smooth muscle cells, with the subsequent activation of dendritic cells and Th2 polarization, re-sulting in allergic inflammation. Ras, MAPK, NF-κB, STAT, AP/1, and ERK cascades mediate the secretion of IL-8, TNF-α and chem-okines by airway epithelial cells, regulating the inflammatory cell cycle. The reduction of responsiveness to infections is mediated by the inhibition of beta defensin-2 and the suppression of the functions of both alveolar MΦ, and NK cells

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Multimodal Molecular Imaging of the Tumour Microenvironment

5

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Abstract

The tumour microenvironment (TME) surrounding tumour cells is a highly dynamic and heterogeneous composition of immune cells, fibroblasts, precursor cells, endothelial cells, signalling molecules and extracellular matrix (ECM) components. Due to the heterogeneity and the constant crosstalk between the TME and the tumour cells, the components of the TME are important prognostic parameters in cancer and determine the response to novel immunotherapies. To improve the characterization of the TME, novel non-invasive imaging paradigms targeting the complexity of the TME are urgently needed.

The characterization of the TME by molecular imaging will (1) support early diagnosis and disease follow-up, (2) guide (stereotactic)

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biopsy sampling, (3) highlight the dynamic changes during disease pathogenesis in a non-invasive manner, (4) help monitor existing therapies, (5) support the development of novel TME-targeting therapies and (6) aid stratification of patients, according to the cellular composition of their tumours in correlation to their therapy response.

This chapter will summarize the most recent developments and applications of molecular imaging paradigms beyond FDG for the characterization of the dynamic molecular and cellular changes in the TME.

Keywords

Tumour microenvironment · PET · MRI · Molecular imaging · Cancer · TAM · GAMM · Myeloid derived suppressor cells · Tumour infiltrating lymphocytes · Immunotherapy · Glioma · Cancer-Associated Fibroblasts · TSPO · Vasculature · TME

5.1 Composition of the Tumour Microenvironment and Possible Imaging Targets

As detailed by Hanahan and Weinberg in "Hallmarks of cancer: next generation", tumours are considered as complex dynamic tissues with an important interplay of distinct cell types, altogether forming the tumour microenvironment (TME) [1]. The TME hosts multiple cell types, some with stem cell-like capacity, some originating from the peripheral immune system and others known to be tumour-associated parenchymal cells, such as vascular cells and tumour-associated fibroblasts as well as various immune cells and their precursor [2] (Fig. 5.1).

The non-invasive characterization of the tumour microenvironment by molecular imaging is of importance, as it (1) supports early diagnosis and disease follow-up, (2) guides (stereotactic) biopsy sampling, (3) follows the dynamic changes during disease pathogenesis in a non-invasive manner, (4) monitors existing therapies, (5) supports the development of novel tumour-microenvironment-targeting therapies and (6) stratifies patients, according to the cellular composition of tumours in correlation to their therapy response [3]. The term molecular imaging refers to several imaging technologies, namely, positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, optoacoustic, bioluminescence and fluorescence imaging. Preclinical and clinical multimodal imaging studies have dramatically increased our understanding of the tumour biology and disease pathogenesis. Consequently, molecular imaging became clinical standard, especially in oncology [4]. In particular, the high sensitivity of PET in the pico- to the nanomolar range is suitable for the development of tumour-microenvironment-specific tracers and allows to study in vivo the dynamic cellular and molecular changes in the TME. Based on the advantages of this imaging technique, this chapter will mostly focus on the application of PET.

Commonly used positron-emitting isotopes include ^{18}F, ^{11}C, ^{68}Ga and ^{64}Cu, but in the context of antibody (Ab)-based tracers, long-lived isotypes like ^{89}Zr are emerging.

2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) was the first tracer used to image tumours in the 1980s, based on the high glucose metabolism of cancer cells correlating with high FDG accumulation [5]. FDG is therefore routinely used in many types of cancer and inflammatory diseases [6]. However, non-malignant cells, like inflammatory cells also metabolizing glucose, can influence imaging findings. Thus, FDG lacks the necessary specificity that allows the precise imaging of cellular and/or metabolic parameters of the tumour microenvironment.

Cell proliferation is another parameter that can be monitored with PET using the thymidine analogue 3′-deoxy-3′-[^{18}F]fluorothymidine ([^{18}F]FLT) [7]. Its uptake correlates with tumour proliferation and can therefore be used for monitoring therapy-induced changes of cancer treatments, as described in the systematic review of preclinical [^{18}F]FLT studies by Schelhaas et al. [8].

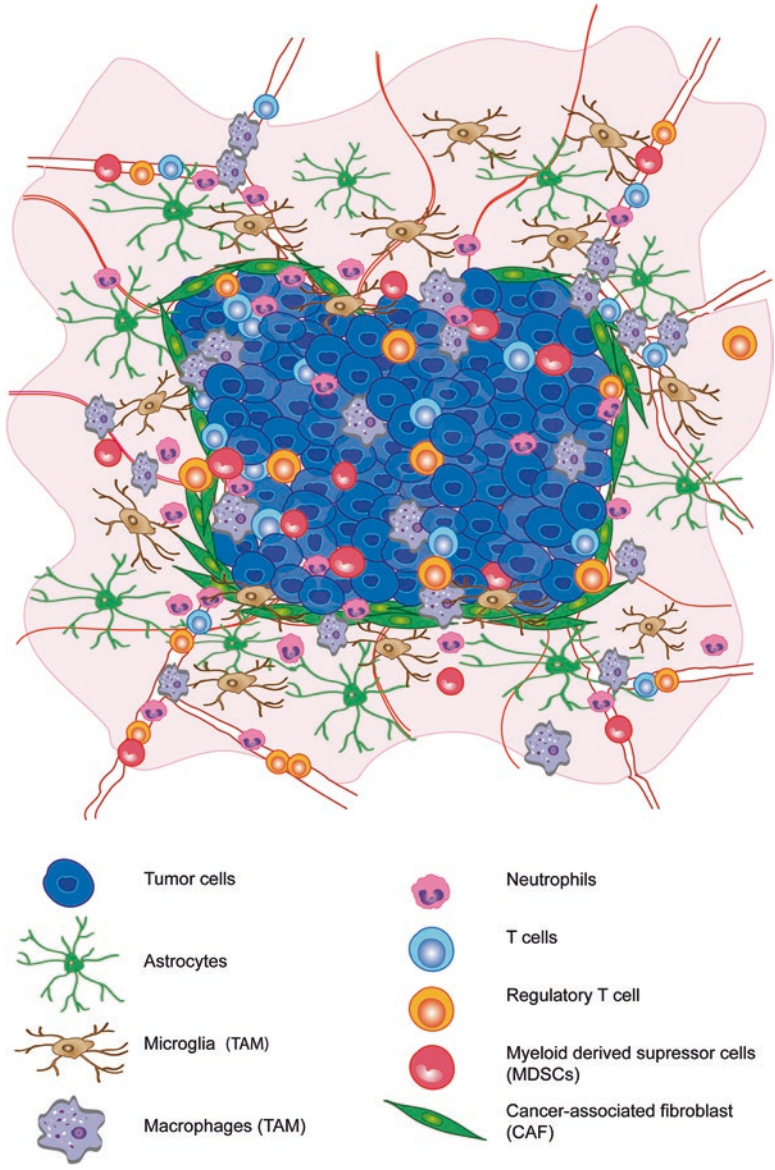

Fig. 5.1 The tumour microenvironment. Typical representation of a brain tumour and its tumour microenvironment (TME), consisting of a heterogeneous pool of cells. Besides tumour cells, cancer-associated fibroblasts (CAFs) and immune cells arrive from the central nervous system reservoir (microglia cells and astrocytes) or are infiltrating from the periphery (macrophages, neutrophils, T-cells, Tregs, MDSCs). Most of the cellular and molecular components of the TME can be efficiently visualized with molecular imaging techniques. This book chapter will review the recent advances in the imaging of the TME

To specifically image the underlying cellular components of the TME and to unravel the dynamic changes in the tumour microenvironment, several different tracers and techniques have been developed and will be discussed in the following paragraphs [9]. This chapter will address general tumour principles, including tumours within the brain. For the latter, one has to consider that the radiotracer has to cross the blood-brain barrier, either by activated transport or by passive diffusion through a disrupted barrier. This is particularly limiting for all antibody-based radiotracers, usually not crossing the intact BBB.

5.2 Imaging Tumour-Associated Inflammation: Tumour-Associated Macrophages (TAMs) and Microglia

The main target for imaging tumour-associated inflammation are tumour-associated macrophages (TAMs). Many imaging studies have been per-

formed in brain tumours, where TAMs can represent up to 30% of the total tumour mass [10]. In the brain, resident specialized macrophages are called microglia. These cells continuously monitor the brain microenvironment, regulating neuronal metabolism and maintaining CNS homeostasis. Under pathological conditions, for example, in the presence of brain tumours, bone marrow-derived peripheral monocytes or macrophages are attracted to the tumour [11]. During tumour growth, a bidirectional crosstalk between tumour cells and TAMs is established depending on the cytokine exposure: cancer cells modulate TAM phenotypes and, in return, recruited TAMs promote tumour progression by releasing pro-tumourigenic and pro-survival factors [12, 13]. This process results in the activation or suppression of different pathways that modulate the escape from the tumour immune response by promoting (1) glioma angiogenesis, (2) growth and invasion, (3) suppression of T cell functions and (4) induction of immunosuppressive regulatory T cells (Tregs) [14].

Several TAM-targeting tracers have been developed. Among the plethora of available tracers, those targeting the translocator protein 18 kDa (TSPO) have gained attention for TAM imaging. TSPO is a protein of the outer mitochondrial membrane and is specifically upregulated in activated microglia cells in response to inflammation and other pathological conditions. The first clinically available TSPO tracer was [^{11}C]PK11195 successfully allowing TSPO imaging in gliomas [15, 16]. However, the application of [^{11}C]PK11195 was limited by the short half-life of ^{11}C and unfavourable tracer kinetics. Consequently, over the last years, several new TSPO-PET tracers with improved characteristics have been developed, such as [^{18}F]PBR28 [17], [^{18}F]PBR111 [18], [^{18}F]DPA-714 [19] and [^{18}F] GE-180 [20]. [^{18}F]DPA-714 has demonstrated high affinity for the target protein, good permeability of the blood-brain barrier and potential to monitor brain tumour growth and inflammation in vivo [21]. Recently, the combination of [^{18}F] DPA-714 with other PET tracers or imaging modalities such as MRI supported the characterization of the TME in preclinical glioma models. In conjunction with immunohistochemistry, [^{18}F] DPA-714 further identified areas of immune cell infiltration, as well as tumour infiltration into the surrounding brain parenchyma [22, 23] (Fig. 5.2).

Despite promising initial results with different TSPO ligands in oncology, the interpretation of PET signals is hindered by the lack of specificity of TSPO to differentiate immune cell (sub-) populations and tumour cells [22, 24] and the existence of mutations in the *tspo* gene in the human population affecting TSPO binding properties of several TSPO tracers [25].

For this reason, novel PET tracers were developed to specifically target activated microglia using radiolabelled molecules binding CX3CR1 or the purinergic receptors P2x7 or P2y12 [3]. The results of preclinical in vitro and in vivo studies show that P2x7 tracers are good candidates for imaging the pro-inflammatory state in neurodegenerative diseases. Unfortunately, the currently available radiolabelled compounds targeting P2Y12R and CX3CR1 are not crossing the BBB [26, 27].

Other tracers were developed to specifically target macrophage phenotypes. Depending on their functional state, macrophages may be categorized in the so-called "classically" activated macrophage (M1) or "antitumour" phenotype and the "alternatively" activated macrophages (M2) or "pro-tumour" phenotype [28], respectively. One example for M2-type macrophage targeting is the use of single-domain antibody fragments (sdAbs) binding the macrophage mannose receptor (MMR or CD206), such as [^{99m}Tc] Tc(CO)3-anti-MMR-sdAb, [^{18}F]FB-anti-MMR-sdAbs and [^{68}Ga]Ga-NOTA-anti-MMR-sdAb [29]. The latter showed high specificity for pro-tumourigenic macrophages and preclinical studies concluded its safety for clinical use [30]. Recently, Horti et al. have developed a new PET tracer targeting the colony-stimulating factor-1 receptor (CSF-1R) expressed on different immune cells of the tumour microenvironment, including TAMs and monocytes [31]. The new tracer [^{11}C]CPPC was investigated in a mouse and baboon model of LPS-induced neuroinflammation, murine models of AD, multiple sclerosis, and post-mortem AD human tissues,

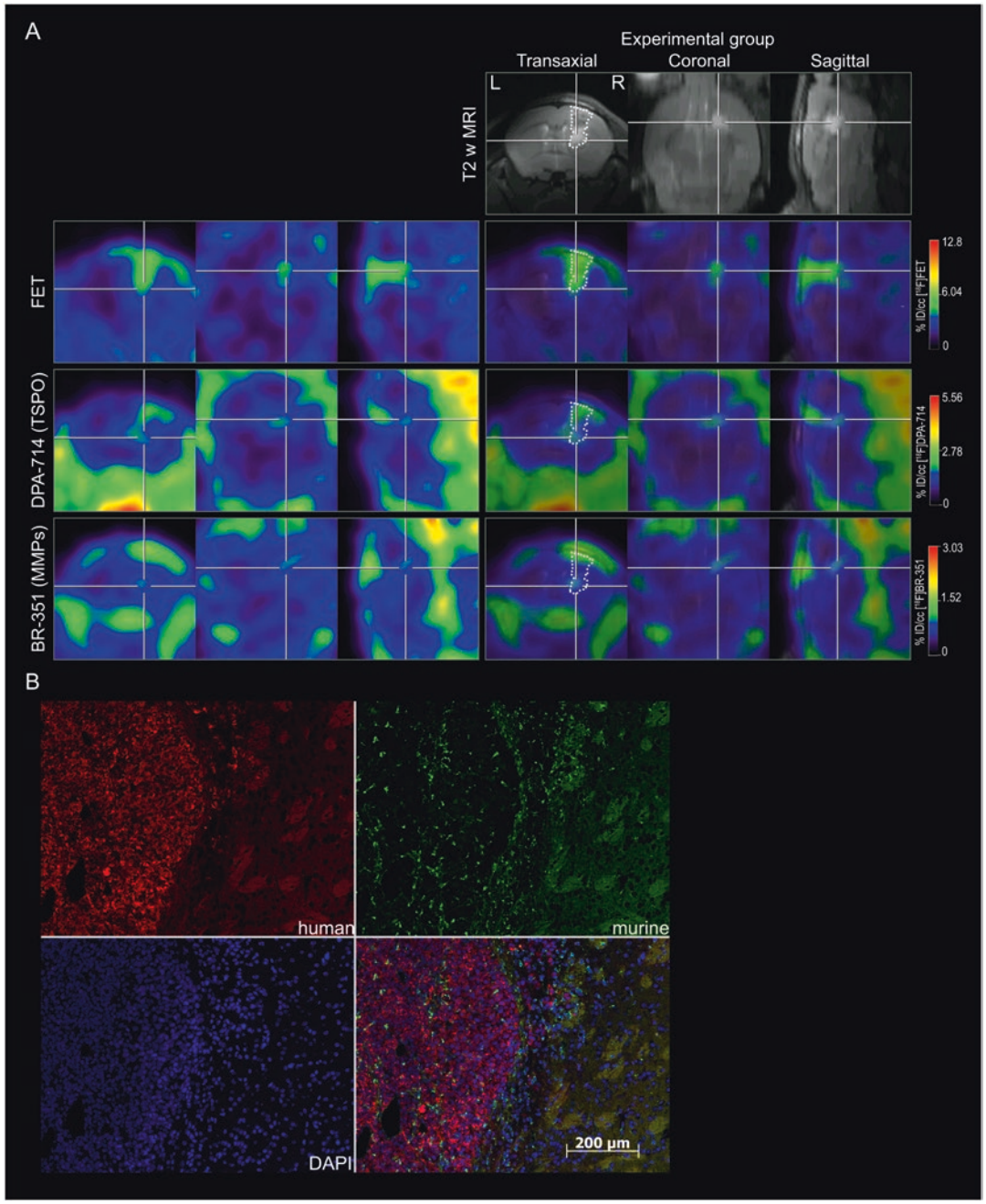

Fig. 5.2 (**a**) Multitracer study of the glioma microenvironment using [^{18}F]FET (amino acid transport), [^{18}F] DPA-714 (TSPO) and [^{18}F]BR-351 (MMPs) in a preclinical mouse model injected with human Gli36ΔEGFR tumour cells. The combination of different tracers targeting distinct parameters of the TME facilitates the non-invasive characterization of the TME. (**b**) Immunofluorescence staining for murine and human TSPO. Human TSPO is highly expressed within the tumour tissue by cancer cells (red—upper left quadrant). The murine TSPO signal (green—upper right quadrant) comes from infiltrating cells. DAPI is the nuclear staining (blue—lower right quadrant). Source: Modified from Zinnhardt et al. (Cancer Res April 15, 2017 (77) (8) 1831–1841; doi: 10.1158/0008-5472.CAN-16-2628) with permission for use

showing in all cases high selectivity and binding specificity [32]. The value of CSF-1R PET in imaging the TME remains to be investigated.

5.3 Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of cells originating in the bone marrow from common hematopoietic progenitors and differentiate in granulocytes, monocytes and dendritic cells.

Based on phenotypic and morphological features, MDSCs can be further classified into two major cell populations: polymorphonuclear (PMN) and monocytic (M-) MDSCs [33, 34]. Both MDSC populations have an important role in the immune regulation in cancer that consists mainly in the suppression of T cell and natural killer cell (NK) responses [35]. By upregulating hypoxia-associated tumour factors such as HIF-1α, Arg-1 and iNOS, MDSCs attract Tregs and increase the expression of inhibitory molecules like PD-L1 [36, 37].

To target MDSC by molecular imaging, some preclinical studies show the feasibility of antibody conjugation with PET tracers to monitor myeloid cell distribution in different tumour models. Cheng and colleagues engineered a single-photon emission computed tomography (SPECT) probe (^{99m}Tc-labelled anti-CD11b antibody) to monitor the inflammatory microenvironment in a model of colorectal cancer, successfully visualizing the origin and migration of CD11b$^+$ MDSCs. It may be a promising tool for early-stage diagnosis [38]. Cao et al. developed the radiotracer ^{64}Cu-labelled anti-CD11b (^{64}Cu-αCD11b) to monitor acute and chronic inflammation locally and systemically. They visualized the immune response of CD11b$^+$ myeloid cells [39].

Recently, another group conjugated an anti-CD11b antibody with a deferoxamine (DFO) chelator and radiolabelled it with ^{89}Zr for PET imaging of glioblastoma. They showed the significantly increased uptake in the tumour area of the brain with high specificity, demonstrating the possibility of non-invasively assessing the neuroinflammation promoted by MDSCs, as well as disease progression [40]. Up to date, no specific tracer is available targeting and distinguishing MDSC subpopulations and more research is needed.

5.4 Tumour-Infiltrating Lymphocytes

Lymphoid cells, represented by T cells, B cells and natural killer (NK) cells, play an important role in tumourigenesis. Different types of T cells have been identified, including CD4$^+$ and CD8$^+$ T cells. CD4$^+$ T cells can be further subdivided into CD4$^+$ T helper 1 (Th1) and CD4$^+$ T helper 2 (Th2), based on their cytokine profiles and the type of cells supported, i.e. Th2 cells support B cell responses [41]. Regulatory T cells (Tregs), a subset of CD4$^+$ cells, have tumour-promoting and immunosuppressive activities. These cells form a highly heterogeneous population; they are indispensable to suppress self-reactive T cells in the periphery and to inhibit the immune response at the resolution phase of inflammation. In cancer, this leads to the suppression of T cells in favour of tumour growth [42].

T cell functions are further controlled by immune checkpoints. In particular, the immune checkpoint receptors programmed death cell protein-1 (PD-1), together with its ligand PD-L1 as well as the cytotoxic T lymphocyte antigen-4 (CTLA-4), have been employed for targeted imaging and therapy [43, 44].

In the era of these targeted immunotherapies, specific visualization of T cell responses is becoming increasingly relevant and new radiotracers were developed to assess the efficacy of antibodies as therapeutic and diagnostic tracers. Many of them are targeting the PD-1/PD-L1 axis [45]. Imaging studies were conducted using ^{111}In-labelled PD-L1 mAbs in breast cancer and melanoma models [46–48]. Atezolizumab, a humanized mAb with high affinity for both human and mouse PD-L1, was evaluated radiolabelled with ^{64}Cu and ^{111}In. The tumour uptake correlated with the levels of PD-L1 expression [49, 50]. ^{89}Zr-labelled Abs against PD-L1 were also tested in head-and-neck squamous cell carci-

noma and melanoma models, also after PD-1 therapy. PET imaging showed a correlation with the upregulation of PD-1 only in the untreated tumours [51]. Natarajan et al. tested a mouse anti-PD-1 antibody labelled with ^{64}Cu demonstrating the possibility to trace tumour-infiltrating lymphocytes at the tumour site [52]. In addition, other groups evaluated ^{64}Cu and ^{89}Zr pembrolizumab and nivolumab for PD-1^{+} T cell imaging, analysing the biodistribution and clearance of the tracers in rodents and in non-human primates. T cell accumulation in the salivary and lacrimal glands of humanized mice engrafted with h-PBMCs developing allogeneic graft versus host disease could be visualized [53, 54]. England and colleagues developed another anti-PD1 PET tracer (^{89}Zc-Df-nivolumab) and characterized it in a model of lung cancer [55]. At the same time, Du et al. described a new PD-1 targeting strategy using IRDye800CW- and ^{64}Cu-labelled liposomes loaded with doxorubicin, conjugated to PD-1 mAbs. They were able to image the inhibition of tumour progression in a breast cancer model, confirming the possibility of using this approach for cancer imaging, image-guided tumour resection and therapy [56, 57]. Finally, the first in-human whole body study for PD-1 and PD-L1 expression has been recently performed in patients with non-small cell lung cancer. Here, ^{89}Zr nivolumab and ^{18}F-BMS-986192 were found to be valuable imaging tracers to evaluate non-

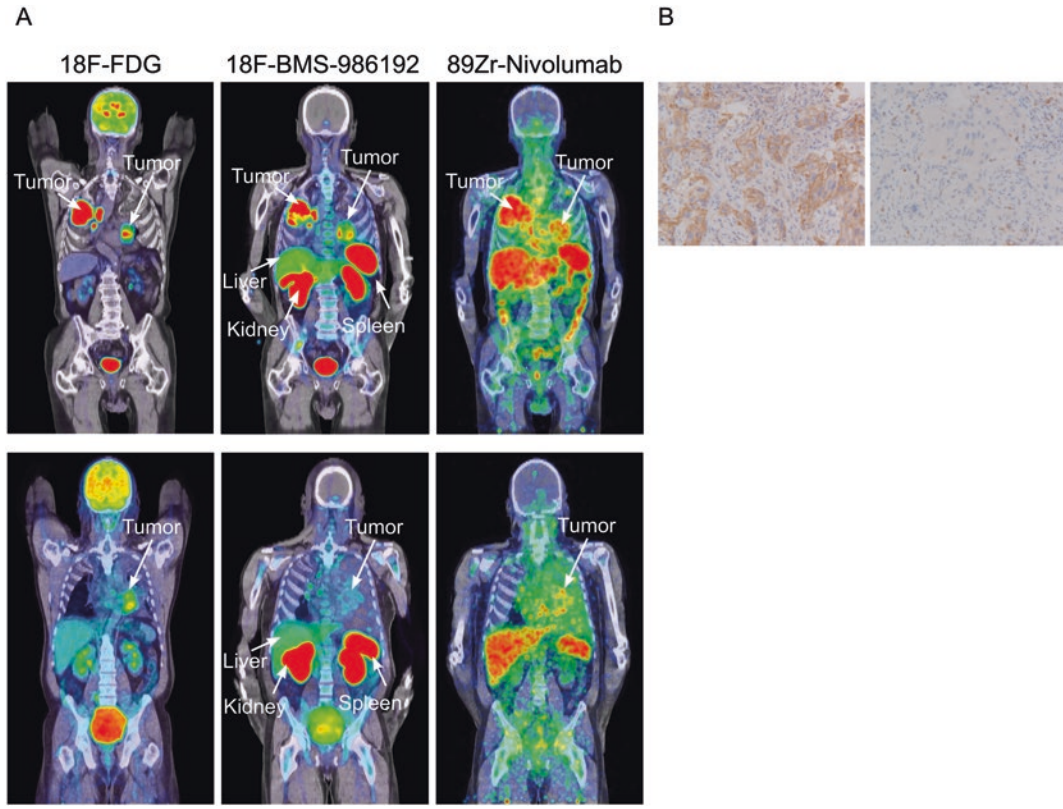

Fig. 5.3 (**a**) First whole body PET study for PD-1 and PD-L1 expression in patients with lung cancer (NSCLCs), prior to treatment with nivolumab. ^{18}F-FDG PET reveals high glucose metabolism of lung tumours; anti-PD-L1 ^{18}F-BMS-986192 PET and ^{89}Zr-labeled nivolumab PET depict heterogeneous tracer uptake in the tumour in line with the expression levels of PD-L1, as shown by the lower uptake in the second patient (lower lane) where PD-L1 expression level is <1%. (**b**) Immunohistochemical staining of PD-1 and PD-L1 in the first patient (**a**—upper lane) in a biopsy of the tumour in the left lower lobe. PD-L1 expression (left picture) is expressed in 95% of the tumour cells, while PD-1 expression in aggregates (right picture) was scored as IC1. Scale bar, 100 μm. Source: Modified from Niemeijer et al. ("Whole body PD-1 and PD-L1 positron emission tomography in patients with non-small-cell lung cancer." *Nature communications* vol. 9, 1, 4664. 7 Nov. 2018, doi: 10.1038/s41467-018-07131-y)

invasive therapy responses and to stratify patients based on imaging parameters [58] (Fig. 5.3).

Immunotherapy targeting CTLA4 receptors shows antitumour efficacy in various cancer patients, but can also lead to serious autoimmune-related side effects. Tracers targeting CTLA4 receptor might be useful to monitor treatment efficacy and to identify the nature of adverse events. This was already demonstrated in preclinical studies in colon carcinoma [59], NSCLC [60] and melanoma [61]. ^{64}Cu-labelled CTLA-4 mAbs, ^{64}Cu-labelled ipilimumab and ^{89}Zr-labelled PEGylated single-domain antibody fragments displayed high tumour uptake and high specificity and were suited for monitoring checkpoint therapy response, respectively.

Besides labelled antibodies, alternative approaches are under investigation. These comprise small PD1-binding moieties such as engineered PD-1 ectodomains [62], peptides and nanobodies [45, 63]. Immuno-PET imaging of $CD4^+$ and $CD8^+$ T cells circulating and infiltrating the tumour area emerged lately and takes advantage of antibodies engineered into bivalent antibody fragments called cys-diabodies (cDb) or minibodies (Mb). These moieties are characterized by (1) rapid clearance for high target-to-background images shortly after injection, (2) high avidity, (3) engineered sites for site-specific conjugation and (4) lack of Fc effector functions. Different studies show positive results in the imaging of T cells in vivo using ^{64}Cu-labelled Mb or ^{89}Zr-labelled cDb, including a high affinity for the target as shown by specific uptake in the spleen and lymph nodes [64]. In addition, these tracers were shown to be a powerful resource to monitor tumour-infiltrating T cells [65], hematopoietic stem cell (HSC) transplantation and therapy response [66].

5.5 The Challenge of Imaging Neutrophils

Neutrophils have an important role in the first line of host immune defence; they help eliminate pathogens by generating reactive oxygen species and releasing antimicrobial and cytotoxic compounds. They also have the ability to create extracellular traps called NETs [67, 68].

Similar to macrophages, neutrophils populating the TME are called tumour-associated neutrophils (TANs). They have been suggested to have a N1 and N2 phenotype, reflecting an anti- or pro-tumourigenic effect [69]. Several studies have reported that neutrophils contribute to cancer proliferation and metastases and that the amount of neutrophils negatively correlates with patients' prognosis and survival [70].

Neutrophils are an abundant part of circulating leucocytes, and therefore, it is challenging to target them with therapeutic agents since this could lead to an unwanted and severe immune suppression exacerbating the disease.

Despite the promising role of neutrophils as drug transporters, there is a lack of specific tracers for this cell type. Clinically different radiotracers are available and used in the diagnosis of infectious or inflammatory events. However, tracers are radiolabelled drugs directly acting on the pathogens, but not specifically on neutrophils [71].

Several radiolabelled peptides have been studied with the purpose of imaging inflammation in vivo, but they had either a low binding affinity or they caused the deregulation of leucocytes and neutropenia [72, 73]. A promising novel ^{64}Cu-labelled peptide was synthesized (cFLFLFK-PEG-^{64}Cu) by Locke and colleagues targeting the formyl peptide receptor (FPR) on leucocytes. The peptide acts as an antagonist of the FPR and is designed to avoid a chemotactic response and unwanted side effects. In vitro and in vivo studies confirmed the specificity of the binding to neutrophils [73]. Recently, the combination of nuclear medicine and nanotechnology led to the development of another interesting PET tracer, the ^{68}Ga-NRT-cFLFLF. First in vitro and in vivo studies were performed in a model of lung LPS-induced inflammation. The results indicated high selectivity for neutrophils and the feasibility of monitoring chronic inflammation [74].

Up to now, however, no neutrophil-targeted tracer was applied in oncology.

5.6 Imaging of Cancer-Associated Fibroblasts

Cancer-associated fibroblasts (CAFs) orchestrate the cellular and molecular organization of the TME including the reorganization of the collagenous extracellular matrix secreting matrix metalloproteinases (MMP), mediating angiogenesis and regulating tumour and immune cell proliferation and migration. Because of their central functional role, CAFs are a promising target for clinical characterization of the molecular and cellular profile of the TME. Although the molecular and metabolic information derived by, e.g. PET imaging are increasingly included in clinical decision-making, the majority of clinical indications are based on the superiority of PET to detect metastases. Considering the high contribution of tumour stroma to the total tumour volume and the abundancy of CAFs, they also compromise an attractive target for sole lesion detection. Depending on whether imaging primarily aims at detection or molecular characterization of lesions, expression of an ideal target structure should be either abundant or restricted to specific CAF subpopulations with a close relationship to functional properties (e.g. tumour-promoting, tumour-restraining, angiogenesis-promoting). However, classification of CAF subpopulations by markers is complex and a matter of intense ongoing research in proteomics [75].

One target structure that has just recently received increasing attention is the fibroblast-activating protein (FAP). FAP expression is, similar to α-smooth muscle actin (αSMA), closely linked to the transition of the active state and it is in this respect a well-characterized histochemical marker for identifying activated fibroblasts/CAFs [76, 77]. Moreover, expression of the transmembrane protein FAP owes a high specificity to activated fibroblasts and low basal expression under physiological conditions [76]. FAP expression has been demonstrated in over 90% of epithelial carcinomas [78], suggesting a high potential sensitivity for FAP imaging in malignant disease. Expression was also found in other pathological conditions including wound healing, fibrotic processes (e.g. liver fibrosis [79], lung fibrosis [80]), atherosclerosis [81] (here in smooth muscle cells) and arthritis [82] (here in myofibroblast-like synoviocytes). On the one hand, these data might reduce the specificity for tumour imaging but, on the other hand, extend the potential indications of FAP imaging to non-malignant pathologies. Different studies demonstrated a correlation between FAP expression and prognostic features in cancer patients with partially conflicting roles in different cancers. For instance, a positive correlation between FAP expression and survival has been demonstrated in a study with breast cancer [83], but FAP expression correlated positively with higher disease stages in another study with colorectal cancer [84]. Despite its non-binary functional role in cancer, different FAP-targeting strategies in anticancer treatment have been proposed and employed in preclinical and few clinical studies using monoclonal antibodies [85] or small molecular inhibitors [86] directed to FAP. These pharmacological developments were picked up by radiochemists to generate radiolabelled FAP-targeting antibodies [87]. Only recently, FAP-targeting small molecule PET tracers [88–91] have been developed. These ^{68}Ga-labelled compounds for PET imaging were introduced by Haberkorn and colleagues and represent the first clinically feasible specific CAF imaging agents. First in vivo results in 28 different kinds of cancer demonstrated excellent tumour uptake at comparable levels or even exceeding that of FDG. These include epidemiologically very important cancers such as breast and pancreatic cancer where FDG or more specific radiotracers could yet not establish a definite clinical role. The novel [^{68}Ga]FAPI ligands display very good molecular specificity and favourable pharmacokinetics as depicted by low levels of tracer retention in normal tissue, resulting in excellent tumour-to-background delineation. Moreover, first clinical data prove the feasibility of using these ligands as theranostic agents, i.e. usage of the same precursor for imaging and radioligand therapy (e.g. ^{90}Y-FAPI) [90]. Although these first clinical studies indicate promising abilities to detect malignant lesions, it is unclear whether FAP imaging provides useful molecular information that can be integrated into

clinical decision-making. Most current preclinical studies worked with cultured cells and patient-derived tumour probes and focused on implications of FAP for therapy rather than for imaging. More specific studies are necessary, to validate FAP on a cellular and histopathological level. This would clarify its clinical potential for (1) diagnosis, (2) monitoring and predicting therapy response and (3) differentiating benign, premalignant and malignant lesions.

5.7 Vascular Parameters/ Endothelium

Tumour angiogenesis results from the lack of oxygen and nutrients within the expanding tumour, leading to the formation of new blood vessels and supporting tumour growth [92]. Neovascularization not only allows tumour cell growth by supplying essential nutrients but also enables waste disposal and metastatic spreading. This fast and uncontrolled process results in a leaky, immature vascular network, characterized by fenestrated vessel walls with poor pericyte coverage and abnormal basement membranes [93, 94]. This specific vascular signature is of relevance for in vivo imaging and targeted cancer therapy.

Non-invasive molecular PET and/or SPECT imaging targeting angiogenesis-related molecular markers were developed. The most investigated as imaging targets are (1) vascular endothelial growth factor (VEGF), (2) cell adhesion molecule integrins (including αvβ3) and (3) hypoxia-inducible factor-1 (HIF-1).

Vascular endothelial growth factor receptors (VEGF1/Flt-1, VEGF2/Flk-1) are tyrosine kinase receptors involved in endothelial cell proliferation and survival. They are expressed by normal vascular endothelial cells and overexpressed in tumour endothelium [95, 96]. To image VEGF and VEGFR expression by PET/SPECT, Rainer et al. reported the first [^{123}I]-VEGF scans in patients with either primary or recurrent brain tumours. They evaluated the differential diagnosis and prognostic value of this radiotracer. Positive [^{123}I]-VEGF scans were associated with grade IV gliomas, while less malignant tumours were [^{123}I]-VEGF negative [97]. However, like other VEGF-based radiotracers, radiolabelling of the small ligand and the competitive binding with endogenous VEGF to the receptor may bias image assessment.

Bevacizumab, an anti-VEGF antibody, has been used as a scaffold for VEGF radiotracer design. It is the most used anti-angiogenetic drug in oncology [98], showing high affinity binding to VEGF. Recently, a ^{89}Zr-labelled bevacizumab immuno-PET tracer has been investigated as a predictive tool of therapy efficiency in children. The results highlighted the benefit of ^{89}Zr-bevacizumab for patient selection in this disease context [99].

Integrins are part of the cell adhesion molecule family involved in cell-cell and cell-ECM interactions, controlling cell migration and survival. Among them, expression of αvβ3 integrin is significantly upregulated in the tumour vasculature, while low expressed on healthy endothelial cells [100]. High expression of αvβ3 in tumour facilitates cell infiltration and metastasis. The Arg-Gly-Asp (RGD) and Arg-Arg-Leu (RRL) sequences are considered as two of the minimal amino acid sequences present in most of the αvβ3 ligand-binding domain. They are of main interest for early detection and treatment of fast-growing tumours [101]. Therefore, they represent an attractive site for in vivo tumour angiogenesis imaging, drug design and drug delivery [102]. For example, [^{18}F]alfatide II allows better detection of brain metastases than [^{18}F] FDG. However, most of those radiolabelled RGD peptides need partially disrupted BBB to be able to reach their target [95, 103, 104].

Another hallmark of tumours is the hypoxic tumour microenvironment. The rapidly growing neovasculature and heterogeneous microcirculation lead to an imbalance between oxygen consumption and supply resulting in poor tissue oxygenation. Hypoxia can be detected by upregulation of hypoxia-inducible factors (HIFs), in particular hypoxia-inducible factor-1. [^{18}F] FMISO (fluoromisonidazole) has been validated for hypoxia imaging, allowing differentiation between low- and high-grade gliomas [105, 106].

Moreover, [^{18}F]FMISO imaging showed increased uptake correlating with primary brain tumour malignancy [107], supporting the use of the tracer as a diagnostic tool in early disease stages. Although [^{18}F]FMISO is the most frequently used hypoxia radiotracer, other radiotracers with improved characteristics are under investigation [108].

Another target for imaging tumour progression, related to the vasculaturization in the TME, is the system-L amino acid transporter and in particular the subunit LAT-1, which is expressed by a large variety of human cancers [109]. Different PET tracers have been synthesized, especially for brain tumour imaging, and the first among them used for this purpose was L-(methyl-[^{11}C])-methionine ([^{11}C]MET) [110]. Due to the very short half-life of ^{11}C (ca. 20 min), other tracers have been developed. Currently, *O*-(2-[^{18}F] fluoroethyl)-L-tyrosine ([^{18}F]FET) is routinely used for diagnosis of brain tumours, monitoring tumour progression and guiding tumour resection. Coupled with MRI and/or CT, PET imaging can also be useful to identify areas of residual tumour or recurrence and to monitor the efficacy of cancer therapies [111, 112]. Recently, a zirconium-labelled antibody ([^{89}Zr]DFO-Ab2) targeting the extracellular domain of LAT-1 has been tested in a model of colorectal cancer; the results show high uptake in the tumour area and high in vivo specificity for the target [113].

Together with nuclear medicine approaches, different MR sequences have been developed, with and/or without the use of exogenous contrast agents (CA), to extract structural information of the vasculature in tumours. MR techniques allow in vivo characterization of tumour neovasculature, including vessel structure, oxygen level and hemodynamics [94, 114]. Among the non-contrast-enhanced MR imaging techniques, arterial spin labelling (ASL) has gained attention for perfusion imaging. On the other hand, perfusion CT, dynamic contrast-enhanced MRI (DCE-MRI) and dynamic susceptibility-enhanced MRI (DSC-MRI) are exogenous paramagnetic contrast agent-based imaging techniques [93].

Dynamic contrast-enhanced MR imaging principles rely on the analysis of tissue response to the inflow of CA, visualized by an enhanced signal [115]. Since this imaging method depends on tissue behaviour in contact with the contrast agent, physiological properties such as vessel permeability, vessel surface area and volume fraction can be derived, depending on the origin of the measured signal and its registration.

MR imaging depends on tissue status, characterized by two physicochemical properties labelled T1 and T2/T2* relaxation times. The inflow of contrast agent through tissue modifies those parameters, inducing the so-called relaxation and susceptibility effects.

Dynamic contrast-enhanced (DCE)-MRI (or T1w-DCE MRI) measures the relaxation effect of CA diffusion into tissues expressed by changes in T1 values and may thus provide information on the integrity of the blood-brain barrier. DCE-MRI-derived parameters (including k-trans and the initial area under the curve (iAUC)) are often used to detect decreased permeability after anti-angiogenic therapy or radiation therapy, reflecting a presumed decrease in tumour neovasculature [116, 117].

Similarly, dynamic susceptibility contrast (DSC)-MRI (or T2*w DCE) exploits the change in the inhomogeneity of magnetic strength during the first pass of the CA, leading to decreased T2/T2* tissue values. The latter is used to calculate estimates of the cerebral blood volume (CBV) and blood flow in tumours.

Of major interest in neuro-oncology, contrast-enhanced MR techniques allow tumour classification into low- and high-grade gliomas using the relative cerebral blood volume as an index (rCBV) [118]. Moreover, these techniques allow to track anti-angiogenic therapy responses by detecting changes in blood-brain barrier permeability and vascular density [119]. In a review, Patel et al. indicated DSC-MR imaging for differentiation between recurrent tumour and post-treatment changes with high sensitivity and specificity [120]. Besides, Barajas et al. reported the use of DSC-MRI-derived rCBV to differentiate between necrotic and viable tumour tissue after radiation therapy [121, 122].

Other than the CA-based MR imaging techniques, arterial spin labelling (ASL) is a non-invasive perfusion-based imaging technique used

to estimate the cerebral blood flow (CBF), like conventional DSC-MR imaging. However, ASL relies on using magnetic labelled arterial blood water as an endogenous tracer [123]. ASL can be used for the classification and grading of different tumour types [124–126]. In particular, ASL is of special interest in paediatric brain tumour imaging [127], since it does not require invasive CA administration.

In summary, tumours are characterized by a specific perfusion phenotype, strongly contrasting from the surrounding healthy tissue. The tumour vasculature exhibits high cellular proliferation, low degree of differentiation, high permeability and low oxygen levels. Those specific parameters are of great interest in in vivo molecular imaging and are actively investigated as prognostic, diagnostic and treatment response feature in oncology.

5.8 Future Trends or Directions

The detailed characterization of the TME remains challenging due to the cellular and molecular heterogeneity. The non-invasive visualization of the ongoing complex processes in the TME by molecular imaging is extremely important for precise diagnosis in oncology, as well as therapy monitoring. Consequently, a variety of targets have been identified for the non-invasive characterization of the TME with and without therapy yielding promising initial results in preclinical and clinical settings. Yet the molecular imaging community needs to overcome several additional challenges for efficient targeting of the TME, including (1) the lack of specificity to distinguish heterogeneous immune cell populations within the TME, and (2) the depiction of the complex spatio-temporal dynamics of the TME.

In the era of targeted immunotherapies, novel molecular (immune) targets and structures need to be identified and utilized to support diagnosis and therapy monitoring of innovative immune therapies. Combinations of tracers and imaging modalities targeting different aspects of importance for specific immunotherapies should be considered and integrated into the clinical workflow. Ideally, the obtained immune-imaging data will be further integrated with detailed immunophenotyping by, e.g. flow cytometry and -omics data to gain a holistic understanding of the patients' individual tumour immune profile.

The emerging application of radioisotopes with a longer half-life, like ^{89}Zr, will support longitudinal imaging to identify spatial and temporal changes in the TME before, during and after therapy with a single injection of a radiotracer. The application of longer half-life isotopes is also of interest in the labelling of novel immune therapeutic antibodies, usually requiring longer circulation times. Radiolabelling of immunotherapies and molecular immuno-imaging will be a powerful tool to select and stratify patients according to the target engagement of a new therapy and the composition of their personalized TME. Further, the combination of diagnostics and radiotherapy (theranostics) with beta and alpha nuclides is rapidly emerging and holds promise to improve personalized cancer therapy. In summary, molecular imaging represents an important parameter in personalized medicine and should be integrated into the clinical workflow for TME-targeted therapies.

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Appendix

Imaging target	Molecular target	Radiotracer	Citation
Tumour-associated macrophages and microglia (TAMMs)	TSPO	[^{11}C]PK11195, [^{18}F]PBR28, [^{18}F]PBR111, [^{18}F]DPA-714, [^{18}F]GE-180	[15–20]
	CXCR1R	–	[27]
	P2X7 and P2Y12	[^{11}C]A-740003, [^{11}C]SMW139, [^{11}C]JNJ-54173717, [^{11}C]GSK1482160, [^{11}C]2	[3, 26, 27]
	M2 macrophages	[^{99m}Tc]Tc(CO)$_3$-anti-MMR-sdAb, [^{18}F]FB-anti-MMR-sdAbs and [^{68}Ga]Ga-NOTA-anti-MMR-sdAb	[28–30]
	CSF-1R	[^{11}C]CPPC	[32]
Myeloid-derived suppressor cells	MDSCs (CD11b$^+$ cells)	[^{99m}Tc]-labelled anti CD11b antibody, ^{64}Cu-antiCD11b, ^{89}Zr anti-CD11b	[38–40]
Tumour-infiltrating lymphocytes	Cancer cell	^{111}In-labelled PD-L1 mAbs, atezolizumab ^{64}Cu and 111In, ^{89}Zr-labelled abs against PD-L1, ^{89}Zr nivolumab and ^{18}F-BMS-986192	[45–51, 58]
	T cells	PD-1 antibody labelled with ^{64}Cu, ^{64}Cu and ^{89}Zr pembrolizumab and nivolumab, ^{89}Zc-Df-nivolumab, IRDye800CW- and ^{64}Cu-labelled liposomes conjugated to PD-1 mAbs, ^{64}Cu-labelled Mb or ^{89}Zr-labelled cDb	[45, 52–57, 62–66]
	CTLA4 receptor	^{64}Cu-labelled CTLA-4 mAbs, ^{64}Cu-labelled ipilimumab and ^{89}Zr-labelled PEGylated single-domain antibody fragments	[59–61]
Neutrophils	Formyl peptide receptor (FPR)	cFLFLFK-PEG-^{64}Cu, ^{68}Ga-NRT-cFLFLF	[73, 74]
Carcinoma-associated fibroblast	Fibroblast-activating protein (FAP)	^{68}Ga-FAPI, ^{90}Y-FAPI	[88–91]
Vasculature and hypoxia	VEGF receptors	[^{123}I]-VEGF, ^{89}Zr-labelled bevacizumab	[97–99]
	αvβ3 ligand-binding domain	[^{18}F]Alfatide II, radiolabelled RGD peptides	[95, 103, 104]
	Tumour hypoxia	[^{18}F]FMISO	[105–108]
	LAT-1	[^{11}C]MET, [^{18}F]FET, [^{89}Zr]DFO-Ab2	[110–113]

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6 Necrosis in the Tumor Microenvironment and Its Role in Cancer Recurrence

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Abstract

Cancer recurrence is one of the most imminent problems in the current world of medicine, and it is responsible for most of the cancer-related death rates worldwide. Long-term administration of anticancer cytotoxic drugs may act as a double-edged sword, as necrosis may lead to renewed cancer progression and treatment resistance. The lack of nutrients, coupled with the induced hypoxia, triggers cell death and secretion of signals that affect the tumor niche. Many efforts have been made to better understand the contribution of hypoxia and metabolic stress to cancer progression and resistance, but mostly with respect to inflammation. Here we provide an overview of the direct anticancer effects of necrotic signals, which are not necessarily mediated by inflammation and the role of DAMPs (damage-associated molecular patterns) on the formation of a pro-cancerous environment.

Keywords

Necrosis · Cancer · Resistance · Recurrence · Metronomic therapy · Hypoxia · DAMPs · Chemotherapy · Angiogenesis · Anti-angiogenic therapy · Tumor microenvironment · Metabolic stress

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6.1 Introduction

Cancer recurrence is one of the most imminent problems in the current world of medicine and is responsible for high rates of cancer-related deaths worldwide. Cancer cells affect their surrounding environment to promote changes that support their growth. As the tumor grows, the need for nutrients and oxygen increases, while the ability of blood vessels to penetrate to the core of the tumor decreases significantly. The lack of blood supply initiates cell death through apoptosis and necrosis which is a result of the hypoxic and metabolic stress [1, 2]. Since many tumors are resistant to apoptosis, for example, through p53 mutations, necrosis is commonly a dominant cell death mechanism [3]. In that regards, antiangiogenic as well as chemotherapies induce tumor death through a significant enhancement of necrosis. Angiogenesis in cancer is an expedited process of blood vessel formation, induced by the increased tissue growth and the high oxygen and nutrient demand. When blocking blood vessels using antiangiogenic treatments, the tumor eventually is "starved to death." Currently, there are dozens of drugs in the clinic, both antiangiogenic and cytotoxic therapeutics, which result in the shrinkage of tumors via induction of necrosis [4–6].

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When considering tissue feedbacks, long-term administration of anticancer cytotoxic drugs may act as a double-edged sword, since necrosis, the desired result of the treatment, may also lead to renewed cancer progression and treatment resistance [7–9]. The lack of nutrients and oxygen causes cell death and consequently secretion of different signals that affect the tumor niche and the tumor microenvironment. In addition to inflammatory effects, which are beyond the scope of this review, necrosis also triggers direct and inflammatory-independent angiogenic effects that play a key role in the "evasion" of anticancer and antiangiogenic drugs. Several intracellular signals, known as damage-associated molecular patterns (DAMPs), are secreted from the necrotic cells, either by a programmable process [10] or due to the disrupted membranes [11], and act as endogenous danger signals that exacerbate inflammatory response and angiogenesis [7]. Although many efforts have been made to better understand the contribution of hypoxia and metabolic stress to cancer progression and resistance, most of them have focused on the effect of each in regards to their role in inflammation [12]: inflammatory-mediated pathways [13], effect of recruited inflammatory cells [14], resolution of inflammation [15], or inflammation caused by dying cell debris [16]. In recent studies, the direct cancer-supporting effects of factors that are released by the dying cancer cells on disease progression are revealed. Identifying these secreted signals and antagonizing them could prolong therapeutic efficacy, reduce tissue-level resistance to anticancer drugs, and prevent the formation of metastases.

6.2 Cancer and the Tumor Microenvironment

The role of the tumor microenvironment in disease progression has been excessively studied throughout the past four decades. The hallmarks of cancer progression as described by Hanahan and Weinberg in 2000 [4] discuss the preservation of proliferation, lack of susceptibility to suppression factors, escaping programmed cell death, the immortality of replication abilities, vasculature formation for oxygen and nutrient supply, and metastasis in distant organs such as the liver, brain, bone, and lungs [17–19]. The metastatic formation is known to be driving the grim statistics of cancer-related deaths; therefore, there is an urgent unmet need for prophylactic treatment that would prevent the development of cancer lesions. This being said, there are currently no efficient preventative therapies available for patients, despite adjuvant therapy that is being used as the standard in many cases [20, 21]. Metarrestin was recently published as a possible drug for metastatic prevention, showing initial effect in mouse models, but it was yet to be tested clinically [22].

Recurrence is one of the biggest challenges in the struggle for eradicating cancer [23]. The effect of cancer recurrence on patients' life extends far beyond the disease itself and is known to increase the severity of physical symptoms as well as cause psychological distress [24]. Many factors are responsible for triggering the rise of subsequent cancer starting with tumor margins that were not fully resected [25], through cancer cells that survive chemotherapy by either DNA modifications [26] or cell dormancy [27] and up to formation of metastatic lesions as a result of disseminated tumor cells that reside in distance organs [28]. Today, there is a clear understanding that tumor progression is not merely the result of intrinsic factors but is also heavily affected by their surrounding environment, known as the tumor microenvironment (TME) [29]. The TME is rich with both cellular and noncellular components—immune and inflammatory cells, stromal cells like fibroblasts or smooth muscle cells, neuroendocrine cells, and blood and lymphatic network—altogether providing the needed support for the tumors to grow and form secondary metastatic lesions [30–33].

6.3 Angiogenesis and Cancer Therapy

The constant supply of oxygen and nutrients to an organism through the blood vessels is fundamental for maintaining the homeostasis of the living tissue. Due to the diffusion limit of oxy-

gen, which is 100–200 μm, most living cells can be found in the vicinity of blood capillary [34]. Thus, for an organ to keep developing, it must recruit new blood vessels which would support the growing demand for both nutrients and oxygen [34, 35]. Angiogenesis is the process referred to as the formation of new blood vessels out of preexisting ones [36]. Angiogenesis plays an important role in physiologic processes such as wound healing [37] and embryonic development [38] and also in many pathologies like macular degeneration [39], diseases which are associated with the vascular system [40], or inflammatory diseases [41]. Tumors, which are masses of cancer cells that keep dividing uncontrollably, require an increasing influx of nutrients and oxygen, which they acquire through the blood flow. The rapid growth of these tumors causes large gaps between the endothelial cells, which altogether lead to a more porous structure of the blood vessesl [42].

The special structure of the newly grown blood vessels can be exploited for enhancing drug delivery due to the enhanced permeability and retention effect [43]. Yet the rapid growth of the tumor can also apply stress on the tissue and cause compression of the blood vessels in the vicinity of the tumor. Such stress can compress the blood vessels and cause lower blood perfusion and, as a result, reduce the levels of oxygen and nutrients. This affects the surrounding environment of the tumor, causing it to be more acidic and compromise the therapeutic treatment [44]. The hypoxic environment, often the resulting from such treatments, is a fertile surface for harboring cancer-promoting processes. It was known, for example, that oxygen diffusion may play a role in the prediction of patients' survival post-anticancer therapy. Rakesh Jain presented data in which patients with increased tumor perfusion or oxygenation presented a better reaction to anticancer treatment (radiology, immunology, chemotherapy, etc.). Therefore, increased perfusion leads to increased tumor oxygen levels and as a result promotes normalization of tumor vasculature which manifests in an immune-supportive microenvironment [36].

In the 1970s, Prof. Judah Folkman, also known as "the father of angiogenesis," proposed the notion that suppression of angiogenesis may be used as a possible target for cancer therapy [45, 46]. This hypothesis later became one of the standard approaches in cancer therapy as well as in therapies of other vascular diseases [4, 5]. The idea is that preventing the tumor mass of normal blood perfusion would lower oxygen and nutrient influx, causing cancer cells to undergo necrotic cell death [47, 48].

One of the first antiangiogenic drugs to be approved by the US Food and Drug Administration (FDA) and prescribed broadly was bevacizumab (Avastin) in 2008. At first, it was approved for treating colorectal cancer, but later it was expanded as first-line treatment for many other types of cancer such as lung, breast, renal, brain (glioblastoma), and just recently ovarian cancer as well. Many other antiangiogenic drugs were approved by the FDA in the past 15 years, and there are currently over a dozen FDA-approved antiangiogenic compounds for cancer therapy, like sorafenib and sunitinib [36, 49], with many more under different stages of clinical development [50, 51]. The main cellular targets of these drugs are endothelial cells, with the most common therapeutic strategy being the blockade of the vascular endothelial growth factor (VEGF) pathway [52–55]. Interestingly, bevacizumab recently showed to be effective in treating radiation necrosis in brain cancer patients. One of the downsides of radiation to the brain is necrosis of the surrounding tissue, considered to be the result of cytokine release, which causes severe headaches and nausea. Radiation causes increased hypoxia which drives an upregulation in VEGF, a known mediator of cerebral edema in radiation necrosis. Bevacizumab was shown to be effective in reducing necrosis of the normal tissue surrounding that of the tumor and presented an improvement in neurological symptoms of the patients [48, 56].

6.4 Tissue-Level Evasion Mechanism and Resistance

Though acquired resistance as a result of anticancer therapy manifests at the cellular level, cancer-promoting factors may also give rise to modulations of the TME, causing tissue-level resistance [57, 58]. While resistance to anticancer therapy can be at the cell level, as cancer cells acquired resistance when treated with chemotherapy drugs which results in high genetic versatility and instability of cancer cells [59], there is also the tissue-level resistance, mainly as a result of cancer-promoting factors in the tumor microenvironment and the contribution of stromal cells [58, 60].

Long-term administration of antiangiogenic drugs often results with acquired therapeutic resistance, a phenomenon which poses a great problem and causes immense concern for physicians and researchers as one [61]. Antiangiogenic therapy, as well as different chemotherapies, leads to metabolic deficiency alongside accelerated low levels of oxygen supply, which evidently promotes tumor death by necrosis [1]. Despite the initial positive response to treatment, in the form of successful inhibition of tumor cell progression, the resistance mechanisms cause normalization of the vasculature and even improves vascular function [59, 62–64].

TME is tremendously affected by the course of antiangiogenic treatment, and the resulting changes are responsible for the rise of tumor resistance. One of these mechanisms is the "tumor evasion" or "tumor escape" which is caused by the direct selection of clonal cell populations [65, 66]. These populations have the capacity to rapidly upregulate alternative proangiogenic pathways, causing an intrinsic resistance that eventually results in nonresponsive endothelial cells [67]. Mechanisms of endothelial resistance arise depending on the tumor type and microenvironment: different stromal components and functions that contribute to the full resistance phenotype [62–64]; upregulation of alternative proangiogenic signals leading to revascularization [62]; cancer-associated fibroblasts and macrophages that mimic cell growth [68, 69]; co-option of existing vasculature [67]; renewal of pericyte-covered tumor vessels encouraging recruitment of inflammatory and angiogenic cells, which shield them from anti-VEGF treatments [70–72]; and epigenetic regulations causing tumor cells to be more invasive and hypoxic [69]. The clinical outcomes were unexpected since endothelial cells, the target of those drugs, are considered genetically stable and therefore were not foreseen to develop such resistance [73, 74], as opposed to cancer cells which are highly versatile and are known to obtain resistance when treated with different chemotherapies [75, 76].

6.5 Hypoxia and Metabolic Stress

Hypoxia, the accelerated diminished supply of oxygen to the cells, is a mechanism which was widely studied in the past years and shown to promote metastases and upregulate tumor-promoting factors and inflammation [77–84]. In addition to cell proliferation and differentiation, hypoxia is also associated with apoptotic and necrotic cell death [2, 81, 85–87]. Though there is great knowledge of hypoxia and metabolic stress in cancer progression, mapping of the components that are affected by these processes and influence cancer recurrence was yet to be performed, and the knowledge in this arena is lacking and insufficient.

6.6 Cell Necrosis

Several cellular death mechanisms are described in the literature, like apoptosis, necrosis, and others [88–91]. Many kinds of cancer cells are known to be resistant to apoptosis as a result of a mutation in p53 [3, 92]. Therefore, necrosis is the main cell death pathway in cancer therapy. Necrosis plays an important role in the mechanism of cancer cell death [8]. Anticancer therapy often induces tissue hypoxia and metabolic depletion, two processes which drive necrosis (Fig. 6.1a) [8, 93]. Cytotoxicity leads to accelerated necrosis, where the content of the cells

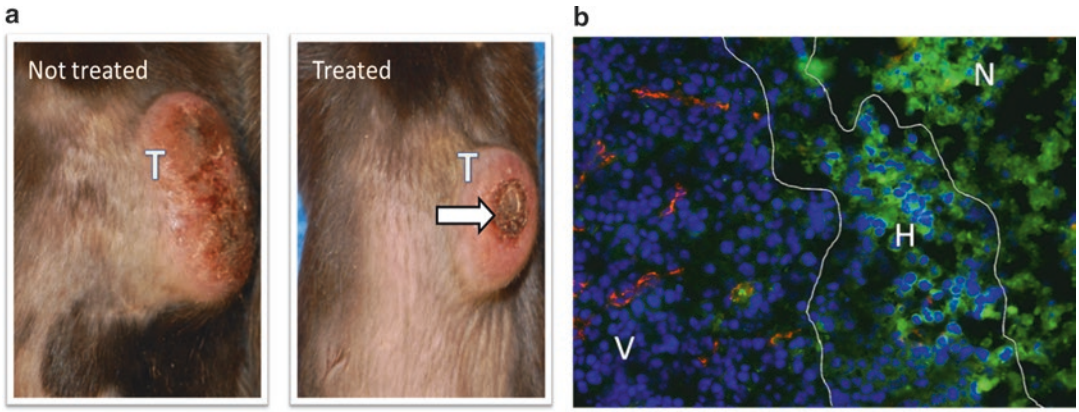

Fig. 6.1 Tumor necrosis induced by antiangiogenic therapy: (**a**) subcutaneous Lewis lung carcinoma in the backs of C57/BL mice. Mice treated with an anticancer drug (fumagillin) developed a smaller tumor with a deep, open, necrotic center (*Right*) as opposed to the larger tissue damage of the untreated subject (*Left*). (**b**) Tissue-level resistance: long-term anticancer treatment leads to necrosis, causing the secretion of tumor progression-generating factors that facilitate the tumor evasion and support recurrence

triggers the immune system which, in turn, ignites an inflammatory response and eventually promotes cancer progression. Most publications regarding the secretion of cytokines from the necrotic core center their attention on their effect on cancer progression via an inflammatory-dependent mechanism [94–96]; however, some researchers also inspect the inflammatory-independent pathways [8].

6.7 Inflammatory and Noninflammatory Effects

When cell death occurs in response to hypoxia and metabolic stress, often the result of anticancer treatments, the cell content is known to provoke an immune response [97, 98]. The disrupted membrane, a product of the necrotic process, consequently allows for the secretion of several intracellular signals, known as the damage-associated molecular patterns (DAMPs) or alarmins, which are released from the necrotic cells [99, 100]. DAMPs act as endogenous danger signals which exacerbate the inflammatory response in sterile inflammation [7, 101–103]. Unfortunately, DAMPs may act as a double-edged sword; on the one hand, they contribute to tissue repair and wound healing, and on the other hand, under chronic inflammation conditions, they encourage the progression of tumor growth [7, 104].

Though necrosis is the desired endpoint in many studies, increased metabolic deprivation and tumor hypoxia cause secretion of cancer-promoting factors from the necrotic core of the tissue, which evidently increases tumor aggressiveness and modifies the tumor microenvironment (Fig. 6.1b).

A meta-analysis of 21 studies (1663 patients) shows that giving soft tissue sarcoma patients neoadjuvant therapy can be associated with an elevated risk of cancer recurrence and lower rates of overall survival [105]. This could be explained by the increase in vascular formation, as well as cancer progression signals that are secreted by the necrotic tissue, to further support the growth of cancer cells which still reside in the tissue (Fig. 6.2).

6.8 Metronomic Therapy and Combined Treatment

One of the methods that were shown to withhold the formation of new blood vessels in the vicinity of the tumor is the low-dose chemotherapy, or

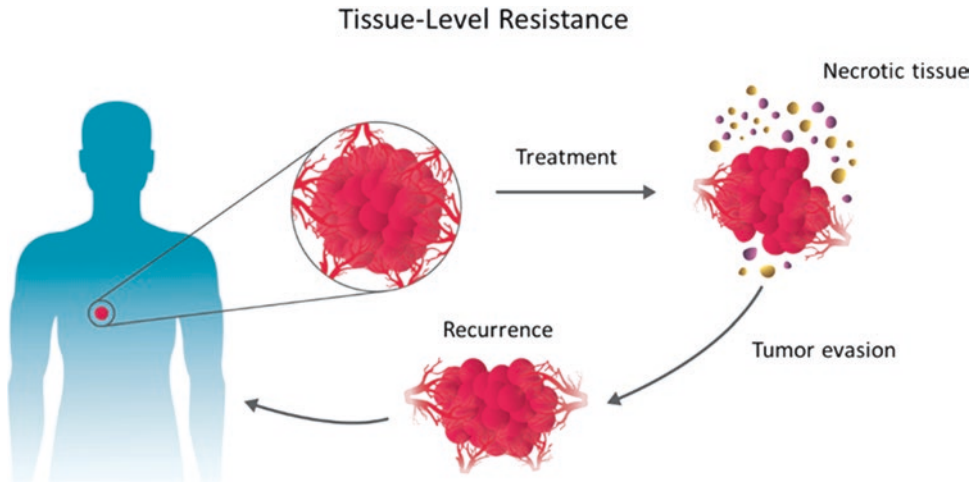

Fig. 6.2 Tissue-level resistance: long-term anticancer treatment leads to necrosis, causing the secretion of tumor progression-generating factors that facilitate the tumor evasion and support recurrence

"metronomic therapy" as was coined by Hanahan [106]. This approach is revolutionary in that it suggests that not only the drug itself but also the dosage and scheme of administration have a role in cancer resistance, though the full effect of this administration methods is yet to be clear [107].

Another approach to avoid the onset of angiogenesis and thereby tumor progression is combined therapy. Different combinations were studied, such as conventional therapies with immunotherapy [108], or targeted anticancer therapies with other drugs [109], showing varying efficacies of such treatment.

While the role of inflammation in cancer progression is widely studied, only little is known on inflammatory-independent pathways. Similarly, even though much work has been done in investigating the effect of hypoxia and metabolic stress on cancer progression and its resistance to treatments, the direct, noninflammatory effects of consequent cell necrosis on these processes remain to be properly determined [78]. Moreover, in most of these studies, researchers focused their attention on the contribution of either nutrient depletion or hypoxia, separately, on cancer progression and angiogenesis [110–116], rather than on the combined effect which drives cell necrosis.

In a recent publication by Karsch-Bluman et al. [8], the researchers studied the inflammatory-independent net total effect of necrosis on cancer progression and recurrence potential. The necrotic cell lysate was analyzed and the various proteins, which are upregulated under these conditions of hypoxia and metabolic deficiency, were mapped. Based on the acquired data, the study offers a combined treatment in which both the anticancer drug, which triggers necrosis, and an antagonist of a known signal that is secreted from the necrotic cell, known to support cancer progression, will be administered as prophylactic therapy.

This is a novel and exciting approach as by analyzing the tumor microenvironment post necrosis, scientists and physicians would be able to provide a prophylactic treatment that would antagonize cancer-promoting signals that are secreted from the dying cell and thereby increase the efficacy of the anticancer treatment and give patients hope for overcoming their sickness.

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7 The Multifaceted Effects of Autophagy on the Tumor Microenvironment

Rui Kang, Herbert Zeh, Michael Lotze, and Daolin Tang

Abstract

The tumor microenvironment is composed of cancer cells, noncancer cells (e.g., immune cells, stromal cells, endothelial cells, and adipocytes), and various mediators (e.g., cytokines, chemokines, growth factors, and humoral factors) that work together to support cancer growth, progression, and resistance to therapies. Autophagy is an evolutionarily conserved degradation mechanism by which various cytosolic cargos (e.g., damaged organelles, unused molecules, or invaded pathogens) are engulfed by double-membrane autophagosomes, and then delivered into the lysosome for degradation and recycling. The level of autophagy is a crucial threshold to either promote cell survival or induce cell death in response to environmental stresses. Autophagy plays a context-dependent role in tumorigenesis and anticancer therapy via shaping the inflammatory, hypoxic, immunosuppressive, and metabolic tumor microenvironment. In particular, impaired autophagy flux is associated with chronic inflammation, immunosuppression, stromal formation, cancer stemness, angiogenesis, metastasis, and metabolic reprogramming in the tumor microenvironment. Understanding the molecular machinery of autophagy and its communication with hallmarks of cancer could lead to potential new anticancer strategies or drugs.

Keywords

Autophagy · Tumor microenvironment · Fibroblast · Immune cells · Cancer stem cells · Hypoxia · Inflammation · Angiogenesis · Metastasis · Metabolic reprogramming · Cell death · Cytokine · Fibroblast · Immunosuppression · Ferroptosis

7.1 Introduction

As the second-leading cause of death globally over the past decades, cancer remains one of the major challenges of modern medical research [1]. Paget's "seed and soil" theory stands among the most famous theories of tumor biology, and has been widely recognized and extended since its first introduction in 1889 [2]. The theory proposes that the occurrence and development of tumors is not only a change in tumor cytogenetics and epigenetics but also depends on a special environment as a "fertile soil" for the growth and breeding of malignant seeds. The interaction

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between fertile environment and malignant seeds facilitates tumor initiation, progression, and metastasis. The tumor microenvironment consists of numerous immune cells, mesenchymal cells, extracellular matrix, and active mediators (e.g., cytokines, chemokines, growth factors, and humoral factors) other than tumor cells [3]. It can be divided into an immunocyte-based immune microenvironment and fibroblast-based nonimmune microenvironment [3]. An abnormal tumor microenvironment is closely linked to resisting cell death, promoting proliferation, evading immune destruction, maintaining inflammation, or inducing angiogenesis, which are major hallmarks of cancer [4]. Understanding the interplay between cancer cells and other components within the tumor microenvironment may result in effective anticancer strategies to suppress tumor growth and metastasis [5].

Autophagy is a homeostatic maintenance mechanism via the degradation of damaged organelles, unused molecules, or invasion pathogens through lysosomes [6, 7]. The word "autophagy" was termed in 1963 by Christian de Duve, who received the Nobel Prize in 1974 for the discovery of lysosomes. Although autophagy has phenotypic plasticity, it has been generally divided into three subtypes, namely macroautophagy, microautophagy, and chaperone-mediated autophagy. Microautophagy is the direct uptake of various substracts into lysosomes for degradation [8]. Chaperone-mediated autophagy is the recognition of proteins with the amino acid motif KFERQ by heat shock proteins (e.g., heat shock protein family A member 8, also known as HSC70) for subsequent lysosomal degradation [9]. Macroautophagy (hereafter known as autophagy) is the best-studied form of autophagy with dynamic changes in the formation of unique membrane structures (e.g., phagophore, autophagosome, and autolysosome) to sequester and engulf substrates for degradation. Dysfunction of autophagy signaling and the autophagy pathway is implicated in various human diseases, including cancer [10–12]. As a mechanism for recycling and balance, autophagy plays a dual role in either promoting or suppressing tumor growth, depending on tumor types, pathologic stages, and the types of substrates [13, 14]. In this chapter, we first introduce the molecular machinery of autophagy and its role in cell survival and death. Then we discuss the multifaceted effects of autophagy on the tumor microenvironment, which are linked to the formation of the hallmarks of cancer (Fig. 7.1). Understanding what cell types in the tumor microenvironment can be modulated by autophagy, and when, may enable significant treatment breakthroughs in tumor therapy.

7.2 Molecular Machinery of Autophagy

Autophagy involves multiple processes, including the formation of phagophores from various membrane resources (e.g., the endoplasmic reticulum, mitochondria, and plasma membrane), the sequestration of cargoes by autophagosomes from phagophores, and the degradation of cargoes by autolysosomes from the fusion of autophagosomes into lysosomes. These dynamic processes in the membrane are fine-tuned by a series of protein complex-mediated molecular mechanisms and signaling transduction. Among the molecular mechanisms, autophagy-related (ATG) proteins play an evolutionarily conserved role in the regulation of the process of autophagy. *ATG* genes were first identified and cloned from the yeast *Saccharomyces cerevisiae* through genetic screening technology 30 years ago [15–17]. Currently, there are 40 *ATG* genes involved in the regulation of autophagy in yeast and half of them are conserved in higher eukaryotes [18, 19]. Due to the growing importance of autophagy in biomedicine and human diseases, Yoshinori Ohsumi received the Nobel Prize in 2016 for the discovery of ATG as the key regulator of autophagy in yeast. Below, we introduce the core autophagy machinery in mammalian cells.

The unc-51-like autophagy-activating kinase (ULK) complex and the class III phosphatidylinositol 3-kinase (PtdIns3K) complex play a central role in the induction of the formation of phagophores, namely, isolated membranes. The ULK complex consists of ULK1, ATG13, and

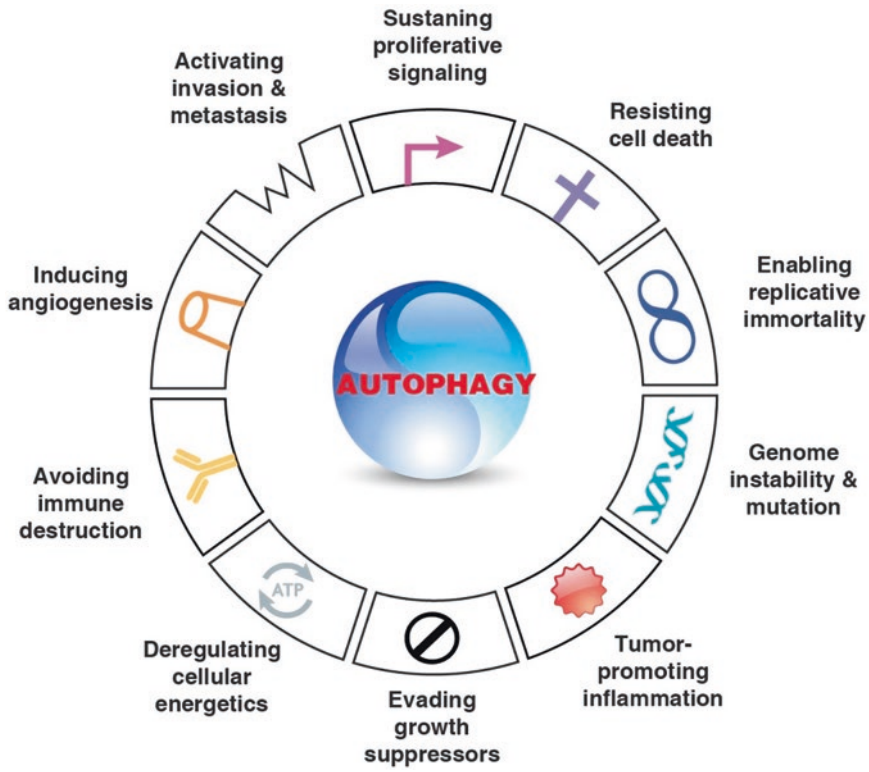

Fig. 7.1 Dual role of autophagy in hallmarks of cancer. Autophagy dysfunction is associated with each of the hallmarks of cancer and plays a dual role in cancer biology

RB1 inducible coiled-coil 1 (RB1CC1, also known as FIP200). ULK1 and RB1CC1 are also known as Atg1 and Atg17, respectively, in yeast. ULK1 is required for amino acid withdrawal-induced phagophore formation at the early stage of autophagy [20]. ULK2 may compensate for the loss of ULK1. Thus, the double knockout of ULK1 and ULK2 can completely block autophagy initiation in some cases. Unlike yeast Atg13, the HORMA domain of ATG13 at the N terminal may play a different role in the regulation of ULK1 complex activity via ATG101. The ULK complex is negatively regulated by the mammalian target of rapamycin (mTOR) and positively regulated by AMP-activated protein kinase (AMPK) [21, 22]. The class III PtdIns3K complex contains phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3), BECN1, and phosphoinositide 3-kinase regulatory subunit 4 (PIK3R4) [23]. PIK3C3, BECN1, and PIK3R4 also are referred to as Vps34, Atg6, and Vps15, respectively, in yeast. The class III PtdIns3K complex phosphorylates phosphatidylinositol to generate phosphatidylinositol 3-phosphate (PtdIns3P) in the isolation membranes and the subsequent formation of autophagosome.

Two ubiquitin-like conjugation systems, the ATG12 and microtubule-associated protein 1 light chain 3 (MAP1LC3) system, are essential for the formation of autophagosomes [24]. Autophagosomes are double-membrane vesicles that generate from the extending isolation membranes to surround cargoes. Half of ATGs are required for the formation and maturation of autophagosomes. AT12 is catalyzed by the E1-like enzyme ATG7 and transferred to the E2-like enzyme ATG10. ATG12 is finally conjugated to ATG5 and can recruit ATG16L1 to form the ATG12-ATG5-ATG16L1 complex. In contrast, the MAP1LC3 (an ortholog of yeast Atg8) is synthesized with additional arginine at its C terminal end, which is cleaved by ATG4 to produce

MAP1LC3Gly-116. This MAP1LC3 is activated by ATG7 and transferred to ATG3. Both these two ubiquitin-like conjugation systems can cause a phosphatidylethanolamine (PE) conjugate with MAP1LC3 (namely MAP1LC3-II) for targeting to autophagosomal membranes. MAP1LC3-II levels therefore correlate with autophagosome numbers.

Autophagosome-lysosome fusion, namely autolysosome formation, depends on the acidic pH as well as on the lysosomal membrane protein, such as lysosomal-associated membrane protein 2 (LAMP2) [25]. Lastly, a number of lysosomal hydrolases and other proteins are essential for the catabolism of autophagic cargoes and the release of macromolecules back into the cytosol. Of note, MAP1LC3-II is finally degraded along with the autophagosome contents by autolysosome. Thus, bafilomycin A1 and chloroquine, which are commonly used autophagy inhibitors that target autolysosome formation, can increase the MAP1LC3-II expression. Although measuring autophagic flux (namely the extent of autophagic degradation activity) remains challenging, the combined use of early- and late-phase autophagy inhibitors are required for autophagic flux assay.

7.3 Autophagy-Dependent Cell Survival and Cell Death

Historically, autophagy was defined as a form of cell death based on the morphological classification system of 1973 [26]. According to morphological criteria in ultrastructural study, cell death is generally divided into three forms, namely types I, II, and III [27]. Type I is often referred to apoptotic cell death with the significant changes of cell shrinkage and membrane blebbing, whereas type III is best known as necrotic cell death with the large occurrence of cell swell and rupture. In contrast, type II corresponds to autophagic cell death with the accumulation of membrane vesicles, such as autophagosome and autolysosome. However, the term autophagic cell death is controversial, and it does not distinguish between cell death induced by autophagy and cell death accompanied by autophagy [28].

Currently, the Nomenclature Committee on Cell Death suggests that autophagy plays a dual role in cell survival and cell death, which is highly contextual [29]. In many cases, increased autophagy is associated with cell death and promotes cell survival. In some cases, excessive autophagy triggers cell death, which is termed as autophagy-dependent cell death [29]. Of note, not all core ATGs are required for autophagy-dependent cell survival and cell death, indicating that ATGs may play an autophagy-dependent or independent role in these processes [30].

As a self-clearance mechanism, autophagy can serve pro-survival functions to remove harmful cytosolic components, such as damaged mitochondria [31, 32]. This increased autophagic flux results in increased recycling from degraded cargos to feed back into the synthesis of amino acids and adenosine triphosphate (ATP), which finally enables rapid protein synthesis and energy production in response to various cellular stresses, including cell death stimuli [31, 32]. In addition to bulk autophagy function as a defense mechanism to promote survival during nutritional stress, selective autophagy can remove cell death effectors such as caspases in response to certain anticancer agents [33]. Thus, the combination of autophagy inhibitors, such as 3-methyladenine and chloroquine, or knockdown of ATG by RNAi can enhance the anticancer activity of chemotherapy agent-induced apoptosis. In contrast, the overexpression of ATG may promote chemotherapy resistance. Autophagy also influences organismal health and aging [34]. Most global ATG-deficient mice are embryonic-lethal and associated with increased cell death and tissue injury, whereas some autophagy inducers (e.g., ramamycin) can prolong animal survival, supporting a pro-survival role of autophagy in vivo [34].

The mechanism of action of autophagy-dependent cell death is complex, involving effects at multiple levels. Excessive bulk autophagy can cause the release and activation of lysosomal hydrolases to trigger death [35]. The excessive removal of normal organelles such as mitochondria could lead to metabolism collapse and oxidative injury [36]. The induction of Na^+-

K^+-ATPase-mediated autosis is required for Tat-Beclin peptide-induced autophagy-dependent cell death in cancer cells [37]. Certain ATGs, such as ATG5 and ATG7, can induce necroptosis through binding to necrosome, whereas ATG3 and ATG12 can form a complex to induce apoptosis through the inhibition of BCL2-like 1 (BCL2L1, also known as BCLXL) [38, 39]. Selective autophagy promotes cell death through the degradation of antideath regulators of apoptosis [40], necroptosis [41], or ferroptosis [42]. Notably, lipid peroxidation-dependent ferroptosis is considered as a type of autophagy-dependent cell death through multiple mechanisms [43], such as the activation of ferritinophagy [42], lipophagy [44], or clockophagy [45], BECN1-mediated system xc^- inhibition [46], or signal transducer and activator of transcription 3 (STAT3)-induced lysosomal cell death [47]. In addition to mediating various neurogenetic disorders, autophagy-dependent cell death can affect tissue development under physiologically relevant conditions [48–50]. These findings indicate a wider role of autophagy-dependent cell death in physiological and pathological conditions.

7.4 Autophagy and the Tumor Microenvironment

7.4.1 Inflammation and Autophagy

Inflammation is the innate immune response to infection and injury, which is usually associated with symptoms of redness, heat, swelling, and pain. Leukocyte-mediated acute inflammation response generally contributes to microbial clearance, wound healing, and tissue regeneration. In contrast, chronic inflammation has been linked to certain human diseases, such as autoimmune disorders and cancer. In particular, leukocyte (e.g., neutrophil, monocyte, and macrophage) infiltration can release various pro-inflammatory cytokines to promote tumor formation through multiple mechanisms, such as causing DNA damage or inducing immune suppression. Chronic inflammation is a well-known risk factor for various types of cancer [51]. For example, people with chronic pancreatitis and inflammatory bowel disease have increased risks of pancreatic cancer and colorectal cancer, respectively. Indeed, an inflammed tumor microenvironment is one of the enabling hallmarks of cancer development [52]. The targeting of cancer-related chronic inflammation by nonsteroidal anti-inflammatory drugs is an important approach to the prevention of malignant disease [52].

In normal cells, autophagy is generally considered as an anti-inflammatory mechanism through the degradation of pro-inflammatory regulators such as inflammasome components [53, 54]. In contrast, autophagy plays a dual role in the regulation of inflammation response in the tumor microenvironment [55]. Autophagy can sustain the inflammatory tumor microenvironment through the production and release of pro-inflammatory cytokines such as high-mobility group box 1 (HMGB1) and interleukin (IL)-6 and recruitment of inflammatory cells [56–58]. HMGB1 is not only a DNA binding protein in the nucleus but also an immunomodulatory cytokine actively secreted from immune cells or passively released by dead or dying cells [59]. HMGB1 also plays a location-dependent role in promoting autophagy in cancer cells [60, 61]. In contrast, autophagy-mediated nonclassical release in immune cells as well as autophagy-dependent cancer cell death can promote HMGB1 release, which further leads to the activation of pro-inflammatory transcription factor nuclear factor-κB (NF-κB) to sustain tumor growth [59, 62]. Oncogenic RAS-induced tumor transformation is associated with increased autophagy, which induces pro-inflammatory IL6 release. IL6 is one of the major cytokines in the tumor microenvironment that drives tumor formation and development through the activation of STAT3. Autophagy-mediated toll-like receptor (TLR) and advanced glycosylation end-product specific receptor (AGER, also known as RAGE) activation can promote inflammation and tumor cell growth in response to bacterial infection or tissue injury [63, 64]. In addition to pro-inflammatory function by autophagy, the conditional knockout of ATG (such as ATG5 and ATG7) in mice promotes tumorigenesis, which is associated with

the upregulation of inflammatory genes [65, 66]. Thus, the interplay between autophagy and inflammatory pathways in the tumor microenvironment is context-dependent, and a better understanding of such a relationship may provide a more precise therapy for cancer patients.

7.4.2 Immunity and Autophagy

Cancer immunoediting is an immune system-mediated dynamic process that consists of three stages: elimination, equilibrium, and escape [67]. In the first elimination phase, tumor cells can be removed from the immune system. In the second equilibrium phase, the immune system can control (but not completely eliminate) tumor growth. In the last escape phase, tumors may escape elimination by recruiting or expressing immunosuppressive cells (e.g., regulatory T cells [Treg] and myeloid-derived suppressor cells [MDSCs]) or molecules (e.g., immune checkpoint) within the tumor microenvironment. Cytotoxic CD8 T lymphocytes (CTLs) and natural killer cells (NKs) play a major role in the elimination of tumor cells [68, 69]. Dendritic cell (DC)-mediated cross-presentation of tumor antigens is generally required for the production and activation of antitumor CD8 T-cell immunity. Immune suppression of the microenvironment is associated with cancer growth and progression. In contrast, a major breakthrough in cancer immunotherapy is the use of immune checkpoint inhibitors, which remove inhibitory signals of CTLs or NKs [70].

Autophagy regulates T-cell activity and function by various mechanisms. The activation of T-cell receptor by cytokines can induce autophagy in naive T cells. In contrast, the knockout of *Atg5*, *Atg7*, or *Atg3* impairs the cell survival and function of various types of T cells, including CD4+ and CD8+ T cells [71]. In addition to modulating normal T-cell functions, autophagy is implicated in T-cell-mediated antitumor immune response. The induction of autophagy by an mTOR inhibitor such as rapamycin increases the production of CTLs [72]. In breast tumor models, the ablation of *Rb1cc1/Fip200* impairs autophagy and increases antitumor immune responses [73]. In lung cancer models, the ablation of *Atg5* impairs autophagy and increases the immune suppression microenvironment via the upregulation of forkhead box P3 (FOXP3)+ Tregs [74]. In contrast, ATG5, AT14, or ATG16L1-mediated autophagy limits antitumor CD8 T-cell immunity via reprogramming glucose metabolism in certain types of cancer, such as syngeneic mammary, prostate, and colorectal tumors [75].

In addition to T cells, autophagy can promote or inhibit tumor growth by control of the activation and function of other immune cells, such as NKs, natural killer T cells (NKTs), B cells, DCs, macrophages, and MDSCs in the tumor microenvironment [76, 77]. For example, autophagy-mediated NKT, DC, and B-cell activation are required for antitumor immunity through the upregulation of antigen presentation or induction of specific IgM and IgG production [78, 79]. Autophagy also affects macrophage polarization and reprogramming to promote or inhibit tumor development via the release of immune-modulatory cytokines, such as IL1, IL2, IL6, IL10, IL12, IL23, transforming growth factor beta (TGFB), nitric oxide synthase 2 (NOS2, also known as iNOS), tumor necrosis factor (TNF), and interferon gamma (IFNG, also known as IFN-γ) [80, 81]. MDSCs exhibit increased autophagy to diminish antitumor immunity [82, 83]. Mitophagy deficiency promotes pancreatic cancer development through the upregulation of inflammation-mediated immunosuppression in the tumor microenvironment [84]. This process relies on HMGB1 release and subsequent immune checkpoint expression [84]. The expression of dominant negative *Atg4B* alleles ($Atg4B^{CA}$) inhibits autophagy and pancreatic ductal adenocarcinoma (PDAC) tumor growth partly through the accumulation of antitumor macrophages in the tumor microenvironment [85].

The dysfunction of the autophagy pathway is also implicated in tumor immunotherapy [86–89]. Immunogenic cell death is a form of regulated cell death caused by chemotherapy or radiation therapy [90]. Autophagy-mediated ATP and HMGB1 release contributes to immunogenic cell death to enhance the effects of immunotherapy [91, 92]. The inhibition of autophagy by the

knockdown of *Becn1* and *Atg5* or by using chloroquine promotes the infiltration of CTLs and NKs as well as degradation of phospho-STAT3 within the tumor microenvironment to suppress tumor growth [89, 93]. Autophagy can inhibit NK activity through the degradation of granzyme B under hypoxia. In addition, the c-Jun N-terminal kinase (JNK)-dependent production of C-C motif chemokine ligand 5 (CCL5) plays a major role in the induction of NK cell infiltration in *Becn1*-defective melanoma cells [94]. Chloroquine also enhances the anticancer activity of IL2 and dopachrome tautomerase (DCT, also known as TRP2) peptide-based immunovaccine [86–89]. In contrast, autophagy-mediated IL10 and TGFB production may limit antitumor immunity through the activation of Treg and MDSC [74]. These findings could perhaps explain the context-dependent consequences of autophagy on tumor immunity, and the inhibition of autophagy may overcome or promote antitumor immune resistance.

7.4.3 Stromal Cells and Autophagy

Stromal cells, the connective tissue cells of organs, are important components in the tumor microenvironment. Fibroblasts are the most common stromal cells in the tumor microenvironment throughout all stages of the cancer [95]. The interplay between cancer cells and cancer-associated fibroblasts (CAFs) can affect the production and infiltration of immune cells via the secretion of a wide variety of factors in the tumor microenvironment [96]. The loss of specific tumor suppressors in CAFs influences the type and character of immune cells in the tumor microenvironment. Consequently, blocking CAF function enhances tumor immunotherapy activity [97]. The anticancer activity of immune checkpoint inhibitors is enhanced by the inhibition of CAFs [98–100]. CAFs also facilitate tumor invasion and metastasis through the production of matrix metalloproteinases as well as extracellular matrix (ECM) components, including collagen, fibronectin, and proteoglycans [96]. These CAF-dependent factors also play a pathologic role in the regulation of the tumor microenvironment.

Autophagy is not only upregulated in CAFs through hypoxia and oxidative stress but also promotes the communications between the tumor and CAFs [101]. Autophagic CAFs can provide nutrition to surrounding cancer cells via the reverse Warburg effect to support tumor proliferation and growth [102–104]. CAFs also use mitophagy, a selective autophagy to remove damaged mitochondria, to produce glutamine to fuel neighboring cancer cells [102, 103, 105]. In addition, autophagic CAFs have the ability to inhibit apoptosis, promote angiogenesis, induce gene instability, and sustain cancer stemness and immunosuppression in the tumor microenvironment. The expression of caveolin-1 in CAFs seems to play a major role in the regulation of autophagy and metabolism within the tumor microenvironment [106–108]. Stromal caveolin-1 is a biomarker for cancer prognosis in breast cancers. In addition, autophagic CAFs can support tumor growth through the upregulation of TP53-induced glycolysis regulatory phosphatase (TIGAR), a TP53-targeted gene that can inhibit glycolysis [105, 109]. These findings indicate an autophagy-dependent metabolic pattern between cancer cells and CAFs in the tumor microenvironment.

7.4.4 Angiogenesis and Autophagy

Tumor growth and metastasis depend on angiogenesis, the process of new blood vessel formation [110, 111]. The new blood vessels can provide oxygen and nutrients and remove unwanted metabolites and CO_2 for tumor growth, invasion, and metastasis. An imbalance between oxygen supply and demand can cause hypoxia, one of the important hallmarks of the tumor microenvironment contributing to therapy resistance to chemotherapy, radiotherapy, and immunotherapy. During tumorigenesis, hypoxia is a critical signal to initiate angiogenesis, which is controlled by the hypoxia-inducible factor (HIF) transcription factor family, especially HIF1A (also known as HIF1α) [112]. Under normoxic conditions, the von Hippel-Lindau syndrome (VHL, also known as pVHL)-mediated ubiquitin

protease pathway promotes HIF1A degradation with a short half-life (<5 min) in an O_2-dependent mechanism. In contrast, hypoxia can block HIF1 degradation through the inhibition of the activity of the Egl-9 family hypoxia-inducible factor 1 (EGLN1, also known as PHD2) and the binding between VHL and HIF1a, which leads to increased HIF1A protein stability and subsequently its translocation from the cytosol to the nucleus [113, 114]. Nuclear HIF1A increases gene expression through binding to HIF-responsive elements (HREs) in the promoter of target genes. HIF2A shares a similar structure and function with H1F1A, whereas HIF3A acts as the inhibitor of HIF1A and HIF2A. HIF1A-mediated gene upregulation plays multiple roles in the regulation of energy metabolism, angiogenesis, metastasis, and other phenotypes in the tumor microenvironment. Notably, HIF1A-induced vascular endothelial growth factor (VEGF) expression is essential for angiogenesis [115]. In addition to HIF1A, mTOR inhibition and unfolded protein response is involved in the induction of autophagy under hypoxia [116–119]. The cooperation between HIF1A and HIF2A and the stem cell self-renewal transcription factor nanog homeobox (NANOG) can induce BNIP3 and BNIP3L expression to trigger mitophagy and autophagy in response to hypoxia [120, 121].

Recent studies indicate that autophagy regulates angiogenesis in tumor development [122–124]. Blood flow restriction increases metabolic stress and subsequent autophagy in endothelial cells, the major components of blood vessels. HIF1A-mediated autophagy promotes angiogenesis and regulates lipid metabolism and the redox homeostasis in endothelial cells in response to hypoxia and other stresses. Lipophagy is selective autophagy involving the degradation of lipid droplets that contribute to vascular lipid homeostasis. The inhibition of autophagy by chloroquine can suppress VEGF expression and promote vessel normalization, which is independent of ATG [125, 126]. Consistently, anti-angiogenic treatment blocks autophagy in endothelial cells. Additionally, autophagy-mediated cell surface molecular cadherin 5 (CDH5) degradation may affect endothelial junction formation and vessel permeability [127]. The manipulation of vascular autophagy might therefore be an attractive anticancer approach to inhibit tumor invasion and metastasis.

7.4.5 Stem Cells and Autophagy

Normal stem cells include embryonic and adult stem cells that can differentiate into other types of cells. Similarly, cancer stem cells are a small subtype of cancer cells within the tumor microenvironment and have the ability to immortalize, continue to divide, and differentiate [128]. These cells can produce a source of new cancers. The notion of cancer stem cells was first proposed in leukemia and $CD34^{+}CD38^{-}$ cells were the first cancer stem cells to be isolated, which came from patients with acute myeloid leukemia. Cancer stem cells are increasingly thought to be one of the important causes of cancer metastasis, recurrence, or tumor resistance to chemotherapy and radiation therapy [129, 130]. Targeting cancer stem cells as well as tumor heterogeneity provides a promising opportunity to treat tumors.

During the last decade, autophagy and mitophagy have been demonstrated to play a potential role in the maintenance of activity and function of cancer stem cells and in preventing their aging in response to various stresses [131, 132]. The level of autophagy is increased in cancer stem cells in many types of tumors, including those of the breast, pancreas, liver, ovaries, and brain. Increased autophagy is important to sustain pluripotency, a key feature of cancer stem cells that allows them to differentiate into new tumor cells [131, 132]. Autophagy and mitophagy also provide metabolic substrates to maintain energy production to support survival, migration, and invasion of cancer stem cells in response to anticancer agents. In contrast, the knockdown of ATG or using chloroquine or bafilomycin A1 suppresses cell proliferation, limits cell migration, and induces cell death in cancer stem cells [133–140]. In addition, the activation of autophagy can protect cancer stem cells against immune elimination through the production of TGFB and

IL4 or the infiltration of immune suppressor cells [133, 141]. Autophagy also plays a dual role in the regulation of ploncolytic virotherapy-mediated clearance of cancer stem cells [134].

7.4.6 Metabolism and Autophagy

A common feature of the metabolism of cancer cells is the ability to obtain energy to support the cells' rapid growth and division. ATP is a substance used in cells to store and transfer chemical energy. It is a high-energy phosphate compound that interacts with adenosine diphosphate (ADP) to achieve energy storage and discharge. ATP is mainly produced by glycolysis in the cytosol and oxidative phosphorylation (the tricarboxylic acid cycle) in the mitochondria. In normal tissues, 90% of ATP is derived from oxidative phosphorylation, only 10% is derived from glycolysis, and glycolysis is inhibited under aerobic conditions, called the Pasteur effect. In 1924, Otto Warburg, a Nobel Prize winner in physiology and medicine, discovered that tumor cells produce more energy and absorb more glucose than normal mature cells through the aerobic glycolysis pathway, but not through the oxidative phosphorylation pathway (named the Warburg effect) [142]. In addition to aerobic glycolysis, cancer cells can rewire many metabolic pathways such as fatty acid metabolism, glutamine metabolism, choline metabolism, serine metabolism, and one-carbon metabolism to shape the tumor microenvironment [143–147].

Autophagy sustains tumor metabolism through recycling cytosolic materials to provide substrates for nucleotide synthesis and energy production. In addition to cancer cells, tumor-associated stromal cells and certain immune cells can generate energy (ATP) via an autophagy-mediated Warburg effect [148–151]. Cancer cells require fatty acid for the synthesis of biological molecules (e.g., phospholipids and second messengers) and fatty acid oxidation for the production of energy [152–154]. Adipocytes in the tumor microenvironment can induce autophagy and mitochondrial fatty acid oxidation to support tumor growth [155]. Moreover, autophagy-deficient genetic tumor models, including RAS-driven lung and pancreatic cancer, exhibited decreased fatty acid oxidation [152, 156–159]. Glutamine, an important amino acid for tumor growth, can be uptaken by membrane transporters (i.e., solute carrier family 1 member 5 [SLC1A5] and solute carrier family 7 member 5 [SLC7A5]) to enter the glutaminolysis pathway for the production of ATP and lactate. Glutaminolysis can promote or inhibit autophagy via the production of the intermediates ammonia or α-ketoglutarate, respectively. Choline is an essential nutrient and aberrant choline metabolism contributes to oncogenesis and tumor progression. Choline kinase alpha, an enzyme catalyzing the first step in the choline pathway for phosphatidylcholine biosynthesis, inhibits autophagy in breast cancer cells [160, 161]. Serine can be cleaved into glycine and 5,10-methylenetetrahydrofolate (a one-carbon unit) by serine hydroxymethyltransferase to support cancer cell growth. Mitophagy has been observed after mitochondrial dysfunction and injury. Serine deficiency may cause mitochondrial injury and induce mitophagy in cancer cells [162]. Additional metabolic pathways, such as the autophagy-mediated production and uptake of alanine from CAFs by tumor cells, promotes PDAC tumor growth [163]. Further investigation is required to define the role of autophagy-based metabolic networks in anticancer therapy.

7.4.7 Metastasis and Autophagy

Cancer metastasis is the process by which cancer cells spread into distant tissues (e.g., liver, lung, and bone) and then form new tumors [164]. Metastatic cancer is a key issue in the clinic, and most cancer patients die from tumor metastasis but not the primary cancer. Although the process of tumor metastasis remains largely unknown, it occurs in a complex tumor microenvironment and can be described as defined steps, including local invasion, intravasation, extravasation, and colonization at the distal sites [165]. Multiple mechanisms of cell migration are beginning to be uncovered and may be beneficial for understanding the phenotype of metastatic cancer.

Changes in autophagy enable tumor cell migration, invasion, and metastasis [166, 167]. Focal adhesions are large macromolecular assemblies that provide the main sites of cell adhesion to the surrounding extracellular matrix. Many kinases, such as Src, FAK, integrin-linked kinase, and phosphatase, are involved in the regulation of focal adhesions during cell migration. Autophagy promotes cell migration through the control of activity of focal adhesion complexes via the selective degradation and turnover of focal adhesion regulators such as paxillin, Src, and Rho [168–174]. These focal adhesion regulators also have the ability to promote or inhibit autophagy as a feedback mechanism. In addition, hypoxia and TGFB-induced autophagy can promote cancer stem cell self-renewal and angiogenesis, which finally result in epithelial-to-mesenchymal transition and tumor metastasis [175–180]. These findings highlight an important role of autophagy in cancer metastasis.

7.5 Conclusions and Perspectives

The complexity and adaptability of the tumor microenvironment have been increasingly recognized as key drivers involved in all stages of cancer development, thereby representing a major direction for the discovery and development of new anticancer targets. Autophagy is an intracellular clearance system that requires the formation of various vacuoles to engulf and deliver cytoplasmic components to lysosomes for degradation. Autophagy and its regulator ATGs play complex and context-dependent roles in tumorigenesis and anticancer therapy. The dysfunction of autophagy has been implicated in all aspects of cancer biology, such as cell survival, death, differentiation, angiogenesis, metastasis, inflammation, immunity, and metabolism. Extensive clinical evidence exists to support the idea of inhibiting autophagy by chloroquine to improve clinical outcomes in certain types of cancer patients (e.g., those with PDAC and glioblastoma). Notably, like ATG, the autophagy inhibitor (e.g., chloroquine) may also play an autophagy-independent role in the tumor microenvironment as well as anticancer therapy. It will be important to distinguish the direct and indirect effects of autophagy-mediated organelle or molecule degradation in the control of tumor initiation and development. Further understanding of the molecular machinery of autophagy and its interplay with other hallmarks of cancer could lead to potential new anticancer strategies or drugs.

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The Sociobiology of Brain Tumors

8

David H. Gutmann

Biological diversity is the key to the maintenance of the world as we know it. Life in a local site struck down by a passing storm springs back quickly: opportunistic species rush in to fill the spaces. They entrain the succession that circles back to something resembling the original state of the environment.

Edward O. Wilson

Abstract

Brain tumors are complex cellular ecosystems, composed of populations of both neoplastic and non-neoplastic cell types. While the contributions of the cancer cells in low-grade and high-grade gliomas have been extensively studied, there is comparatively less known about the contributions of the non-neoplastic cells in these tumors. As such, a large proportion of the non-neoplastic cells in gliomas are resident brain microglia, infiltrating circulating macrophages, and T lymphocytes. These immune system-like stromal cells are recruited into the evolving tumor through the elaboration of chemokines, and are reprogrammed to adopt new cellular identities critical for glioma formation, maintenance, and progression. In this manner, these populations of tumor-associated microglia and macrophages produce growth factors that support gliomagenesis and continued tumor growth. As we begin to characterize these immune cell contributions, future therapies might emerge as adjuvant approaches to glioma treatment.

Keywords

Astrocytoma · Cancer · Chemokine · Glioblastoma · Glioma · Ecosystem · Macrophage · Microglia · Neurofibromatosis type 1 · RAS · T lymphocyte · Tumorigenesis · Tumor microenvironment

Decades of research in the field of cancer have focused on the genetic and genomic alterations that occur within the neoplastic cells, largely ignoring the fact that all solid tumors are multicellular organisms composed of numerous distinct cell types that change over time. In this regard, both benign and malignant tumors grow in a highly adaptive milieu containing immune system-like cells, vascular elements, reactive stromal cells, and stem-like (progenitor) cells, each contributing in unique ways to cancer homeostasis and fitness. As such, cancers operate like cellular societies in which independencies are established between distinct cell types through plasma membrane-bound and soluble (paracrine) mediators. This concept of a tumor ecosystem has gained traction over the past several years, as researchers and clinicians have begun to appreciate the complex interactions between the neoplastic and non-neoplastic cells and their importance for tumor formation, maintenance, and progression.

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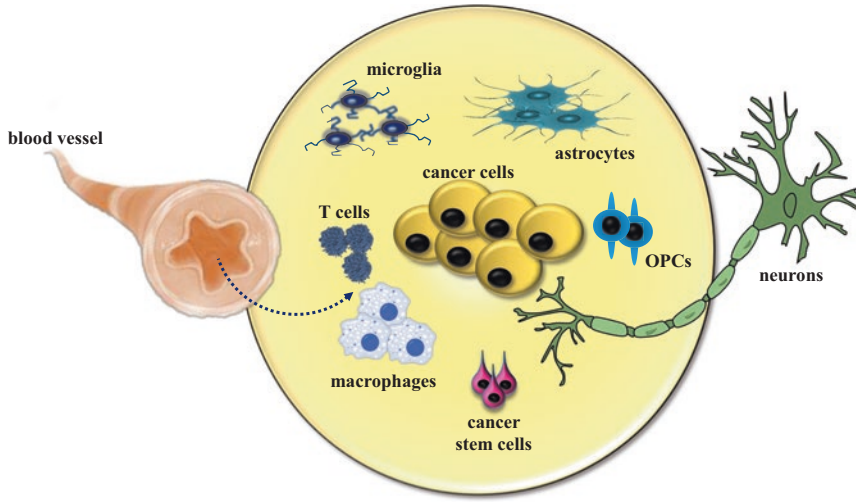

Fig. 8.1 Numerous cell types exist in gliomas. Low-grade and high-grade gliomas are composed of neoplastic (cancer cells and cancer stem cells) and non-neoplastic (astrocytes, oligodendrocyte precursor cells [OPCs], oligodendrocytes [not shown], neurons, T cells, microglia, and macrophages). Whereas T cells and macrophages infiltrate the brain, the other cell types are intrinsic to the central nervous system

The idea of a tumor ecosystem is highly reminiscent of the societies established by some species of insects (ants, bees, and termites). The colonies of these social insects exhibit a reproductive division of labor with cooperative brood care, homeostasis, and emergent behaviors and, in this manner, collectively function as "superorganisms." Each of the different castes of insects has specific functions that synergistically support colony perpetuation and overall fitness: a single queen lays the eggs, which are fertilized by drones (or a single king), while nonreproductive (sterile) worker insects are responsible for nest construction, maintenance, and defense against intruders (soldiers). This caste system is tightly regulated by chemical signals (pheromones) that serve to optimize the number of soldier, worker, or reproductive insects.

Similar to social insects, tumors are composed of distinct cell types that work in unison to facilitate tumor survival, growth, and homeostasis. Within these tumors are neoplastic cellular elements, such as differentiated cancer cells and their stem cell progenitors, the latter of which largely lie dormant unless required to replenish the tumor mass in the setting of chemotherapy or radiation. In addition, there is a panoply of non-neoplastic (stromal) cell types that are either intrinsic to the tissue or are recruited into the evolving tumor. In the setting of brain tumors, these intrinsic cellular elements include neurons, glial cells (astrocytes, oligodendroglial precursor cells [OPCs], and oligodendrocytes) and resident brain macrophages (microglia), as well as T cells and circulating macrophages recruited from the blood (Fig. 8.1). Herein, we propose to use brain tumors, and specifically low-grade and high-grade (malignant) gliomas, to illustrate how the cancer ecosystem is constructed, regulated, and maintained in a collective manner to facilitate the perpetuation and overall fitness of the tumor.

8.1 The Genetics of Pediatric Low-Grade Glioma

Brain tumors are largely categorized by their histological appearance, where pediatric low-grade gliomas express proteins normally found in glial cell populations (e.g., glial fibrillary acidic protein, GFAP) and harbor low proliferative indices (typically <5% of the cells being $Ki67^+$). Unlike their more clinically aggressive and fatal adult counterparts (glioblastoma; see below), these tumors have comparatively few genetic mutations. Among the mutations encountered, the

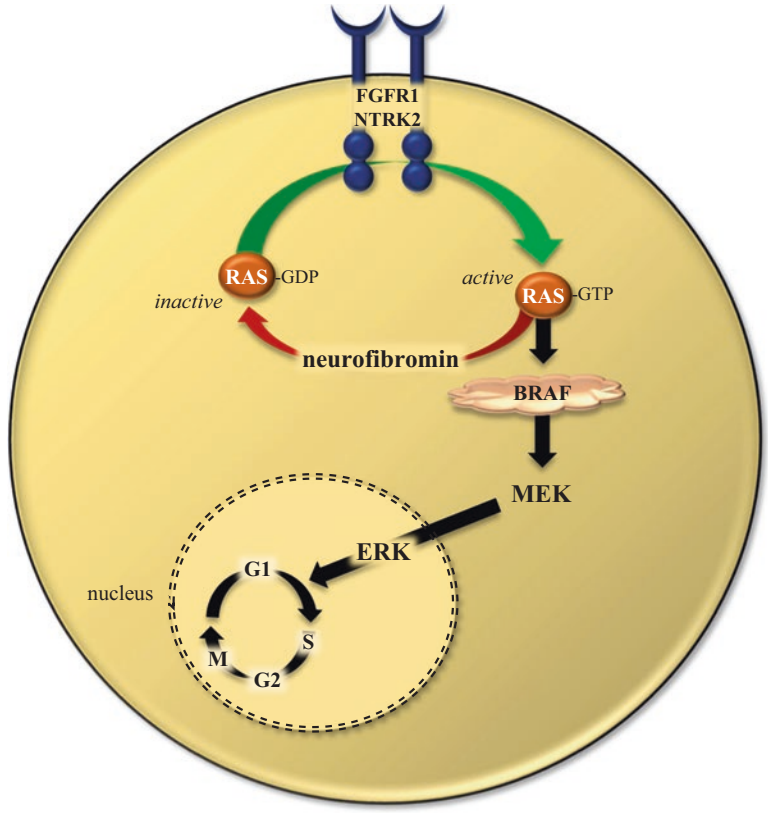

Fig. 8.2 Mutational landscape of low-grade glioma. Few genetic alterations have been reported in pediatric low-grade gliomas. These include activating mutations in receptor tyrosine kinases (*FGFR1*, *NTRK2*), loss of the *NF1* protein (neurofibromin), and alterations in the *BRAF* kinase gene (e.g., activating mutations, *BRAF* genomic rearrangements). Importantly, all of these changes lead to MEK activation and ERK-mediated accelerated progression through the cell cycle and increased cell growth

majority lead to increased growth factor signaling, all converging on the RAS mitogenic pathway. In this respect, the spectrum of causative mutations include chromosomal rearrangements involving the *BRAF* gene (e.g., *KIAA1549:BRAF*), leading to elevated BRAF kinase activity, activating receptor kinase receptor mutations (*NTRK2* and *FGFR1* genes) that increase RAS activity, and biallelic loss of the neurofibromatosis type 1 (NF1) tumor suppressor gene causing greater RAS activation (Fig. 8.2). Importantly, while all of these genomic alterations lead to increased RAS/MEK/ERK activity and cell growth; by themselves, they are not oncogenic (transforming) mutations. As such, they provide a mitogenic or anti-apoptotic advantage, which operates in a context-dependent manner, in concert with other signals from the tumor microenvironment, to increase precursor cell growth and culminate in low-grade glioma formation.

8.2 The Genetics of Malignant Glioma

In contrast to their benign pediatric counterparts, high-grade gliomas (glioblastoma) likely arise from a benign precursor lesion (low-grade glioma) through the successive accumulation of additional genetic alterations. Glioblastomas are divided into those with wild-type isocitrate dehydrogenase 1 or 2 (*IDH1/IDH2*) gene expression and those that harbor a mutant *IDH1* gene. *IDH* mutation leads to the production of alpha-

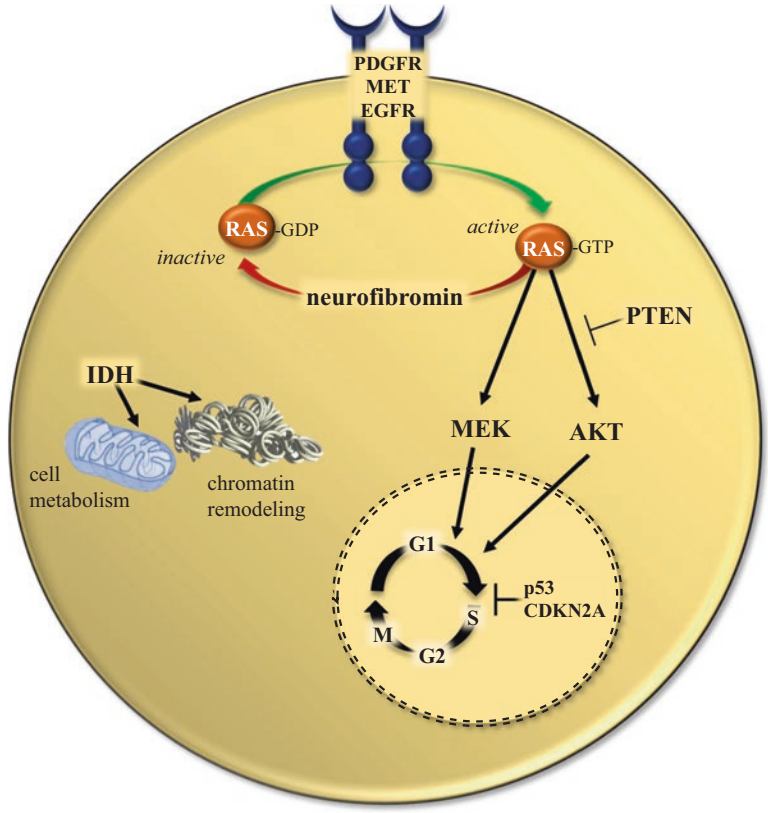

Fig. 8.3 Mutational landscape in high-grade glioma. A number of different genetic alterations have been reported in adult high-grade gliomas. These include activating mutations in or amplifications of receptor tyrosine kinases (*EGFR*, *PDGRF*, *MET*), loss of the *NF1* protein (neurofibromin), and mutations in the *PTEN* negative regulator of AKT activation, and loss of expression/function of cell cycle regulators, like the *TP53* (p53 protein) and *CDKN2A* genes. In addition, some malignant gliomas harbor mutations in the *IDH1* and *IDH2* genes, leading to changes in metabolism and epigenetic changes (chromatin remodeling)

ketoglutarate, which reshapes the metabolism and epigenetic (aberrant histone methylation) status of the cancer cells [1]. The importance of *IDH1* mutation to tumor biology is further underscored by the improved survival of patients harboring these mutations relative to their wild-type *IDH1* counterparts [2]. In the *IDH1* wild-type class of tumors, most glioblastomas share amplification of the platelet-derived growth factor A (*PDGFA*) gene and loss of portions of chromosome 10 containing the *PTEN* tumor suppressor gene, followed next by mutations in the *NF1* and *TP53* (p53) tumor suppressor genes, mutational activation of the epidermal growth factor receptor (EGFR) and c-MET receptor, and loss of the cell cycle regulator *CDKN2A* [3] (Fig. 8.3). These molecular alterations are critical for neoplastic transformation by coupling deregulated mitogenic signaling and increased cell growth with mutations that remove the compensatory mechanisms, which, separately or together, prevent unlimited cell proliferation or survival.

8.3 Tumor Cell Genomic Changes Are Necessary, But Not Sufficient, for Low-Grade Gliomagenesis

Since high-grade gliomas acquire a multiple of genetic and genomic changes, the primary role of the tumor microenvironment in malignant glioma

formation and progression is more difficult to discern. In contrast, the smaller number of molecular alterations in low-grade gliomas has facilitated a detailed exploration of the contributions of stromal cells and signals to gliomagenesis. For this reason, we will initially focus on low-grade gliomas.

As observed in social insect colonies, there is similarly a division of labor, which aims to maximize the reproductive fitness of the tumor, as supplied by non-neoplastic cells that establish and maintain a supportive cancer ecosystem. For example, biallelic *NF1* inactivation and *KIAA1549:BRAF* rearrangement both increase MEK activation, which serves as a major driver of cell cycle progression. However, the cells and tumors harboring these changes have a limited growth potential, and will frequently undergo senescence (growth arrest) in vitro [4–7], suggesting that the signals required for full transformation (tumorigenesis) are lacking. This notion is supported by several experimental findings. First, biallelic *Nf1* loss in neuroglial progenitors in mice is not sufficient by itself for murine *Nf1* optic glioma development in vivo [8, 9]. Second, impaired monocyte function abrogates *KIAA1549-BRAF*-induced tumorigenesis in mice [10]. Third, *Nf1* inactivation or *KIAA1549-BRAF* expression does not lead to increased growth in all neuroglial cell types [11–14], suggesting that not all cells are susceptible to the effects of low-grade glioma-associated molecular alterations. Fourth, *Nf1* loss or *KIAA1549:BRAF* expression activates MEK and increases cell growth in a brain region-specific manner [11–14]. Taken together, these observations raise the intriguing possibility that low-grade glioma-associated molecular changes merely prime the pre-neoplastic cells for tumorigenesis, but that transformation requires additional signals from the tumor microenvironment in order to bypass the natural physiologic induction of growth arrest. This two-step requirement provides a natural protective barrier, which allows the brain to contain the growth advantage conferred by stromal signaling molecules, and likely mirrors what occurs during brain development, where growth signals must be tightly regulated in order to ensure proper and orderly organogenesis [15].

8.4 Microglia and Macrophages Are Abundant in Glioma

Gliomas are composed of various populations of neoplastic (differentiated cancer cells and cancer stem cells) and non-neoplastic cells (monocytes, lymphocytes, astrocytes, neurons, and blood vessels). Numerous studies have highlighted the importance of blood vessels to the maintenance of brain tumors, culminating in the use of anti-angiogenic therapies as adjuvant treatments for central nervous system (CNS) malignancies (e.g., bevacizumab) [16]. In addition to these vascular elements, as much as 50% of the cellular content of gliomas is accounted for by immune system-like cells [17]. These immune system-like cells include T lymphocytes (T cells) and macrophages that enter the tumor from the blood through either passive efflux or active chemoattraction, as well as resident macrophages (microglia) that colonize the brain during embryonic (fetal) development, and serve as integral cellular homeostatic sensors in the healthy brain [18, 19]. In this manner, microglia are important for maintaining neuronal integrity, clearing cellular debris, and providing instructive signals to astroglial lineage cells (astrocytes, neural stem cells, oligodendrocyte precursors). However, in the setting of CNS pathology, especially brain tumors, they adopt specialized roles that actively dictate disease progression [20, 21].

8.5 Microglia and Macrophages Are Recruited by Glioma-Produced Chemokines

The recruitment of microglia and macrophages into gliomas operates through the elaboration of chemokines, such as CSF1, CCL2, CXCL12, and CX3CL1, frequently produced by the tumor cells [22–25] (Fig. 8.4). For example, in mouse models of NF1-associated low-grade glioma, *Nf1* optic glioma stem cells produce the chemokine

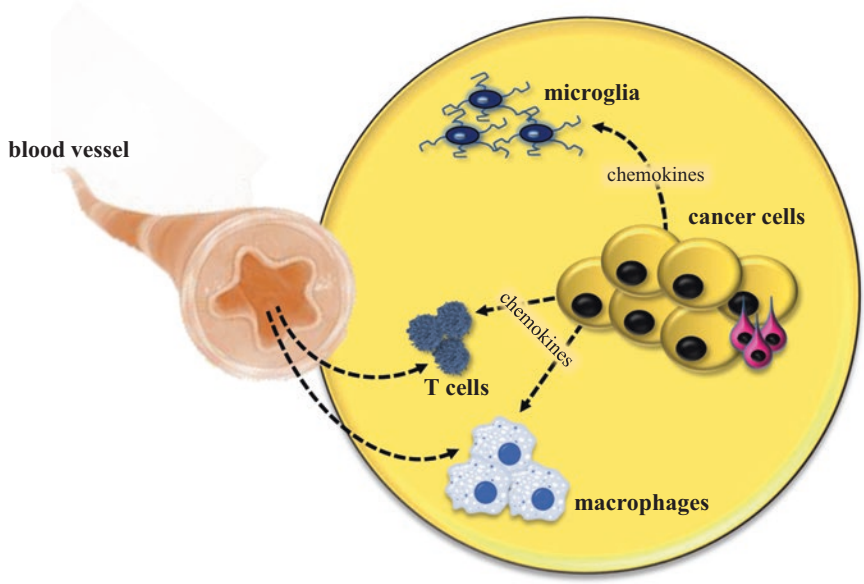

Fig. 8.4 Microglia, T cells, and macrophages are recruited by neoplastic glioma cells. Glioma cells can attract resident microglia in the brain, as well as T cells and macrophages from blood, through the elaboration of chemokines

CX3CL1, which attracts microglia through its cognate receptor (CX3CR1) expressed on microglia [26]. Genetically eliminating this chemotactic receptor on microglia in mice is sufficient to inhibit murine *Nf1* low-grade glioma formation following optic glioma stem cell transplantation [24, 27]. Similarly, in a murine model of sporadic low-grade glioma, *KIAA1549:BRAF*-expressing cerebellar neural stem cells elaborate CCL2, which attracts monocytes. Genetic ablation of the CCL2 receptor (CCR2) expressed on these infiltrating monocytes blocks low-grade glioma formation [10]. In this manner, glioma tumor cells are capable of establishing a microenvironment supportive of their own growth, thus facilitating tumor expansion and maintenance.

Analogously, in high-grade glioma, monocytes can be recruited by cancer cells in a mutation-specific manner, typically mediated by chemokines, including CCL2 and CX3CL1, which act as directional migratory signals for macrophages and microglia expressing the CCR2 and CX3CR1 receptors, respectively [22, 28, 29]. In this regard, *IDH*-mutant tumors have reduced monocyte (microglia and macrophage) and T cell content relative to their *IDH* wild-type counterparts, likely as a result of reduced expression of key immune system chemokines, like CCL2, CXCL1, GM-CSF, and CXCL4 [22, 28, 30]. In addition, *NF1* deficiency in high-grade glial neoplasms is associated with greater monocyte infiltration [31], perhaps through the secretion of unique chemokine and cytokine modules [32].

8.6 Microglia and Macrophages Are Required for Gliomagenesis and Progression

In the setting of glioma, microglia and macrophages have emerged as essential conductors that orchestrate tumor development and progression (Fig. 8.5). Each of these monocyte populations adopts new functions in their role as modulators of glioma progression, including cytokine and growth factor production [33–36]. In the setting of experimental murine glioblastoma, microglia and infiltrating macrophages often occupy distinct intra-tumoral locations and exhibit different gene expression patterns. As such, macrophages in human glioblastoma are localized to perivas-

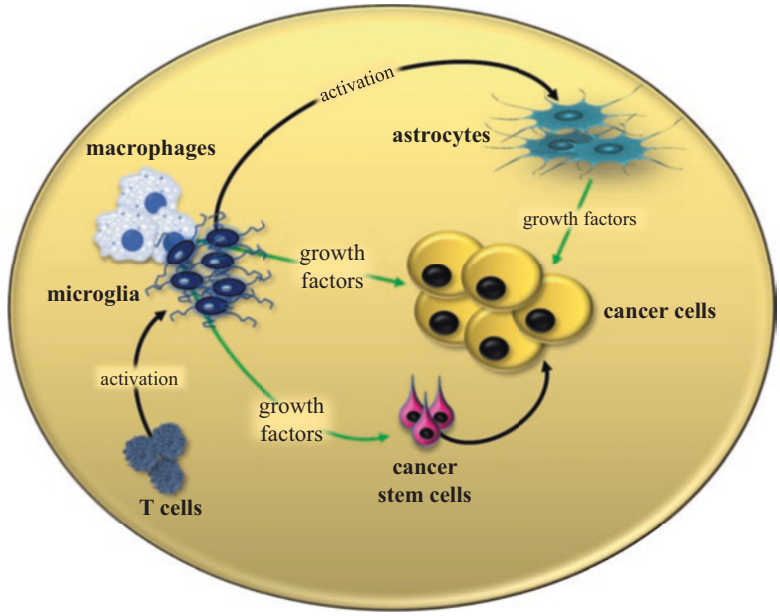

Fig. 8.5 T cells, microglia, and macrophages are key regulators of glioma growth. Recruited microglia and macrophages are reprogrammed in the setting of glioma to produce growth factors that increase the growth of the neoplastic cells (cancer cells and cancer stem cells). In addition, microglia can activate brain astrocytes to further support glioma growth through the generation of mitogenic and survival factors. Similarly, T cells can prime resident microglia to produce growth/survival factors that promote glioma homeostasis and expansion

cular and necrotic regions of the tumor, where they have increased expression of immunosuppressive cytokines [37]. Moreover, glioma-associated monocytes can induce a paracrine circuit involving monocyte production of factors that negatively and positively regulate glioblastoma cell growth through the activation of numerous signaling pathways, acting on receptors expressed on glioma cells [34, 36, 38, 39].

In experimental *Nf1* low-grade glioma models, silencing of microglia function, using either genetic or pharmacologic strategies, reduces tumor growth and delays glioma formation in vivo [24, 40, 41]. In *Nf1* optic gliomas, tumor-associated microglia secrete CCL5, which serves as a potent mitogen for the neoplastic glioma cells. As such, treatment of tumor-bearing mice with Ccl5-neutralizing antibodies reduces optic glioma growth in vivo [42]. Moreover, *Nf1* optic glioma stem cells do not form tumor-like lesions following injection into the brains of mice lacking Ccl5 [27], firmly establishing Ccl5 as a key microglia mitogen for *Nf1* low-grade glioma formation and growth.

In addition to monocytes, converging evidence from numerous laboratories has underscored the notion that the brain is not an immune privileged organ [43]. Trafficking of T cells through the neuroaxis in both health and in the setting of CNS diseases is facilitated by the presence of a unique brain lymphatic system and transmeningeal entry [44–46]. Leveraging an optic glioma stem cell (o-GSC) transplantation model, tumors readily form in wild-type mice, but not in athymic (nude) mice lacking mature T cells [27]. The absence of T cells results in impaired microglia function, notably reduced phagocytosis, and dramatically decreased levels of CCL5 production. Importantly, wild-type mouse T cells induce athymic mouse microglia to produce CCL5 through paracrine mechanisms, suggesting that T cells "educate" microglia to create a microenvironment supportive of tumor cell engraftment and growth. These T cells are also recruited through chemokines produced by the tumor cells [26], thus establishing an oncoimmune

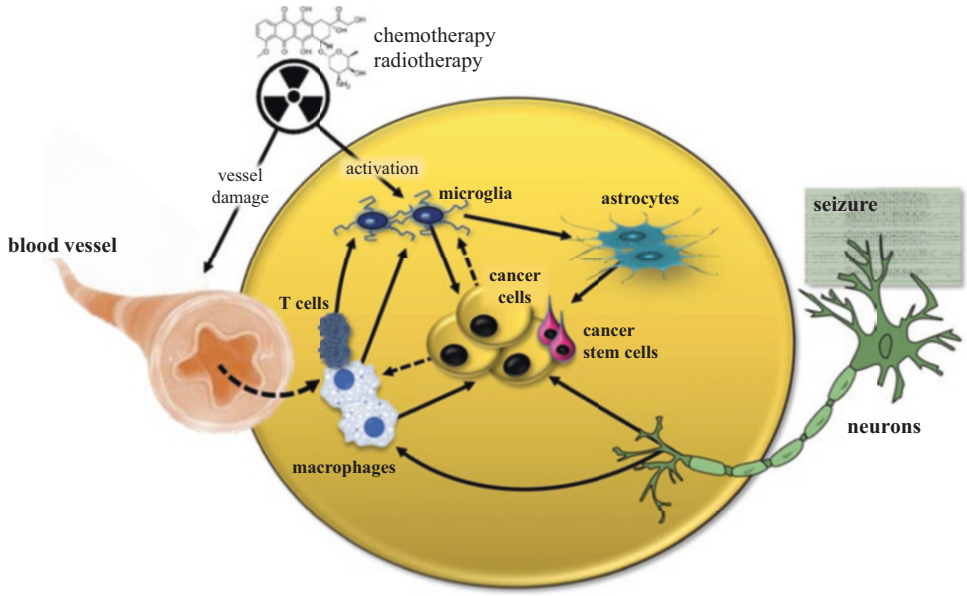

Fig. 8.6 Adaptive responses to glioma treatment. Radiation and chemotherapy can lead to vascular damage, causing increased entry of immune system cells (T cells and macrophages) into the glioma. Furthermore, these treatments can act directly on microglia to establish new functional states that lead to astrocyte activation. Similarly, changes in neuronal activity, through tumor-associated seizures, can additionally influence glioma biology through the elaboration of chemokines to attract immune system cells or paracrine factors that increase glioma growth

axis that drives tumor growth and maintains the low-grade glioma ecosystem. Consistent with the idea that T lymphocytes are important for glioma biology, human *NF1*-mutant gliomas exhibit T cell infiltration, where the majority of these recruited T cells are $CD8^+$ lymphocytes [47]. Importantly, since these T cells originate outside the brain, they are theoretically capable of transducing signals from the rest of the body, especially in the context of conditions that reprogram and activate T cells (e.g., asthma, atopic skin conditions).

8.7 Future Directions

As we begin to unravel the complexity that is inherent in the cancer ecosystem, new opportunities to improve the management of people afflicted with these neoplasms will likely emerge. In this regard, one of the major considerations is the cellular and molecular adaptations that occur in the tumor ecosystem during cancer homeostasis and in response to treatment (Fig. 8.6).

In this regard, as tumors evolve, their cellular composition and the molecular signals that govern tumor growth change. As such, macrophages initially populate malignant gliomas, residing in the perivascular niches, whereas microglia appear later and predominate at the peri-tumoral edges [23]. In addition, as tumors progress from low-grade to high-grade malignancies, the cancer cells themselves often express the very growth factors originally provided by the tumor microenvironment. One example of this phenomenon is observed in *NF1*-mutant tumors, where the low-grade tumors require CCL5 from microglia in their microenvironment, but their high-grade counterparts produce their own CCL5 to drive tumor growth in an autocrine fashion [48].

Additionally, in the setting of treatment (either chemotherapy or radiation therapy), the composite cell populations respond by changing their cellular interactions and transcriptional profiles. Microglia, in particular, are sensitive to the effects of ionizing radiation and chemotherapy. As such, some studies have shown that cranial irradiation induces a transient accumulation of

microglia followed by microglia loss [49, 50], as well as microglia transcriptional changes similar to those found in the aging rodent brain [51]. Similarly, microglia can be persistently activated by chemotherapy (e.g., methotrexate) in both the rodent and human brain [52]. These alterations in microglia could have profound consequences on the activation of astrocytes, neuronal function, and the elaboration of growth and survival factors essential for overall glioma fitness. In addition, brain tumor therapy changes the milieu in which the cancer stem cells reside, leading to expansion of normally quiescent glioma stem cells, which repopulate the tumor following therapy [53]. Moreover, treatment has the capacity to enrich for relatively treatment-resistant clones, which then could later emerge as the predominant cancer species.

Taken together, attacking tumors at their most vulnerable nodes offers the greatest opportunity to disrupt this adaptive architecture. Future studies aimed at defining the critical cellular and molecular interdependencies within these cancers and determining how they adapt in the face of chemotherapy and/or radiation may usher in a new era of cancer therapeutics in which emergent behaviors and new homeostatic states that maintain the tumor are abrogated.FundingThe author was funded by a Research Program Award grant from the National Institutes of Health (1-R35-NS07211-01).

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9 Tumor Microenvironment Conditioning by Abortive Lytic Replication of Oncogenic γ-Herpesviruses

Christian Münz

Abstract

Epstein Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KSHV) constitute the human γ-herpesviruses and two of the seven human tumor viruses. In addition to their viral oncogenes that primarily belong to the latent infection programs of these viruses, they encode proteins that condition the microenvironment. Many of these are early lytic gene products and are only expressed in a subset of infected cells of the tumor mass. In this chapter I will describe their function and the evidence that targeting them in addition to the latent oncogenes could be beneficial for the treatment of EBV- and KSHV-associated malignancies.

Keywords

Kaposi sarcoma-associated herpesvirus · Epstein Barr virus · Viral IL-6 · Viral IL-10 · Viral MIP · Viral miRNA · CCL5 · Lytic replication · Angiogenesis · Kaposi sarcoma · Primary effusion lymphoma · Multicentric Castleman's disease · Hodgkin's lymphoma · Burkitt's lymphoma · Nasopharyngeal carcinoma

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9.1 Introduction to Human γ-Herpesviruses

Among human herpesviruses, oncogenesis is confined to the γ-herpesviridae [1]. These contain the lymphocryptovirus Epstein Barr virus (EBV) or human herpesvirus 4 (HHV4) and the rhadinovirus Kaposi sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8). Both viruses share a tropism for human B cells and stimulate these into lymphoproliferations in some of which even both viruses are present at the same time [2]. In addition they are, however, also associated with tumors that originate from other cell types, including epithelial, NK/T, and smooth muscle cells for EBV and endothelial cell for KSHV [3, 4]. The oncogenic potential of these two viruses is thought to originate from their need to differentiate B cells into long-lived memory compartments for persistence, memory B cells for EBV and plasma cells for KSHV [4, 5]. These B cells get infected by the two viruses in submucosal secondary lymphoid tissues after transmission via saliva exchange and possibly transcytosis across the mucosal epithelium [6]. Latent infection by the two viruses is then thought to lead to B cell activation and proliferation. For EBV mainly 8 latent gene products, more than 40 miRNAs, and 2 EBV-encoded small RNAs (EBERs) are involved in this task [5]. Differentiation from this activated B cell stage to memory B cells by follicular and extrafollicular

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routes allows for EBV persistence without any viral protein expression [7, 8]. KSHV contains also three latent gene products which together with the viral miRNAs induce B cell proliferation after overexpression in mice [9]. In addition, however, expression of some lytic KSHV gene products without overt infectious particle production augments B cell activation and differentiation, like the plasmablast or plasma cell differentiation that is induced by viral IL-6 (vIL-6) expression from the K2 locus [2]. B cell proliferations that are driven by these viral programs can be found in human immunodeficiency virus (HIV)-infected patients with acquired immunodeficiency syndrome (AIDS) as immunoblastic lymphoma that is associated with all EBV latent gene expression and multicentric Castleman's disease (MCD) that is associated with latent KSHV gene expression but also some lytic KSHV virus production [10, 11]. These programs extensively shape the phenotype of the infected B cells to activated lymphoblasts by EBV and plasmablasts by KSHV, as well as modify their B cell receptor, introducing addition somatic hypermutation in the case of EBV, and switch to λ-light chain expression in the case of KSHV [12, 13]. From these stages both extrafollicular and germinal center-dependent routes most likely lead to persistence with no viral protein and only viral non-translated RNA expression. At least EBV expresses a restricted set of latent proteins in centroblast and centrocytes to rescue infected B cells from cell death in germinal centers [14]. From this reservoir both viruses reactivate into lytic infectious particle replication upon plasma cell differentiation for EBV and most likely also in plasma cells for KSHV [11, 15]. If this occurs at submucosal secondary lymphoid tissues, infectious virus can find its way into the saliva for further transmission, possibly after an additional amplification in mucosal epithelial cells at least for EBV [16]. These life cycles of EBV and KSHV utilize B cell immunobiology to both disseminate in their host, establish persistence in long-lived cells, and allow reactivation in submucosal tissues for further transmission.

However, this lifestyle also forces them to induce B cell lymphoproliferations and channel infected cells through differentiation stages with increased somatic mutations. The oncogenic capacity of EBV and KSHV gene products and host gene mutations that emerge in the process of B cell differentiation leads to tumors associated with the two γ-herpesviruses. In this chapter I will discuss the different EBV- and KSHV-associated malignancies and how their microenvironments are conditioned for both pro-proliferative and immune evasive functions.

9.2 Classical Oncogenes of EBV and KSHV

EBV is associated with tumors of B, epithelial, NK/T, and smooth muscle cell origin [3]. In these malignancies, EBV expresses a variable amount of latent viral proteins, and the respective gene expression patterns are called latencies I, II, and III. B cell-derived immunoblastic lymphomas and posttransplant lymphoproliferative diseases (PTLDs) express all 8 latent EBV genes and are primarily observed during severe immune suppression, for example, during advanced HIV infection and iatrogenic immune system inhibition [17]. EBV-associated smooth muscle tumors harbor also latency III [18]. Latency II tumors like Hodgkin's lymphoma of B cell origin and nasopharyngeal carcinoma of epithelial cell origin express only one of the six nuclear antigens of EBV (EBNAs), namely, EBNA1, and the two latent membrane proteins LMP1 and 2 [1, 19]. This latency II or even less latent viral protein expression can also be found in NK/T cell lymphomas that are associated with EBV [20]. Finally, only EBNA1 is expressed in latency I which is found in Burkitt's lymphoma and primary effusion lymphoma (PEL), the latter being in the majority of cases also co-infected with KSHV [1]. In contrast to these distinct latent EBV gene expression patterns, KSHV rarely expresses only its three latent viral proteins, latency-associated nuclear antigen (LANA), viral FLICE-like inhibitory protein (vFLIP) and viral cyclin (vCYC), and viral miRNAs [4]. The adjacent kaposin locus K12 is also often expressed, as well as in decreasing frequency the K15, the K2,

and the non-translated polyadenylated nuclear RNA (PAN) encoding KSHV genome region [21, 22]. This variable gene expression is seen in both endothelial and B cell-derived tumors that are associated with KSHV, namely, Kaposi sarcoma or multicentric Castleman's disease (MCD) and PEL [23–25]. It might represent a variable frequency of cells undergoing abortive and productive lytic KSHV replication in the respective tumors [26]. Thus, variable viral gene expression patterns can be observed in γ-herpesvirus-associated malignancies. These segregate with tumor entities for EBV and subdivide tumor entities for KSHV.

Both viruses contain bona fide oncogenes, which upon expression in mice cause tumors. This has been shown for the viral latency locus and vFLIP of KSHV [9, 27, 28] and for EBNA1 and LMP1 of EBV [29, 30]. Interestingly, both viruses activate c-myc and NF-κB to induce B cell activation and proliferation. EBV achieves this via EBNA2-assisted c-myc transcription [31] and LMP1-mediated constitutive NF-κB activation [32]. In some EBV-associated lymphomas that express only EBNA1, the c-myc expression is achieved by cellular mutations that are thought to compensate for EBNA2 absence, like c-myc translocation into the immunoglobulin loci for Burkitt's lymphoma [33] and c-myc gene amplification in lymphomas that emerge in mice upon EBNA1 expression in B cells [34]. For KSHV, LANA amplifies c-myc activity [35, 36] and vFLIP activates NF-κB [37, 38]. These pro-proliferative functions are paired with anti-apoptotic mechanisms, such as for EBV the EBNA3C-mediated inhibition of pro-apoptotic $p16^{INK4a}$ and BIM expression [39, 40] and the pro-survival B cell receptor-like signaling of LMP2 [41]. In PEL the p16INK4a locus is sometimes mutated to presumably compensate for the absence of an active mechanism to suppress this pro-apoptotic protein, which is induced by the cell cycle driving activity of vCYC [42]. One can also speculate that the B cell receptor modifying activities, somatic hypermutation by EBNA3C-mediated activation-induced deaminase (AID) induction [12] and λ-chain usage driven by vFLIP [28], might improve tonic signaling for infected B cell survival, similar to LMP2 function. In addition to these immunoblastic features of latent EBV and KSHV infection, the leaky lytic KSHV gene product expression, mainly from the K2 locus encoding vIL-6 (Fig. 9.1), induces plasma cell features in MCD and PEL [43, 44]. However, the functions of latent γ-herpesvirus proteins and leaky presumably abortive lytic gene expression go much further than just transforming EBV and KSHV-infected cells. They also heavily condition the microenvironment of the associated tumors and this regulation will be discussed next.

9.3 Conditioning of the Tumor Microenvironment by Lytic and Latent EBV and KSHV Gene Expression

During their co-evolution with the human host, both EBV and KSHV have reached a stalemate with the immune system that in the vast majority of the more than 90% of adults that are persistently infected with EBV and of the more than 75% of adults that have encountered KSHV in some sub-Saharan countries leads to persistence of both viruses, but also does not cause pathology [45, 46]. On the contrary KSHV and EBV seem to even promote this equilibrium. For example, EBV encodes with EBNA3B a tumor suppressor [47]. This latent viral nuclear antigen induces transcription of CXCL9 and 10, two inflammatory chemokines that recruit lymphocytes via their CXCR3 receptor. EBV deficient in EBNA3B causes lymphomas at increased frequencies with diminished inflammatory infiltrates, and restoration of CXCL10 secretion by transfection also reinstalls T cell-mediated immune control of lymphoma cells with EBNA3B-deficient EBV. Not only latent gene products but also early lytic EBV proteins foster leucocyte recruitment to the vicinity of infected cells (Fig. 9.1). Along these lines CCL5 production has been reported in lymphomas with higher lytic EBV replication [48]. This chemokine facilitates macrophage recruitment via CCR5 binding into the tumor microenvironment [49]. Similarly, KSHV encodes three macrophage inflammatory protein

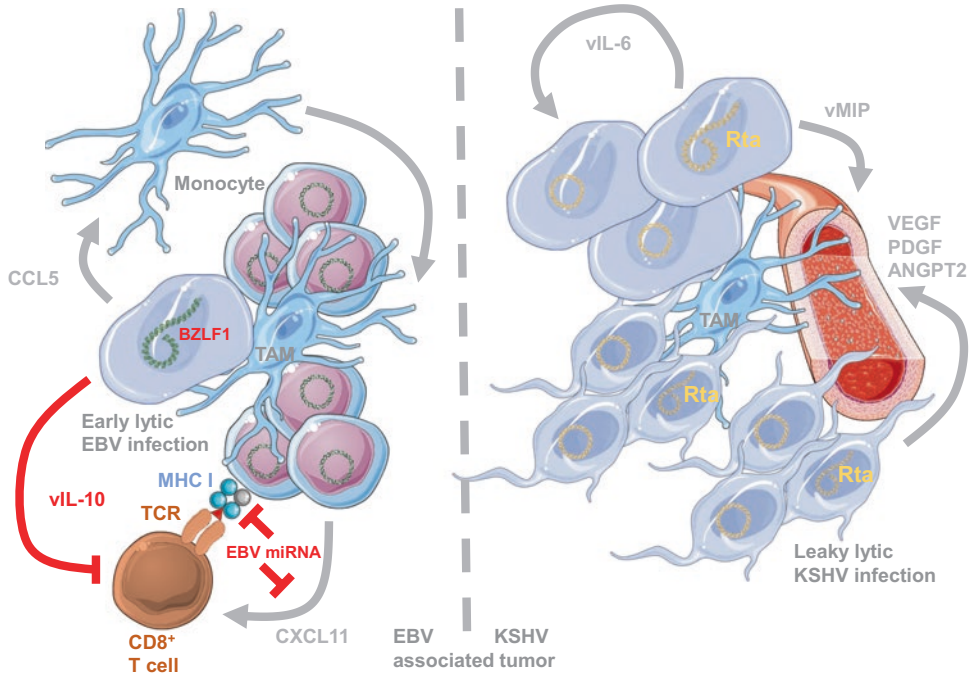

Fig. 9.1 Conditioning of the tumor microenvironment of EBV- and KSHV-associated malignancies**.** The microenvironment of both EBV (left)- and KSHV (right)-associated malignancies is composed of a mixture of latently and early lytically (BZLF1 or Rta) infected cells. Early lytic replication conditions the microenvironment of both EBV- and KSHV-associated malignancies by attracting monocytes to differentiate into immune suppressive tumor-associated macrophages (TAM) via CCL5 or viral macrophage inflammatory proteins (vMIP). Furthermore, viral IL-10 (vIL-10) suppresses immune activation in the microenvironment of EBV-associated malignancies, and viral IL-6 (vIL-6) induces plasma cell differentiation in KSHV-associated malignancies. EBV further suppresses $CD8^+$ T cell-mediated immune control by blocking CXCL11-mediated attraction of $CD8^+$ T cells and downregulation of MHC class I-restricted antigen presentation with its miRNAs that are expressed during latency and lytic infection. Early lytic KSHV infection is pro-angiogenic, triggering vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and angiopoietin 2 (ANGPT2) production. This figure was created in part with modified Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 unported license: https://smart.servier.com.

(MIP) homologues (vCCL1–3) as early lytic gene products. These are thought to recruit myeloid cells into the microenvironment of KSHV-infected cells via CCR8 (vCCL1 and 2) and XCR1 (vCCL3) [50]. These inflammatory infiltrates protect persistently EBV- and KSHV-infected hosts probably most of the time from γ-herpesvirus-associated pathologies.

However, in virus-associated tumors these infiltrates are turned into tumor cell nurturing and immunosuppressive leucocytes. Along these lines EBV encodes viral IL-10 [51, 52] and KSHV-associated lymphomas are also dominated by IL-10 production [53]. IL-10 suppresses T cell-mediated restriction of EBV-transformed B cells [54, 55] (Fig. 9.1). In addition, tumor cells as well as inflammatory infiltrates produce TGF-β in Hodgkin's lymphoma [56], which is presumably involved in the induction of regulatory T cells rosetting around the malignant Reed-Sternberg cells in this tumor entity [57]. Furthermore, EBV-encoded viral miRNAs also compromise MHC class I-restricted antigen presentation to $CD8^+$ T cells and their CXCL11-mediated attraction into the tumor microenvironment [58–61], further dampening T cell-mediated immune control (Fig. 9.1). While EBV miRNAs compromise MHC-restricted antigen presentation both during latency and lytic replication, early lytic gene products of both viruses further compromise MHC class I-restricted $CD8^+$ T cell stimulation. These are

the KSHV K3 and K5 gene products that downregulate MHC class I molecules [62] and the EBV BGLF5, BNLF2a, and BILF1 gene products that inhibit MHC class I transcription, peptide loading, and surface expression, respectively [63]. Both viruses contain with KSHV vIRF1–4 and EBV BZLF1, BRLF1, and BGLF4 also lytic gene products that block interferon signaling [46, 63], but at least for EBV there is little evidence that type I IFN influences its infection in vivo [64]. Thus, lytic gene expression of γ-herpesviruses in a subset of cells in the associated tumors and often not resulting in productive replication of infectious particles, conditions the respective tumor microenvironment to be immune suppressive and amplifies this immune suppression by infiltrating leucocyte polarization.

In addition to immune modulation in the tumor microenvironment, lytic KSHV gene product also contributes to angiogenesis and thereby further supports tumor growth. KSHV G protein-coupled receptor (vGPCR), K1 and K15 stimulate angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and angiopoietin 2 (ANGPT2) [26, 46] (Fig. 9.1). This pro-angiogenic functions of KSHV might be an adaptation to the viral life cycle in endothelial cells, giving rise to Kaposi sarcoma, even so it remains unclear why KSHV requires endothelial cell infection for persistence.

9.4 Targeting Lytic Replication for the Treatment of EBV- and KSHV-Associated Malignancies

Even so lytic viral replication induction should intuitively destroy tumor cells; the above-discussed contributions of early lytic gene products to paracrine microenvironment conditioning for optimal tumor growth might make it attractive to inhibit lytic γ-herpesvirus infection as a treatment for EBV- and KSHV-associated malignancies. Along these lines EBV deficient in lytic infection induction causes less tumors in mice with from $CD34^+$ hematopoietic progenitor cells reconstituted human immune system compartments (humanized mice) [65, 66]. Furthermore, EBV strains with increased lytic replication are enriched in malignancies that are associated with this virus [67–69]. Similarly in KSHV-associated MCD, inhibition of the lytic cycle-associated viral DNA polymerase with a combination of zidovudine and valganciclovir was clinically efficacious in the majority of cases [70]. Furthermore, in HIV-infected individuals that were treated with the herpesviral DNA polymerase inhibitor ganciclovir for human cytomegalovirus (HCMV) reactivation, Kaposi sarcoma incidence was significantly reduced [71, 72]. Finally, herpesviral DNA polymerase inhibition has also been successful in individual cases of PELs [73]. These studies indicate that lytic EBV and KSHV replication might enhance virus-associated tumorigenesis and should be targeted for treatment.

However, instead of inhibition of overall lytic γ-herpesviral infection, individual effects of lytic EBV and KSHV gene expression can also be targeted. Along these lines CCR5 that has been suggested to mediate recruitment of myeloid cell into the tumor microenvironment of Hodgkin's lymphoma has been inhibited with maraviroc in combination with blocking antibodies against its ligand CCL5 [74]. CCL5 is thought to be elicited by early lytic EBV infection [48]. Blocking CCL5 binding to CCR5 inhibited Hodgkin's lymphoma growth in a xenograft model. Similarly, VEGF that is induced by KHSV vGPCR, K1, and K15 has been blocked with the recombinant antibody bevacizumab in Kaposi sarcoma patients [75]. This led to a clinical response in around 30% of treated individuals. Furthermore, the early lytic KSHV gene product vIL-6 is thought to drive plasma cell differentiation in MCD and PEL [2]. Plasma cell differentiation renders tumors susceptible to proteasome inhibition, as seen for multiple myeloma [76]. Indeed, combining the proteasome inhibitor bortezomib with chemotherapy successfully treated PEL in one patient [77]. IL-6 receptor was also directly targeted for treatment of MCD with clinical efficacy in a few patients [78, 79]. These initial encouraging results suggest that also individual lytic γ-herpesvirus gene products and their effects can be inhibited for therapeutic benefit.

These individual lytic EBV and KSHV proteins can also be used as active or passive vaccine antigens to target the above-discussed paracrine functions. Along these lines the protective value of late lytic EBV antigen-specific $CD4^+$ T cell responses has been explored in a humanized mouse model [80]. The respective viral antigens also sensitized neighboring latently infected cells for $CD4^+$ T cell recognition after transfer from the subset of lytically EBV replicating cells. However, in active vaccination with EBV-derived viral particles, addition of the latent EBNA1 antigen improved protective vaccine efficacy [81]. Nevertheless, lytic EBV antigens should be considered in combination with latent antigens for an optimal vaccine formulation to elicit protective T cell responses.

9.5 Conclusions and Future Outlook

Human γ-herpesviruses contain some of the most oncogenic pathogens. Apart from their oncogenes, some of the EBV- and KSHV-associated malignancies, however, heavily rely also on their inflammatory infiltrates to sustain tumor growth. This is probably most dramatic in Hodgkin's lymphoma in which only around 1% of the tumor mass represents the malignant Reed–Sternberg cells [57]. It has become apparent in the recent years that paracrine conditioning of this tumor microenvironment by a small subset of cells undergoing lytic γ-herpesvirus infection serves functions in the recruitment of immune cells, immune suppression, and angiogenesis. We now need to capitalize on these findings for new treatments of EBV- and KSHV-associated malignancies that are more specific for these viruses than B cell depletion and overall inhibition of herpesviral DNA polymerases.

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The Microbiome as a Component of the Tumor Microenvironment

10

Tünde Kovács, Edit Mikó, Gyula Ujlaki, Zsanett Sári, and Péter Bai

Abstract

Microbes, which live in the human body, affect a large set of pathophysiological processes. Changes in the composition and proportion of the microbiome are associated with metabolic diseases (Fulbright et al., PLoS Pathog 13:e1006480, 2017; Maruvada et al., Cell Host Microbe 22:589–599, 2017), psychiatric disorders (Macfabe, Glob Adv Health Med 2:52–66, 2013; Kundu et al., Cell 171:1481–1493, 2017), and neoplastic diseases (Plottel and Blaser, Cell Host Microbe 10:324–335, 2011; Schwabe and Jobin, Nat Rev Cancer 13:800–812, 2013; Zitvogel et al., Cell 165:276–287, 2016). However, the number of directly tumorigenic bacteria is extremely low. Microbial dysbiosis is connected to cancers of the urinary tract (Yu, Arch Med Sci 11:385–394, 2015), cervix (Chase, Gynecol Oncol 138:190–200, 2015), skin (Yu et al., J Drugs Dermatol 14:461–465, 2015), airways (Gui et al., Genet Mol Res 14:5642–5651, 2015), colon (Garrett, Science 348:80–86, 2015), lymphomas (Yamamoto and Schiestl, Int J Environ Res Public Health 11:9038–9049, 2014; Yamamoto and Schiestl, Cancer J 20:190–194, 2014), prostate (Yu, Arch Med Sci 11:385–394, 2015), and breast (Flores et al., J Transl Med 10:253, 2012; Fuhrman et al., J Clin Endocrinol Metab 99:4632–4640, 2014; Xuan et al., PLoS One 9:e83744, 2014; Goedert et al., J Natl Cancer Inst 107:djv147, 2015; Chan et al., Sci Rep 6:28061, 2016; Hieken et al., Sci Rep 6:30751, 2016; Urbaniak et al., Appl Environ Microbiol 82:5039–5048, 2016; Goedert et al., Br J Cancer 118:471–479, 2018). Microbial dysbiosis can influence organs in direct contact with the microbiome and organs that are located at distant sites of the body. The altered microbiota can lead to a disruption of the mucosal barrier (Plottel and Blaser, Cell Host Microbe 10:324–335, 2011), promote or inhibit tumorigenesis through the modification of immune responses (Kawai and Akira, Int Immunol 21:317–337, 2009; Dapito et al., Cancer Cell 21:504–516, 2012) and microbiome-derived metabolites, such as

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estrogens (Flores et al., J Transl Med 10:253, 2012; Fuhrman et al., J Clin Endocrinol Metab 99:4632–4640, 2014), secondary bile acids (Rowland, Role of the gut flora in toxicity and cancer, Academic Press, London, p x, 517 p., 1988; Yoshimoto et al., Nature 499:97–101, 2013; Xie et al., Int J Cancer 139:1764–1775, 2016; Shellman et al., Clin Otolaryngol 42:969–973, 2017; Luu et al., Cell Oncol (Dordr) 41:13–24, 2018; Miko et al., Biochim Biophys Acta Bioenerg 1859:958–974, 2018), short-chain fatty acids (Bindels et al., Br J Cancer 107:1337–1344, 2012), lipopolysaccharides (Dapito et al., Cancer Cell 21:504–516, 2012), and genotoxins (Fulbright et al., PLoS Pathog 13:e1006480, 2017). Thus, altered gut microbiota may change the efficacy of chemotherapy and radiation therapy (McCarron et al., Br J Biomed Sci 69:14–17, 2012; Viaud et al., Science 342:971–976, 2013; Montassier et al., Aliment Pharmacol Ther 42:515–528, 2015; Buchta Rosean et al., Adv Cancer Res 143:255–294, 2019). Taken together, microbial dysbiosis has intricate connections with neoplastic diseases; hereby, we aim to highlight the major contact routes.

Keywords

Microbiome · Breast cancer · Tumor microenvironment · Bacterial metabolite · Bacterial metabolism · Antitumor immunity · Tumor metabolism · Epithelial-mesenchymal transition · Tumorigenesis · Metastasis · Chemotherapy

10.1 The Human Microbiome

The human body harbors different kinds of symbiotic, commensal, and pathogenic bacteria that live on the surface and the cavities of the body. Microbiota is a collective term that refers to the group of microbes colonizing the human body, and the collection of genes they encode is known as our microbiome [36]. The number of colonizing microbial cells (>10^{14}) is 10 times more than the total sum of human somatic and germ cells. Therefore, their collective genome—called the metagenome—contains a large number of genes that exceed the human genome by 150 times. This metagenome performs key functions relevant to human health [37].

Each anatomical niche possesses a unique mixture of microbial populations (gut, skin, vagina, mouth, nose, and conjunctiva) that have important and functionally relevant individual variability (at the levels of genus, species, and strain) [5]. The great majority of microorganisms live in the gastrointestinal (GI) lumen. These microbes compete and collaborate with other organisms in this niche, resulting in a functionally and genetically plastic metagenome [5]. The GI microbiota plays a crucial role in digestion, maturation, immune response, protection against pathogen overgrowth, maintenance of intestinal barrier function, regulation of intestinal endocrine functions, neurologic signaling, bone density, biosynthesis of vitamins, neurotransmission, metabolism of bile salts, reaction or modification of drugs, elimination of exogenous toxins, and maintenance of the energy homeostasis of the host [38].

10.2 Bidirectional Microbiome-Host Connection

There is increasing evidence for complex and dynamic microbial interactions with hosts. The microbe-human symbiotic connection is a result of millions of years of coevolution, coadaptation, and codependence. Bacterial colonization begins at birth and progresses through childhood to adulthood. The adaptation process is nonrandom [39] and depends on the body habitat, lifestyle, physiological conditions, genotype of the host, and presence of other microbes in the niche [40]. The function and composition of the microbiome are determined by the diet of the host, probiotic or antibiotic consumption, stress, and short- or long-term travel. Besides these external factors, the host can affect the dynamics of the microbiome through its genetics, immune system, and per-

sonal hygiene [38]. Given the diverse functional repertoire of the microbiome, it is not surprising that dysbiosis is associated with a broad range of diseases from neurological disorders to metabolic diseases and cancer [12]. Numerous studies highlight the relationship between changes in the function, composition, and proportion of microbes—also called microbial dysbiosis—and the progression of certain diseases. Koch's concept that one microbe is responsible for the formation of one disease ("one microbe-one disease hypothesis") was shown to be an oversimplification. Recent advances have shown that the loss of balance in microbial communities and the global change in our microbiome are directly or indirectly connected to carcinogenesis, rather than the presence of a single causative microbe [41]. Nevertheless, there are directly tumorigenic bacteria, although their number is extremely low, including about 10 species (e.g., *Helicobacter pylori* promote the development of gastric cancer). Dysbiosis is associated with cancers of the urinary tract, cervix, skin, airways, colon, lymphomas, prostate, and breast [42]. However, it is still unclear whether cancer is the product of alterations of the microbiota or modifications in the "normal" microbiome are the consequences of cancer progression.

10.3 The Tumor Microenvironment

Cancers are not just masses of homogenous malignant cells. Tumors have been recognized as complex organs, whose complexity may exceed that of normal healthy tissues. Interactions between malignant and recruited non-transformed cells create the tumor microenvironment (TME). Nonmalignant cells include immune cells, cells of the vasculature and lymphatic system, cancer-associated fibroblasts, pericytes, and adipocytes [43]. The role of nonmalignant cells in the TME is to support cancer growth. Nonmalignant cells have a dynamic tumor-promoting function at all stages of carcinogenesis. The communication between cell types is driven by an extremely complex network of cytokines, chemokines, growth factors, other inflammatory mediators, and matrix remodeling enzymes [44]. Cancer cell metabolism is strictly regulated by the tumor microenvironment. The microbiome is a new component of the tumor microenvironment that impairs tumor cell metabolism by maintaining a healthy barrier, inducing inflammation, and producing genotoxins and bacterial metabolites with different features. Below, we review the modalities of how dysbiosis interferes with carcinogenesis (Fig. 10.1).

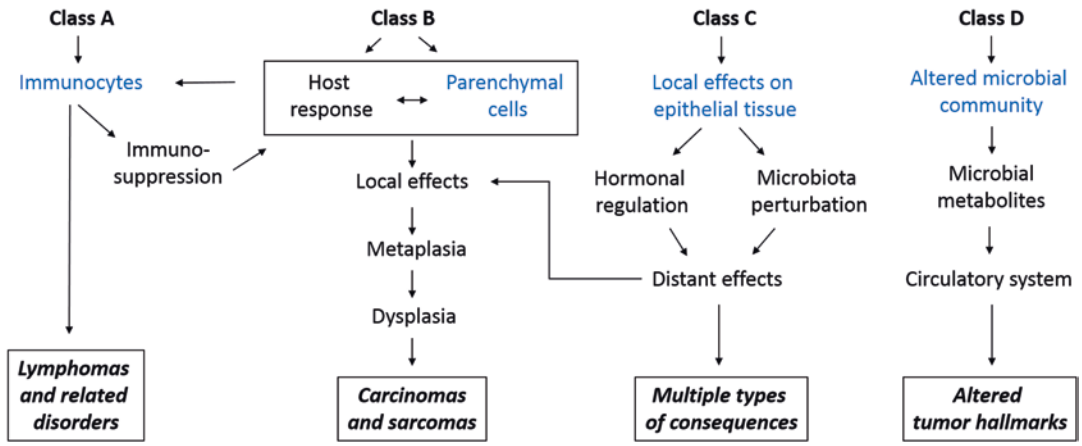

Fig. 10.1 Schematic picture of the classification of microbiota-associated human malignancies. Class A is defined by the involvement of the immune response, Class B requires direct microbial interactions with parenchymal cells, Class C covers distant effects from local interactions, and Class D shows the consequences of altered microbiome composition. (Modified figure from [5])

10.4 Bacteria-Driven Carcinogenesis Through Physical Interaction

The most relevant pathomechanism for microbiome-derived carcinogenesis is *barrier failure*. In healthy humans, numerous commensal bacteria are found in the intestinal lumen, where some bacteria are in direct association with the epithelium. The microbiota is vital in preserving the functional luminal barrier, by maintaining epithelial cell turnover, facilitating mucin production, and competing for resources and, thereby, suppressing the growth of pathogens [45]. The physical and chemical barrier of gut epithelial cells prevents microbial translocation to the underlying connective tissue. Defects in protein-coding genes (e.g., laminin) that are essential for the maintenance of a normal barrier, infections, inflammation, carcinogenesis, or microbial dysbiosis may induce barrier failure. Inflammation and carcinogenesis may trigger barrier failure, but barrier failure also promotes inflammation and carcinogenesis, suggesting a forward-amplifying loop [6]. Breakdown of the intestinal barrier leads to translocation of bacteria and the development of a systemic inflammatory response [46].

10.5 Microbiome-Immune System Interactions in Tumorigenesis

Microbiome-immune system interactions play multifaceted roles in tumorigenesis. The microbiome may promote tumorigenesis by inducing chronic inflammation, disrupting the balance between cell proliferation and cell death, and triggering immune responses. The physical loss of the natural gut epithelial barrier—barrier failure—or the loss of the antibacterial defense system enables the movement of cellular components and microbes across the barrier, where they cause an innate inflammatory response. The mammalian immune system detects the presence of microbial infection through *pattern recognition receptors (PRRs). Toll-like receptors (TLRs)* and *NOD-like receptors (NLR)* belong to the PRR family and recognize different but overlapping microbial components. They are expressed in different cellular compartments (cell surface, cytoplasm, lysosome, and endosome) and activate specific signaling pathways that promote inflammation, tumor proliferation, or resistance to cell death [23].

TLRs are one of the most powerful pro-inflammatory stimuli. These structures recognize microbe-associated molecular patterns, such as lipopolysaccharides (LPS), peptidoglycan, flagella, or microbial DNA/RNA. TLR2 recognizes peptidoglycan and lipoteichoic acid (bacterial cell wall components) and promotes gastric cancer, while TLR4 detects LPS (Gram-negative cell wall component) and contributes to skin, pancreas, liver, and colon cancer development [6]. Carcinogenesis is promoted through TLRs of epithelial cells, macrophages, and fibroblasts. TLR induction leads to the production of pro-inflammatory cytokines, such as interleukins and TNFα. Downstream effectors of TLR signaling induce cell survival and suppress apoptosis through NF-κB (nuclear factor-κB) and STAT3 signaling, which is in line with the role of MYD88 mutations that induce NF-κB and STAT3 in many human lymphomas [24]. Tumor formation is reduced by pharmacologic inhibition of interleukins (IL-17 and IL-23), antibiotic treatment, or MYD88 inactivation [6].

Although a direct link between endogenous bacteria and tumor-associated angiogenesis has not been shown, the microbiome is required for normal development of the vasculature. LPS, produced by the microbiome, may promote angiogenesis through TLRs. IL-17 is produced by T-helper-17 (Th17), suggesting that bacteria also impact the tumor microenvironment by stimulating Th17 lymphocytes. A connection between breast cancer and immunoglobulins has been established. Secretory immunoglobulin A (IgA) helps to maintain the integrity of the mucosal barrier, attenuates the host immune response, and regulates the composition of the gut microbial community.

Several bacterial species induce immunity in tumor development. *Lactococcus* species help

maintain the cytotoxic activity of natural killer (NK) cells, while *Sphingomonas yanoikuyae* have an important role in maintaining breast tissue health. Cytotoxic immune cells (cytotoxic T lymphocytes) are essential for identifying and destroying precancerous and cancerous cells; *Fusobacterium nucleatum* destroy this protective mechanism and enable tumor progression, while others stimulate anticancer immunity. *Bifidobacterium*, *Bacteroides thetaiotaomicron*, and *Bacteroides fragilis* enhance dendritic cell function and antitumor cytotoxic T cell immunity [1]. TLRs may also promote cancer cell proliferation through different growth factor receptor ligands (amphiregulin, epiregulin, and hepatocyte growth factors), which exert both local and long-distance effects.

In carcinogenesis, the microbiota induce activation of NOD-like receptors (NLRs) as well. Many studies focus on NOD2, because loss of NOD2 activity is connected with Crohn's disease. NOD2 has a key role in the activation of NF-κB signaling and the formation of a bacterial community. Thus, NOD2 loss-of -function mutations may lead to intestinal dysbiosis and an enhanced risk of developing colorectal carcinoma (CRC). Genetically induced CRC is also evoked by NOD1 deficiency, which plays an important role in intestinal defense against bacteria. NLRP6, another NLR, is important in microbiota-tumorigenesis interactions. NRRP6 is a component and key activator of inflammasomes (multiprotein oligomers responsible for the activation of inflammatory responses), which are downregulated in dysbiosis-driven carcinogenesis, together with decreased IL-18 production [6].

Immunotherapy is used to eliminate residual cancer cells after chemotherapy or radiation therapy. In therapy, monoclonal antibodies target molecules, such as anti-T-lymphocyte-associated antigen 4 (CTLA-4) and anti-programmed death 1 (PD-1) or its ligand anti-PD-L1. The advantage of immunotherapy is that it stimulates and supports the immune system of the host to fight cancer cells. The gut microbiome can stimulate the T cell response and improve inflammatory signaling through PRRs that potentiate the immune system to directly eliminate cancer cells. Antibodies against immune checkpoints improve T cell function and proliferation and, thereby, improve the anticancer immune response, providing an effective therapeutic approach in patients with various types of cancers, such as in advanced melanoma [47], renal cell carcinoma [48], or non-small cell lung cancer [49]. Alterations in commensal gut bacteria influence therapeutic responses to inhibition of CTLA-4 and PD-1. Following CTLA-4 therapy, the microbial composition shifts; *Bacteroidales* and *Burkholderiales* abundance decreases and *Bacteroides* and *Clostridiales* are enriched [50]. *Bacteroides fragilis* is capable of promoting T-helper 1 (Th1) responses and activating antigen-presenting cells (dendritic cells) through the induction of IL-12. Thus, an improvement in anti-CTLA-4 effectiveness may be partially due to the enrichment of *Bacteroides fragilis.* Improved effectiveness of anti-CTLA-4 therapy was observed in melanoma patients with increased abundance of *Bacteroides*, *Bacteroides thetaiotaomicron*, and *Bacteroides fragilis* [50]. The main bacterial component driving these processes was found to be the LPS of *Bacteroides* species. Thus, inhibition of CTLA-4 can alter the composition of the gut microbiome that in turn influences responsiveness to immunotherapy. Studies on anti-PD-1 or anti-PD-L1 therapy showed similar bacteria-driven differences in tumor outgrowth. In a mouse model of melanoma, increased effectiveness of anti-PD-L1 therapy was associated with enhanced *Bifidobacterium* (*Bifidobacterium longum* and *B. breve*) abundance in the gut and a consequent activation of dendritic cells [51]. In metastatic melanoma patients receiving anti-PD-1 and anti-PD-L1 treatment, patients with greater alpha diversity with an enrichment of *Clostridiales*, *Faecalibacterium*, and *Ruminococcaceae* species and decrement in *Bacteroidales* had longer survival. These beneficial effects were partly due to an enhanced T cell response (connected mainly to $CD8^+$ T lymphocytes) and the upregulation of antigen-presenting pathways [52]. Increased $CD8^+$ T cell activation was shown in another study in advanced melanoma patients. Patients

that responded to anti-PD-L1 therapy had elevated levels of *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*. Moreover, all patients that responded to treatment carried *Akkermansia muciniphila* [53]. Better survival was shown in urothelial carcinoma, renal cell carcinoma, or non-small cell lung carcinoma patients undergoing anti-PD-1 treatment who did not receive antibiotics during or after treatment and carried elevated levels of *Akkermansia* and *Alistipes* species. These findings were mainly connected to $CD4^+$ T cell activation [54] and demonstrated that antibiotic-induced dysbiosis could negatively influence responses to immunotherapy.

However, the mechanisms that contribute to dysbiosis and changes in the microbial community are not well understood. Host-driven immune and inflammatory responses are important driving factors that shape the bacterial community composition. The composition of the microbiome, innate immunity, and inflammation determine the outgrowth of different types of specific bacteria by changing the production of metabolites, such as nitrate. Nitrate may provide a unique energy source for facultative anaerobic bacteria (e.g., *Enterobacteriaceae*). Inflammation may promote bacterial fitness and adaptation by inducing the expression of stress-response genes in bacteria (e.g., *Escherichia coli*) [6].

10.6 Genotoxins and Microbiota-Driven Genomic Instability

Inflammation enhances tumorigenesis by inducing DNA damage and altering the mechanism of DNA repair. Macrophage release of reactive oxygen species (ROS) in response to inflammatory cytokines directly induces DNA breakage and mutations, and their downstream pathways stimulate transcription factors (NRF2, NF-κB) that impair cellular growth to produce cancer [36]. *Enterococcus faecalis* can generate large amounts of superoxide, while *Fusobacteria* species and *Deltaproteobacteria* produce hydrogen sulfide; both *Fusobacteria* species and *Deltaproteobacteria* are associated with CRC.

Hydrogen sulfide is a product of sulfate reduction from dietary taurine and sulfur-containing amino acids and has a wide effect on the host. Hydrogen sulfide is highly inflammatory and toxic to colonocytes. Furthermore, hydrogen sulfide can enhance colonocyte proliferation through the ERK1/2 pathway [55], inhibit mucus synthesis and butyrate oxidation while impairing the activity of cytochrome oxidase, and generate free radicals that lead to genotoxicity.

Although the ability of microorganisms to produce ROS [56] contributes to tumorigenesis, bacteria can also release specific *toxins* that induce DNA damage responses, which also contribute to tumorigenesis (Fig. 10.2). Damaged barrier function may also allow the bacteria to transfer or deliver toxins, including cytolethal distending toxin (CDT), colibactin, cytotoxic necrotizing factor 1 (CNF1), and *Bacteroides fragilis* toxin. CDT and colibactin are true genotoxins, which directly damage the DNA and activate the ataxia signaling pathway and histone phosphorylation, which lead to G2/M cell cycle arrest [6]. CDT is created by Gram-negative bacteria (*E. coli, Helicobacter species,* and *Salmonella typhi*) and is relevant to colorectal, gastric, and gallbladder cancer. Colibactin is produced by *E. coli*, *Enterobacteriaceae*, *Proteus mirabilis*, and *Klebsiella pneumoniae* and is important in the development of CRC. Colibactin produced by *E. coli* induces DNA double-strand brakes, cell cycle arrest, and improper cell division [1]. *Bacteroides fragilis* toxin activates the Wnt/β--catenin signaling pathway, which promotes epithelial proliferation, by promoting the cleavage of the adhesion molecule, E-cadherin. The cleavage of E-cadherin leads to β-catenin translocation to the nucleus and enables the transcription of proto-oncogene c-myc, leading to colonic epithelial hyperplasia [1].

10.7 Bacterial Metabolites in Carcinogenesis

A major pathway in microbiome-host signaling is the production of bacterial metabolites. These metabolites, which are synthesized by the

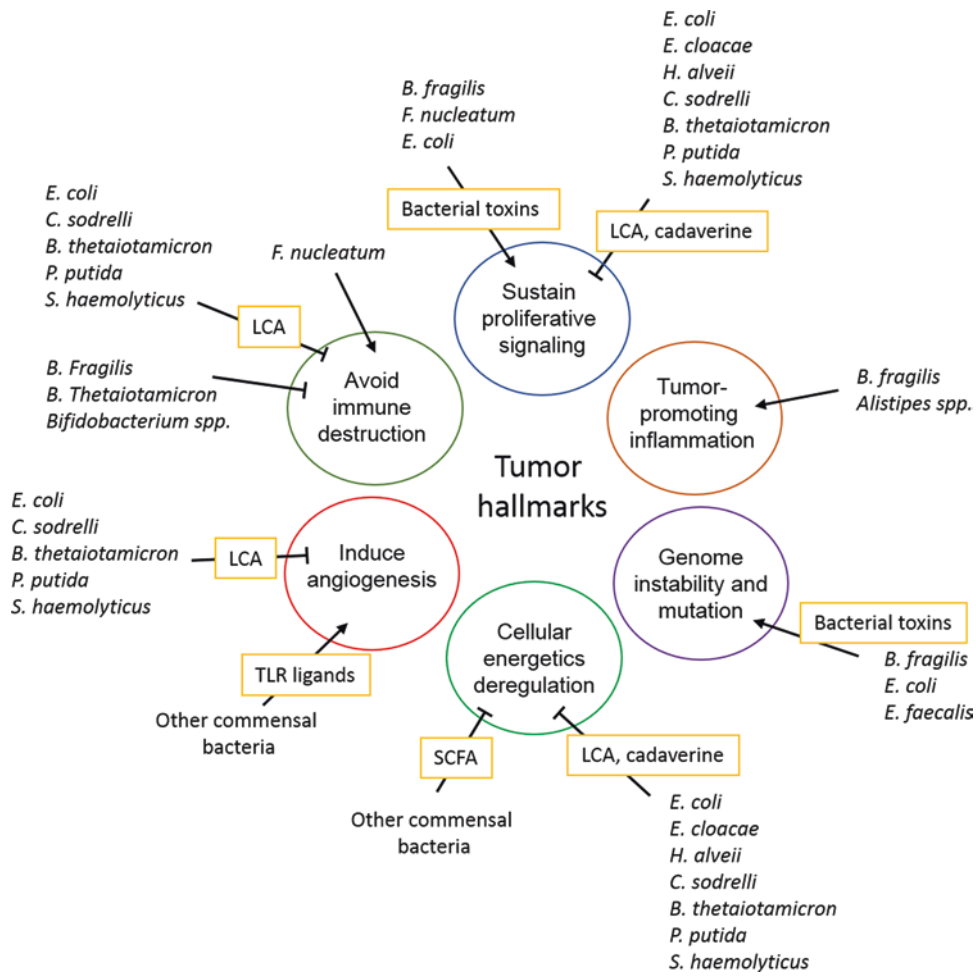

Fig. 10.2 The intestinal microbiota can modulate several hallmarks of cancer through different mechanisms

microbiome, enter the circulation at the site of production and travel to distant organs, where they exert their biological effects [57]. Bacterial metabolites behave like human hormones in the sense that they are synthesized by an "organ" (the microbiome) and are then transferred to the site of action by the circulation [57].

Microbiota have the potential to metabolize hormones, such as *estrogen*. The gut microbiome is a key determinant of estrogen levels in the body. β-Glucuronidases are the enzymes responsible for estrogen deconjugation. Deconjugation of excreted estrogen is important in estrogen reuptake and, thus, modulation of systemic estrogen availability and the regulation of estrogen-associated pathways. Numerous bacterial species can express β-glucuronidases, including *Firmicutes and Bacteroidetes*: *Alistipes, Bacteroides, Bifidobacterium, Citrobacter, Clostridium, Collinsella, Dermabacter, Edwardsiella, Escherichia, Faecalibacterium, Lactobacillus, Marvinbryantia*, *Propionibacterium, Roseburia*, and *Tannerella.* Thus, these bacterial species affect circulating and excreted estrogen levels. Reactivated estrogen increases the serum estrogen levels and act through estrogen receptors (ERα and ERβ) to modulate the expression of several genes, including mitochondrial genes. Elevated oxidative phosphorylation was shown to support metastasis [58], contribute to therapy failure [59], and, thereby, render the tumors more aggressive. Taken together, bacterial estrogen deconjuga-

tion promotes breast cancer progression and changes the risk for development and progression of estrogen-dependent cancers [6, 57].

The fermentation of nondigestible carbohydrates is beneficial for the host due to the generation of *short-chain fatty acids (SCFAs)*, such as acetate, butyrate, formate, lactate, and propionate. SCFAs are novel potential targets for the management of obesity, metabolic disorders, and lipomas, due to their ability to influence adipocyte differentiation [60]. SCFAs have known anti-inflammatory, antiproliferative, and antineoplastic effects. In addition, SCFAs can regulate autophagy. Thus, SCFAs have a protective effect on the colonic mucosa and play a significant role in the protection against colon and liver cancer [6]. In the gut, acetate, butyrate, and propionate production are associated with a large group of bacteria. Acetate production is widespread, while the production of butyrate is connected to *Faecalibacterium prausnitzii, Eubacterium hallii, Eubacterium rectale, Roseburia faecalis, Odoribacter*, and *Anaerotruncus* species. The majority of propionate production is associated with *Bacteroidetes, Lachnospiraceae*, and *Negativicutes* species, as well as to *Roseburia inulinivorans* and *Ruminococcus obeum.* In line with this, the abundance of *Akkermansia muciniphila*, a propionate-producing bacterium, is associated with the richness of the gut microbiome [61]. SCFAs have both positive and negative effects on breast cancer. Stroma and cancer cells have free fatty acid receptors, through which SCFAs modulate several hallmarks of cancer: cell proliferation, invasion, apoptosis, metabolism, and the expression level of certain genes. Lactate can be used as a direct energy substrate; thus, the inhibition of lactate metabolism reduces cancer cell viability. Butyrate enhances mitochondrial ROS level, induces apoptosis, and inhibits histone deacetylases, which lead to elevated anticancer activity [57].

The intestinal microbiota regulate *bile acid* metabolism and are involved in producing the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), through the deconjugation, oxidation, and dehydroxylation of primary bile acids. The enzyme responsible for the conversion of primary bile acids to secondary bile acids is 7α/β hydroxysteroid dehydrogenase (HSDH). Conversion to secondary bile acids increases the hydrophobicity of bile salts allowing recovery through the colonic epithelium. Secondary bile acids have both pro- and anticancer activity. The consumption of a high-fat diet changes the gut microbiome and enhances the level of DCA via 7/α-dehydroxylase, which is produced by bacteria, mainly clostridia. DCA is a promoter of carcinogenesis in certain cancers. DCA-elicited cell signaling is connected to protein kinase C and ERK1/2 signaling through epidermal growth receptors, resulting in enhanced cell proliferation. DCA is known to increase CRC development and promote colon and esophageal cancers [6]. Moreover, bile acids disrupt cell membranes through their amphipathic properties and the generation of ROS and reactive nitrogen species. Bile acids also exert antimicrobial activity that changes the composition of the intestinal community. LCA is synthesized through 7α-dehydroxylation of chenodeoxycholic acid (CDCA) or 7β-dehydroxylation of ursodeoxycholic acid (UDCA). The enzyme responsible for LCA synthesis is encoded by the bile acid-inducible (baiH) operon and expressed by aerobic and anaerobic bacteria, including *Bacteroides fragilis, Bacteroides intestinalis, Clostridium scindens, Clostridium sordellii, Clostridium hylemonae*, and *E. coli*. These bacteria belong to the phyla *Bacteroides, Firmicutes*, and *Proteobacteria.* LCA inhibits the epithelial-to-mesenchymal transition, vascular endothelial growth factor (VEGF) production, and metastasis formation of breast cancer cells, changes the metabolic features of the cells, and enhances antitumor immunity of the host [30]. In line with these observations, human serum levels of LCA and the ability of the microbiome to produce LCA are largely reduced in breast cancer; this is most pronounced in in situ and early stage carcinoma (stages 0 and 1) [30]. LCA can potentially exert its effects through the farnesoid X receptor (FXR), liver X receptor (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D receptor (VDR), and

G-protein-coupled bile acid receptor 1 (TGR5). In breast cancer, the main receptor is TGR5. Activation of TGR5 signaling was shown to induce OXPHOS, mitochondrial biogenesis through NRF1, AMPK, and PGC-1β signaling. The expression of mitochondrial proteins (cytochrome c, atp5g1, and ndufb5) consequently increases mitochondrial activity and exerts anti-Warburg effects in breast cancer models [30]. In supraphysiological concentrations (>1 μM), LCA was shown to inhibit fatty acid production and induce cell death and the expression of multidrug-resistant proteins [62].

When undigested dietary compounds reach the large intestine, they are fermented through anaerobic respiration. High protein consumption is associated with elevated colonic fermentation. *Bioactive products*, similar to bile salts, can produce or inhibit carcinogenesis. Cadaverine, a *biogenic amine*, is synthesized from L-lysine by bacterial lysine decarboxylase enzymes (LdcC and CadA). Cadaverine also has a human origin, but it seems that bacterial production is more important as it highly exceeds human biosynthesis. The main cadaverine-producing bacteria include *Aeromonas veronii, Clostridium perfringens, E. coli, Enterobacteriaceae bacteria, Edwardsiella tarda, Hafnia alvei, Raoultella ornithinolytica, Staphylococcus*, and *Streptomyces* species. These species belong to the *Acinetobacteria, Bacteroides, Firmicutes, Fusobacteria,* and *Proteobacteria* phyla. Trace amine-associated receptors (TAARs) were shown to be responsible for mediating cadaverine-elicited effects. Through TAARs, cadaverine inhibits epithelial-to-mesenchymal transition, proliferation, movement, and invasion of breast cancer cells. Moreover, cadaverine treatment inhibits primary tumor infiltration to the surrounding tissue and reduces the proportion of cancer stem cells [42].

Many bacteria in the GI tract have alcohol dehydrogenase activity, which enables the bacteria to metabolize ethanol and produce reactive and toxic *acetaldehyde*. The most important gastric pathogen, *H. pylori,* and some skin bacteria have high alcohol dehydrogenase activity. The colonic mucosa has a low aldehyde dehydrogenase activity, resulting in acetaldehyde accumulation in the colon. High acetaldehyde levels contribute to the pathogenesis of alcohol-induced diarrhea and the increased risk of colon polyps and colon cancer [63] (Fig. 10.3).

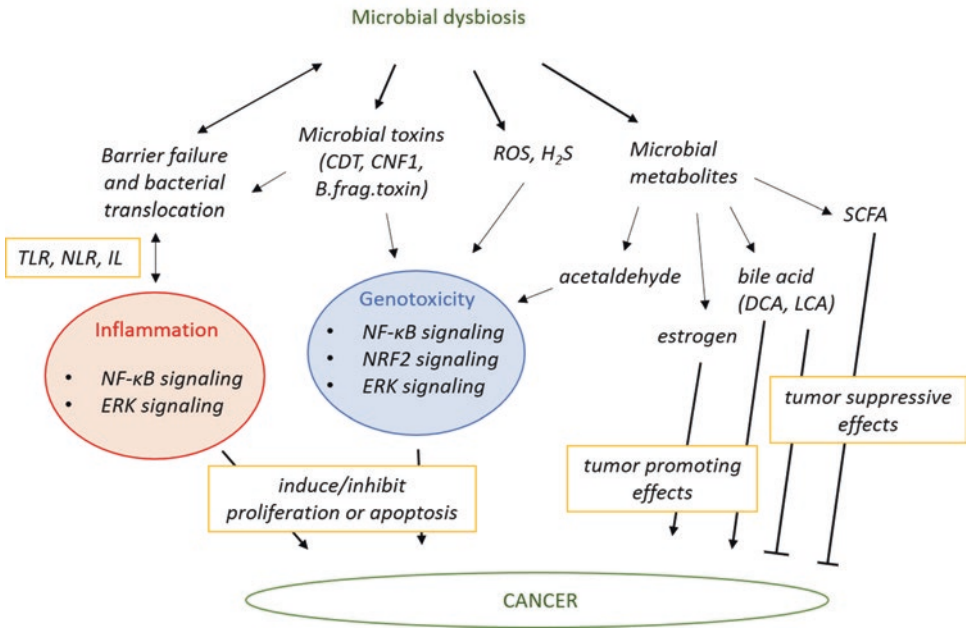

Fig. 10.3 Mechanisms by which microbial dysbiosis modulates carcinogenesis

10.8 The Interference of the Microbiome with Chemotherapy

Bacteria of the intestinal microbiome can interfere with therapeutic agents during cancer treatment and management. The microbiome can modulate the efficacy of both chemotherapy and radiotherapy. Bacteria can inactivate or activate chemotherapeutic drugs, alter immune responses, or interfere with the side effects of the therapy. The relationship is reciprocal, as tumor therapy can influence the composition and function of the microbiome [57].

Chemotherapeutic compounds, such as cisplatin or oxaliplatin, exert their cytotoxic effects through DNA damage, the upregulation of apoptotic pathways, or the promotion of antitumor immune responses (through a TLR4-dependent mechanism). The antitumor effects of *platinum compounds* significantly decrease upon broad-spectrum antibiotic treatment or in microbiota-deficient mice. In addition, tumor-infiltrating cells show reduced production of ROS after antibiotic treatment [35]. In this scenario, commensal microbes prime tumor-infiltrating cells for ROS production through the connection to PRRs, with the involvement of MYD88 signaling (described previously) [6, 56]. *Lactobacillus acidophilus* supplementation can restore the antitumor effects of cisplatin in mice [11]. *Cyclophosphamides* have been used for anticancer therapy for almost 60 years. In high doses, cyclophosphamides are immunosuppressive, while in low doses, cyclophosphamides promote the antitumor immune response through activation of cytotoxic T cells and induction of immunogenic cell death [33]. Cyclophosphamides are used in the therapy of breast cancer; however, cyclophosphamides cause damage to the gut mucosa, making the gut leaky and allowing gut bacteria to enter the circulation. A rich microbiome and elevated levels of *Lactobacillus plantarum* are protective against cyclophosphamide-induced mucosal injury [57]. Cyclophosphamide treatment causes the overrepresentation of Gram-negative species, such as *Barnesiella intestinihominis* that enhance effector T cells (cytotoxic $CD8^+$ T cell), and *Enterococcus hirae*, a Gram-positive bacteria that enhance MYD88-dependent $CD8^+$ T cell activation in a tumor-specific manner. Both bacteria are regulated by intestinal NOD2 receptors that promote a pro-inflammatory tumor environment and drive antitumor immune responses [35]. T cell-mediated immune responses against *B. intestinihominis* and *E. hirae* have clinical relevance in chemotherapy-treated patients with lung and ovarian cancers.

In addition to cyclophosphamides, anthracyclines, selective estrogen receptor modulators (SERMs), taxanes, and antimetabolites have key roles in breast cancer therapy. *Anthracyclines* are produced by *Streptomyces* species. Anthracyclines act mainly by intercalating into DNA and interfering with DNA metabolism and RNA production, or by generating excessive ROS. Anthracyclines can be bacteriostatic; they decrease the abundance of *Acinetobacter* species [32]. No bacterial drug metabolism was associated with SERMs (tamoxifen, raloxifene). Tamoxifen can modulate the composition of the microbiome, while tamoxifen resistance can also be modulated by the microbiome. SERMs are toxic to different species in the GI tract, including *Acinetobacter baumannii, Bacillus stearothermophilus, Enterococcus faecium, Klebsiella pneumoniae, Porphyromonas gingivalis, Pseudomonas aeruginosa,* and *Streptococcus mutans* [57]. *Taxanes* (paclitaxel, docetaxel) are widely used as chemotherapy agents. Taxanes disrupt microtubule formation and, hence, block cell division and proliferation. Taxanes may change the composition of the microbial community or interfere with bacterial LPS, while activating the immune system. PARP inhibitors are drugs used in the treatment of ovarian cancer with a potential to be used for other neoplasias (e.g., breast cancer, prostate cancer). PARP inhibitors were shown to induce the diversity of the gut microbiome [64].

Drugs are often used in combinations to enhance treatment efficacy. *Irinotecan* is used to treat colon cancer and small cell lung carcinoma. For treating colon cancer, irinotecan is generally used in combination with 5-fluorouracil (5FU),

whereas for the treatment of small cell lung cancer, irinotecan is combined with cisplatin. Bacterial reactivation of irinotecan by bacterial β-glucuronidase leads to severe side effects, such as diarrhea, vomiting, bone marrow suppression, hair loss, shortness of breath, and fever. Antibiotic treatment or β-glucuronidase inhibition prevents most of these side effects [6]. When *5FU* is used in combination with irinotecan, dysbiosis-induced mucositis leads to bacterial translocation from the GI tract. Both 5FU and *gemcitabine* undergo bacterial activation and bacterial deactivation. In human pancreatic ductal adenocarcinoma, *Gammaproteobacteria* was found to be the most important player in deactivating gemcitabine. In tumors, levels of *Gammaproteobacteria* were elevated in tumor patients as compared to healthy individuals, underlining its role in the regulation of gemcitabine availability. Both 5FU and gemcitabine have bactericidal properties; therefore, they can alter the composition of the GI microbial community [57].

Chemotherapy is often not specific for one or two bacterial species, but change the proportion and diversity of the microbiome. After chemotherapy, both the alpha diversity, which represents species richness (the number of different species in a sample), and beta diversity, which refers to the diversity in the microbial community between different environments, are altered as compared to samples without chemotherapy. These changes are independent of covariates (age, sex, previous antibiotic consumption, and previous chemotherapeutic treatment) and show increases in *Citrobacter, Enterococcus, Klebsiella, Megasphaera,* and *Parabacteroides* species, while showing decrements in the abundance of *Adlercreutzia, Anaerostipes, Bifidobacterium, Blautia, Clostridium, Collinsella, Coprococcus, Dorea, Lachnospira, Roseburia*, and *Ruminococcus* species. Some bacteria showed resistance to chemotherapy; thus their abundance did not change upon treatment, including *Actinomyces, Erysipelotrichaceae, Mobiluncus, Mitsuokella, Oxalobacter, Prevotella, Scardovia*, and *Slackia* [34].

Besides inducing taxonomic dysbiosis, chemotherapy can disrupt microbial function. Several metabolic pathways can be suppressed by chemotherapy, including amino acid, carbohydrate, and nucleotide metabolism, as well as the metabolism of vitamins and cofactors. Other pathways are enhanced by chemotherapy, including signal transduction, xenobiotic degradation, and glycan metabolism. Glycan metabolism, together with disrupted carbohydrate and amino acid metabolism, contributes to enhanced intestinal inflammation [65] and upregulation of nitrogen, sulfate, and riboflavin pathways, which is associated with inflammatory diseases, increased ROS production, and bacterial translocation [66]. Moreover, chemotherapy increases bacterial motility proteins and flagella assembly (essential for bacterial pathogenesis, motility, adhesion, and invasion).

Dysregulated microbiota plays a significant role in the development of GI mucositis. Mucositis is a painful inflammation of the mucous membranes of the digestive system, usually as an unpleasant side effect of chemotherapy and radiotherapy for cancer. In the first step of this process, the microbiome enhances the activation of NF-κB and TNFα signaling, leading to long-lasting inflammation. Several bacteria are reduced after chemotherapy, including *Bifidobacterium, Coprococcus, Clostridium, Dorea, Faecalibacterium, Lachnospira, Roseburia*, and *Ruminococcus*, which inhibit inflammation through blocking NF-κB and produce mucosa-protecting metabolites (SCFAs), whereas *Citrobacter* and other species, which participate in LPS biosynthesis and enhance intestinal inflammation, are increased during chemotherapy [34]. Subsequently, GI mucositis barrier dysfunction develops, leading to increased intestinal permeability, which coincides with a decrease in the amount of the previously mentioned protective bacteria. The microbiome may modulate the composition of the mucus layer, as the terminal step of mucositis induction. *Citrobacter*, which increases after chemotherapy, may participate in the degradation of the mucosal barrier through the expression of mucus-

degrading enzymes (mucinase, glycosidase), and *Enterobacteriaceae* can disrupt the mucus layer. Butyrate-producing bacteria protect the mucin layer, as butyrate increase mucin synthesis. A decrement in cysteine, proline, and methionine metabolism, which occurs during chemotherapy, can also be responsible for altered mucin composition and the development of GI mucositis after chemotherapy [34].

Radiation therapy is used as a primary treatment in cancers that are localized to one area of the body to prevent tumor recurrence after surgery or applied together with chemotherapeutic agents. Radiation itself is genotoxic, resulting in cancer cell death. However, radiation can also abolish nontarget cells due to the activation of the immune system by radiation-induced inflammation. The microbiota is known to be involved in these off-target effects due to intestinal mucosa damage and toxicity. Radiotherapy decreases both the diversity and the total amount of gut bacteria, particularly *Bacteroidetes, Enterobacteriaceae, Firmicutes*, and *Lactobacillus* species, while enriching *Fusobacterium* and *Proteobacteria*, which are connected with increased production of proinflammatory cytokines [35].

10.9 Modulation of the Microbiome to Enhance the Efficacy of Chemotherapy

Probiotics and prebiotics are widely used to shift the composition of the microbiome, and these interventions are potentially useful in restoring the microbiome after chemotherapy. Probiotics contain live bacteria that can be administered orally, while prebiotics (dietary prebiotics) are compounds in food, which provide substrates that stimulate the growth or activity of advantageous bacteria colonizing the gut. Prebiotics and probiotics prevent infection and moderate the side effects of cancer treatment. Administration of various strains of *Lactobacillus*, such as *Lactobacillus acidophilus,* is associated with enhanced cisplatin sensitivity and longer survival in lung cancer [35]. *Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus casei*, and *Lactobacillus rhamnosus* decrease the toxicity associated with 5FU chemotherapy and, consequently, reduce abdominal discomfort and diarrhea. In addition, *Bifidobacterium* and *Lactobacillus* species in combination were able to moderate the side effects after radiation treatment. Current clinical trials are focused on the efficacy of probiotic treatment for colorectal, kidney, breast, gynecologic, and lung cancer [35].

Fecal microbiota transplantation (FMT), also known as stool transplantation, is the process of transplanting fecal bacteria from a healthy individual into a diseased subject. FMT is an effective therapy to shift the composition of the microbiome. FMT is effective in the treatment of *Clostridium difficile,* where FMT is curative through enhancement of the diversity of the microbiome [67]. FMT could be potentially effective after chemotherapy or radiotherapy in cancer patients by avoiding gut toxicity or preventing infections. However, FMT has numerous side effects (fever, diarrhea, vomiting), including serious side effects, such as GI bleeding or perforation, that limit its applicability in cancer patients [35].

As a developing future therapy, bacterial engineering offers the opportunity to treat cancer without reconfiguring the gut microbiome. Biologically engineered bacteria could be applied effectively to target cancer cells or to deliver therapeutic agents, thereby avoiding serious side effect-eliciting anticancer therapies. Bacterial cells can be easily and rapidly transfected with vectors encoding interfering RNAs, cytokines, toxins, antiangiogenic factors, or antibodies. *Listeria* and *Shigella* species could invade hypoxic tumor tissues, and, given their quick replication rate, these bacteria could amplify their transgene(s) within the tumor microenvironment. Upon the application of bacteria, finding a good balance is necessary; one must seed a sufficient number of bacteria to elicit therapeutic effect but should avoid suppressing the immune system at the same time [35] (Fig. 10.4).

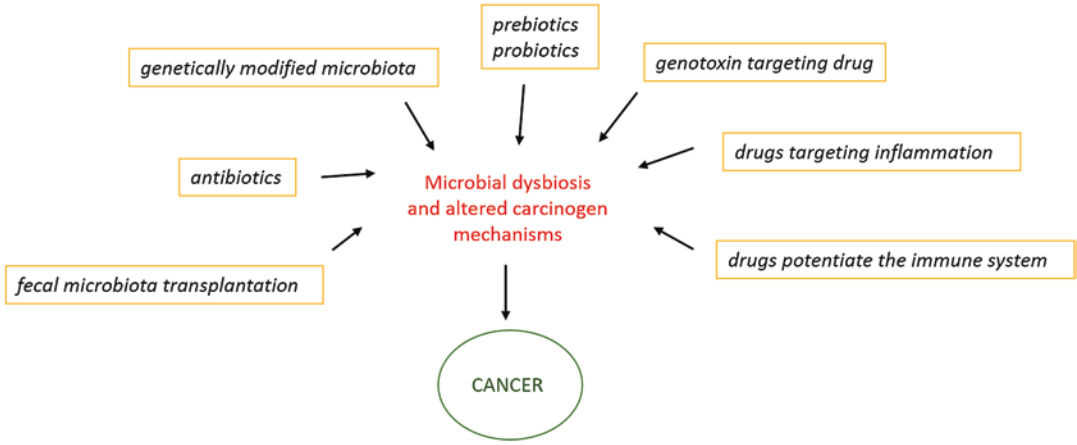

Fig. 10.4 Targeting the microbiome for modulation of carcinogenesis

10.10 Type of Cancers Related to Microbial Dysbiosis

Besides the GI tract, other organs are colonized by a unique microbial community, such as the skin, oral cavity, and germinal tracts. Growing evidence confirms a significant relevance of bacterial microbiota in the carcinogenesis of the colon, liver, breast, lung, oral cavity, and pancreas.

The liver receives 70% of its blood supply from the intestinal vein. This close functional relationship between the liver and GI tract results in constant exposure to nutrients, toxins, microbial metabolites, and microbes. Various types of immune cells (NK cells, macrophages, lymphocytes) defend this organ against harmful agents derived from the intestine. An altered microbiome may contribute to the development of *hepatocellular carcinoma* (HCC), which is preceded by chronic liver disease, fibrosis, and cirrhosis [68]. The disrupted microbiome may drive this process through the loss of intestinal barrier function, the activation of the NF-κB pathway, the production of pro-inflammatory cytokines, and increased anti-apoptotic signals.

Pancreatic cancer is an aggressive cancer type with low therapeutic success and survival rate. Periodontal disease, low oral hygiene, obesity, smoking, and alcohol consumption are well-known risk factors for pancreatic cancer, because they facilitate the translocation of bacteria through disrupted barrier layers. Bacteria can reach the pancreas through the circulation. Furthermore, although the pancreas does not have a microbiome, carcinogenesis of this organ is enhanced by distant dysbiotic microbiota [6], through the involvement of inflammatory responses, LPS expression, and TLR4 activation [69].

About 90% of all lung cancer cases are attributed to smoking, while only 15% of smokers develop *lung cancer*, suggesting other mechanisms and influences. The interface of the lung is continuously connected to the outside environment, and the microbiota of the lung reflect the microaspiration of oral microbiota. The lung has a unique microbiome with different species of *Proteobacteria*. The connection between lung cancer and chronic pulmonary disease is assigned to toxic pro-inflammatory and neoplasia-causing compounds. Different bacteria species, such as *Moraxella catarrhalis, Haemophilus influenza*, and *Streptococcus pneumoniae*, are associated with 50% of chronic pulmonary disease, and their presence can elicit chronic inflammatory responses [70].

The oral cavity harbors diverse individual microbiota. Moreover, the composition of the microbiota differs between microenvironments within the oral cavity; the lateral and dorsal tongue and tooth surface all have unique microbial communities. The normal oral microbiome

includes *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Haemophilus, Neisseria, Prevotella, Proteobacteria, Streptococcus*, and *Veillonella* species. *Capnocytophaga gingivalis, Prevotella melaninogenica,* and *Streptococcus mitis* are found in *oral squamous cell carcinoma (OSCC)* and are considered biomarkers of this disease. Risk factors for OSCC, which are connected to anaerobic, Gram-negative bacteria that liberate inflammatory markers, include smoking, heavy alcohol consumption, poor oral hygiene, and periodontal disease [71].

Genetic factors, infection, inflammation, and diet are well-known risk factors for *colorectal carcinoma* (CRC). CRC is associated with other diseases, such as inflammatory bowel disease, autoimmune, allergic reactions, obesity, and diabetes. Despite the great diversity of bacterial species of the GI tract, CRC is closely related to changes in the diversity and activity of microbes. Microbes produce metabolically active molecules that alter homeostasis or carcinogenesis [72]. The microbiota may contribute to CRC through different mechanisms that result in an imbalance between cellular proliferation and apoptosis pathways, such as PRR signaling and inflammation, metabolites that induce DNA damage and chromosome instability, or the loss of protective metabolites (due to microbial dysbiosis), such as SCFAs, secondary bile acids, or bioactive amines [73].

Recent research showed a strong correlation between gut microbiome dysbiosis and *breast cancer*. In addition to the gut microbiome, the breast has a unique microbiome that shows drastic changes in breast cancer. The microenvironment of breast cancer cells is modulated by bacterial metabolites (SCFAs, secondary bile acids, amino acid degradation products, and estrogen derivatives) that are produced in the intestine and reach cancer cells of the breast via the circulatory system. In breast cancer, various pathways are disrupted or altered in addition to the general changes in glycolysis and mitochondrial function, including glutamine, fatty acid, cholesterol metabolism, protein translation, and glutamine-serine pathways in cancer cells. These changes are the consequence of the rearrangement of a complex homeostatic system and energy sensors and lead to changes in cell proliferation and angiogenesis. Microbial dysbiosis occurs in both the fecal flora and the breast microbiome in breast cancer [20]. Fecal samples of breast cancer patients contain increased levels of *Clostridiaceae, Faecalibacterium*, and *Ruminococcaceae* and decreased levels of *Dorea* and *Lachnospiraceae* species [18]. Moreover, the microbiota composition differs not only between cancerous persons and healthy volunteers but also between breast cancer stages and grades and according to different tumor subtypes (triple-negative breast cancer associated with unique microbiome) [74]. For example, patients with grade III cancer have an increased number of *Blautia* species, compared with grade I patients, and samples from stage II/III showed elevated absolute numbers of *Bacteroidetes, Clostridium*, and *Blautia* species [75].

10.11 Future Prospects

The recent emergence of studies on the microbiome in various diseases highlights the importance of bacterial dysbiosis in different cancers. Despite the increasing literature on colorectal cancer, the data and observations on those cancers that are not in direct contact with the (gut) microbiome are limited and the available studies are often restricted to observational studies. Hence, mechanistic studies are largely missing. Minor microbiome compartments are understudied, in terms of the number of bacteria (e.g., lower airways). These caveats will need to be filled in the future.

The currently available data suggest that prebiotics and probiotics may have beneficial effects in restoring/preventing the microbiome dysbiosis, but these findings will have to be assessed in well-controlled clinical studies. Along those same lines, the use of antibiotics in cancer patients will need to be assessed in detail. Finally, the microbiome-drug interactions, a key element in cancer-related personalized medicine, will need to be precisely mapped.

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Correction to: Multimodal Molecular Imaging of the Tumour Microenvironment

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Unfortunately the book was published with the incorrect citations in the appendix section of chapter 5. Now the citation has been updated.

The updated online version of this chapter can be found at https://doi.org/10.1007/978-3-030-35727-6_5

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