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Pawel Kalinski *Editor*

Tumor Immune Microenvironment in Cancer Progression and Cancer Therapy

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Preface

Tumor microenvironment (TME) and the patterns of local interactions between malignant cells, tumor stroma, and inflammatory infiltrate have been extensively demonstrated to be essential for the survival of cancer cells, their proliferation and invasion into surrounding tissues, and formation of distant metastasis, as well as their resistance to treatments.

More recent works demonstrate that the immune component of the TME (iTME) and the interactions between different types of immune cells, tumor-associated fibroblasts, and cancer cells are critical for the success, and frequent failure, of the spontaneously arising anticancer immunity and for the effectiveness of different forms of immunotherapy. Most strikingly, recent reports documented the critical role of the immune system and iTME in the effectiveness of radio-and chemotherapy, the pillars of cancer treatment that were traditionally considered as immunosuppressive, rather than immunostimulatory.

This book *Tumor Immune Microenvironment in Cancer Progression and Cancer Therapy* compiles 14 chapters discussing the roles of different iTME components (cells, proteins, and nonprotein mediators) in tumor progression, metastatic process, and different forms of cancer treatment, as well as newly arising opportunities for their modulation to enhance the overall therapeutic benefit of the comprehensive cancer care.

After decades of controversies regarding the role of immune system in controlling cancer growth, fueled by frequent dissociation between the levels of systemic immunity against tumor-related antigens (observed in the blood) and cancer progression, recent clinical and animal studies have demonstrated that the immune component of the TME is the key predictor of cancer progression and treatment outcomes. A striking example is the effectiveness of checkpoint inhibitors, where the clinical responses and long-lasting therapeutic benefit of individual patients, even patients with advanced cancer, can be predicted by the levels of infiltration with cytotoxic T cells (CTLs) within the TME. However, the current methods of evaluation of different iTME components, and evaluating their intrinsic plasticity and context-dependent roles at different stages of antitumor responses are not absolutely reliable. This warrants the development of new methods of TME analysis to optimally define predictive markers and targets for cancer therapy.

I wholeheartedly thank all the authors, leaders in their respective fields, who took time from their labs and other duties to share with you their insights on different aspects of iTME during the progression of cancer and its treatment.

I hope that you will find this book interesting, thought provoking, and helpful in your research.

Buffalo, NY, USA Pawel Kalinski

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List of Abbreviations

1-MT	1-Methyl-tryptophan
5-HTP	Hydroxytrytophan
AHR	Aryl hydrocarbon receptor
CCL	CC-chemokine ligand
CD	Cluster designation
COX2	Cyclooxygenase-2
CSF	Colony stimulating factor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CYP1a1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1b1	Cytochrome P450, family 1, subfamily B, polypeptide 1
D-1-MT	1-Methyl-D-tryptophan
DC	Dendritic cell
DCs	Dendritic cells
FICZ	6-Formlindolo [3,2-b] carbazole
GCN2	General control non-derepressible 2
GIST	Gastrointestinal stromal tumors
G-MDSC	Granulocyte myeloid derived suppressor cell
IDO	Indoleamine-2,3-dioxygenase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
KYN	Kynurenine
L-1-MT	1-Methyl-L-tryptophan
L-NAME	N^G-nitro-L-arginine methyl ester
Ly6C	Lymphocyte antigen 6 complex
MDSC	Myeloid derived suppressor cell
M-MDSC	Monocyte myeloid derived suppressor cell
mTOR	Mammalian target of rapamycin
NADPH	Dihydronicotinamide-adenine dinucleotide phosphate

PD-1	Programmed cell death-1
PDE-5	Phosphodiesterase-5
PGE	Prostaglandin E
RAGE	Receptor for advanced glycation end products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TDO	Tryptophan-2,3-dioxygenase
TILs	Tumor infiltrating lymphocytes
TLR4	Toll like receptor 4
Tregs	Regulatory T cells
Treg cell	Regulatory T cell
TRP	Tryptophan
VEGF	Vascular endothelial growth factor

Tumor Immuno-Environment in Cancer Progression and Therapy

1

Pawel Kalinski and James E. Talmadge

1.1 Introduction

The immune environment of tumor tissues is a determinant of tumor progression and the overall effectiveness of cancer treatments, including not only immunotherapy but also chemotherapy and radiotherapy [1–12]. Systematic efforts to utilize the immune system to treat cancer dates back to the 1890s and the work of William Coley who used heat inactivated *Streptococcus erysipelas* and *Serratia marcescens* (Coley's toxins) [13, 14] in patients with sarcoma and other cancers. However, the outcomes were not consistent and could not be confirmed, resulting in a century-long controversy on whether the immune system has any role in cancer rejection. After decades of inconsistent clinical results testing different forms of immune therapy [15] and studies showing frequent dissociation between the effectiveness of systemic immunization against tumor-associated antigens (TAAs) and cancer progression [16, 17], recent preclinical, correlative and epidemiologic studies have focused on immune events in the tumor microenvironment (TME), as the key factor involved in cancer progression and treatment outcomes. This modified perspective and the undisputed success of several forms of immune therapy have together addressed the century-long dispute on the ability of the immune system to control cancer progression and allowed key insights into rational design of immune therapies and other forms of cancer treatment which depend on the activity of the immune system. This observation does not trivialize the therapeutic activity of BCG for bladder cancer [18] and IL-2 and IFNα for melanoma and renal cell carcinoma [19]. However, the response rates for IL-2 and IFNα are limited (8–12%) and generally of limited duration, while BCG has a higher response rate and can be effective longer, especially at early stage of the disease. This contrasts with checkpoint inhibitors which have higher response rates and often result in long-lasting responses, even in patients with advanced cancer. However, even checkpoint inhibitors are typically effective only in patients with inflamed "hot" tumors, which show baseline infiltration with anti-cancer immune cells, such as cytotoxic T cells (CTLs), highlighting the importance of the TME in the effectiveness of anti-cancer immunity and overall patient outcomes [5–8, 20–23].

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1.2 Cancer Cells and Non-cancer Components of the TME: Key Players

Cancer cells provide the critical initial stimulus to the establishment of TME and tumor formation, but they typically constitute only a minor part of the overall tumor mass and critically depend on the remaining elements of the TME (Fig. 1.1) for survival, expansion, and metastatic process [8]. The percentage of the actual cancer cells within a tumor can vary significantly, depending on the histologic type, site within the tumor architecture (invasive margin or the hypoxic and often necrotic center of more advanced lesions), disease stage and prior treatment(s), but with the exception of transplantable mouse tumors, cancer cells represent only a small proportion of the total number of tumor cells and an even smaller portion of the overall tumor mass.

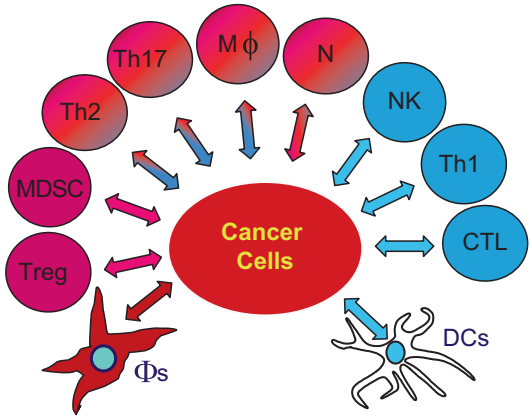

Fig. 1.1 Non-cancer component of tumor tissues: key players and their functions. *CTL* cytotoxic T cells, *DCs* dendritic cells, *Φs* fibroblasts, *MDSC* myeloid-derived suppressor cells, *Mϕ* macrophages, *NK* natural killer cells, *N* neutrophils, *Th1* Type-1 T helper cells, *Th2* Type-2 T helper cells, *Th17* Type-17 T helper cells, *Treg* regulatory T cells. Predominant pro- or anti-tumor-functions of the cells are depicted using the following color code: *Brown:* Critical for tumor cell survival; *Magenta*: Tumor-promoting; *Blue:* Uniformly anti-tumor; *Transitional:* Predominantly pro-tumor, but with anti-tumor potential. Please note that, in addition to directly interacting with cancer cells and being affected by them, all of the above non-mutated tumor-associated cancer cells affect each-others' function. All of them are also modulated by different forms of cancer treatments and can be ch of them can also be targets for immune therapy

The non-cancer elements of the TME involve tumor-infiltrating inflammatory cells and tumor-associated stromal fibroblasts, which are the key source of the non-cellular matrix, which together with cancer-cell produced mucins constitute the main non-cellular tumor component and the main component of overall tumor mass [8, 24].

Inflammatory (or immune) cells belong to myeloid and lymphoid lineages. Myeloid cells include tumor-associated macrophages (TAMs), monocytic and granulocytic myeloid-derived suppressor cells (MDSCs), granulocytes and dendritic cells (DCs).

TAMs include type-1 macrophages (M1 cells) which secrete TNFα and express additional TNF/TNF-R family members on their membrane, resulting in antitumor effector functions, and type-2 (M2) macrophages, which, similar to myeloid-derived suppressor cells (MDSCs) express multiple immunosuppressive and tumor promoting factors, such as prostaglandin E2, VEGF and IL-10, which can suppress antitumor immunity and promote tumor growth via non-immune mechanisms, which include enhance induction of tumor blood vessels and anti-apoptotic activity, which enhance cancer cell proliferation and promote resistance to killing by immune mechanisms, as well as hypoxia, chemo- and radiotherapy [1–4, 8, 25, 26].

Another myeloid subset, DCs are the key inducers of immune responses. DCs specialize in local uptake of antigen from dying or distressed cancer cells, and their presentation both locally (enhancing effector functions of CTLs and Th1 cells) and in draining lymph nodes (where DCs actively migrate following activation), and initiate central (lymphoid) antigen-specific immunity by activating naive and memory CD8+ and CD4+ T cells and inducing their differentiation into typer-1 effector cells (CTLs and Th1 cells).

The above subsets of myeloid cells show a significant degree of intrinsic plasticity and can also cross-regulate each other's functions. For example, suppressive TMEs can redirect the differentiation of circulating early myeloid

progenitors and monocytes from DCs to MDSCs and differentiate M1 macrophages to become M2 macrophages. Reciprocally, activation of type-1 immunity (see below) by DCs a can reduce the frequency of M2 macrophages, by NK or CTL cytotoxicity or re-differentiation toward a M1 phenotype, as well as their replacement by new M1-like cells, due to a change in the chemokine production pattern in TME.

Lymphoid component of the TME, often referred to as tumor infiltrating lymphocytes (TILs), involves T and B lymphocytes as well as natural killer (NK) cells. Tumor-infiltrating T cells include varying numbers and ratios of $CD8^+$ T cells (which involve CTLs, believed to be key anti-tumor effector cells) and $CD4^+$ T cells, whose subset composition and role is more complex. Type-1 $CD4^+$ T cells (T helper-1 or Th1 cells), secrete high levels of effector cell-activating cytokines, including IL-2, TNFα and IFNγ, that contribute to effective antitumor immunity, by supporting CTL functions and inducing antitumor effector functions by TAMs (type-1 or M1 macrophages). An opposing functional type of $CD4^+$ T cells, regulatory (Treg) cells produce such factors as TGFβ and IL-10 that can suppress CTL functions directly (by the above soluble factors, by depleting CTL-supporting growth factor, such as IL-2, or in a contact-dependent manner), or by inducing and activating suppressive functions of type-2 (M2) macrophages and MDSCs. Additional subsets of $CD4^+$ T cells, Th2 and Th17 cells, are believed to play mostly tumor-promoting functions, although they can also suppress tumor growth in some situations. Similar, activated B cells play mainly tumor-promoting factors, partially mediated by IL-10 [27, 28], although their presence in tumor-associated lymphoid structures may also have a positive prognostic value [29].

Similar to myeloid cells, $CD4^+$ T cell display significant level of plasticity, such that Th1 cells can be differentiated into Th17, Th2, and Treg cells upon repetitive, particularly chronic (re) stimulation in suppressive TMEs. The concept of chronic activation is critical to $CD4^+$ T-cell mediated immune suppressions, as chronically activated $CD4^+$ T cells frequently express checkpoint inhibitor molecules which contribute to T-cell suppression [30, 31].

NK cells show a unique ability to kill cancer cells upon initial interaction, without need for prior "education". Specific NK cell activating stimuli can enhances their ability to kill tumor cells [32–34], although are not strictly required. Although NK cells are very effective in controlling cancer cell survival in the circulation (liquid cancers; such as lymphomas and leukemia's and circulating solid tumor cell), but they are relatively ineffective in controlling established solid tumors, where there are inactivated themselves by a hostile TMEs.

Lymphoid cells are present in TME as both disseminated cells, and as lymphoid foci formed by precursor cell proliferation and formation of organized lymph-node-like structures [35–40]. Their relative prevalence and localization within TME is regulated by their distinct patterns of migration (see Figs. 1.2 and 1.3), as well as survival and functional plasticity.

1.3 Establishment of the Tumor Microenvironment During Cancer Progression

The process of tumor initiation and progression is regulated in part by the TME. Malignant tumors once initiated and following slow progression to a malignant status became invasive, and can have disparate morphologies from their tissue of origin. Further, they can metastasize, i.e., "the transfer of disease from one organ or part to another not directly connected to it" a term originally coined by Recamier in 1829 [41]. Metastasis provides a critical clinical challenge as its onset is unpredictable, is often hard to detect and significantly reduces the chances of patients' survival.

The TME and the associated inflammatory processes have a key role in the process of tumor progression. The TME has been identified as a critical part of the soil in the "seed and soil" hypothesis such that the "seed" is the tumor pro-

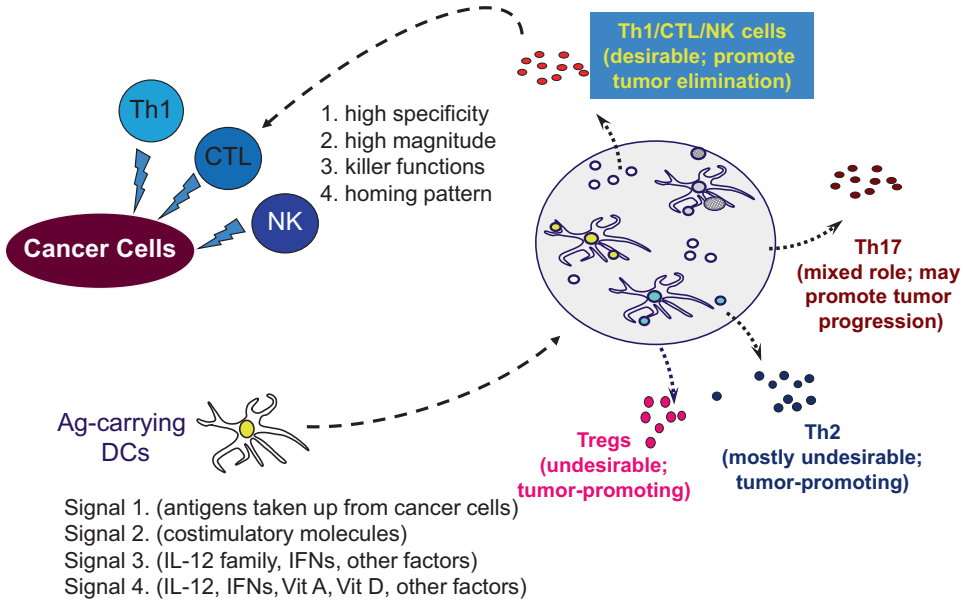

Fig. 1.2 Dendritic cells as orchestrators of immune cell communication within TME and lymphoid tissues: Signals and mediators. *DCs* dendritic cells, *MDSC* myeloid-derived suppressor cells, *NK* natural killer cells, *Th1* Type-1 T helper cells, *Th2* Type-2 T helper cells, *Th17* Type-17 T helper cells, *Treg* regulatory T cells

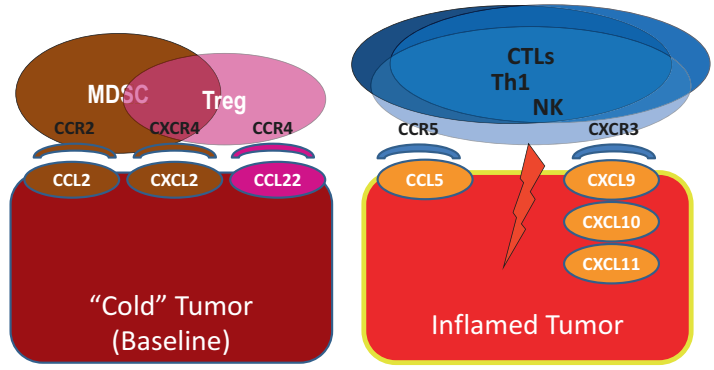

Fig. 1.3 Reprogramming of the immune component of TME for enhanced effectiveness of cancer therapies. Differential expression of chemokine receptors on anti-tumor effector cells and tumor-promoting suppressor cells allows for their therapeutic targeting to enhance the effects of cancer immunotherapy and other forms of cancer treatment

genitor cell, tumor initiating cell, "cancer stem cell", or metastatic cell, while the "soil" includes host factors, such as tumor infiltrating inflammatory cells, and the tissue/organ microenvironment. Tumor progression depends on multiple interactions between tumor and host cells such that it is a process that is both sequential and selective with multiple stochastic elements [42].

Role of the TME in the pathogenesis of tumor initiation, progression, and metastasis. Cancer progression incorporates a series of interrelated steps such that a failure at any step may interrupt/delay/block the process of cancer progression to metastasis. The general steps involved in tumor initiation, progression, and metastasis are similar for all tumors; requiring the development of a

vascular network, evasion of host immunity, and other interactions with the TME. However, there is an inherent redundancy to the process such that if one factor is blocked, an alternative mechanism may be utilized.

Clinical observations suggest that metastasis tends to occur in association with specific host cells and within tumor specific organs. During tumor growth, tumor cells invade into the organ parenchyma, a process that maybe facilitated by infiltrating myeloid cells and hypoxia [43]. This provides cellular and enzymatic interactions that disrupt cell:cell junctions and facilitate tumor cell invasion. Due to the inherent plasticity of tumor infiltrating myeloid cells, their response to the soluble factors and chemokines released during tumor growth by both tumor cells and infiltrating cells within the TME varies. Invading tumor cells move along pre-existing tissue planes in association with proteolytic degradation. As one form of metastasis, invading tumor cells can grow within pleural and peritoneal cavities forming an effusion.

The invasive properties of malignant cells are associated with upregulation of metalloproteases, which can degrade collagen, serine protease that can activate coagulation mediators and chemokines as well as enzymes that degrade other stromal components including basal laminar molecules. This was initially identified by Liotta and co-workers [44] who recognized the importance of basement membrane type IV collagen degradation (by matrix metallopeptidase 9 (MMP-9)) during tumor invasion and its association with metastatic potential. Since this initial observation, numerous enzymatic activities by myeloid cells have been associated with tumor invasion [45]. Unfortunately, to date, most clinical studies with collagenase inhibitors have provided minimal therapeutic benefit [46], perhaps due to redundant enzymatic mechanisms associated with invasion and/or challenges in achieving effective local concentrations [47].

Metastasis can occur early in tumor progression, but is rarely diagnosed until after a prolonged latency period. Metastatic process can show a delayed onset following "curative" surgical resection. This reflects prolonged latency period when cancer cell survives in a quiescent state prior to the acquisition of competency for growth. As part of the metastatic process, different organs impose varying selective pressures, both positive and negative, such that the establishment of metastatic colonies is frequently tumor specific. Further, tumor cells may survive in selected organs following survival in the circulation and subsequent organ arrest. Once tumor cells have extravasated, their growth may be regulated by the local milieu where they are retained. This has been identified as the metastatic "niche". These niches are controlled, in part, by the target organ environment; including the site-specific secretion of chemokines and growth factors, which attract and activate both cancer cells and different classes of immune cells, which either support or limit cancer growth. Further, increased numbers of myeloid and vascular precursor cells in tumor bearing (TB) hosts exist in an organ specific manner, including the, lung, bone marrow (BM), brain and liver, and, as such, support metastasis to these sites in association with extramedullary hematopoiesis (EMH). The mechanism(s) that support metastasis may also be regulated by lifestyle mediators, including, but not limited to, hormonal therapy, diet; especially dietary fat and simple sugars intake, as well as psychologic stress reaction. Primary tumors secrete factors that can control EMH at distant environments [48], thereby facilitating the growth of metastatic cells. Survival of circulating tumor cells, following arrest, requires a supportive microenvironment that can include reprogrammed fibroblasts and immune cells (Fig. 1.1).

1.4 Roles of Distinct TME-Associated Immune Cells and Their Modulation During Tumor Progression

Tumor-Associated Macrophages (TAMs). TAM can have direct tumoricidal activity and can also support antitumor T-cells, but in most situations they have been found to suppress type-1 immunity mediated

by CTLs, Th1- and NK cells, thus promoting tumor growth. Numerous studies have demonstrated that activated macrophages can kill tumor cells *in vitro*, especially if they have been activated by immune adjuvants, such as interferons or TNFα. Such type-1 TAMs show M1 cytokine- and chemokine profiles, produce cytotoxic factors, including NO, reactive oxygen intermediates (ROIs), and other type-1 mediators, which makes them potent effectors capable of killing tumor cells, as well as attracting additional anti-cancer effector cells to TME.

However, most of macrophages present in tumors have a pro-tumorigenic/immunosuppressive M2 phenotype [49]. M2 cells are induced and expand in response to macrophage-colony stimulating factor (M-CSF), IL-4, IL-10, IL-13, IL-21, and Activin A, as well as corticosteroids, prostaglandins (PGs), and vitamin D3 [50, 51], and counteract M1 macrophage function [52–57]. M2 TAMs contribute to tumor escape from host immunity by producing immune-suppressive cytokines, such as PGE_2 [58], interleukin (IL)-10 and transforming growth factor beta (TGFβ) [59–61]. They can also suppress DC differentiation and inhibit DC functions through IL-10 production [62].

In addition to their immunosuppressive functions, TAMs are also involved in facilitating tumor invasion into surrounding tissues [63], being a primary source of proteolytic enzymes that facilitate tumor cell invasion [59, 64]. They also directly stimulate the proliferation and survival of cancer cells [65, 66] and promote neoangiogenesis through secretion of VEGF [59, 64–68].

Although the general prognostic value of TAM remains controversial [69–71], most of the current studies indicate that high TAM infiltration can predict distant metastasis formation [72] and is associated with accelerated cancer progression and overall poor prognosis [59, 73–80].

Myeloid-derived suppressor cells (MDSCs). The numbers of MDSCs are enhanced both in the circulation of tumor-bearing hosts and within their tumor tissues [52–54, 57, 81]. Murine MDSCs are $CD11b^+Gr\text{-}1^+$ [82], while human MDSCs, originally described in the peripheral blood (PB) of head and neck cancer patients [83] are a heterogeneous cellular population, showing an lineage-negative ($CD3^-$, $CD56^-$, $CD19^-$, $CD13^{-/Dull}$) immature myeloid phenotype ($HLA\text{-}DR^-CD11b^+$). Some subsets express $CD33^+$, $CD14^+$, $CD15^+$, $CD34^+$and $CD31^+$, depending on commitment pathway and extent of differentiation. The immunosuppressive activity of MDSCs (both murine and human) occurs through multiple mechanisms including the upregulation of PGE_2, IDO, reactive oxygen species (ROS), nitric oxide (NO) production and arginase levels, as well as the secretion of immunosuppressive cytokines [84–88].

Granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF), in a tumor-dependent manner, are directly associated with MDSC numbers and tumor burden [89, 90]. Vascular endothelial growth factor (VEGF) has also been directly linked with MDSC expansion, tumor progression, and immune suppression [91] through its inhibitory effect on dendritic cell (DC) differentiation [92]. A correlation has been reported between plasma VEGF levels in cancer patients, poor prognosis [93], and abnormal DC differentiation [94], including an inverse correlation with circulating DC frequency [95]. Further, the injection of neutralizing VEGF antibodies can reverse the VEGF-induced abnormalities in DC differentiation, but has not reduced the circulating MDSC frequency, perhaps due to compensatory GF secretions [96].

Another factor shown to be associated with the numbers of monocytic MDSCs in cancer patients and controlling their suppressive function (including the production of IDO, NOS2, arginase, IL-10 and CXCL12 (SDF-1), is PGE_2 [84–87].

High frequencies of MDSCs (predominantly $CD34^+CD33^+CD11b^+CD14^+CD15^-$) have been found responsible for suppression of tumor-specific CTL responses and associated with the resistance to treatments and overall poor outcomes in cancer patients [97–103].

Dendritic Cells (DCs): Facilitators of Intercellular communication within TME and lymphoid tissues. In contrast to other types of myeloid cells, intratumoral densities of DCs, particularly

mature DCs, represent some of the strongest predictors of improved survival in multiple groups of cancer patients [37, 104–113]. These observations and the frequently observed dysfunction of the DC system in cancer-bearing hosts, prompted a widespread use of *ex vivo* generated DCs in cancer immunotherapy [5, 114–120].

DCs orchestrate the induction- and effector phases of tumor-specific immunity delivering at least four types of signals to T cells [84]. The first signal (Signal 1) is the presentation of processed antigen in the context of major histocompatibility complex (MHC) molecules by DCs to naïve T cells via the T cell receptor (TCR) [121]. DCs show unique ability to take up different forms of antigens, process, and then cross-present the antigens to both $CD4^+$ and $CD8^+$ T cells. Signal 2 are the costimulatory signals that complement the TCR signal to ensure effective T cell activation. This amplification signal is provided by B7 family molecules, such as CD80 and CD86, by binding to CD28 on the T cell [114, 122]. The molecules involved in costimulation are upregulated upon DC maturation, the process which also enhances the ability of DC to migrate to lymphoid tissues and interact with naïve and memory T cells [123, 124]. Signal 3 is mediated by the DC-produced cytokines which provide differentiation signals to the T cells affected the character of the resulting immune response generated (Th1, Th2, Th17, induction of killer function in $CD8^+$ T cells). A prototypical example of a Signal 3 cytokine that promotes cell-mediated immunity is IL-12p70 [125]. An additional signal (Signal 4) delivered to T cells by DCs is the induction of different sets of chemokine receptors and integrins, which affect the pattern of their homing to different tissues [114]. *In vitro* and *ex vivo* studies have demonstrated that DCs isolated from various tissues can differentially modulate the T cell expression of integrins and chemokine receptors, thereby directing activated T cells back to the tissues of the DCs origin [126, 127].

Numerous reports have demonstrated that animals with established tumors and patients with advanced cancer show significant levels of local (intratumoral) and systemic dysfunction of the DC system [128–130], which includes DC arrest at immature stage of development, reduced ability to migrate to draining lymph nodes, and impaired delivery Signal 2 and Signal 3 (Reviewed in [84, 131, 132]). Among multiple tumor-produces factors which negatively affect DC functions, IL-10, PGE_2, TGFβ, IL-6, and VEGF have been studied most extensively and shown to be implicated in the immunopathology of multiple cancers [84, 129, 131–135].

Tumor infiltrating T-cells (TILs): Key role of the balance between CTLs and Tregs. The type, location, and density of tumor-infiltrating lymphocytes (TILs), especially $CD8^+$ CTLs in many groups of cancer patients are strong predictors of survival, independent of tumor histopathologic and metastatic status and the stage of disease [11, 37, 136–140]. In particular, the presence of effector and effector/memory $CD8^+$ T cells within tumor nests predict improved progression-free survival (PFS) and overall survival (OS) in patients with multiple cancer types [136, 138, 139, 141–145].

In contrast, high numbers of intratumoral $CD4^+$ T*regs* in tumor tissues [139] and tumor-associated ascites [146], are associated with poor tumor response to therapy, and accelerated progression [29, 108, 146–155]. T*regs* include both natural and adaptive T*regs* [156]. The natural T*reg* population found within the thymus is involved in Ag-specific regulatory responses and may protect the host against potentially harmful immune responses. Adaptive T*regs* develop from mature, circulating $CD4^+$ T-cells in response to Ag-specific responses and regulate host immunity via production of suppressive cytokines [156]. The presence or absence of Foxp3 has been used to characterize T*regs* and is critical to their suppressive function [157].

In accordance with the complexity of the $CD4^+$ T cell population, which includes the desirable Th1 cells with distinct anti-cancer role, but also Th2, Th17, and Tregs, which show different levels of tumor-promoting activity, the overall infiltration of cancer tissues with $CD4^+$ T cells does not clearly correlate with patients' prognosis. The overall numbers of $CD4^+$ TILs in head and neck (SCCHN) and renal cell cancer (RCC) tumors, has been found correlated with improved patients'

overall survival (OS) [158, 159]. In contrast, another study in resectable RCC patients found that the frequency of $CD4^+$ T-cells infiltrating the tumor was associated with poor survival, independent of tumor grade [159].

Some of the above controversies may reflect the functional heterogeneity of T cells and different immune context of different tumors. An interesting recent study in gastric cancer indicated a better predictive value of infiltration of T*bet*-positive T cells (effector CTLs and Th1 cells), rather than total $CD8^+$ T cells, with an unexpected additional benefit of B cell infiltration [160].

Natural-killer (NK) cells. Presence of functional NK cells has been shown to predict improved treatment outcomes and longer OS in some groups of cancer patients, while the paucity of functional NK cells is associated with poor prognosis [161–171]. In addition to their ability to kill cancer cells, NK cells can also play a helper role during the DC-driven induction of type-1 immunity, by activating DCs [172–174] and promoting their differentiation into mature, high IL-12p70 producing type-1 polarized (non-exhausted) DCs with enhanced capacity to induce Th1 and CTL responses [175], desirable against cancer. These observations explain the documented role of NK cells during the induction of anti-cancer Th1 and CTL-mediated responses *in vivo* [176–179]. In addition to their helper role in the development of type-1 immunity, NK cells can also perform suppressive functions. While the mechanism of suppressive activity of NK cells is most likely multifactorial, and can involve such factors as IL-13, IL-10, or TGFβ, the Pfn-dependent elimination of antigen (Ag)-carrying dendritic cells (DCs) by activated NK cells can act as a suppressive mechanism, providing negative feedback controlling the scope of immune responses [180].

1.5 Key Role of Immune TME in the Outcomes of Cancer Treatments

Clinical efficacy of immune therapies requires tumor-infiltrating CTLs. Immune checkpoint blockade therapies (such as PD-1/PDL-1 or CTLA-4 blockade), which are highly effective in multiple forms of advanced cancer, show only marginal effectiveness in most forms of CRC and OvCa [12, 181–185]. The responsiveness to PD-1/PD-L1 blockers has been shown to critically depend on the presence of intratumoral accumulation of CTLs (and associated local PD-L1 expression in the TME, which is induced by CTL-produced IFNγ) [6, 7, 10, 12, 20, 21, 23, 186]. Notably, despite its overall aggressive character, the subset of CRC with mismatch repair deficiency and high microsatellite instability (MSI^{high} tumors) shows surprisingly high responsiveness to PD-1/PD-L1 blockade, compared to MSI^{low} CRC [10, 12, 20, 22, 23, 187], which reflects its very high CTL infiltration of MSI^{high} tumors at baseline [184, 188]. Likewise, enhanced infiltration of $CD8^+$ T cells following intratumoral injection of a STING activator (cGAMP) improves spontaneous control of tumor growth and the effectiveness of PD-1 and CTLA-4 blockade in mouse models of CRC and other cancers [189]. Similar to their importance for the effectiveness of checkpoint blockade, the levels of intratumoral CTL infiltration can predict patients' clinical response to cancer vaccines [190, 191].

Clinical efficacy of radio- and chemotherapy depends on immune infiltration following treatment. Recent studies demonstrated the key role of immune system in antitumor effectiveness of radio- and chemo-therapy, two main pillars of comprehensive cancer care, which have been traditionally considered as immunosuppressive and relying solely on direct cytotoxic effect against cancer cells [2, 3, 192, 193]. The earliest indication that radiation therapy may have an immunogenic component resulted from sporadic observations (about 5% of patients) of the regression of distant, non-irradiated, tumors in patients receiving localized radiation therapy (abscopal effect [194]), but only recently it became clear that immunosuppressed individuals (or immunodeficient mice) show impaired responses to radio- and chemotherapies [11, 12, 169, 170]. Some of the most striking observations come from HIV positive patients, where radiation has

over 3× higher failure rate in elimination of cervical cancer than in HIV-negative population [195]. The importance of local events in the irradiated (or chemotherapy-exposed) TME is underscored by the observations that the levels of enhanced infiltration with $CD4^+$ and $CD8^+$ T cells post-therapy predicted prolonged PFS, with $CD8^+$ T cell infiltration also predicting prolonged OS, in patients with resectable cancer [196–199], while preferential intratumoral infiltration with Tregs in the course of radio-chemotherapy predicts enhanced risk of relapse [1, 200, 201]. In accordance with the impaired ability of radiation to eliminate residual cervical cancer cells in immunocompromised individuals [195], the predictive role of chemo-radiotherapy-induced TILs appears particularly strong in patients with HPV-associated malignancies, where it can predict long-term recurrence free survival and overall therapeutic activity of the combination therapy [9, 202].

Over the past 10 years, a wide range of immune events occurring in irradiated- or chemotherapy-exposed cancer tissues were identified which include type-1 inflammation, attraction of both lymphocytes and antigen-presenting cells (APCs), such as DCs and macrophages, leading to uptake and cross-presentation of antigens released from cancer cells and systemic immunization [203, 204]. The resulting intratumoral accumulation of CTLs, Th1, and NK cells promotes elimination of persisting cancer cells, preventing their survival, even in suboptimally-irradiated tissues [1, 3, 205]. In line with these observations, immunotherapy can sensitize patients to radiotherapy [192], jointly providing strong rationale for in-depth evaluation of the patient-specific immune events in the course of traditional non-immune therapies, and for the combined application of immunotherapy with other modalities of cancer treatment.

Importantly, the same considerations also implicate the intrinsic limitations of the evaluation of antitumor efficacy of any type of cancer treatments in preclinical models which do not involve the immune system, such as cancer cell lines or xenotransplant mouse models, which involve immunodeficient mice.

1.6 Feasibility of Reprograming Tumor Microenvironments for Therapeutic Purposes

Different chemokine receptor usage between type-1 effector cells (CTL, Th1, and NK cells), which typically home to sites of acute inflammation (such as acute viral infections), versus T*regs* and MDSCs, which accumulate at late and chronic stages of inflammation to promote tissue healing and regeneration, make the chemokine system an interesting target for the TME reprograming for enhanced therapy outcomes (Fig. 1.3).

Reports from multiple groups, including ours, indicate that functionally different classes of immune cells share commonalities in their expression of chemokine receptors, and preferentially respond to specific classes of chemokines. The key chemokines attracting CCR5- and CXCR3-espressing CTLs, Th1- and NK cells are CCL5 (ligand for CCR5) and CXCL9, CXCL10 and CXCL11 (ligands for CXCR3). In contrast, intratumoral production of CCL2, CCL22, and CXCL12, promotes attraction of $CXCR4^+$, and $CCR2^+$ MDSCs and tumor-promoting/type-2 macrophages, as well as $CCR4^+$/$CXCR4^+$ human T*regs*.

High tumor production of CCL5/RANTES (ligand for CCR5) and CXCL9/MIG, CXCL10/IP10, and CXCL11/ITAC (three known ligands for CXCR3) is associated with high CTL infiltration in CRC [206] and other cancers [126, 207]. Our own studies [208] showed tight correlation between intratumoral production of CCL5, CXCL9 and CXCL10, and local infiltration with $CD8^+GrB^+$ CTLs, with over 95% of CRC-infiltrating CTLs expressing at least one of these chemokine receptors.

Both the spontaneously-occurring and vaccination-induced tumor-specific CTLs express high levels of CCR5 and CXCR3 [209], and migrate in response to the chemokine ligands produced by inflamed tissues and subsets of tumors [208–210]. For this reason, the therapeutic benefit of checkpoint blockers, cancer vaccines (or adoptive T cell transfer therapies [ACT]) is likely to be enhanced by therapies that selectively enhance

CXCL10 (and other CXCR3 ligands), as well as CCR5 ligands (such as CCL5/RANTES), thus promoting migration of CTLs and other effector cells (spontaneously-arising, vaccination-induced or adoptively-transferred) to tumor tissues. In this regard, our prior mouse studies confirmed the antitumor role of CXCL10 in the effectiveness of therapeutic cancer vaccines, and the synergy between vaccines and the CXCL10-inducing factor poly-I:C (ligand for toll like receptor (TLR)-3) [211–213]. In our phase I/II study in patients with high-grade malignant glioma [214], in which peptide antigen-loaded DC-vaccines were combined with poly-IC:LC (stabilized poly-I:C variant), 9 of 19 patients experienced prolonged time to progression (TTP; historically 2–4 months in this group of patients) to at least 12 months, with 4 patients manifesting long-term objective clinical responses (two complete and two partial). However, poly-I:C, alone, although superior to many alternative TLR ligands, is an ineffective inducer of CTL-attracting chemokines, and can amplify the intratumoral expression of COX2 and production of COX2-dependent T*reg*/MDSC-attracting chemokines [208, 210, 215, 216]. Two options to enhance its effectiveness are either to combine poly-I:C with a COX2 blocker and IFNα, which uniformly reprogram the patterns of poly-I:C-induced chemokine production in several types of cancer [187, 189, 194, 195] or to apply modified TLR ligands which avoid activation of COX2 and COX2-dependent suppressive events (Theodoraki, Kalinski et al., *manuscript in preparation*).

Additional clinically-applicable factors with previously demonstrated TME-reprogramming activities include ligands for other TLRs, such as TLR4-binding monophosphoryl lipid A (MPLA); the TLR7 agonist, imiquimod; TLR9 agonists, CpGs, as well as cytokines (e.g., interferon-α, interleukin-2, or granulocyte macrophage colony-stimulating factor (GM-CSF)), and whole microorganism-based adjuvants, such as Bacille Calmette-Guérin (BCG) [215].

1.7 Conclusions

Comprehensive cancer care has traditionally focused on reducing the bulk of disease through surgery, chemotherapy and radiation. Despite the increasing effectiveness of these modalities and increasingly high cure rates of multiple cancer forms, cancer still remains a leading cause of death [217]. Recent entry of cancer immunotherapy as a standard element of comprehensive cancer care and our increasing understanding of the immune effects (and immune dependence) of chemo- and radiotherapy provide promising new tools to further enhance the overall success rate of oncologic treatments.

Recent clinical and laboratory evidence underscores the importance of local events within tumor microenvironments in regulating cancer progression, metastatic process and response to different forms of immunotherapy. Altering the tumor microenvironment to selectively enhance CTL infiltration and reduce T_{reg} and MDSC migration, together with limiting secondary suppressive mechanisms induced in the TME by activated CTLs [218] is likely to enhance the current response rate of immune checkpoint- and other immune therapies, as well as other elements of comprehensive cancer care.

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References

1. Weichselbaum RR, Liang H, Deng L, Fu YX. Radiotherapy and immunotherapy: a beneficial liaison? Nat Rev Clin Oncol. 2017;14(6):365–79.
2. Galluzzi L, Buque A, Kepp O, Zitvogel L, Kroemer G. Immunological effects of conventional chemotherapy and targeted anticancer agents. Cancer Cell. 2015;28(6):690–714.
3. Demaria S, Golden EB, Formenti SC. Role of local radiation therapy in cancer immunotherapy. JAMA Oncol. 2015;1(9):1325–32.
4. Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer. 2015;15(7):409–25.

5. Weber JS. Current perspectives on immunotherapy. Semin Oncol. 2014;41(Suppl 5):S14–29.
6. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515(7528):568–71.
7. Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 2014;515(7528):563–7.
8. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. Nature. 2013;501(7467):346–54.
9. Gadducci A, Guerrieri ME, Greco C. Tissue biomarkers as prognostic variables of cervical cancer. Crit Rev Oncol Hematol. 2013;86(2):104–29.
10. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443–54.
11. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298–306.
12. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366(26):2455–65.
13. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med. 1910;3(Surg Sect):1–48.
14. Nauts HC, Swift WE, Coley BL. The treatment of malignant tumors by bacterial toxins as developed by the late William B. Coley, reviewed in the light of modern research. Cancer Res. 1946;6(4):205–16.
15. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med. 2004;10(9):909–15.
16. Astsaturov I, Petrella T, Bagriacik EU, et al. Amplification of virus-induced antimelanoma T-cell reactivity by high-dose interferon-alpha2b: implications for cancer vaccines. Clin Cancer Res. 2003;9(12):4347–55.
17. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol. 2005;175(9):6169–76.
18. Bassi P. BCG (Bacillus of Calmette Guerin) therapy of high-risk superficial bladder cancer. Surg Oncol. 2002;11(1–2):77–83.
19. Kirkwood JM, Butterfield LH, Tarhini AA, Zarour H, Kalinski P, Ferrone S. Immunotherapy of cancer in 2012. CA Cancer J Clin. 2012;62(5):309–35.
20. Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. Cancer Discov. 2015;5(1):43–51.
21. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372(26):2509–20.
22. Kroemer G, Galluzzi L, Zitvogel L, Fridman WH. Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy? Oncoimmunology. 2015;4(7):e1058597.
23. Taube JM, Klein A, Brahmer JR, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res. 2014;20(19):5064–74.
24. Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer. 2016;16(9):582–98.
25. Harper J, Sainson RC. Regulation of the anti-tumour immune response by cancer-associated fibroblasts. Semin Cancer Biol. 2014;25:69–77.
26. Hill RP. The changing paradigm of tumour response to irradiation. Br J Radiol. 2017;90(1069):20160474.
27. Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ, Selvan SR. Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy. Immunol Res. 2011;51(2–3):170–82.
28. Sarvaria A, Madrigal JA, Saudemont A. B cell regulation in cancer and anti-tumor immunity. Cell Mol Immunol. 2017;14(8):662–74.
29. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin Cancer Res. 2006;12(18):5423–34.
30. Karasar P, Esendagli G. T helper responses are maintained by basal-like breast cancer cells and confer to immune modulation via upregulation of PD-1 ligands. Breast Cancer Res Treat. 2014;145(3):605–14.
31. Wang C, Hillsamer P, Kim CH. Phenotype, effector function, and tissue localization of PD-1-expressing human follicular helper T cell subsets. BMC Immunol. 2011;12:53.
32. Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. Nat Immunol. 2016;17(9):1025–36.
33. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nat Rev Immunol. 2012;12(4):239–52.
34. Whiteside TL, Herberman RB. The role of natural killer cells in immune surveillance of cancer. Curr Opin Immunol. 1995;7(5):704–10.
35. Kroeger DR, Milne K, Nelson BH. Tumor-infiltrating plasma cells are associated with tertiary lymphoid structures, cytolytic T-cell responses, and superior prognosis in ovarian cancer. Clin Cancer Res. 2016;22(12):3005–15.
36. Joshi NS, Akama-Garren EH, Lu Y, et al. Regulatory T cells in tumor-associated tertiary lymphoid

structures suppress anti-tumor T cell responses. Immunity. 2015;43(3):579–90.
37. Goc J, Germain C, Vo-Bourgais TK, et al. Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating CD8+ T cells. Cancer Res. 2014;74(3):705–15.
38. Kim KH, Kim TM, Go H, et al. Clinical significance of tumor-infiltrating FOXP3+ T cells in patients with ocular adnexal mucosa-associated lymphoid tissue lymphoma. Cancer Sci. 2011;102(11):1972–6.
39. Alam I, Frahad K, Griffiths PA, Hurley M. Simultaneous gastrointestinal stromal tumor and mucosa-associated lymphoid tissue lymphoma of the stomach. J Clin Oncol. 2007;25(9):1136–8.
40. Schrama D, thor Straten P, Fischer WH, et al. Targeting of lymphotoxin-alpha to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. Immunity. 2001;14(2):111–21.
41. Recamier JC. Recherches sur la Traitment du Cancer sur la Compression Methodique Simple ou Combinee et sure l'Histoire Generale de la Meme Maladie, vol. 21829. Paris: Gabon; 1829.
42. Price JE, Aukerman SL, Fidler IJ. Evidence that the process of murine melanoma metastasis is sequential and selective and contains stochastic elements. Cancer Res. 1986;46(10):5172–8.
43. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124(2):263–6.
44. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature. 1980;284(5751):67–8.
45. Bauvois B. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. Biochim Biophys Acta. 2012;1825(1):29–36.
46. Rosenbaum E, Zahurak M, Sinibaldi V, et al. Marimastat in the treatment of patients with biochemically relapsed prostate cancer: a prospective randomized, double-blind, phase I/II trial. Clin Cancer Res. 2005;11(12):4437–43.
47. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat Rev Cancer. 2006;6(3):227–39.
48. Gross S, Marymont JH Jr. Extramedullary hematopoiesis and metastatic cancer in the spleen. Am J Clin Pathol. 1963;40:194–6.
49. Eccles SA, Alexander P. Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature. 1974;250(5468):667–9.
50. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004;25(12):677–86.
51. Mantovani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. Eur J Immunol. 2007;37(1):14–6.
52. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005;5(12):953–64.
53. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005;23(4):344–6.
54. Goerdt S, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity. 1999;10(2):137–42.
55. Gordon S. Alternative activation of macrophages. Nat Rev Immunol. 2003;3(1):23–35.
56. Anderson CF, Mosser DM. A novel phenotype for an activated macrophage: the type 2 activated macrophage. J Leukoc Biol. 2002;72(1):101–6.
57. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol. 2009;86(5):1065–73.
58. Kalinski P. Regulation of immune responses by prostaglandin E2. J Immunol. 2012;188(1):21–8.
59. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454(7203):436–44.
60. Sinha P, Clements VK, Ostrand-Rosenberg S. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. Cancer Res. 2005;65(24):11743–51.
61. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002;23(11):549–55.
62. Elgert KD, Alleva DG, Mullins DW. Tumor-induced immune dysfunction: the macrophage connection. J Leukoc Biol. 1998;64(3):275–90.
63. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med. 2001;193(6):727–40.
64. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2008;9(4):239–52.
65. Hamada I, Kato M, Yamasaki T, et al. Clinical effects of tumor-associated macrophages and dendritic cells on renal cell carcinoma. Anticancer Res. 2002;22(6C):4281–4.
66. Lewis C, Murdoch C. Macrophage responses to hypoxia: implications for tumor progression and anticancer therapies. Am J Pathol. 2005;167(3):627–35.
67. Talmadge JE, Key M, Fidler IJ. Macrophage content of metastatic and nonmetastatic rodent neoplasms. J Immunol. 1981;126(6):2245–8.
68. Steidl C, Lee T, Shah SP, et al. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med. 2010;362(10):875–85.
69. Key M, Talmadge JE, Fidler IJ. Lack of correlation between the progressive growth of spontaneous

metastases and their content of infiltrating macrophages. J Reticuloendothel Soc. 1982;32(5):387–96.
70. Evans R, Lawler EM. Macrophage content and immunogenicity of C57BL/6J and BALB/cByJ methylcholanthrene-induced sarcomas. Int J Cancer. 1980;26(6):831–5.
71. Steele RJ, Eremin O, Brown M, Hawkins RA. A high macrophage content in human breast cancer is not associated with favourable prognostic factors. Br J Surg. 1984;71(6):456–8.
72. Robinson BD, Sica GL, Liu YF, et al. Tumor microenvironment of metastasis in human breast carcinoma: a potential prognostic marker linked to hematogenous dissemination. Clin Cancer Res. 2009;15(7):2433–41.
73. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer. 2004;4(1):71–8.
74. Qian B-Z, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141(1):39–51.
75. Kang JC, Chen JS, Lee CH, Chang JJ, Shieh YS. Intratumoral macrophage counts correlate with tumor progression in colorectal cancer. J Surg Oncol. 2010;102(3):242–8.
76. Sun XF, Zhang H. Clinicopathological significance of stromal variables: angiogenesis, lymphangiogenesis, inflammatory infiltration, MMP and PINCH in colorectal carcinomas. Mol Cancer. 2006;5:43.
77. Chen JJ, Lin YC, Yao PL, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. J Clin Oncol. 2005;23(5):953–64.
78. Ryder M, Ghossein RA, Ricarte-Filho JC, Knauf JA, Fagin JA. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. Endocr Relat Cancer. 2008;15(4):1069–74.
79. Zhu XD, Zhang JB, Zhuang PY, et al. High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. J Clin Oncol. 2008;26(16):2707–16.
80. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol. 2002;196(3):254–65.
81. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 2009;27:451–83.
82. Bronte V, Wang M, Overwijk WW, et al. Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. J Immunol. 1998;161(10):5313–20.
83. Kusmartsev S, Gabrilovich DI. STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. J Immunol. 2005;174(8):4880–91.
84. Kalinski P. Dendritic cells in immunotherapy of established cancer: roles of signals 1, 2, 3 and 4. Curr Opin Investig Drugs. 2009;10(6):526–35.
85. Obermajer N, Kalinski P. Key role of the positive feedback between PGE(2) and COX2 in the biology of myeloid-derived suppressor cells. Oncoimmunology. 2012;1(5):762–4.
86. Obermajer N, Muthuswamy R, Lesnock J, Edwards RP, Kalinski P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. Blood. 2011;118(20):5498–505.
87. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. Cancer Res. 2011;71(24):7463–70.
88. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162–74.
89. Young MR, Lathers DM. Myeloid progenitor cells mediate immune suppression in patients with head and neck cancers. Int J Immunopharmacol. 1999;21(4):241–52.
90. Vasconcelos ZF, Dos Santos BM, Farache J, et al. G-CSF-treated granulocytes inhibit acute graft-versus-host disease. Blood. 2006;107(5):2192–9.
91. Ohm JE, Carbone DP. VEGF as a mediator of tumor-associated immunodeficiency. Immunol Res. 2001;23(2–3):263–72.
92. Ellis LM, Takahashi Y, Liu W, Shaheen RM. Vascular endothelial growth factor in human colon cancer: biology and therapeutic implications. Oncologist. 2000;5(Suppl 1):11–5.
93. Toi M, Kondo S, Suzuki H, et al. Quantitative analysis of vascular endothelial growth factor in primary breast cancer. Cancer. 1996;77(6):1101–6.
94. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med. 1996;2(10):1096–103.
95. Saito H, Tsujitani S, Ikeguchi M, Maeta M, Kaibara N. Relationship between the expression of vascular endothelial growth factor and the density of dendritic cells in gastric adenocarcinoma tissue. Br J Cancer. 1998;78(12):1573–7.
96. Rodriguez PC, Ernstoff MS, Hernandez C, et al. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res. 2009;69(4):1553–60.
97. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol. 2001;166(1):678–89.
98. Filipazzi P, Valenti R, Huber V, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol. 2007;25(18):2546–53.

99. Arina A, Bronte V. Myeloid-derived suppressor cell impact on endogenous and adoptively transferred T cells. Curr Opin Immunol. 2015;33:120–5.
100. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. Clin Exp Immunol. 2012;167(2):195–205.
101. Marvel D, Gabrilovich DI. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. J Clin Invest. 2015;125(9):3356–64.
102. Nagaraj S, Gabrilovich DI. Tumor escape mechanism governed by myeloid-derived suppressor cells. Cancer Res. 2008;68(8):2561–3.
103. Nagaraj S, Gabrilovich DI. Myeloid-derived suppressor cells in human cancer. Cancer J. 2010;16(4):348–53.
104. Dieu-Nosjean MC, Antoine M, Danel C, et al. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. J Clin Oncol. 2008;26(27):4410–7.
105. Elliott B, Scolyer RA, Suciu S, et al. Long-term protective effect of mature DC-LAMP+ dendritic cell accumulation in sentinel lymph nodes containing micrometastatic melanoma. Clin Cancer Res. 2007;13(13):3825–30.
106. Gallo O, Bianchi S, Giannini A, Gallina E, Libonati GA, Fini-Storchi O. Correlations between histopathological and biological findings in nasopharyngeal carcinoma and its prognostic significance. Laryngoscope. 1991;101(5):487–93.
107. Giannini A, Bianchi S, Messerini L, et al. Prognostic significance of accessory cells and lymphocytes in nasopharyngeal carcinoma. Pathol Res Pract. 1991;187(4):496–502.
108. Kashimura S, Saze Z, Terashima M, et al. CD83(+) dendritic cells and Foxp3(+) regulatory T cells in primary lesions and regional lymph nodes are inversely correlated with prognosis of gastric cancer. Gastric Cancer. 2012;15(2):144–53.
109. Kobayashi M, Suzuki K, Yashi M, Yuzawa M, Takayashiki N, Morita T. Tumor infiltrating dendritic cells predict treatment response to immmunotherapy in patients with metastatic renal cell carcinoma. Anticancer Res. 2007;27(2):1137–41.
110. Mansuet-Lupo A, Alifano M, Pecuchet N, et al. Intratumoral immune cell densities are associated with lung adenocarcinoma gene alterations. Am J Respir Crit Care Med. 2016;194(11):1403–12.
111. Reichert TE, Scheuer C, Day R, Wagner W, Whiteside TL. The number of intratumoral dendritic cells and zeta-chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma. Cancer. 2001;91(11):2136–47.
112. Tsukayama S, Omura K, Yoshida K, Tanaka Y, Watanabe G. Prognostic value of CD83-positive mature dendritic cells and their relation to vascular endothelial growth factor in advanced human gastric cancer. Oncol Rep. 2005;14(2):369–75.
113. Zeid NA, Muller HK. S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. Pathology. 1993;25(4):338–43.
114. Kalinski P, Muthuswamy R, Urban J. Dendritic cells in cancer immunotherapy: vaccines and combination immunotherapies. Expert Rev Vaccines. 2013;12(3):285–95.
115. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. Lancet Oncol. 2014;15(7):e257–67.
116. Czerniecki BJ, Roses RE, Koski GK. Development of vaccines for high-risk ductal carcinoma in situ of the breast. Cancer Res. 2007;67(14):6531–4.
117. Fong L, Engleman EG. Dendritic cells in cancer immunotherapy. Annu Rev Immunol. 2000;18:245–73.
118. Gilboa E. DC-based cancer vaccines. J Clin Invest. 2007;117(5):1195–203.
119. Kirkwood JM, Tarhini AA, Panelli MC, et al. Next generation of immunotherapy for melanoma. J Clin Oncol. 2008;26(20):3445–55.
120. Simons JW. Prostate cancer immunotherapy: beyond immunity to curability. Cancer Immunol Res. 2014;2(11):1034–43.
121. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245–52.
122. Andersen BM, Ohlfest JR. Increasing the efficacy of tumor cell vaccines by enhancing cross priming. Cancer Lett. 2012;325(2):155–64.
123. de Vries IJ, Lesterhuis WJ, Scharenborg NM, et al. Maturation of dendritic cells is a prerequisite for inducing immune responses in advanced melanoma patients. Clin Cancer Res. 2003;9(14):5091–100.
124. Adema GJ, de Vries IJ, Punt CJ, Figdor CG. Migration of dendritic cell based cancer vaccines: in vivo veritas? Curr Opin Immunol. 2005;17(2):170–4.
125. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today. 1999;20(12):561–7.
126. Kunz M, Toksoy A, Goebeler M, Engelhardt E, Brocker E, Gillitzer R. Strong expression of the lymphoattractant C-X-C chemokine Mig is associated with heavy infiltration of T cells in human malignant melanoma. J Pathol. 1999;189(4):552–8.
127. Calzascia T, Masson F, Di Berardino-Besson W, et al. Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. Immunity. 2005;22(2):175–84.
128. Almand B, Resser JR, Lindman B, et al. Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res. 2000;6(5):1755–66.
129. Della Bella S, Gennaro M, Vaccari M, et al. Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br J Cancer. 2003;89(8):1463–72.

130. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12(4):265–77.
131. Pinzon-Charry A, Maxwell T, Lopez JA. Dendritic cell dysfunction in cancer: a mechanism for immunosuppression. Immunol Cell Biol. 2005;83(5):451–61.
132. Wen H, Schaller MA, Dou Y, Hogaboam CM, Kunkel SL. Dendritic cells at the interface of innate and acquired immunity: the role for epigenetic changes. J Leukoc Biol. 2008;83(3):439–46.
133. Melief CJ. Cancer immunotherapy by dendritic cells. Immunity. 2008;29(3):372–83.
134. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. 2007;25:267–96.
135. Uchida K, Schneider S, Yochim JM, et al. Intratumoral COX-2 gene expression is a predictive factor for colorectal cancer response to fluoropyrimidine-based chemotherapy. Clin Cancer Res. 2005;11(9):3363–8.
136. Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960–4.
137. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. Cancer Res. 2007;67(5):1883–6.
138. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348(3):203–13.
139. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102(51):18538–43.
140. Fridman WH, Galon J, Pages F, Tartour E, Sautes-Fridman C, Kroemer G. Prognostic and predictive impact of intra- and peritumoral immune infiltrates. Cancer Res. 2011;71(17):5601–5.
141. Formica V, Cereda V, di Bari MG, et al. Peripheral CD45RO, PD-1, and TLR4 expression in metastatic colorectal cancer patients treated with bevacizumab, fluorouracil, and irinotecan (FOLFIRI-B). Med Oncol. 2013;30(4):743.
142. Yamada N, Oizumi S, Kikuchi E, et al. CD8+ tumor-infiltrating lymphocytes predict favorable prognosis in malignant pleural mesothelioma after resection. Cancer Immunol Immunother. 2010;59(10):1543–9.
143. Anraku M, Cunningham KS, Yun Z, et al. Impact of tumor-infiltrating T cells on survival in patients with malignant pleural mesothelioma. J Thorac Cardiovasc Surg. 2008;135(4):823–9.
144. Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med. 2005;353(25):2654–66.
145. Naito Y, Saito K, Shiiba K, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res. 1998;58(16):3491–4.
146. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942–9.
147. Chaput N, Louafi S, Bardier A, et al. Identification of CD8+CD25+Foxp3+ suppressive T cells in colorectal cancer tissue. Gut. 2009;58(4):520–9.
148. Clarke SL, Betts GJ, Plant A, et al. CD4+CD25+FOXP3+ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. PLoS One. 2006;1:e129.
149. Michel S, Benner A, Tariverdian M, et al. High density of FOXP3-positive T cells infiltrating colorectal cancers with microsatellite instability. Br J Cancer. 2008;99(11):1867–73.
150. Brigati C, Noonan DM, Albini A, Benelli R. Tumors and inflammatory infiltrates: friends or foes? Clin Exp Metastasis. 2002;19(3):247–58.
151. Fu J, Xu D, Liu Z, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology. 2007;132(7):2328–39.
152. Carreras J, Lopez-Guillermo A, Fox BC, et al. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. Blood. 2006;108(9):2957–64.
153. Tzankov A, Meier C, Hirschmann P, Went P, Pileri SA, Dirnhofer S. Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. Haematologica. 2008;93(2):193–200.
154. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2009;29(8):1093–102.
155. Siddiqui SA, Frigola X, Bonne-Annee S, et al. Tumor-infiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma. Clin Cancer Res. 2007;13(7):2075–81.
156. Gershenwald JE, Thompson W, Mansfield PF, et al. Multi-institutional melanoma lymphatic mapping experience: the prognostic value of sentinel lymph node status in 612 stage I or II melanoma patients. J Clin Oncol. 1999;17(3):976–83.
157. Elder DE, Gimotty PA, Guerry D. Cutaneous melanoma: estimating survival and recurrence risk based on histopathologic features. Dermatol Ther. 2005;18(5):369–85.
158. Badoual C, Hans S, Rodriguez J, et al. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. Clin Cancer Res. 2006;12(2):465–72.
159. Bromwich EJ, McArdle PA, Canna K, et al. The relationship between T-lymphocyte infiltration, stage, tumour grade and survival in patients undergoing

curative surgery for renal cell cancer. Br J Cancer. 2003;89(10):1906–8.
160. Hennequin A, Derangere V, Boidot R, et al. Tumor infiltration by Tbet+ effector T cells and CD20+ B cells is associated with survival in gastric cancer patients. Oncoimmunology. 2016;5(2):e1054598.
161. Xu B, Chen L, Li J, et al. Prognostic value of tumor infiltrating NK cells and macrophages in stage II+III esophageal cancer patients. Oncotarget. 2016;7(46):74904–16.
162. Donadon M, Hudspeth K, Cimino M, et al. Increased infiltration of natural killer and T cells in colorectal liver metastases improves patient overall survival. J Gastrointest Surg. 2017;21(8):1226–36.
163. Introna M, Allavena P, Biondi A, Colombo N, Villa A, Mantovani A. Defective natural killer activity within human ovarian tumors: low numbers of morphologically defined effectors present in situ. J Natl Cancer Inst. 1983;70(1):21–6.
164. Li T, Zhang Q, Jiang Y, et al. Gastric cancer cells inhibit natural killer cell proliferation and induce apoptosis via prostaglandin E2. Oncoimmunology. 2016;5(2):e1069936.
165. Sznurkowski JJ, Zawrocki A, Biernat W. Subtypes of cytotoxic lymphocytes and natural killer cells infiltrating cancer nests correlate with prognosis in patients with vulvar squamous cell carcinoma. Cancer Immunol Immunother. 2014;63(3):297–303.
166. Granzin M, Wagner J, Kohl U, Cerwenka A, Huppert V, Ullrich E. Shaping of natural killer cell antitumor activity by ex vivo cultivation. Front Immunol. 2017;8:458.
167. Introna M, Mantovani A. Natural killer cells in human solid tumors. Cancer Metastasis Rev. 1983;2(4):337–50.
168. Pross HF, Lotzova E. Role of natural killer cells in cancer. Nat Immun. 1993;12(4–5):279–92.
169. Tatsumi T, Takehara T. Impact of natural killer cells on chronic hepatitis C and hepatocellular carcinoma. Hepatol Res. 2016;46(5):416–22.
170. Whiteside TL, Vujanovic NL, Herberman RB. Natural killer cells and tumor therapy. Curr Top Microbiol Immunol. 1998;230:221–44.
171. Yang Y, Cao JZ, Lan SM, et al. Association of improved locoregional control with prolonged survival in early-stage extranodal nasal-type natural killer/T-cell lymphoma. JAMA Oncol. 2017;3(1):83–91.
172. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, Munz C. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med. 2002;195(3):343–51.
173. Piccioli D, Sbrana S, Melandri E, Valiante NM. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med. 2002;195(3):335–41.
174. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med. 2002;195(3):327–33.
175. Mailliard RB, Son YI, Redlinger R, et al. Dendritic cells mediate NK cell help for Th1 and CTL responses: two-signal requirement for the induction of NK cell helper function. J Immunol. 2003;171(5):2366–73.
176. Kelly JM, Darcy PK, Markby JL, et al. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. Nat Immunol. 2002;3(1):83–90.
177. Strbo N, Oizumi S, Sotosek-Tokmadzic V, Podack ER. Perforin is required for innate and adaptive immunity induced by heat shock protein gp96. Immunity. 2003;18(3):381–90.
178. Mocikat R, Braumuller H, Gumy A, et al. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity. 2003;19(4):561–9.
179. Westwood JA, Kelly JM, Tanner JE, Kershaw MH, Smyth MJ, Hayakawa Y. Cutting edge: novel priming of tumor-specific immunity by NKG2D-triggered NK cell-mediated tumor rejection and Th1-independent CD4+ T cell pathway. J Immunol. 2004;172(2):757–61.
180. Cudkowicz G, Hochman PS. Do natural killer cells engage in regulated reactions against self to ensure homeostasis? Immunol Rev. 1979;44:13–41.
181. Tse BW, Collins A, Oehler MK, Zippelius A, Heinzelmann-Schwarz VA. Antibody-based immunotherapy for ovarian cancer: where are we at? Ann Oncol. 2014;25(2):322–31.
182. Hamanishi J, Mandai M, Ikeda T, et al. Safety and antitumor activity of anti-PD-1 antibody, nivolumab, in patients with platinum-resistant ovarian cancer. J Clin Oncol. 2015;33(34):4015–22.
183. Speetjens FM, Zeestraten EC, Kuppen PJ, Melief CJ, van der Burg SH. Colorectal cancer vaccines in clinical trials. Expert Rev Vaccines. 2011;10(6):899–921.
184. de Weger VA, Turksma AW, Voorham QJ, et al. Clinical effects of adjuvant active specific immunotherapy differ between patients with microsatellite-stable and microsatellite-instable colon cancer. Clin Cancer Res. 2012;18(3):882–9.
185. Koido S, Ohkusa T, Homma S, et al. Immunotherapy for colorectal cancer. World J Gastroenterol. 2013;19(46):8531–42.
186. Ji RR, Chasalow SD, Wang L, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol Immunother. 2012;61(7):1019–31.
187. Gatalica Z, Snyder C, Maney T, et al. Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. Cancer Epidemiol Biomark Prev. 2014;23(12):2965–70.
188. Phillips SM, Banerjea A, Feakins R, Li SR, Bustin SA, Dorudi S. Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite

instability are activated and cytotoxic. Br J Surg. 2004;91(4):469–75.
189. Demaria O, De Gassart A, Coso S, et al. STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity. Proc Natl Acad Sci U S A. 2015;112(50):15408–13.
190. Vasaturo A, Halilovic A, Bol KF, et al. T-cell landscape in a primary melanoma predicts the survival of patients with metastatic disease after their treatment with dendritic cell vaccines. Cancer Res. 2016;76(12):3496–506.
191. Fong L, Carroll P, Weinberg V, et al. Activated lymphocyte recruitment into the tumor microenvironment following preoperative sipuleucel-T for localized prostate cancer. J Natl Cancer Inst. 2014;106(11):1–9.
192. Sharabi AB, Lim M, DeWeese TL, Drake CG. Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy. Lancet Oncol. 2015;16(13):e498–509.
193. Vanpouille-Box C, Pilones KA, Wennerberg E, Formenti SC, Demaria S. In situ vaccination by radiotherapy to improve responses to anti-CTLA-4 treatment. Vaccine. 2015;33(51):7415–22.
194. Nobler MP. The abscopal effect in malignant lymphoma and its relationship to lymphocyte circulation. Radiology. 1969;93(2):410–2.
195. Gichangi P, Bwayo J, Estambale B, et al. HIV impact on acute morbidity and pelvic tumor control following radiotherapy for cervical cancer. Gynecol Oncol. 2006;100(2):405–11.
196. Kotoula V, Chatzopoulos K, Lakis S, et al. Tumors with high-density tumor infiltrating lymphocytes constitute a favorable entity in breast cancer: a pooled analysis of four prospective adjuvant trials. Oncotarget. 2016;7(4):5074–87.
197. Rao N, Qiu J, Wu J, et al. Significance of tumor-infiltrating lymphocytes and the expression of topoisomerase IIalpha in the prediction of the clinical outcome of patients with triple-negative breast cancer after taxane-anthracycline-based neoadjuvant chemotherapy. Chemotherapy. 2017;62(4):246–55.
198. Wang K, Xu J, Zhang T, Xue D. Tumor-infiltrating lymphocytes in breast cancer predict the response to chemotherapy and survival outcome: a meta-analysis. Oncotarget. 2016;7(28):44288–98.
199. Andre F, Dieci MV, Dubsky P, et al. Molecular pathways: involvement of immune pathways in the therapeutic response and outcome in breast cancer. Clin Cancer Res. 2013;19(1):28–33.
200. Vacchelli E, Semeraro M, Enot DP, et al. Negative prognostic impact of regulatory T cell infiltration in surgically resected esophageal cancer post-radiochemotherapy. Oncotarget. 2015;6(25):20840–50.
201. Frey B, Hehlgans S, Rodel F, Gaipl US. Modulation of inflammation by low and high doses of ionizing radiation: implications for benign and malign diseases. Cancer Lett. 2015;368(2):230–7.
202. Martin D, Rodel F, Balermpas P, Rodel C, Fokas E. The immune microenvironment and HPV in anal cancer: rationale to complement chemoradiation with immunotherapy. Biochim Biophys Acta. 2017;1868(1):221–30.
203. Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007;13(1):54–61.
204. Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13(9):1050–9.
205. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. Cell Death Differ. 2014;21(1):15–25.
206. Musha H, Ohtani H, Mizoi T, et al. Selective infiltration of CCR5(+)CXCR3(+) T lymphocytes in human colorectal carcinoma. Int J Cancer. 2005;116(6):949–56.
207. Ohtani H, Jin Z, Takegawa S, Nakayama T, Yoshie O. Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. J Pathol. 2009;217(1):21–31.
208. Muthuswamy R, Berk E, Junecko BF, et al. NF-kappaB hyperactivation in tumor tissues allows tumor-selective reprogramming of the chemokine microenvironment to enhance the recruitment of cytolytic T effector cells. Cancer Res. 2012;72(15):3735–43.
209. Watchmaker PB, Berk E, Muthuswamy R, et al. Independent regulation of chemokine responsiveness and cytolytic function versus CD8+ T cell expansion by dendritic cells. J Immunol. 2010;184(2):591–7.
210. Muthuswamy R, Urban J, Lee JJ, Reinhart TA, Bartlett D, Kalinski P. Ability of mature dendritic cells to interact with regulatory T cells is imprinted during maturation. Cancer Res. 2008;68(14):5972–8.
211. Fujita M, Zhu X, Ueda R, et al. Effective immunotherapy against murine gliomas using type 1 polarizing dendritic cells—significant roles of CXCL10. Cancer Res. 2009;69(4):1587–95.
212. Zhu X, Fallert-Junecko BA, Fujita M, et al. Poly-ICLC promotes the infiltration of effector T cells into intracranial gliomas via induction of CXCL10 in IFN-alpha and IFN-gamma dependent manners. Cancer Immunol Immunother. 2010;59(9):1401–9.
213. Okada H. Brain tumor immunotherapy with type-1 polarizing strategies. Ann N Y Acad Sci. 2009;1174:18–23.
214. Okada H, Kalinski P, Ueda R, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients

with recurrent malignant glioma. J Clin Oncol. 2011;29(3):330–6.
215. Muthuswamy R, Wang L, Pitteroff J, Gingrich JR, Kalinski P. Combination of IFNalpha and poly-I:C reprograms bladder cancer microenvironment for enhanced CTL attraction. J Immunother Cancer. 2015;3:6.
216. Muthuswamy R, Corman JM, Dahl K, Chatta GS, Kalinski P. Functional reprogramming of human prostate cancer to promote local attraction of effector CD8(+) T cells. Prostate. 2016;76(12):1095–105.
217. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. CA Cancer J Clin. 2017;67(1):7–30.
218. Wong JL, Obermajer N, Odunsi K, Edwards RP, Kalinski P. Synergistic COX2 induction by IFNgamma and TNFalpha self-limits type-1 immunity in the human tumor microenvironment. Cancer Immunol Res. 2016;4(4):303–11.

2 Cancer Immunotherapy Targets Based on Understanding the T Cell-Inflamed Versus Non-T Cell-Inflamed Tumor Microenvironment

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2.1 T Cell-Inflamed Versus Non-T Cell-Inflamed Tumor Microenvironment

Interrogation of the tumor microenvironment in metastatic melanoma was initially pursued to test the hypothesis that resistance mechanisms downstream from T cell priming in response to melanoma vaccines might dominate and enable tumor escape [1, 2]. Baseline biopsies of melanoma metastases were obtained and evaluated by gene expression profiling, to correlate with clinical outcome from vaccination. Two major subsets of tumor microenvironment could be identified that were largely characterized by the presence or absence of a transcriptional profile indicative of a pre-existing T cell infiltrate (Fig. 2.1). The T cell-inflamed subset of tumors was dominated by T cell markers and chemokines that likely mediate effector T cell recruitment [3–5]. Expression of the chemokines CCL2, -3, -4, -5 and CXCL9, -10 was observed to correlate with T cell presence, and each of these chemokines was sufficient to recruit $CD8^+$ effector T cells in vitro [3]. Recently, CXCR3-binding chemokines (such as CXCL9 and CXCL10) were found to be critical and necessary for trafficking of activated $CD8^+$ T cells into tumor sites [6]. Immunohistochemistry confirmed the presence of $CD8^+$ T cells, macrophages, as well as some B cells and plasma cells in the T cell-inflamed lesions [3]. This subset of tumors was remarkably distinct from the non-T cell inflamed subset, and the biology suggests that spontaneous T cell priming and recruitment into the tumor microenvironment had occurred in those patients, even prior to any therapy. Interestingly, reflecting back onto the original goal of this analysis, the clinical responders to vaccination were seen among patients with the T cell-inflamed phenotype [4]. Thus, it appears as though tumors capable of supporting recruitment of activated $CD8^+$ T cells are those that stand to benefit from interventions that increase the frequency of tumor antigen-specific T cells in the circulation, such as vaccination.

The T cell-inflamed subset of melanoma metastases is remarkably similar to the phenotype described in early stage colon cancer and other tumors in which activated T cells have been

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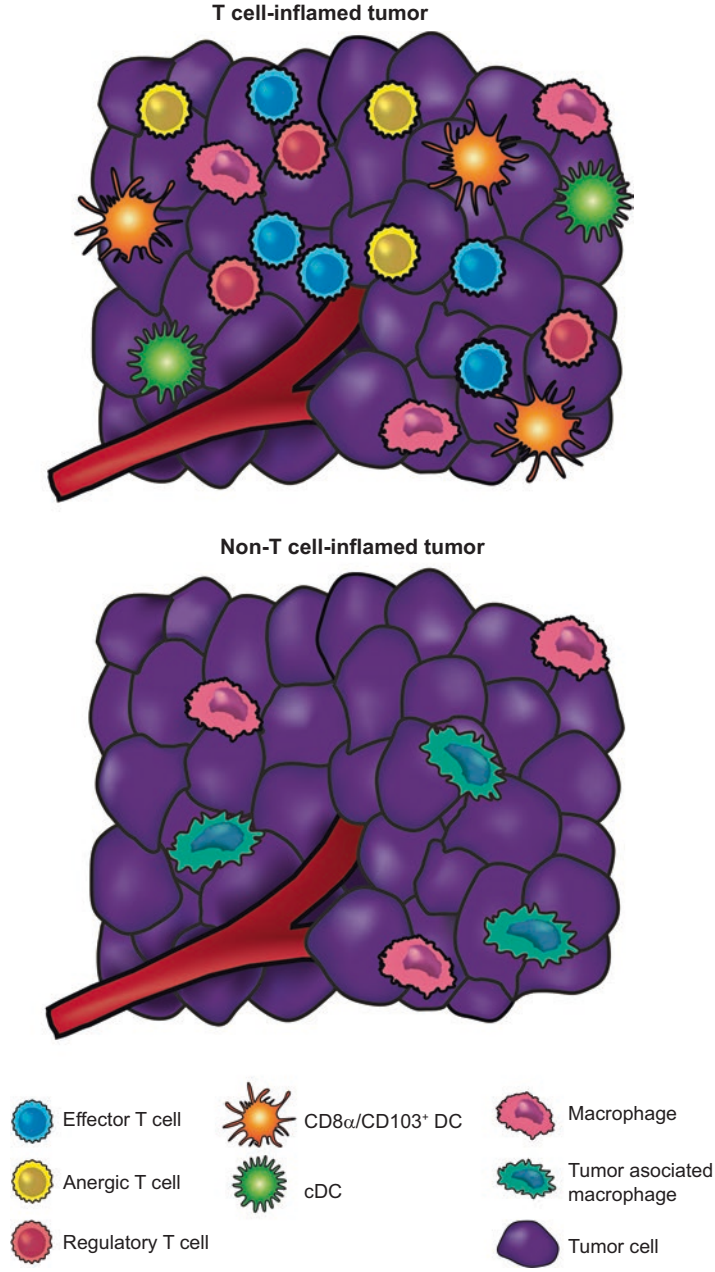

Fig. 2.1 Immunologic composition of the T cell-inflamed versus non-T cell-inflamed tumor microenvironments. The T cell-inflamed tumors contain variable numbers of $CD8^+$ T cells and CD8α/CD103-lineage DCs, but also possess the highest density of $FoxP3^+$ Tregs. In addition, many of the conventional T cells have a dysfunctional anergic phenotype. In contrast, the non-T cell-inflamed tumors lack these elements but still contain blood vessels, fibroblasts, and macrophages that help support tumor growth. Recruitment of $CD8^+$ effector cells is largely dependent on the chemokines CXCL9 and CXCL10, which engage the receptor CXCR3. Treg recruitment is primarily driven by CCL22, which is in part produced by activated $CD8^+$ T cells

associated with favorable prognosis [7–9]. In several small studies of $HLA\text{-}A2^+$ patients, $CD8^+$ T cells specific for melanoma differentiation antigens such as Melan-A were identified from tumor sites using peptide-HLA-A2 tetramer analysis [10–12]. Therefore, at least a subset of T cells specific for tumor antigens is present among these infiltrates. The fact that the starting point for adoptive T cell approaches utilizing tumor-infiltrating lymphocytes (TIL), which have demonstrated approximately a 50% response rate in metastatic melanoma [13], is T cells harvested from the tumor, also supports the notion that tumor-specific T cells are present. However, the function of these T cells in situ is impaired. Various degrees of dysfunction of tumor

antigen-specific T cells have been described upon analysis directly ex vivo [10–12]. Together, these results suggest that the reason for tumor progression despite the presence of specific adaptive immunity in this subset of patients is likely secondary to immune suppressive mechanisms acting at the level of the tumor microenvironment. Interestingly, in some cases the presence of memory virus-specific $CD8^+$ T cells also has been observed in T cell-inflamed melanomas. However, their function seems to be intact [10, 14], and these probably represent non-specifically recruited activated T cells migrating along chemokine gradients but not participating in tumor recognition. These observations suggest that a component of T cell dysfunction in the tumor microenvironment is antigen-specific and restricted to tumor-reactive T cells.

In contrast to the rich set of immune genes expressed in the T cell-inflamed tumor microenvironment phenotype, the non-T cell-inflamed tumors lacked this broad signature. In particular, there was a lack of T cell markers and of chemokines that can mediate T cell recruitment [3]. These tumors still contain macrophages and vascular endothelial cells, and work from others has indicated the presence of fibroblasts and extracellular matrix, and in some cases immature dendritic cells [15–19]. It is not yet certain whether tumors that lack spontaneous T cell infiltration are defective only at the level of initial T cell priming against tumor antigens or whether there are additional mechanisms that exclude activated T cells from migrating into the tumor microenvironment, but is seems plausible that both processes may be operational. The idea that both the priming and the effector phases of the anti-tumor immune response are defective in non-T cell-inflamed tumors is supported by recent data in genetically engineered mouse models. Tumors with poor T cell infiltration appear to have higher expression of several angiogenic factors [3, 19]. Vascular endothelial cells from T cell-infiltrated versus T cell-non-infiltrated tumors have been reported to have distinct gene expression profiles, and the endothelin B receptor has been identified as a vascular target in an ovarian cancer context [19]. Thus, effector T cell trafficking into the tumor microenvironment is complex, and depends on adhesion molecules and homing receptors expressed on vascular endothelial cells in concert with chemokines produced by tumor cells and/or stromal cells within the tumor microenvironment. This process is likely necessary for clinical response to immunotherapies in most instances.

It has been argued that non-T cell-inflamed tumors might lack neoantigens for T cell recognition and therefore might not be immunogenic because they are not antigenic. A recent report has suggested that patients who failed to derive clinical benefit from the anti-CTLA-4 mAb ipilimumab might have fewer mutations and lack the antigens present in the tumors of responding patients [20]. However, we have recently analyzed exome sequencing versus germline data from the metastatic melanoma samples that are among The Cancer Genome Atlas data set. RNAseq data were used to categorize patients as having a T cell-inflamed versus a non-T cell-inflamed tumor microenvironment. The frequency of non-synonymous mutations, expression of cancer-testis antigen genes, and the expression of melanoma differentiation antigens were enumerated between these groups. No differences were observed with any of these parameters between the two cohorts of patients [21]. These data indicate that lack of antigen expression is unlikely to explain the non-T cell-inflamed tumor microenvironment phenotype in melanoma. These data are encouraging, as they suggest that strategies to overcome the barrier of T cell migration into tumor sites might ultimately enable immunotherapy efficacy in non-T cell-inflamed tumors.

2.2 Negative Regulatory Pathways Impeding Immune Efficacy in T Cell-Inflamed Tumors

Because of the presence of dysfunctional T cells in the same microenvironment as antigen-expressing tumors cells, the T cell-inflamed subset of tumors was probed for candidate

immune-inhibitory mechanisms that might contribute to T cell dysfunction in situ. Gene expression profiling data revealed the presence of transcripts encoding indoleamine-2,3-dioxygenase (IDO) in these tumors, a molecule that had previously been demonstrated to contribute to peripheral tolerance [22]. Interrogation for additional candidates revealed that these tumors also expressed PD-L1 and Foxp3 transcripts [23, 24]. Quantitative analysis of individual tumors revealed that the expression level of each of these three transcripts was significantly correlated, and that the degree of expression was also associated with T cell markers. Immunohistochemistry confirmed that PD-L1 and IDO protein expression, and also nuclear $Foxp3^+CD4^+$ cells, were found within T cell-inflamed tumors in the same region as $CD8^+$ T cells (Fig. 2.2). However, non-T cell-inflamed melanomas generally lacked these factors. These observations suggested that these immune suppressive mechanisms might not be a property of the tumor cells themselves but rather immune-intrinsic negative feedback processes that follow the recruitment of activated $CD8^+$ T cells. Indeed, mouse mechanistic studies confirmed that $CD8^+$ T cells were required for the upregulation of all of these three factors within the tumor microenvironment. For PD-L1 and IDO induction, the requisite factor produced by the $CD8^+$ T cells was IFN-γ. For $FoxP3^+$ Tregs, production of the chemokine CCL22 was identified, which mediated Treg recruitment into tumor sites [24]. Using laser capture microdissection, a correlation between IFN-γ production by TILs and local PD-L1 expression also was observed by Taube and colleagues in human tumors [25], supporting the notion that infiltrating T cells become activated by specific antigen and consequently produce IFN-γ and upregulate PD-L1 expression. The fact that these immune evasion mechanisms are part of the host response implies that targeting these pathways therapeutically should have an increased likelihood of efficacy because they are less dependent on tumor cell properties and the associated mutability that can frequently lead to therapeutic resistance.

Preclinical studies targeting CTLA-4, PD-L1, and IDO have indicated that the therapeutic effect is associated with re-activation of $CD8^+$ T cells directly within the tumor microenvironment [26]. The major biologic correlate that is restored with blockade of these pathways is the ability of tumor-infiltrating $CD8^+$ T cells to produce IL-2 and to proliferate when analyzed ex vivo. In order to test whether the therapeutic effect required influx of new T cells at all, the drug FTY720 was utilized, which prevents new T cell egress from lymph nodes. In fact, both restoration of IL-2 production and proliferation of TIL as well as tumor regression were preserved despite FTY720 administration [26], arguing that the immediate functional effects of checkpoint blockade can all be explained by re-activation of T cells already present within the tumor microenvironment. Consistent with these data, clinical response with anti-PD-1 mAb in metastatic melanoma was found to occur predominantly in patients with pre-existing T cell infiltrates in the region of PD-L1 upregulation [25, 27, 28]. Following anti-PD-1 administration, these $CD8^+$ T cells seemed to proliferate and expand, as indicated by Ki67 expression, and to penetrate deeply throughout the tumor [28]. Thus, the preponderance of clinical response with active immunotherapies also is likely mediated through restored function of pre-existing TIL.

In addition to the presence of PD-L1, IDO, and Tregs that likely mediate extrinsic suppression, a T cell-intrinsic mechanism is also likely contributing to tumor escape in the T cell-inflamed cancers. This phenomenon is similar to T cell anergy that has been characterized extensively using in vitro model systems, an observation which has provided a tool for identifying additional immune regulatory targets on dysfunctional T cells within the tumor microenvironment. In vitro experiments using $CD4^+$ Th1 clones as a model system have identified the transcription factor EGR2 as a critical mediator of T cell dysfunction [29]. EGR2 is induced following TCR ligation alone, and leads to upregulation of the lipid phosphatase diacylglycerol kinase, which in turn inhibits TCR-mediated Ras pathway activation [30]. Conditional EGR2-knockout T cells have shown improved anti-tumor activity in vivo [29], arguing for a functional relevance of this pathway in anti-tumor immunity. With this

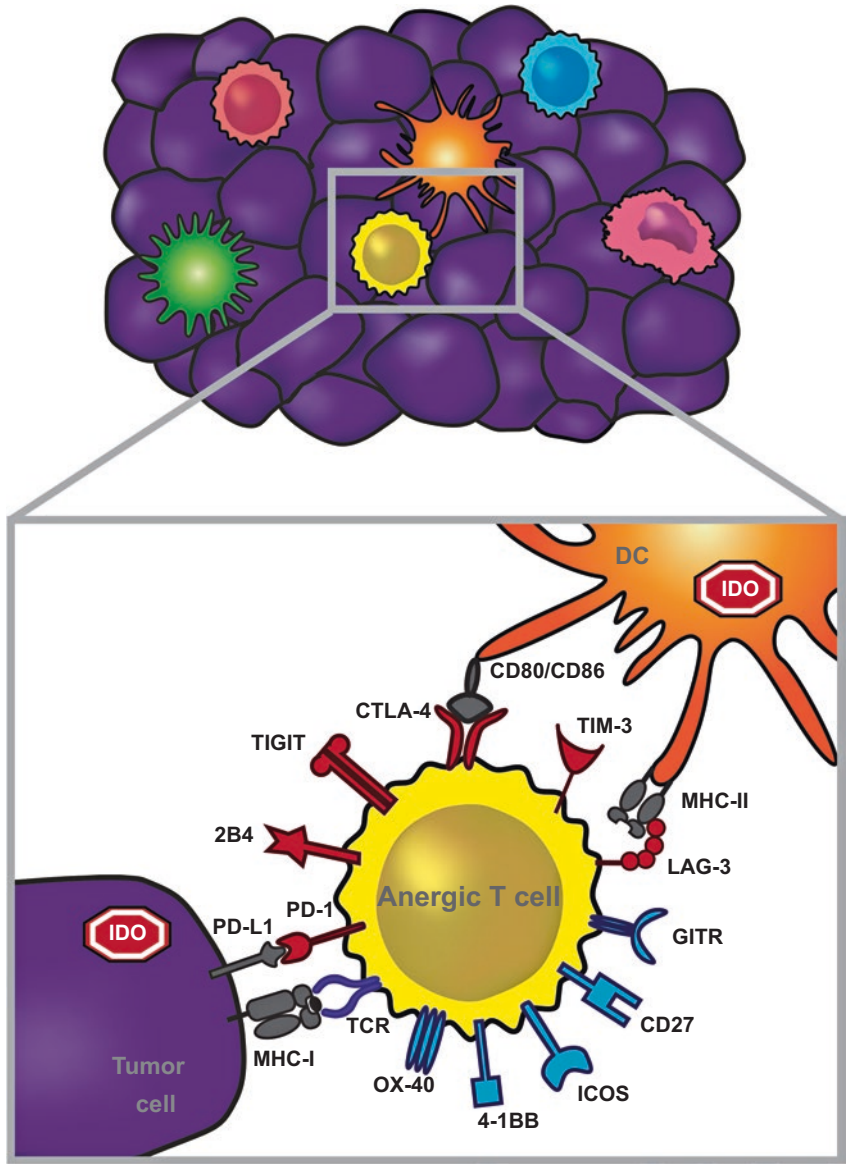

Fig. 2.2 Immunotherapeutic targets that are preferentially relevant for the T cell-inflamed tumor microenvironment subset. T cell-inflamed tumors contain activated $CD8^+$ T cells but also express IDO and PD-L1, which inhibit T cell function. The dysfunctional/anergic T cells in the tumor microenvironment also can express an array of additional inhibitory receptors, including LAG-3, Tigit, Tim3, and 2B4. But in addition, these T cells also paradoxically express costimulatory receptors, including 4-1BB, Ox40, ICOS, GITR, and CD27. Both blockade of inhibitory receptors and ligation of costimulatory receptors are being developed as cancer therapeutics. Additional candidate immune suppressive factors not shown here that have yet to be effectively targeted clinically include TGF-β, IL-10, iNOS, and PGE_2

functional importance in mind, experiments were conducted to identify the full spectrum of EGR2 target genes in anergic T cells. Gene expression profiling of wild-type versus EGR2-deleted T cells was performed, to identify EGR2-dependent genes. In parallel, a genome-wide ChIPseq study was performed, to identify genes directly bound by EGR2. Merging these two datasets revealed

50 genes that characterized the EGR2 transcriptome [31]. Interestingly, several of these target genes encode surface receptors that allow phenotyping using flow cytometry, including LAG-3 and 4-1BB. LAG-3 is an inhibitory receptor with homology to CD4 that recognizes at least class II MHC as a ligand [32]. 4-1BB is a costimulatory receptor of the TNFR superfamily that engages the NF-κB pathway [33]. Returning to the tumor microenvironment, flow cytometric analysis revealed that a major population of $CD8^+$ TIL co-expressed LAG-3 and 4-1BB. All of these cells were also PD-1-positive. By cell sorting and stimulation in vivo, it was found that the LAG-3^+4-$1BB^+$ subset was the most dysfunctional as reflected by IL-2 production and proliferation. The majority of tumor-specific T cells were found to fall into this subset. Thus, these likely represent important markers for identifying the dysfunctional tumor antigen-specific T cell subset within the tumor microenvironment (Williams and Gajewski, manuscript in preparation). Administration of a blocking mAb against LAG-3 along with an agonistic Ab against 4-1BB showed profound anti-tumor activity in vivo. Anti-4-1BB also synergized therapeutically with either anti-CTLA-4 or anti-PD-L1 mAbs (Horton and Gajewski, unpublished data). Interestingly, all of these combination therapies also depend upon re-activation of T cells already present within the tumor microenvironment. These data suggest that thorough phenotypic analysis of dysfunctional TIL should reveal the total array of immune regulatory receptors amenable to in vivo therapeutic targeting.

Because of the presence of multiple immune regulatory factors in the same T cell-inflamed tumor microenvironment, and based on preclinical evidence for synergistic efficacy, multiple phase I/II trials are underway to evaluate key combinations. These include an IDO inhibitor combined with either anti-CTLA-4 or anti-PD-1 mAbs, anti-LAG-3 + anti-PD-1, and anti-4-1BB mAb in various combinations. The potential for combination immunotherapy to have superior efficacy is supported by recent data using anti-CTLA-4 + anti-PD-1, which revealed a higher response rate than either agent alone in metastatic melanoma, albeit with a higher rate of adverse events [34]. Over time, additional combinations that have comparable efficacy and perhaps decreased toxicity will hopefully be identified, both for melanoma and for other cancer types showing a fraction of patients characterized by the T cell-inflamed tumor microenvironment.

2.3 Innate Immune Mechanisms Bridging to Spontaneous Anti-Tumor T Cell Responses

Expanding the efficacy of immunotherapies will require a better understanding of the mechanisms mediating the non-T cell-inflamed tumor microenvironment. As a first approach, possible innate immune pathways involved in generating the T cell-inflamed tumor microenvironment when it does occur have been pursued. In general, in order for adaptive T cell responses to be induced against an antigen, dendritic cells (DCs) or other involved antigen-presenting cells (APCs) need to be activated themselves for productive adaptive immunity. In the context of infectious agents, this is typically through stimulation of Toll-like receptors (TLRs) by pathogen-associated molecular patterns (PAMPs), such as endotoxin that is recognized by TLR4 [35]. However, it had not been clear what factors might provide innate immune signaling in the context of sterile tumors in which infectious agents are not implicated. Melanoma gene expression profile data were interrogated for evidence of innate immune pathway activation. A major clue came from the observation that tumors that contained a T cell infiltrate also showed evidence for a transcriptional signature known to be induced by type I IFNs [3, 36]. Armed with that information, mouse mechanistic experiments were carried out to determine whether type I IFN signaling on host cells was necessary for spontaneous priming of $CD8^+$ T cells against tumor-associated antigens. In fact, type I $IFNR^{-/-}$ mice, or mice deficient in the downstream transcription factor Stat1, showed markedly reduced T cell responses against tumor-associated antigens in multiple transplantable tumor models [36]. The requirement for type I IFN signaling was mapped to the level of APCs, and indeed specific deletion of the

type I IFNR in DCs was sufficient to reproduce this defect. Mixed bone marrow chimera experiments demonstrated that the specific subset of DCs involved in this effect was the Batf3-driven lineage that expresses CD8α or CD103 [36–38]. In addition, IFN-β production was found to be induced in DCs upon implantation of a tumor in vivo. These data suggest that early innate immune recognition of cancer cells in vivo involved activation of a pathway that results in IFN-β production, which in turn was necessary for complete DC activation and $CD8^+$ T cell priming to give rise to the T cell-infiltrated tumor microenvironment phenotype [39].

These observations led to the next level question of identifying the receptor system and putative ligands that induce IFN-β production by host DCs in the context of a growing tumor in vivo. By using a series of knockout mice specifically lacking TLRs, the adaptors MyD88 or Trif, the intracellular RNA sensing pathway involving MAVS, or the extracellular ATP sensing receptor P2X7R, most of the innate immune pathways that have been implicated in various infectious disease models to promote type I IFN production were ruled out as being essential. By process of elimination, this pointed to the STING pathway as an important candidate. STING is an adapter that is activated by cyclic dinucleotides generated by cGAS, which in turn is directly activated by cytosolic DNA [40–42]. This pathway has been implicated in the sensing of DNA viruses, but also in selected autoimmune models [43, 44]. Moreover, activating mutations of STING have been identified in human patients with a vasculitis/pulmonary inflammation syndrome characterized by increased type I IFN production [45]. Indeed, the use of $STING^{-/-}$ mice revealed that spontaneous T cell priming against tumor antigens was markedly reduced in vivo, and rejection of immunogenic tumors was ablated [46]. Moreover, tumor-derived DNA was detected within the cytosol of a major population of tumor-infiltrating DCs, which was associated with STING pathway activation and IFN-β production. Therefore, the host STING pathway appears to be a major innate immune sensing pathway that is activated in the tumor context to drive DC activation and subsequent T cell priming against tumor-associated antigens in vivo. Several additional tumor model systems have confirmed a role for the STING pathway in antitumor immunity in vivo [47–49].

The realization of the importance of this particular innate immune pathway in the cancer context is generating new therapeutic strategies that might be utilized to activate or mimic the cGAS/STING axis for promoting immune-mediated tumor control, particularly in the non-inflamed tumor subset. Recent studies have pursued intratumoral injection of STING agonists. 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a flavonoid compound that was previously shown to have anti-tumor activity in mouse models [50]. This drug ultimately failed in humans when combined with chemotherapy in a Phase 3 trial in non-small cell lung cancer [51]. Structure-function studies demonstrated that DMXAA is a direct ligand for mouse STING [52, 53]. However, sequence differences in human STING rendered it unable to bind DMXAA, therefore abrogating its activity in human cells and explaining the lack of clinical activity of this compound. Recent evidence has confirmed that DMXAA is a strong agonist of the mouse STING pathway in vitro and in vivo. Intratumoral injection of DMXAA markedly augmented endogenous priming of tumor antigen-specific $CD8^+$ T cells and caused complete tumor elimination. Rejection was completely dependent on host STING, and most of the effect depended on T cells and type I IFNs. New STING agonists that stimulate all known human STING polymorphic variants have been developed that also bind mouse STING and showed similarly potent efficacy in preclinical tumor models [54]. These agents will be attractive for clinical translation as a potential strategy to initiate de novo inflammation, DC activation, and T cell priming especially in non-T cell-inflamed tumors.

An alternative approach for promoting appropriate innate immune activation in the tumor microenvironment is through targeted radiation. Directed radiation to the tumor site also appears to induce type I IFN production, augment specific T cell priming, and support T cell-mediated

tumor control in vivo [55]. Based on the observation that the STING pathway was critical for spontaneous innate immune sensing of tumors in vivo, it was of interest to determine whether the STING pathway was also important for the therapeutic effect of radiation. Indeed, recent data have revealed that the therapeutic efficacy of radiation was largely ablated in STING$^{-/-}$ hosts. This defect was associated with blunted type I IFN induction and markedly reduced T cell priming. In contrast, no defect in the therapeutic effect of radiation was observed using mice lacking specific TLR signaling pathways [56]. Thus, radiation may facilitate the proper acquisition of tumor-derived DNA by host DCs in the tumor microenvironment, thereby leading to improved T cell priming as well as coordination of the effector phase of the anti-tumor immune response.

2.4 Reverse-Translational Research to Identify New Therapeutic Angles for Non-T Cell-Inflamed Tumors

An additional major strategy for identifying molecular mechanisms that control the presence or absence of a T cell-inflamed tumor microenvironment is to interrogate categories of genomic heterogeneity directly from patients. By clustering patients has having a T cell-inflamed versus non-T cell-inflamed tumor microenvironment using gene expression profiling as a defined phenotype, reproducible genetic or genomic patterns can be identified as a correlation. Because the T cell-inflamed tumor microenvironment is also a good predictive biomarker for response to immunotherapies such as anti-PD-1, this question can also be viewed as a pharmacogenomic analysis for mechanisms of primary resistance to these agents. Based on these notions, three potential sources of inter-patient heterogeneity could be envisioned that have the potential to influence whether a tumor in a given subject might contain or lack spontaneous T cell infiltration. These categories are differences in accessory oncogene pathways within the tumor cells based on somatic mutational events, germline polymorphisms in immunoregulatory genes that could set thresholds for immune cell activation, or environmental differences that could have global effects on immune functionality. Regarding the latter category, the major phenomenon that has recently garnered interest is the impact of the intestinal microbiome on systemic immune responses in the host. Importantly, each of these parameters is measurable in individual patients. Somatic heterogeneity in tumors can be assessed through exome sequencing and pathway analysis, germline heterogeneity in the host can be evaluated using SNP arrays on peripheral blood cells, and patterns of differences in the intestinal microbiome can be identified through 16S ribosomal RNA sequencing on stool samples. Associations between individual sequences and either the T cell-inflamed tumor microenvironment or clinical outcome to immunotherapy can then be investigated. These analyses require prospective tissue collection from patients embarking on immunotherapy treatments—fresh tumor biopsies, peripheral blood specimens, and stool samples. Broad-based tissue banking from cancer patients participating in immunotherapy clinical trials should be supported and represents a rich discovery opportunity to identify mechanisms of immunotherapy success versus resistance.

Such an analysis has been initiated in metastatic melanoma patients, focusing first on somatic differences at the level of the tumor itself. Using a series of 266 melanoma metastases, tumors were categorized based on the presence or absence of the gene signature indicative of the T cell-inflamed tumor microenvironment [3]. These same tumors were also subjected to exome sequencing, as well as pathway analysis using the Ingenuity platform based on gene expression patterns in the non-T cell-inflamed subset. Strikingly, these data indicated that nearly one-half of the non-T cell-inflamed tumors showed evidence of activation of the Wnt/β-catenin pathway. Some tumors had activating mutations in β-catenin itself, some had inactivating mutations in negative regulators of β-catenin, and some showed over-expression of Wnt7B or Frizzled 3. Using a genetically engineered mouse model in which melanomas were

induced that either did or did not include conditionally expressed active β-catenin, mechanistic experiments confirmed that tumor cell-intrinsic β-catenin activation was sufficient to exclude T cell infiltrates in vivo. The molecular mechanism was narrowed down to a loss of chemokines that mediate recruitment of Batf3-lineage DCs into the tumor microenvironment, leading to defective T cell priming. The therapeutic activity of anti-CTLA-4 + anti-PD-L1 mAb normally seen was lost when tumors additionally expressed active β-catenin [57]. Thus, the Wnt/β-catenin pathway is the first identified tumor-intrinsic oncogene pathway to result in immune exclusion and resistance to immunotherapy. These data suggest that pharmacologic strategies to block β-catenin activity might not only be directly therapeutic against tumor cells, but additionally might support a positive interaction with host immunity.

Ongoing work also investigates germline polymorphisms as they relate to the presence or absence of the T cell-inflamed tumor microenvironment. There is precedent for germline genetic differences influencing response to immunotherapy. A SNP in the gene encoding the chemokine receptor CCR5 was identified that was associated with clinical response to high-dose IL-2 [58]. More recently, a polymorphism in the IRF5 gene was identified that was associated with clinical benefit in a cohort of patients treated with T cell-adoptive transfer [59]. Numerous polymorphisms have been identified in immune regulatory genes that are associated with various types of autoimmunity, including lupus [60], and many patients who are treated with immune-potentiating drugs do develop autoimmune-like adverse events. Thus, it is attractive to consider that specific germline SNPs might be associated either with clinical response or with side effects upon treatment with anti-CTLA-4 or anti-PD-1 mAbs.

The third category of biomarkers is the composition of the intestinal microbiota. The group of Trinchieri and colleagues has found in a mouse model that treatment with anti-bacterial antibiotics, which altered intestinal microbial composition, reduced the therapeutic efficacy of immunotherapy with the TLR9 agonist CpG combined with anti-IL-10R antibody in a transplantable tumor model [61]. In addition, Zitvogel and colleagues reported that the immune-potentiating effect of cyclophosphamide is associated with translocation of commensal bacteria [62]. These early data have prompted a comprehensive analysis of the intestinal microbiota using 16S rRNA sequencing from patients undergoing treatment with immunotherapeutics. Restoring the presence of specific commensal that maximize anti-tumor immunity, such as through the use of a probiotic, represents an additional future immunotherapeutic strategy.

2.5 Conclusions and Future Directions

The paradigm of the T cell-inflamed and non-T cell inflamed tumor microenvironment has provided a useful working model for identifying therapeutic targets for immunotherapy, understanding mechanisms of response versus resistance, and pursuing new strategies for overcoming resistance to expand the range of immunotherapy efficacy. Inasmuch as many of these concepts have been explored predominantly in melanoma, there is a rich opportunity to investigate these principles similarly in other tumor types. A summary of candidate interventions to improve immunotherapy efficacy to include the non-T cell-inflamed tumor microenvironment based on these principles is illustrated in Fig. 2.3. One could envision intratumoral administration of innate immune activators such as STING agonists, to trigger de novo DC activation and T cell priming and recruitment. Tumor-focused radiation also may have these effects. If specific oncogene pathways are activated such as β-catenin, then targeted inhibitors could be administered to block such pathways and restore immune cell entry. If unfavorable germline genetics are identified, specific gene products might be amenable to pharmacologic manipulation as well. Finally, if commensal bacteria are identified that might amplify host anti-tumor immunity, then probiotics could be developed to improve T cell infiltra-

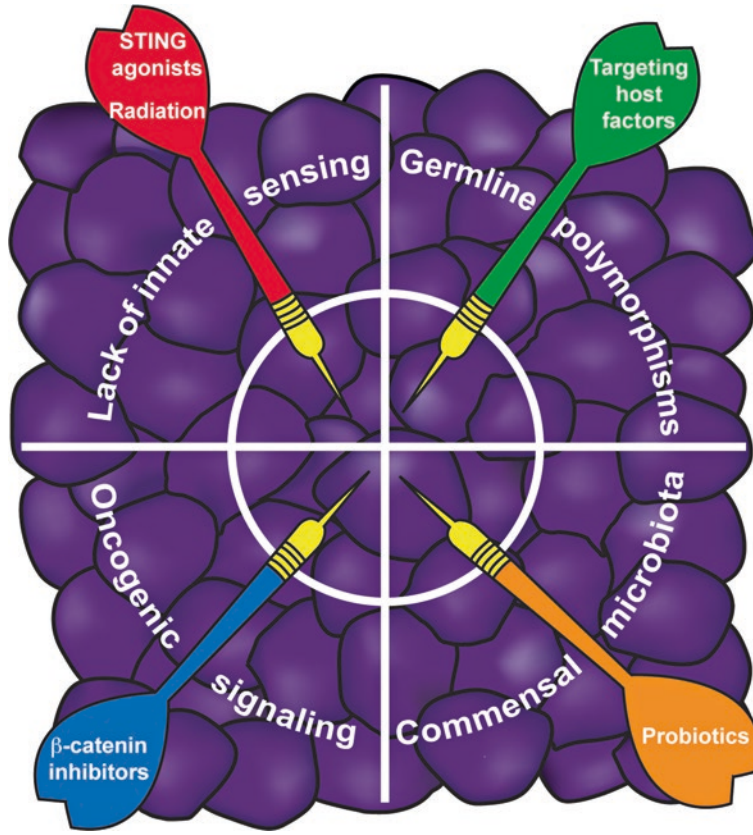

Fig. 2.3 Summary of four types of strategies that could be considered to overcome the barrier of the non-T cell-inflamed tumor microenvironment. It is envisioned that intratumoral administration of innate immune activators or local tumor radiation, modulators of host polymorphic gene products, blockade of immune-exclusionary oncogene pathways, and delivery of probiotics that amplify anti-tumor immunity all could be considered for ultimate clinical translation

tion and clinical efficacy of immunotherapies such as anti-PD-1. Ultimately, combination therapies will likely provide the broadest and deepest clinical benefit.

References

1. Peterson AC, Harlin H, Gajewski TF. Immunization with melan-A peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma. J Clin Oncol. 2003;21:2342–8.
2. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006;213:131–45.
3. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, McKee M, Gajewski TF. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res. 2009;69:3077–85.
4. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. Cancer J. 2010;16:399–403.
5. Ulloa-Montoya F, Louahed J, Dizier B, Gruselle O, Spiessens B, Lehmann FF, Suciu S, Kruit WH, Eggermont AM, Vansteenkiste J, Brichard VG. Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. J Clin Oncol. 2013;31:2388–95.
6. Mikucki ME, Fisher DT, Matsuzaki J, Skitzki JJ, Gaulin NB, Muhitch JB, Ku AW, Frelinger JG, Odunsi K, Gajewski TF, Luster AD, Evans SS. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. Nat Commun. 2015;6:7458.
7. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Galon J. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med. 2005;353:2654–66.
8. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313:1960–4.

9. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, Bruneval P, Trajanoski Z, Fridman WH, Pages F, Galon J. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol. 2011;29:610–8.
10. Harlin H, Kuna TV, Peterson AC, Meng Y, Gajewski TF. Tumor progression despite massive influx of activated CD8(+) T cells in a patient with malignant melanoma ascites. Cancer Immunol Immunother. 2006;55:1185–97.
11. Mortarini R, Piris A, Maurichi A, Molla A, Bersani I, Bono A, Bartoli C, Santinami M, Lombardo C, Ravagnani F, Cascinelli N, Parmiani G, Anichini A. Lack of terminally differentiated tumor-specific CD8+ T cells at tumor site in spite of antitumor immunity to self-antigens in human metastatic melanoma. Cancer Res. 2003;63:2535–45.
12. Zippelius A, Batard P, Rubio-Godoy V, Bioley G, Lienard D, Lejeune F, Rimoldi D, Guillaume P, Meidenbauer N, Mackensen A, Rufer N, Lubenow N, Speiser D, Cerottini JC, Romero P, Pittet MJ. Effector function of human tumor-specific CD8 T cells in melanoma lesions: a state of local functional tolerance. Cancer Res. 2004;64:2865–73.
13. Rosenberg SA, Dudley ME. Cancer regression in patients with metastatic melanoma after the transfer of autologous antitumor lymphocytes. Proc Natl Acad Sci U S A. 2004;101(Suppl 2):14639–45.
14. Kaufman HL, Kim DW, DeRaffele G, Mitcham J, Coffin RS, Kim-Schulze S. Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma. Ann Surg Oncol. 2010;17:718–30.
15. Kraman M, Bambrough PJ, Arnold JN, Roberts EW, Magiera L, Jones JO, Gopinathan A, Tuveson DA, Fearon DT. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science. 2010;330:827–30.
16. Salmon H, Franciszkiewicz K, Damotte D, Dieu-Nosjean MC, Validire P, Trautmann A, Mami-Chouaib F, Donnadieu E. Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J Clin Invest. 2012;122:899–910.
17. Demoulin S, Herfs M, Delvenne P, Hubert P. Tumor microenvironment converts plasmacytoid dendritic cells into immunosuppressive/tolerogenic cells: insight into the molecular mechanisms. J Leukoc Biol. 2013;93:343–52.
18. Watkins SK, Zhu Z, Riboldi E, Shafer-Weaver KA, Stagliano KE, Sklavos MM, Ambs S, Yagita H, Hurwitz AA. FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer. J Clin Invest. 2011;121:1361–72.
19. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med. 2008;14:28–36.
20. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014;371:2189–99.
21. Gajewski TF, Zha Y-y, Hernandez K, Li Y, Bao R, Alexieff P, Andrade J, Luke JJ, Spranger S. Density of immunogenic antigens and presence or absence of the T cell-inflamed tumor microenvironment in metastatic melanomal. J Clin Oncol. 2015;33(suppl; abstr):3002.
22. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C, Mellor AL. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281:1191–3.
23. Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res. 2007;13:5256–61.
24. Spranger S, Spaapen R, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF. Upregulation of PD-L1, IDO and Tregs in the melanoma tumor microenvironment is driven by CD8+ T cells. Sci Transl Med. 2013;5(200):200ra116.
25. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL, Chen L. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med. 2012;4:127ra137.
26. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. J Immunother Cancer. 2014;2:3.
27. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443–54.
28. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, Chmielowski B, Spasic M, Henry G, Ciobanu V, West AN, Carmona M, Kivork C, Seja E, Cherry G, Gutierrez AJ, Grogan TR, Mateus C, Tomasic G, Glaspy JA, Emerson RO, Robins H, Pierce RH, Elashoff DA, Robert C, Ribas A. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515:568–71.

29. Zheng Y, Zha Y, Driessens G, Locke F, Gajewski TF. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. J Exp Med. 2012;209:2157–63.
30. Zheng Y, Zha Y, Gajewski TF. Molecular regulation of T-cell anergy. EMBO Rep. 2008;9:50–5.
31. Zheng Y, Zha Y, Spaapen RM, Mathew R, Barr K, Bendelac A, Gajewski TF. Egr2-dependent gene expression profiling and ChIP-Seq reveal novel biologic targets in T cell anergy. Mol Immunol. 2013;55:283–91.
32. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, Hercend T. LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med. 1990;171:1393–405.
33. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A, Mittler RS. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J Exp Med. 1997;186:47–55.
34. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, Linette GP, Meyer N, Giguere JK, Agarwala SS, Shaheen M, Ernstoff MS, Minor D, Salama AK, Taylor M, Ott PA, Rollin LM, Horak C, Gagnier P, Wolchok JD, Hodi FS. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. N Engl J Med. 2015;372:2006–17.
35. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004;5:987–95.
36. Fuertes MB, Kacha AK, Kline J, Woo SR, Kranz DM, Murphy KM, Gajewski TF. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. J Exp Med. 2011;208(10):2005–16.
37. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml BU, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science. 2008;322:1097–100.
38. Diamond MS, Kinder M, Matsushita H, Mashayekhi M, Dunn GP, Archambault JM, Lee H, Arthur CD, White JM, Kalinke U, Murphy KM, Schreiber RD. Type I interferon is selectively required by dendritic cells for immune rejection of tumors. J Exp Med. 2011;208:1989–2003.
39. Fuertes MB, Woo SR, Burnett B, Fu YX, Gajewski TF. Type I interferon response and innate immune sensing of cancer. Trends Immunol. 2013;34:67–73.
40. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature. 2008;455:674–8.
41. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science. 2013;339:786–91.
42. Zhang X, Shi H, Wu J, Sun L, Chen C, Chen ZJ. Cyclic GMP-AMP containing mixed phosphodiester linkages is an endogenous high-affinity ligand for STING. Mol Cell. 2013;51:226–35.
43. Ahn J, Gutman D, Saijo S, Barber GN. STING manifests self DNA-dependent inflammatory disease. Proc Natl Acad Sci U S A. 2012;109:19386–91.
44. Ahn J, Ruiz P, Barber GN. Intrinsic self-DNA triggers inflammatory disease dependent on STING. J Immunol. 2014;193:4634–42.
45. Liu Y, Jesus AA, Marrero B, Yang D, Ramsey SE, Montealegre Sanchez GA, Tenbrock K, Wittkowski H, Jones OY, Kuehn HS, Lee CC, DiMattia MA, Cowen EW, Gonzalez B, Palmer I, DiGiovanna JJ, Biancotto A, Kim H, Tsai WL, Trier AM, Huang Y, Stone DL, Hill S, Kim HJ, St Hilaire C, Gurprasad S, Plass N, Chapelle D, Horkayne-Szakaly I, Foell D, Barysenka A, Candotti F, Holland SM, Hughes JD, Mehmet H, Issekutz AC, Raffeld M, McElwee J, Fontana JR, Minniti CP, Moir S, Kastner DL, Gadina M, Steven AC, Wingfield PT, Brooks SR, Rosenzweig SD, Fleisher TA, Deng Z, Boehm M, Paller AS, Goldbach-Mansky R. Activated STING in a vascular and pulmonary syndrome. N Engl J Med. 2014;371:507–18.
46. Woo S-R, Fuertes MB, Corrales L, Spranger S, Furdyna MJ, Leung MYK, Duggan R, Wang Y, Barber GN, Fitzgerald KA, Alegre M-L, Gajewski TF. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. Immunity. 2014;41:830–42.
47. Ahn J, Xia T, Konno H, Konno K, Ruiz P, Barber GN. Inflammation-driven carcinogenesis is mediated through STING. Nat Commun. 2014;5:5166.
48. Ohkuri T, Ghosh A, Kosaka A, Zhu J, Ikeura M, David M, Watkins SC, Sarkar SN, Okada H. STING contributes to antiglioma immunity via triggering type I IFN signals in the tumor microenvironment. Cancer Immunol Res. 2014;2(12):1199–208.
49. Zhu Q, Man SM, Gurung P, Liu Z, Vogel P, Lamkanfi M, Kanneganti TD. Cutting edge: STING mediates protection against colorectal tumorigenesis by governing the magnitude of intestinal inflammation. J Immunol. 2014;193:4779–82.
50. Baguley BC, Ching LM. Immunomodulatory actions of xanthenone anticancer agents. BioDrugs. 1997;8:119–27.
51. Lara PN Jr, Douillard JY, Nakagawa K, von Pawel J, McKeage MJ, Albert I, Losonczy G, Reck M, Heo DS, Fan X, Fandi A, Scagliotti G. Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. J Clin Oncol. 2011;29:2965–71.
52. Conlon J, Burdette DL, Sharma S, Bhat N, Thompson M, Jiang Z, Rathinam VA, Monks B, Jin T, Xiao TS, Vogel SN, Vance RE, Fitzgerald KA. Mouse, but not human STING, binds and signals in response to the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid. J Immunol. 2013;190:5216–25.

53. Gao P, Ascano M, Zillinger T, Wang W, Dai P, Serganov AA, Gaffney BL, Shuman S, Jones RA, Deng L, Hartmann G, Barchet W, Tuschl T, Patel DJ. Structure-function analysis of STING activation by c[G(2′,5′)pA(3′,5′)p] and targeting by antiviral DMXAA. Cell. 2013;154:748–62.
54. Corrales L, Glickman LH, McWhirter SM, Kanne DB, Sivick KE, Katibah GE, Woo SR, Lemmens E, Banda T, Leong JJ, Metchette K, Dubensky TW Jr, Gajewski TF. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. Cell Rep. 2015;11:1018–30.
55. Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, Fu YX, Auh SL. The efficacy of radiotherapy relies upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res. 2011;71:2488–96.
56. Deng L, Liang H, Xu M, Yang X, Burnette B, Arina A, Li X-D, Mauceri H, Beckett M, Darga T, Huang X, Gajewski TF, Chen ZJ, Fu Y-X, Weichselbaum RR. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. Immunity. 2014;41:843–52.
57. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. Nature. 2015;523(7559):231–5.
58. Ugurel S, Schrama D, Keller G, Schadendorf D, Brocker EB, Houben R, Zapatka M, Fink W, Kaufman HL, Becker JC. Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. Cancer Immunol Immunother. 2008;57:685–91.
59. Uccellini L, De Giorgi V, Zhao Y, Tumaini B, Erdenebileg N, Dudley ME, Tomei S, Bedognetti D, Ascierto ML, Liu Q, Simon R, Kottyan L, Kaufman KM, Harley JB, Wang E, Rosenberg SA, Marincola FM. IRF5 gene polymorphisms in melanoma. J Transl Med. 2012;10:170.
60. Kariuki SN, Franek BS, Kumar AA, Arrington J, Mikolaitis RA, Utset TO, Jolly M, Crow MK, Skol AD, Niewold TB. Trait-stratified genome-wide association study identifies novel and diverse genetic associations with serologic and cytokine phenotypes in systemic lupus erythematosus. Arthritis Res Ther. 2010;12:R151.
61. Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, Molina DA, Salcedo R, Back T, Cramer S, Dai RM, Kiu H, Cardone M, Naik S, Patri AK, Wang E, Marincola FM, Frank KM, Belkaid Y, Trinchieri G, Goldszmid RS. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science. 2013;342:967–70.
62. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, Enot DP, Pfirschke C, Engblom C, Pittet MJ, Schlitzer A, Ginhoux F, Apetoh L, Chachaty E, Woerther PL, Eberl G, Berard M, Ecobichon C, Clermont D, Bizet C, Gaboriau-Routhiau V, Cerf-Bensussan N, Opolon P, Yessaad N, Vivier E, Ryffel B, Elson CO, Dore J, Kroemer G, Lepage P, Boneca IG, Ghiringhelli F, Zitvogel L. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science. 2013;342:971–6.

3 Regulation of CTL Infiltration Within the Tumor Microenvironment

Sarah E. Church and Jérôme Galon

3.1 The Predictive Capability of Tumor Infiltrating Lymphocytes

3.1.1 T-Lymphocyte Infiltration in the Tumor and Immunoscore

Classical methods for determining malignant disease prognosis are based upon the morphology and location of tumor cells at the primary sites and in lymph node tissues, and the existence of distant metastases. While this analysis provides important information about a patient's disease it fails to capture the biological complexity of the tumor microenvironment and the contribution of the anti-tumor immune response. Immunohistochemical and gene analyses of immune cells, particularly $CD3^+$ T lymphocytes in the primary tumor, provides a prognostic biomarker that is highly statistically accurate for predicting clinical outcome in the vast majority of cancer types including colorectal, lung, melanoma, ovarian, head and neck, breast, urothelial, hepatocellular, gallbladder, and esophageal (reviewed in [1–3]). Furthermore, basic histological quantification of T lymphocyte density, cytotoxicity, and memory by CD3, CD8, and CD45RO, respectively, has demonstrated that increased infiltration of T lymphocytes is associated with statistically significant improvement in patients' disease-free survival (DFS) and overall survival (OS) [2, 4, 5]. In colorectal carcinoma (CRC), further delineating the location of cytotoxic T lymphocytes (CTL--$CD3^+$, $CD8^+$) into two areas within the primary tumor, the center (CT) and the invading margin (IM), provides a statistically accurate prediction of clinical outcome [4]. Quantification of the density, phenotype, and location (CT or IM) of T lymphocytes has been termed Immunoscore [6–8]. In fact, for the first time it was shown that analysis of a marker, CD3, surpassed the gold standard of diagnostics via tumor-stage, lymph node, and metastatic invasion. Immunoscore defines patients into five categories (I0-I4) based on the distinct location (CT and IM) of $CD3^+$ and $CD8^+$ T lymphocytes within the primary tumor, where I0 has no $CD3^+$ or $CD8^+$ cells and I4 has high densities of both

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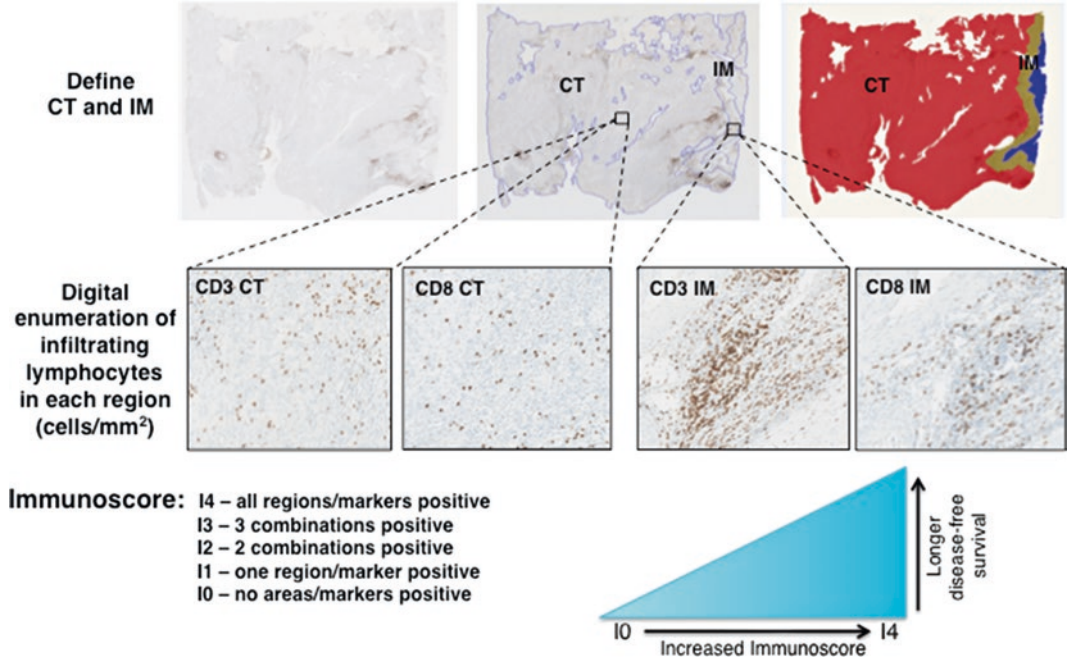

Fig. 3.1 Overview of Immunoscore. Immunoscore classifies tumors by density and location of CD3- and CD8-positive T lymphocytes. Paraffin-embedded tumor tissue is stained for CD3 or CD8. Stained tissue samples are analyzed for tumor and normal epithelium. The tumor center (CT) and invasive margin (IM) are then defined using digital software. The software then enumerates infiltrating lymphocytes in each region. Immunoscore is calculated based on the density of each marker in both regions. Patients with high Immunoscore (I4) have significantly longer disease-free survival compared to patients with low Immunoscore (I0)

$CD3^+$ and $CD8^+$ cells in the CT and IM (Fig. 3.1) [9, 10]. Immunoscore utilizes simple analysis of two markers to accurately predict a patient's clinical outcome. By combining Immunoscore with quantification of additional immune components associated with the tumor microenvironment, as a part of the immune contexture, we continue to enrich our understanding of why tumors become resistant, avoid elimination, or fail to generate a tumor-specific cytotoxic response. In particular, identifying characteristics of the tumor microenvironment that lead to low densities of immune infiltrates and consequently low Immunoscore (I0 and I1) could dramatically improve the selection of personalized treatments for cancer. In the cases with high Immunoscore, patients might be more likely to respond to immunotherapies that stimulate an existing immune response, such as checkpoint blockades, while patients with low Immunoscore would need therapy that primes a *de novo* antitumor response or facilitates trafficking of CTL to the tumor site.

3.1.2 Memory and Cytotoxic Lymphocytes Indicate Improved Prognosis

The importance of memory and CTL in the tumor microenvironment is well established. The presence of effector memory T cells in primary colorectal tumors is negatively correlated with signs of early metastatic invasion, as defined by presence of vascular emboli, lymphatic invasion, and perineural invasion [5]. This observation is supported by phenotypic analysis by flow cytometry of effector memory T lymphocytes, where patients with signs of early metastatic invasion have significantly fewer $CD3^+CD8^+CD45RO^+$

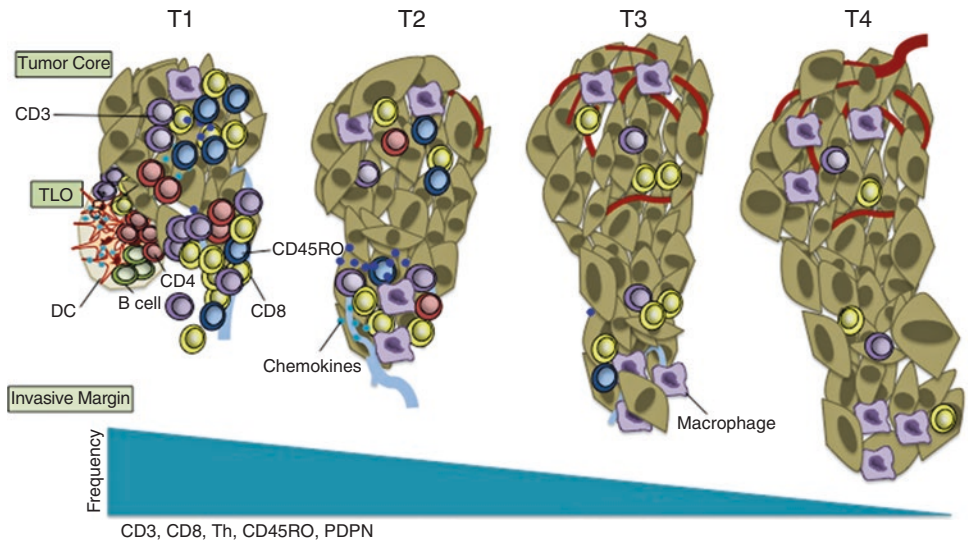

Fig. 3.2 The tumor microenvironment by progressive tumor stage. Depiction of the microenvironment of tumors from stage T1 to T4. Early stage tumors are smaller with many CD3, CD8, CD4, CD45RO, and TLO at the invasive margin and in the tumor center. T2 and T3 tumors are larger and progressively have less T lymphocytes, less lymphatic vessels (PDPN) and more angiogenesis. T4 tumors have very few T lymphocytes, increased macrophages and increased angiogenesis. The presence of CTL, memory and helper lymphocytes at the invasive margin, tumor center and in TLO predicts better clinical outcomes

and $CD3^+CD8^+CCR7^-$ cells in the tumor, as well as by immunohistochemistry measuring CD45RO, where patients with metastatic progression have significantly fewer CD45RO-positive cells in the primary tumor compared to non-invasive disease [5]. Furthermore, a high density of $CD45RO^+$ cells in the primary tumor significantly predicts better overall and disease-free survival compared to patients with low density of $CD45RO^+$ expression within their tumors [5]. High expressers had median OS of 36.5 months and DFS of 53.2 months, compared to 11.1 and 20.6 months, respectively, for low density CD45RO. This indicates that not only are CTL an excellent biomarker for determining patient disease-related survival, but also further delineates the importance of the transition of CTL into memory phenotype, which can help refining the predictive capability of intratumoral CTL on patients' clinical outcome, as illustrated in Fig. 3.2. Two important mechanisms of memory T lymphocyte development and maintenance are determined by cytokine stimulation and help by $CD4^+$ T lymphocytes [11–14]. The role of these two mechanisms in maintaining anti-tumor memory and cytotoxic lymphocytes are discussed in the following sections.

3.1.3 Th1 and Th17 Have Opposing Effects on Disease-Specific Survival

It has been previously shown that incorporating the subtype and location of T helper (Th) lymphocytes, in addition to CTL, improves the accuracy of disease-specific survival prediction [15]. Primary CRC tumors from 125 patients for the expression of 45 immune genes representing four T helper populations, Th1, Th2, Th17 and regulatory T lymphocytes (Treg) were analyzed. Hierarchical clustering revealed eight categories of Th genes as follows: Th1 cytotoxic (IRF1, GZMB, IL27, GNLY, PRF1, CCL5, CD8a, STAT1), Th1 (IL12RB1, CD28, CCR5, HLA-DMB, IL12RB2, CD38, CXCR6, TBX21), Th17 (RORC, IL-17A), Th2 (IL4, IL5, IL13) or (CCR7, CD3E, CD40LG, CCL19, CCR4,

GATA3) or (IFNGR1/2, STAT3, IL10RB, IL4R, STAT6), Th2/Treg (FOXP3, CTLA-4, CCL17, CCL22) or Tregs (IL-10, TGFB). Interestingly, when disease-free survival (DFS) was assessed based on expression of Th1 cytotoxic genes, patients with high expression had significantly increased time to relapse versus patients with low expression (78 *versus* 18 months, $p = 0.01$). Conversely, patients with low expression of Th17 genes had prolonged disease-free survival with 80% of patients not experiencing relapse after 9 years. Since the Th1 cytotoxic and Th17 gene profiles exhibited this extreme contrast in prediction of DFS, the two gene profiles were assessed for complementarity. The patients were separated into 4 groups based on high or low Th1 cytotoxic or Th17 gene expression, Th1-Hi Th17-Hi, Th1-Hi Th17-Lo, and Th1-Lo Th17-Lo. Remarkably, the few patients with Th1-Hi and Th17-Lo had no tumor recurrence at 5 years, while patients with Th1-Hi and Th17-Hi had a DFS of 65% at 5 years and patients with Th1-Lo and Th17-Hi tumors had the worst outcome with DFS of 40% at 5 years. These findings were confirmed at the protein level by immunohistochemistry (IHC) analysis of IL-17 and CD8. Density and location (CT or IM) of IL17- and CD8-positive cells were analyzed where "high" is positive in both the CT and IM, "heterologous" has high density in either the CT or IM, and "low" has low densities of cells in both regions. DFS analysis showed the vigorously augmenting effect of IL17 expression on incidence of relapse, whereas IL17-low CD8-heterologous, IL17-heterologous CD8-heterologous and IL17-high and CD8-heterologous had 8, 40, and 80% observed relapse, respectively ($p < 0.001$). These data demonstrate the benefit of complementary analysis of Th1, Th17, and CTL in the tumor microenvironment.

The finding that high density of IL-17 expressing cells in the primary CRCs is a negative prognostic biomarker is not unprecedented because IL-17 production by T lymphocytes (Th, NK, Tc, γδ), NK, neutrophil, and innate lymphoid cells has been associated with colon tumorigenesis [16]. One mechanism for this is via commensal bacteria that skew Th17-directed inflammation, leading to hyperplasia of normal colon cells and eventually colon cancer [17]. IL-17 also induces colorectal cell lines and primary cells to secrete pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and can cause resistance to anti-angiogenic therapies [18, 19]. This pro-angiogenic stimulation likely also prevents trafficking of tumor-specific CTL to the tumor site, discussed in further detail below. Additional support for Th17 cells as a negative prognostic factor is that IL-17 expression defines patients with decreased disease-specific survival for pancreatic, breast, and gastric cancer [19–21], as well as increased tumor growth for intraocular lymphoma [22].

Contrary to Th17, Th1 cells are slowly becoming recognized for their important role in anti-tumor immunity for multiple types of cancer. Expression of $CD4^+$ cells within the primary tumor correlates with improved prognosis in esophageal squamous carcinoma and non-small cell lung cancer, with statistically significant additive predictability when combined with enumeration of CTL [23, 24]. Helper $CD4^+$ T lymphocytes, particularly tumor antigen-specific Th, guide CTL trafficking and maintain their cytolytic function within the tumor [25–27]. Tumor antigen-specific Th1 lymphocytes produce IFN-γ in the tumor microenvironment leading to the expression of CTL chemoattractants including CXCL9, CXCL10, CCL2, CCL3 and CCL5 [25]. Tumor-specific Th cells also produce IL-2, which is critical for CTL survival and can inhibit PD-1 mediated exhaustion of tumor-specific CTL leading to better efficacy of adoptive immunotherapy [27]. Additionally, mesenchymal stromal cells in the tumor microenvironment can directly activate CD4 T lymphocytes to become Th1 cells via IL-12 and consequently make tumor cells more vulnerable to CTL mediated destruction [28].

Regulatory T cells (Tregs) have an important role in the immune system to prevent uncontrolled immune responses to self-antigens. In cancer, this can lead to immunosuppression of the anti-tumor immune response due to self-antigens present on tumor cells. It has been shown that Treg gene signatures were not correlated to patient outcome; however, high densities

of FoxP3 protein expression was associated with increased disease-free survival of patients with colorectal cancer [15]. This is supported by other studies suggesting that in colorectal cancer, Tregs are not correlated with immunosuppression of the anti-tumor immune response and are significantly associated with high densities of CTL and Th1 T cell infiltration in the tumor [15, 29]. The effectiveness of Tregs as a prognostic maker has been variable between cancer types [30–32]. The comparisons of effector to Treg ratios, in hepatocellular and ovarian cancer showed that increased ratio of effector T cells to Tregs has positive prognostic value [33, 34]. Altogether this suggests that Tregs are a complicated biomarker for predicting patient outcome.

Finally, follicular T helper (Tfh) lymphocytes should be mentioned. Tfh cells are specialized to provide help to T and B lymphocytes, maintain memory B lymphocytes and produce IL-21 [35]. It has been previously reported that expression of the Tfh cells markers, CXCL13, CXCR5, and IL21 in the tumor were significantly correlated with prolonged disease-free survival [36]. Furthermore patients with aberration in the CXCL13 gene leading to gene deletion and dysfunction had significantly shorter disease-free survival compared to CRC patients with no aberration. High density of Tfh infiltration in the primary tumor has also been associated with prolonged disease-free survival in breast cancer [37]. The role of CXCL13 and IL-21 on CTL function will be further discussed in the following sections.

3.2 Factors Regulating Tumor Infiltration of Lymphocytes

3.2.1 T-Cell Homing Molecules Mediate Migration of CTL to Tumors

Chemokines have an important role in orchestrating both innate and adaptive immune cells chemotaxis and localization within the tumor. Chemokines can direct development and maintenance of tertiary lymphoid organs (TLO) that prime tumor-specific CTL at the tumor site, which has been described in multiple cancer types including non-small cell lung cancer, melanoma, and colorectal carcinoma [36, 38–40].

We examined the predictive capability of chemokines using data integration of gene expression in primary tumors from CRC patients [41]. We discovered a significant prolongation of DFS in patients with high expression of the chemokines CX3CL1, CXCL10, and CXCL9. CX3CL1, also known as fractalkine, mediates T lymphocyte and monocytes migration and promotes strong adhesion to endothelial cells [42]. CXCL10, also named IFN-γ protein 10, and CXCL9 are closely related cytokines in the monokine-induced by IFN-γ family. CXCL10 and CXCL9 facilitate migration of CTL, monocytes, NK and dendritic cells, inhibit angiogenesis, and have anti-tumor properties [43, 44]. CRC patients with elevated gene expression of one of these three chemokines had increased percentage and density of $CD3^+CD8^+$ T lymphocytes in the tumor as assessed by flow cytometry and immunohistochemistry [41]. Analysis by location, CT or IM, within the tumor microenvironment showed that: (I) patients with high intra-tumoral CXC3CL1 expression also had significantly increased density of effector-activated CTL ($GZMB^+$) and Th1 ($T\text{-}Bet^+$) cells; (II) tumors of patients with high CXCL9 and CXLCL10 expression levels contained a significantly increased number of memory T lymphocytes ($CD45RO^+$) and macrophage ($CD68^+$). TCR repertoire analysis of ten patients randomly selected from the same cohort showed that the TCR repertoire of patients with a high CX3CL1 level was clearly distinguishable from the repertoire of patients with low CX3CL1 expression level. One cluster with CX3CL1, CXCL9, or CXCL10 gene expression levels correlated with a specific CTL repertoire (Vb5.2L08, Vb2L03, Vb2L07), thus suggesting that these chemokines attract clonal CTL with distinct tumor-specificity. Strikingly, when CRC tumors had high expression of any of these three TCRs the patients overall 3-year survival was 100%, as opposed to 28% with low expression of these TCRs. This suggests that CX3CL1, CXCL9, and CXCL10 in the tumor

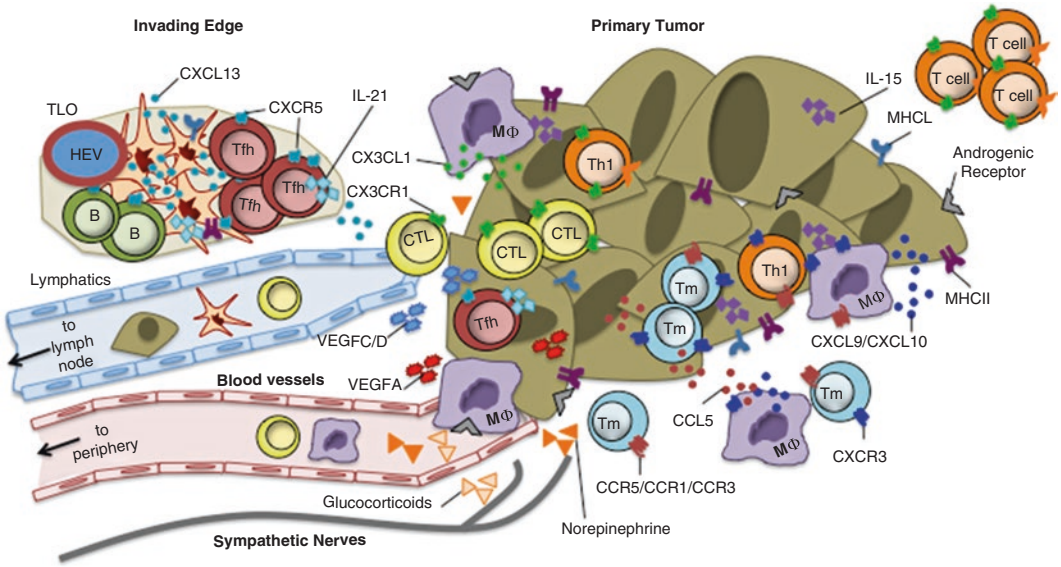

Fig. 3.3 Modulators of T lymphocytes in the tumor microenvironment. Overview of lymphatics, blood vessels, nerves, tertiary lymphoid organs (TLO), immune and tumor cells that produce cytokines, chemokines, hormones, and immunosuppressive factors that regulate function and trafficking of lymphocytes in tumors. Expression of many of these factors can predict prognosis of patients with cancer, described in Table 3.1

microenvironment recruit tumor-specific CTL to eliminate malignant cells, and tumors become resistant to CTL-mediated death when these chemokines are not present. High expression density of CXCL9 and CXCL10 also accurately predicts prolonged disease-specific survival in melanoma patients [39, 45]. Pre-clinical studies with melanoma show that blocking CXCL9 or CXCL10 substantially reduces the ability of CTL to traffic to the primary tumor and distant metastatic legions [45], which may be due to their role in directing CTL homing to the tumor by CD4 T lymphocyte help.

Another chemokine, CXCL13, was recently found to be associated with follicular helper T (Tfh) lymphocytes and also predicts patients' clinical outcome. CXCL13 is produced by and has been associated with generation of tertiary lymphoid organs (TLO) within the invasive margin of primary tumors [38, 46]. The presence of TLO in primary tumors is positively correlated to prolonged disease-free survival in multiple cancers [38, 39]. It is hypothesized that priming and activation of tumor-specific CTL is orchestrated by dendritic cells presenting tumor-antigens within these TLO. In conjunction with this observation, CXCL13 as a single biomarker can accurately predict patients' clinical outcome [36]. Earlier it was discussed that low protein expression density or chromosomal aberration of CXCL13 is associated with worse clinical prognosis in CRC [36]. Similarly, in specific subtypes of breast cancer, elevated expression of CXCL13 in the tumor is associated with increased disease-free survival compared to tumors with low expression of CXCL13 [37]. Additionally, there is evidence supporting that high or low density CXCL13 expression can accurately predict patients' response to chemotherapy [47, 48]. It seems that the CXCL13-CXCR5 axis has the highest predictive score in HER2-positive breast cancers as opposed to other breast cancer subtypes [49]. This may be due to potential immunogenicity of HER2 for generating HER2-specific T helper and CTL immune response against the tumor. Figure 3.3 shows an overview of many

Table 3.1 Factors regulating lymphocyte infiltration into tumors

	Tumor immune factor	Prognosis	Ref
Chemokines			
CX3CL1	Mediates T-lymphocyte and monocyte migration to tumors and adhesion to endothelial cells	Good	[35, 36]
CXCL9/10 CCL5	Induced on tumor cells and MΦ by IFN-γ to promote CTL, monocyte, NK, and dendritic cell migration to the tumor and anti-angiogenic properties	Good	[25, 35, 37, 38]
CXCL13	Produced by dendritic cells in the TLO and signals through the CXCR5 receptor on B cells and Tfh cells controlling the organization of TLO	Good	[30, 32, 40]
Cytokines			
IL-17	Associated with tumorigenesis. Induces tumor and primary cells to secrete pro-angiogenic factors	Poor	[15, 18, 19]
IL-15	Regulates memory T lymphocyte maintenance and homing capabilities. Shown to rescue tolerant T cells and augment tumor-reactive CTL function and survival	Good	[11, 48, 50–52]
IL-21	Produced by NKT and Th cells. Activates and prevents exhaustion of tumor-specific CTL	Good	[30, 48, 55]
Angiogensis/Lymphatics			
VEGFA	Generates leaky vasculature that prevents trafficking of leukocytes to the tumor. Stimulates suppressive Tregs and MDSC and induces immune checkpoints on endothelium (PD-L1, B7-H3, and TIM3)	Poor	[71–74]
VEGFC/D	Generates lymphatic vessels that are dysfunctional in fluid mechanics. Associated with chronic inflammation and induces secretion of immunosuppressive factors	Poor	[75, 77]
TLO/HEV	Facilitates priming, maintenance, and migration of lymphocytes in tumors. Presence in stroma correlates with high density of T and B cells	Good	[32, 78]
Neural			
Glucocorticoids	Induce expression of chemokine, cytokine, complement family members, innate immune-related genes, and TLR and repress adaptive immune-related genes. Reduce adaptive immune gene expression and skew Th1 cells to a Th2 phenotype. Upregulate IL-7Ra, enhance IL-7-mediated signaling and function, and inhibit apoptosis	Both	[82–84, 86]
Norepinephrine AR	Downregulates MHC-I, co-stimulatory molecules and increases production of IDO by tumor cells via beta-AR. AR signaling enhances Treg-mediated suppression, polarizes MΦ to a M2 phenotype and increases infiltration of MDSC	Poor	[87–92]

MΦ macrophage, *Th* T helper lymphocytes, *CTL* cytotoxic T lymphocytes, *TLO* tertiary lymphoid organ, *MDSC* myeloid-derived suppressor cells, *MHCI* MHC Class I, *AR* androgenic receptors

prognostic chemokines involved in T lymphocyte recruitment to the tumor. It is becoming increasing clear that chemokines have an essential role in trafficking CTL to the tumor site. Furthermore, the addition of chemokine expression to Immunoscore has potential to predict patient response to chemotherapy [48].

3.2.2 Cytokines Contribute to the Distribution of CTL within the Tumor

An immense number of studies have been performed to investigate the components of the cytokine milieu that regulate lymphocytes in the tumor microenvironment. Interferon-gamma (IFN-γ) is well appreciated for its capacity to

prevent tumor growth during cancer immunoediting [50]. Detection of an IFN-γ signature within the tumor has been associated with prolonged disease-specific survival in melanoma, colorectal, gastrointestinal, and ovarian cancer [51, 53]. To dissect the role of cytokines in tumor progression, a large cohort of CRC primary tumors has been analyzed for copy number variations in cytokines and cytokine receptors [54]. Fifty-nine soluble and membrane-bound proteins and their corresponding receptors from the IFN, IL, transforming growth factor (TGF), and tumor necrosis factor (TNF) families were analyzed. The majority (75%) of CRC patients displayed no difference in genomic alterations in the cytokine gene and receptor families studied. Of the remaining patients, the highest level of gain was in *IL29* and loss was in *IL15.* Furthermore, clinically advanced patients with distant metastases displayed a higher frequency of deletions in the interleukin family members *IL2, IL8, IL15,* and *IL21*. Interestingly, three of these deletions are in cytokines from the common γ-chain family, which has essential functions for maintenance, proliferation, and migration of memory, CTL, and Th lymphocytes [55]. Most strikingly, only patients with deletions in *IL2, IL15,* and *IL21* had significantly higher risk of tumor relapse [54]. On the other hand, gains or deletions of suppressive cytokines genes, *IL8, IL10,* and *TGFβ* did not correlate to tumor recurrence.

Considering the previously discussed importance of localization and density of memory $CD8^+$ T lymphocytes in the CRC tumor microenvironment, and the predominant role of IL-15 in the regulation of memory T lymphocyte homing and maintenance [56, 57], the cellular source of IL-15 within the CRC tumor microenvironment was investigated, both *in silico* and *in vitro*, by ClueGo and CluePedia, and IHC, respectively. It was discovered that tumor and myeloid cells were the source of IL-15, and increased IL-15 expression could significantly predict prolonged DFS [54]. IL-15 has been shown to rescue tolerant T lymphocytes [58] and augment the therapeutic efficacy of tumor-reactive CTL [11]. Moreover, patients with high expression of IL-15 in the tumor microenvironment have increased immune cell density, immune gene expression, and DFS compared to medium or low expressing CRC tumors [54]. These data show that deletion of the *IL15* gene and reduced production of IL-15 by tumor cells is a substantial mechanism in preventing CTL infiltration and elimination of tumor cells. Furthermore, IL-15 signaling is highly effective in augmenting the anti-tumor CTL response, both as a mechanism to enhance CD4 T cell help and to maintain adoptively-transferred CTL survival [11, 58–60].

Expression of another common receptor λ-chain cytokine family member, IL-21, also predicts clinical outcome in CRC patients. CRC patients with chromosomal aberration of the *IL21* gene leading to deletion had higher risk of relapse than those without a deletion [54]. Overrepresentation of *IL2*, *IL15*, and *IL21* deletions was seen in patients with metastases, suggesting that these cytokines may be involved in putative anti-tumor immune mechanisms. IL-21 has a broad range of therapeutic anti-cancer properties, including activating and preventing exhaustion of tumor-specific CTL [61]. IL-21 is produced by NKT cells, Th1, Th17, and Tfh cells, again suggesting a role of Tfh as a substantial player in orchestrating the CTL response in the tumor.

3.3 Global Factors that Contribute to the Immune Contexture of Tumors

3.3.1 Mutagenesis and CTL Specificity

In melanoma, historically one of the most immune responsive cancers, it is known that the most potent tumor-specific T lymphocytes are directed toward neoantigens expressed by melanoma cells [62, 63]. Similarly, in other cancer types it has been documented that CTL specific for tumor neoantigens are extremely effective at immunosurveillance, elimination of tumor cells, and predicting clinical outcome [64, 65]. Recently, Alexandrov and colleagues reported extensive somatic mutational analysis describing 30 types of human cancer, where highly immunogenic cancers including melanoma, lung and

colorectal carcinoma had the highest prevalence of somatic mutations in their genome [66], thus integrating the paradigm that increased frequency of tumorogenic mutations provides better tumor-specific CTL targets. From this, it might be hypothesized that somatic mutations could be used as predictive biomarkers of cancer patient survival and response to therapy; however, the data up to this point has been inconsistent. Enumeration of total numbers of somatic mutations does not always predict prolonged disease-free survival, however presence of selected immunogenic mutations can distinguish patients with better clinical outcome [65, 67–69]. On the other hand, low numbers of genomic mutations can predict the presence of immunosuppressive mechanisms within the tumor [70]; furthermore, immunogenic mutational gene signatures have been shown to accurately predict benefit from CTLA-4 and PD-1 blocking immunotherapies [68, 69].

A few studies have reported that cancer-driver mutations are associated with immune gene signatures in the microenvironment, most notably in the RAS and EGFR genes. Interestingly, both of these genes have been linked to immune regulatory pathways. The presence of RAS mutations in colorectal carcinoma has been associated with decreased immunogenicity of tumors [71, 72]. Evidence also suggests that mutations in KRAS correlate with downregulation of MHC Class I molecules on tumor cells [71]. Additionally, it has been found that 20 immune genes encompassing checkpoint (CTLA-4, PD-1/L1/L2, TIM3 and LAG3), MHC class II, and Th1 genes were significantly under expressed in patients with KRAS mutations, independently of microsatellite stable or unstable disease [72]. EGFR mutations in NSCLC have been linked with upregulation of PD-L1 on tumor cells leading to inhibition of T lymphocyte response [73, 74].

This suggests that mutations in cancer driver genes themselves are not the most accurate measurement of patient prognosis, but the enumeration of immune-related gene mutations, particularly those involved in MHC-processing [70]. The predictive capability of expression of genes regulating immune cells and MHC-processing has been reported in colorectal, lung, ovary, breast, brain, and renal cancers [67, 75, 76].

3.3.2 Intratumoral Blood and Lymphatic Vessels Modulate CTL Trafficking

Tumor-stimulated angiogenesis is a well-established target for anti-cancer therapies because of the necessity for tumors to obtain a sufficient supply of nutrients. However, because blood vessels generated by tumor-induced angiogenesis lack structure causing blood flow to be leaky, leukocytes are unable to traffic properly [77]. Furthermore, many of the angiogenic tumor-derived factors have promiscuous functions in stimulating suppressive immune mechanisms, such as chemotaxis of Tregs and myeloid-derived suppressor cells to the tumor [78]. Angiogenic promoters also reduce endothelial adhesion molecules, preventing CTL from attaching to the vascular walls and migrating into the tumor [79]. The early inflammatory response driven by TNF-α induces adhesion molecules such as VCAM-1 in normal endothelium, however, when pro-angiogenic factors such as basic fibroblast growth factor are present, TNF-α loses the ability to invoke adhesion molecules [79]. Lastly, pro-angiogenic factors can also induce expression of immune checkpoints, including PD-L1/L2, B7-H3, galectin-1, and TIM3, on the endothelium putting the brakes on CTL activation [79, 80]. A few studies have demonstrated that combination therapy using angiogenesis inhibitors with anti-tumor immune stimulation can restore the migratory potential of CTL [81].

Tumors not only stimulate angiogenesis, but also the generation of new lymphatic vasculature by lymphangiogenesis via production of vascular endothelial growth factor C (VEGFC) and VEGFD [82]. VEGFC and VEGFD are often associated with poor clinical prognosis and increased cancer progression. Tumor-induced lymphatic vessels are important factor for dissemination of tumor cells into the lymph node and distant metastases. These tumor-induced

vessels have dysfunctional fluid mechanics that augment chronic inflammation and secrete immunosuppressive factors, such as TGF-β [83].

On the other hand, the presence of well-ordered lymphatic structures, including TLO and high endothelial venules (HEV) have been demonstrated to facilitate priming, maintenance, and migration of lymphocytes into solid tumors in melanoma, breast, ovarian, colon, and lung cancer [38, 84]. The presence of HEVs in the tumor stroma strongly correlates with increased infiltration of T and B lymphocytes. In breast cancer, high density of tumor HEVs is associated with the extravasation of Th1, CTL, and effector memory T lymphocytes into the tumor [84]. Furthermore patients with high density of tumor HEVs have longer metastasis-free survival, DFS, and OS. These observations were independent of the density of blood vessels within the tumor. In conjunction with these reports, the Authors have observed that patients with high density of lymphatic vessels, as measured by podoplanin (PDPN), in the IM of colorectal tumors are less likely to have metastatic invasion [85]. It is possible that high ordered lymphatic vessels facilitate CTL infiltration into the tumor at the edge of the invading tumor where activated CTL function to prevent metastatic dissemination.

3.3.3 Neuromodulators in Tumor Microenvironment

Chronic exposure to hormones, such as norepinephrine, progesterone, glucocorticoids, and androgenic receptor signaling have been linked to tumorgenesis and metastatic invasion of multiple cancer types (reviewed in [86, 87]). Glucocorticoids potential for immunosuppression is harnessed as an anti-inflammatory treatment for uncontrolled immune cells in patients with autoimmune disease [88]. Glucocorticoids are the major immunomodulatory agents used in clinical medicine. However, their actions as anti-inflammatory and immunosuppressive drugs are both beneficial and deleterious. Glucocorticoids induce the expression of chemokine, cytokine, complement family members, and innate immune-related genes, including scavenger and Toll-like receptors [89]. In contrast, glucocorticoids repress the expression of adaptive immune-related genes [90]. Glucocorticoids modulate T helper lymphocyte differentiation by blocking IL-12-induced Stat4 phosphorylation without altering IL-4-induced Stat6 phosphorylation, therefore leading to suppressive action on the Th1 cellular immune response and a shift toward the Th2 humoral immune response [91]. However, glucocorticoids, in addition to their immunosuppressive function, enhance T-lymphocyte responses [92]. Glucocorticoids up-regulate IL-7RA and enhance IL-7-mediated signaling and function. Moreover, IL-7-mediated inhibition of apoptosis is increased in the presence of glucocorticoids, in a concentration-dependent manner, suggesting enhanced cell sensitivity to IL-7 following glucocorticoid exposure. These observations provide a mechanism by which glucocorticoids may also have a positive influence on T lymphocyte survival and function. Norepinephrine has been shown to downregulate expression of MHC class I molecules and co-stimulatory receptors, as well as increase production of IDO by tumor cells via beta-androgenic receptors [93]. Beta-androgenic receptor signaling has also been implicated in enhanced Treg suppression, polarization of macrophages to the M2 phenotype, and increased infiltration of myeloid-derived suppressor cells [94–98]. These hormones can be produced by cells in the tumor microenvironment or enter through the tumor vasculature. Altogether this suggests tumors that develop in conditions of chronic stress leading to hormone release may be preconditioned to an immunosuppressive immune contexture, which might lead to decreased infiltration of CTL.

3.4 Predicting Patients' Response to Treatment

The ultimate goal of predicting patient survival by immune gene signature and by the presence of immune cells in the tumor microenvironment is to accurately determine which personalized treatment will result in optimal tumor regression.

More and more clinical trials are incorporating immune cell quantification by immunohistochemistry, exome sequencing, gene expression, and flow cytometry to delineate why patients respond to therapy.

In the previous section we describe the use of immunogenic mutational analysis of tumors to predict response to CTLA-4 and PD-1 blocking immunotherapy. Recently there have been a number of reports using immunohistochemical analysis to study immune cells, particularly CTL before and after therapy. A study in patients with melanoma investigated the expression of CD8 and PD-1/PD-L1 in the tumor center and invasive margin of tumor biopsies prior to and following treatment with a humanized blocking antibody to PD-1 [99]. Patients exhibiting beneficial response had significantly higher expression of CD8, PD-1, and PD-L1 in their invasive margin before treatment than patients whose tumors progressed following treatment. This data suggests that therapy blocking the PD-1/PD-L1 pathway would be most beneficial to patients that have pre-existing CTL in the tumor microenvironment. Even though one might assume that patients with high density of immune cells would respond better to immunotherapies targeting the immune system, the presence of immune genes and TILs also predicts patients' response to traditional chemotherapies [48, 100–102]. Analysis of colorectal cancer tumors from 1156 stage III patients treated with 5-fluorouracil based chemotherapy found that patients with TILs at the time of treatment had a better survival advantage after treatment than patients lacking TILs [102]. Additionally, two studies in breast cancer compare the predictive value of TILs for three types of therapy, docetaxel, doxorubicin, and trastuxumab [100, 101]. In the first study, patients with HER2-positive breast cancer were compared for high and low density of TILs. Patients with tumors containing high densities of TILs had significantly longer disease-free (5-year 78.6 *vs*. 47%) and overall (5-year 92.9 *vs*. 70.7%) survival after treatment with doxorubicin than patients with low densities of TILs. Interestingly, this was not the case when doxorubicin and docetaxel were administered in conjunction. In another study it was shown that patients with HER2 positive breast cancer and high density TILs had better response to treatment with the anti-HER2 antibody, trastuzumab. These studies portray the importance of characterizing the immune microenvironment of tumors to determine optimal personalized beneficial treatments.

3.5 Conclusions

We ascertain that the evaluation of the CTL densities in primary tumors is a superior method of predicting patient survival for the majority cancer types. Moreover, patients that lack CTL in their primary tumor have the worst clinical prognosis and have tumors that are resistant to CTL killing because CTL are not able to traffic to the tumor site. We propose this is due to the lack of T helper lymphocytes, memory promoting cytokines, and chemoattractants, as well as dysfunctional blood and lymphatic flow preventing CTL from getting into the tumor microenvironment. We also hypothesize that potential immunosuppressive factors from stress-related hormones deters CTL from the tumor. These mechanisms are illustrated in Fig. 3.3. Plainly, if CTL are not present at the tumor site they are not able to eliminate tumor cells.

The difficult question remains, how do we induce CTL trafficking to the tumor for patients with low Immunoscore (I0-I1)? Currently systemic treatment of both recombinant human IL-15 and IL-21 are being tested in clinical trials with favorable results [57, 103]. In addition, IL-15 and IL-21 are being used in combination with adoptive immunotherapy to stimulate CTL *ex vivo* or as supplemental systemic administration. Initial studies using intratumoral injection of membrane-anchored chemokine fusion proteins, including CXCL10, are being used as a method to induce CTL trafficking to the tumor site [104, 105]. Another potential target to improve CTL migration to the tumor is by combination immunotherapy with angiogenesis-inhibitors. Inhibition of angiogenesis improves the organization of the vasculature allowing for better extravasation and migration of CTL into

the tumor [78, 79, 81, 106]. Angiogenesis inhibition has improved the therapeutic efficacy of both adoptive immunotherapy and vaccine-induced anti-tumor immunity. Finally, it will be essential that clinical studies incorporate tumor immune microenvironment analysis, such as Immunoscore, to fully understand the factors managing tumor-specific CTL trafficking to the tumor and quality of response to cancer therapy.

References

1. Angell H, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. Curr Opin Immunol. 2013;25:261–7.
2. Fridman W-H, Pagès F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12:298–306.
3. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity. 2013;39:11–26.
4. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoué F, Bruneval P, Cugnenc P-H, Trajanoski Z, Fridman W-H, Pagès F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313:1960–4.
5. Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, Cugnenc P-H, Trajanoski Z, Fridman W-H, Galon J. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Eng J Med. 2005;353:2654–66.
6. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, Lugli A, Zlobec I, Hartmann A, Bifulco C, Nagtegaal ID, Palmqvist R, Masucci GV, Botti G, Tatangelo F, Delrio P, Maio M, Laghi L, Grizzi F, Asslaber M, D'Arrigo C, Vidal-Vanaclocha F, Zavadova E, Chouchane L, Ohashi PS, Hafezi Bakhtiari S, Wouters BG, Roehrl M, Nguyen L, Kawakami Y, Hazama S, Okuno K, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Wang Y, Kopetz S, Sinicrope FA, Scripcariu V, Ascierto PA, Marincola FM, Fox BA, Pagès F. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J Pathol. 2014;232:199–209.
7. Galon J, Pages F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Mihm M, Vidal-Vanaclocha F, Allison JP, Gnjatic S, Hakansson L, Huber C, Singh-Jasuja H, Ottensmeier C, Zwierzina H, Laghi L, Grizzi F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O'Donnell-Tormey J, Lagorce C, Pawelec G, Nishimura MI, Hawkins R, Lapointe R, Lundqvist A, Khleif SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D'Arrigo C, Kopetz S, Sinicrope FA, Trinchieri G, Gajewski TF, Ascierto PA, Fox BA. Cancer classification using the Immunoscore: a worldwide task force. J Transl Med. 2012;10:205.
8. Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA, Gajewski TF, Ascierto PA. The immune score as a new possible approach for the classification of cancer. J Transl Med. 2012;10:1.
9. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, Bruneval P, Trajanoski Z, Fridman W-H, Pagès F, Galon J. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol Off J Am Soc Clin Oncol. 2011;29:610–8.
10. Pagès F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G, Lagorce C, Wind P, Marliot F, Bruneval P, Zatloukal K, Trajanoski Z, Berger A, Fridman W-H, Galon J. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. J Clin Oncol. 2009;27:5944–51.
11. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. Proc Natl Acad Sci U S A. 2004;101:1969–74.
12. Sun JC, Bevan MJ. Defective CD8 T cell memory following acute infection without CD4 T cell help. Science. 2003;300:339–42.
13. Sprent J, Surh CD. Cytokines and T cell homeostasis. Immunol Lett. 2003;85:145–9.
14. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. Nature. 2003;421:852–6.
15. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, Berger A, Bruneval P, Fridman WH, Pages F, Galon J. Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. Cancer Res. 2011;71:1263–71.
16. Wu D, Wu P, Huang Q, Liu Y, Ye J, Huang J. Interleukin-17: a promoter in colorectal cancer progression. Clin Dev Immunol. 2013;2013:436307.
17. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis

via activation of T helper type 17 T cell responses. Nat Med. 2009;15:1016–22.
18. Chung AS, Wu X, Zhuang G, Ngu H, Kasman I, Zhang J, Vernes JM, Jiang Z, Meng YG, Peale FV, Ouyang W, Ferrara N. An interleukin-17-mediated paracrine network promotes tumor resistance to anti-angiogenic therapy. Nat Med. 2013;19:1114–23.
19. Liu J, Duan Y, Cheng X, Chen X, Xie W, Long H, Lin Z, Zhu B. IL-17 is associated with poor prognosis and promotes angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma. Biochem Biophys Res Commun. 2011;407:348–54.
20. Liu T, Peng L, Yu P, Zhao Y, Shi Y, Mao X, Chen W, Cheng P, Wang T, Chen N, Zhang J, Liu X, Li N, Guo G, Tong W, Zhuang Y, Zou Q. Increased circulating Th22 and Th17 cells are associated with tumor progression and patient survival in human gastric cancer. J Clin Immunol. 2012;32:1332–9.
21. He S, Fei M, Wu Y, Zheng D, Wan D, Wang L, Li D. Distribution and clinical significance of Th17 cells in the tumor microenvironment and peripheral blood of pancreatic cancer patients. Int J Mol Sci. 2011;12:7424–37.
22. Galand C, Donnou S, Crozet L, Brunet S, Touitou V, Ouakrim H, Fridman WH, Sautes-Fridman C, Fisson S. Th17 cells are involved in the local control of tumor progression in primary intraocular lymphoma. PLoS One. 2011;6:e24622.
23. Hiraoka K, Miyamoto M, Cho Y, Suzuoki M, Oshikiri T, Nakakubo Y, Itoh T, Ohbuchi T, Kondo S, Katoh H. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. Br J Cancer. 2006;94:275–80.
24. Cho Y, Miyamoto M, Kato K, Fukunaga A, Shichinohe T, Kawarada Y, Hida Y, Oshikiri T, Kurokawa T, Suzuoki M, Nakakubo Y, Hiraoka K, Murakami S, Shinohara T, Itoh T, Okushiba S, Kondo S, Katoh H. CD4+ and CD8+ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res. 2003;63:1555–9.
25. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer Res. 2010;70:8368–77.
26. Wong SB, Bos R, Sherman LA. Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells. J Immunol. 2008;180:3122–31.
27. Church SE, Jensen SM, Antony PA, Restifo NP, Fox BA. Tumor-specific CD4+ T cells maintain effector and memory tumor-specific CD8+ T cells. Eur J Immunol. 2014;44:69–79.
28. Jin P, Civini S, Zhao Y, De Giorgi V, Ren J, Sabatino M, Jin J, Wang H, Bedognetti D, Marincola F, Stroncek D. Direct T cell-tumour interaction triggers TH1 phenotype activation through the modification of the mesenchymal stromal cells transcriptional programme. Br J Cancer. 2014;110:2955–64.
29. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol. 2009;27:186–92.
30. Adams SF, Levine DA, Cadungog MG, Hammond R, Facciabene A, Olvera N, Rubin SC, Boyd J, Gimotty PA, Coukos G. Intraepithelial T cells and tumor proliferation: impact on the benefit from surgical cytoreduction in advanced serous ovarian cancer. Cancer. 2009;115:2891–902.
31. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10:942–9.
32. Merlo A, Casalini P, Carcangiu ML, Malventano C, Triulzi T, Menard S, Tagliabue E, Balsari A. FOXP3 expression and overall survival in breast cancer. J Clin Oncol. 2009;27:1746–52.
33. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol. 2007;25:2586–93.
34. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102:18538–43.
35. Schmitt N, Ueno H. Human T follicular helper cells: development and subsets. Adv Exp Med Biol. 2013;785:87–94.
36. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman W-H, Becker C, Pagès F, Speicher MR, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39:782–95.
37. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, Ravoet M, Le Buanec H, Sibille C, Manfouo-Foutsop G, Veys I, Haibe-Kains B, Singhal SK, Michiels S, Rothe F, Salgado R, Duvillier H, Ignatiadis M, Desmedt C, Bron D, Larsimont D, Piccart M, Sotiriou C, Willard-Gallo K. CD4(+) follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest. 2013;123:2873–92.
38. de Chaisemartin L, Goc J, Damotte D, Validire P, Magdeleinat P, Alifano M, Cremer I, Fridman WH, Sautes-Fridman C, Dieu-Nosjean MC. Characterization of chemokines and adhesion molecules associated with T cell presence in tertiary

lymphoid structures in human lung cancer. Cancer Res. 2011;71:6391–9.
39. Messina JL, Fenstermacher DA, Eschrich S, Qu X, Berglund AE, Lloyd MC, Schell MJ, Sondak VK, Weber JS, Mule JJ. 12-chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? Sci Rep. 2012;2:765.
40. Coppola D, Nebozhyn M, Khalil F, Dai H, Yeatman T, Loboda A, Mule JJ. Unique ectopic lymph node-like structures present in human primary colorectal carcinoma are identified by immune gene array profiling. Am J Pathol. 2011;179:37–45.
41. Mlecnik B, Tosolini M, Charoentong P, Kirilovsky A, Bindea G, Berger A, Camus M, Gillard M, Bruneval P, Fridman W-H, Pagès F, Trajanoski Z, Galon J. Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology. 2010;138:1429–40.
42. Umehara H, Bloom E, Okazaki T, Domae N, Imai T. Fractalkine and vascular injury. Trends Immunol. 2001;22:602–7.
43. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol. 2002;168:3195–204.
44. Romagnani P, Annunziato F, Lazzeri E, Cosmi L, Beltrame C, Lasagni L, Galli G, Francalanci M, Manetti R, Marra F, Vanini V, Maggi E, Romagnani S. Interferon-inducible protein 10, monokine induced by interferon gamma, and interferon-inducible T-cell alpha chemoattractant are produced by thymic epithelial cells and attract T-cell receptor (TCR) alphabeta+ CD8+ single-positive T cells, TCRgammadelta+ T cells, and natural killer-type cells in human thymus. Blood. 2001;97:601–7.
45. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, McKee M, Gajewski TF. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res. 2009;69:3077–85.
46. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41:529–42.
47. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, Pfitzner BM, Salat C, Loi S, Schmitt WD, Schem C, Fisch K, Darb-Esfahani S, Mehta K, Sotiriou C, Wienert S, Klare P, Andre F, Klauschen F, Blohmer JU, Krappmann K, Schmidt M, Tesch H, Kummel S, Sinn P, Jackisch C, Dietel M, Reimer T, Untch M, Loibl S. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol. 2015;33:983–91.
48. Stoll G, Enot D, Mlecnik B, Galon J, Zitvogel L, Kroemer G. Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. Oncoimmunology. 2014;3:e27884.
49. Razis E, Kalogeras KT, Kotoula V, Eleftheraki AG, Nikitas N, Kronenwett R, Timotheadou E, Christodoulou C, Pectasides D, Gogas H, Wirtz RM, Makatsoris T, Bafaloukos D, Aravantinos G, Televantou D, Pavlidis N, Fountzilas G. Improved outcome of high-risk early HER2 positive breast cancer with high CXCL13-CXCR5 messenger RNA expression. Clin Breast Cancer. 2012;12:183–93.
50. Kaplan DH, Shankaran V, Dighe AS, Stockert E, Aguet M, Old LJ, Schreiber RD. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A. 1998;95:7556–61.
51. Delahaye NF, Rusakiewicz S, Martins I, Menard C, Roux S, Lyonnet L, Paul P, Sarabi M, Chaput N, Semeraro M, Minard-Colin V, Poirier-Colame V, Chaba K, Flament C, Baud V, Authier H, Kerdine-Romer S, Pallardy M, Cremer I, Peaudecerf L, Rocha B, Valteau-Couanet D, Gutierrez JC, Nunes JA, Commo F, Bonvalot S, Ibrahim N, Terrier P, Opolon P, Bottino C, Moretta A, Tavernier J, Rihet P, Coindre JM, Blay JY, Isambert N, Emile JF, Vivier E, Lecesne A, Kroemer G, Zitvogel L. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat Med. 2011;17:700–7.
52. Duncan TJ, Rolland P, Deen S, Scott IV, Liu DT, Spendlove I, Durrant LG. Loss of IFN gamma receptor is an independent prognostic factor in ovarian cancer. Clin Cancer Res. 2007;13:4139–45.
53. Lee JE, Abdalla J, Porter GA, Bradford L, Grimm EA, Reveille JD, Mansfield PF, Gershenwald JE, Ross MI. Presence of the human leukocyte antigen class II gene DRB1*1101 predicts interferon gamma levels and disease recurrence in melanoma patients. Ann Surg Oncol. 2002;9:587–93.
54. Mlecnik B, Bindea G, Angell HK, Sasso MS, Obenauf AC, Fredriksen T, Lafontaine L, Bilocq AM, Kirilovsky A, Tosolini M, Waldner M, Berger A, Fridman W-H, Rafii A, Valge-Archer V, Pagès F, Speicher MR, Galon J. Functional network pipeline reveals genetic determinants associated with in situ lymphocyte proliferation and survival of cancer patients. Sci Transl Med. 2014;6:228ra237.
55. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol. 2009;9:480–90.
56. Wilkinson PC, Liew FY. Chemoattraction of human blood T lymphocytes by interleukin-15. J Exp Med. 1995;181:1255–9.
57. Jakobisiak M, Golab J, Lasek W. Interleukin 15 as a promising candidate for tumor immunotherapy. Cytokine Growth Factor Rev. 2011;22:99–108.
58. Teague RM, Sather BD, Sacks JA, Huang MZ, Dossett ML, Morimoto J, Tan X, Sutton SE, Cooke MP, Ohlén C, Greenberg PD. Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. Nat Med. 2006;12:335–41.

59. Oh S, Perera LP, Terabe M, Ni L, Waldmann TA, Berzofsky JA. IL-15 as a mediator of CD4+ help for CD8+ T cell longevity and avoidance of TRAIL-mediated apoptosis. Proc Natl Acad Sci U S A. 2008;105:5201–6.
60. Yang T, Wall EM, Milne K, Theiss P, Watson P, Nelson BH. CD8+ T cells induce complete regression of advanced ovarian cancers by an interleukin (IL)-2/IL-15 dependent mechanism. Clin Cancer Res. 2007;13:7172–80.
61. Petrella TM, Tozer R, Belanger K, Savage KJ, Wong R, Smylie M, Kamel-Reid S, Tron V, Chen BE, Hunder NN, Hagerman L, Walsh W, Eisenhauer EA. Interleukin-21 has activity in patients with metastatic melanoma: a phase II study. J Clin Oncol. 2012;30:3396–401.
62. Lennerz V, Fatho M, Gentilini C, Frye RA, Lifke A, Ferel D, Wolfel C, Huber C, Wolfel T. The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc Natl Acad Sci U S A. 2005;102:16013–8.
63. Gubin MM, Zhang X, Schuster H, Caron E, Ward JP, Noguchi T, Ivanova Y, Hundal J, Arthur CD, Krebber WJ, Mulder GE, Toebes M, Vesely MD, Lam SS, Korman AJ, Allison JP, Freeman GJ, Sharpe AH, Pearce EL, Schumacher TN, Aebersold R, Rammensee HG, Melief CJ, Mardis ER, Gillanders WE, Artyomov MN, Schreiber RD. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature. 2014;515:577–81.
64. Matsushita H, Vesely MD, Koboldt DC, Rickert CG, Uppaluri R, Magrini VJ, Arthur CD, White JM, Chen YS, Shea LK, Hundal J, Wendl MC, Demeter R, Wylie T, Allison JP, Smyth MJ, Old LJ, Mardis ER, Schreiber RD. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature. 2012;482:400–4.
65. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348:69–74.
66. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL, Boyault S, Burkhardt B, Butler AP, Caldas C, Davies HR, Desmedt C, Eils R, Eyfjord JE, Foekens JA, Greaves M, Hosoda F, Hutter B, Ilicic T, Imbeaud S, Imielinski M, Jager N, Jones DT, Jones D, Knappskog S, Kool M, Lakhani SR, Lopez-Otin C, Martin S, Munshi NC, Nakamura H, Northcott PA, Pajic M, Papaemmanuil E, Paradiso A, Pearson JV, Puente XS, Raine K, Ramakrishna M, Richardson AL, Richter J, Rosenstiel P, Schlesner M, Schumacher TN, Span PN, Teague JW, Totoki Y, Tutt AN, Valdes-Mas R, van Buuren MM, van 't Veer L, Vincent-Salomon A, Waddell N, Yates LR, Zucman-Rossi J, Futreal PA, McDermott U, Lichter P, Meyerson M, Grimmond SM, Siebert R, Campo E, Shibata T, Pfister SM, Campbell PJ, Stratton MR. Signatures of mutational processes in human cancer. Nature. 2013;500:415–21.
67. Brown SD, Warren RL, Gibb EA, Martin SD, Spinelli JJ, Nelson BH, Holt RA. Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. Genome Res. 2014;24:743–50.
68. Snyder A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade. N Engl J Med. 2015;372:783.
69. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015;348:124–8.
70. Angelova M, Charoentong P, Hackl H, Fischer ML, Snajder R, Krogsdam AM, Waldner MJ, Bindea G, Mlecnik B, Galon J, Trajanoski Z. Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. Genome Biol. 2015;16:64.
71. Atkins D, Breuckmann A, Schmahl GE, Binner P, Ferrone S, Krummenauer F, Storkel S, Seliger B. MHC class I antigen processing pathway defects, ras mutations and disease stage in colorectal carcinoma. Int J Cancer. 2004;109:265–73.
72. Lal N, Beggs AD, Willcox BE, Middleton GW. An immunogenomic stratification of colorectal cancer: implications for development of targeted immunotherapy. Oncoimmunology. 2015;4:e976052.
73. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, Mikse OR, Cherniack AD, Beauchamp EM, Pugh TJ, Wilkerson MD, Fecci PE, Butaney M, Reibel JB, Soucheray M, Cohoon TJ, Janne PA, Meyerson M, Hayes DN, Shapiro GI, Shimamura T, Sholl LM, Rodig SJ, Freeman GJ, Hammerman PS, Dranoff G, Wong KK. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer Discov. 2013;3:1355–63.
74. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, Matsumoto K, Takayama K, Takamori S, Kage M, Hoshino T, Nakanishi Y, Okamoto I. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. Ann Oncol. 2014;25:1935–40.
75. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell. 2015;160:48–61.
76. Bindea G, Mlecnik B, Angell HK, Galon J. The immune landscape of human tumors: implications for cancer immunotherapy. Oncoimmunology. 2014;3:e27456.
77. Folkman J. Angiogenesis. Annu Rev Med. 2006;57:1–18.
78. Motz GT, Coukos G. The parallel lives of angiogenesis and immunosuppression: cancer and other tales. Nat Rev Immunol. 2011;11:702–11.

79. Griffioen AW, Damen CA, Blijham GH, Groenewegen G. Tumor angiogenesis is accompanied by a decreased inflammatory response of tumor-associated endothelium. Blood. 1996;88:667–73.
80. Melero I, Rouzaut A, Motz GT, Coukos G. T-cell and NK-cell infiltration into solid tumors: a key limiting factor for efficacious cancer immunotherapy. Cancer Discov. 2014;4:522–6.
81. Griffioen AW. Anti-angiogenesis: making the tumor vulnerable to the immune system. Cancer Immunol Immunother. 2008;57:1553–8.
82. Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. Cell. 2010;140:460–76.
83. Swartz MA, Lund AW. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. Nat Rev Cancer. 2012;12:210–9.
84. Martinet L, Garrido I, Filleron T, Le Guellec S, Bellard E, Fournie JJ, Rochaix P, Girard JP. Human solid tumors contain high endothelial venules: association with T- and B-lymphocyte infiltration and favorable prognosis in breast cancer. Cancer Res. 2011;71:5678–87.
85. Mlecnik B, Bindea G, Kirilovsky A, Angell HK, Obenauf AC, Tosolini M, Church SE, Maby P, Vasaturo A, Angelova M, Fredriksen T, Mauger S, Waldner M, Berger A, Speicher MR, Pages F, Valge-Archer V, Galon J. The tumor microenvironment and Immunoscore are critical determinants of dissemination to distant metastasis. Sci Transl Med. 2016;8(327):327ra26.
86. Eng JW, Kokolus KM, Reed CB, Hylander BL, Ma WW, Repasky EA. A nervous tumor microenvironment: the impact of adrenergic stress on cancer cells, immunosuppression, and immunotherapeutic response. Cancer Immunol Immunother. 2014;63:1115–28.
87. Garcia SB, Stopper H, Kannen V. The contribution of neuronal-glial-endothelial-epithelial interactions to colon carcinogenesis. Cell Mol Life Sci. 2014;71:3191–7.
88. Coutinho AE, Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol. 2011;335:2–13.
89. Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat Rev Immunol. 2006;6:318–28.
90. Galon J, Franchimont D, Hiroi N, Frey G, Boettner A, Ehrhart-Bornstein M, O'Shea JJ, Chrousos GP, Bornstein SR. Gene profiling reveals unknown enhancing and suppressive actions of glucocorticoids on immune cells. FASEB J. 2002;16:61–71.
91. Franchimont D, Galon J, Gadina M, Visconti R, Zhou Y, Aringer M, Frucht DM, Chrousos GP, O'Shea JJ. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. J Immunol. 2000;164:1768–74.
92. Franchimont D, Galon J, Vacchio MS, Fan S, Visconti R, Frucht DM, Geenen V, Chrousos GP, Ashwell JD, O'Shea JJ. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. J Immunol. 2002;168:2212–8.
93. Wang L, Liu H, Chen X, Zhang M, Xie K, Ma Q. Immune sculpting of norepinephrine on MHC-I, B7-1, IDO and B7-H1 expression and regulation of proliferation and invasion in pancreatic carcinoma cells. PLoS One. 2012;7:e45491.
94. Bernard AC, Fitzpatrick EA, Maley ME, Gellin GL, Tsuei BJ, Arden WA, Boulanger BR, Kearney PA, Ochoa JB. Beta adrenoceptor regulation of macrophage arginase activity. Surgery. 2000;127:412–8.
95. Cosentino M, Fietta AM, Ferrari M, Rasini E, Bombelli R, Carcano E, Saporiti F, Meloni F, Marino F, Lecchini S. Human CD4+CD25+ regulatory T cells selectively express tyrosine hydroxylase and contain endogenous catecholamines subserving an autocrine/paracrine inhibitory functional loop. Blood. 2007;109:632–42.
96. Flierl MA, Rittirsch D, Nadeau BA, Sarma JV, Day DE, Lentsch AB, Huber-Lang MS, Ward PA. Upregulation of phagocyte-derived catecholamines augments the acute inflammatory response. PLoS One. 2009;4:e4414.
97. Guereschi MG, Araujo LP, Maricato JT, Takenaka MC, Nascimento VM, Vivanco BC, Reis VO, Keller AC, Brum PC, Basso AS. Beta2-adrenergic receptor signaling in CD4+ Foxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner. Eur J Immunol. 2013;43:1001–12.
98. Qin JF, Jin FJ, Li N, Guan HT, Lan L, Ni H, Want Y. Adrenergic receptor B2 activation by stress promotes breast cancer progression through macrophages M2 polarization in tumor microenvironment. BMB Rep. 2015;48(5):295–300.
99. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, Chmielowski B, Spasic M, Henry G, Ciobanu V, West AN, Carmona M, Kivork C, Seja E, Cherry G, Gutierrez AJ, Grogan TR, Mateus C, Tomasic G, Glaspy JA, Emerson RO, Robins H, Pierce RH, Elashoff DA, Robert C, Ribas A. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515:568–71.
100. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, Kellokumpu-Lehtinen PL, Bono P, Kataja V, Desmedt C, Piccart MJ, Loibl S, Denkert C, Smyth MJ, Joensuu H, Sotiriou C. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. Ann Oncol. 2014;25:1544–50.
101. Loi S, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, Rouas G, Francis P, Crown JP, Hitre E, de Azambuja E, Quinaux E, Di Leo A, Michiels S, Piccart MJ, Sotiriou C. Prognostic and predic-

tive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol. 2013;31:860–7.
102. Morris M, Platell C, Iacopetta B. Tumor-infiltrating lymphocytes and perforation in colon cancer predict positive response to 5-fluorouracil chemotherapy. Clin Cancer Res. 2008;14:1413–7.
103. Santegoets SJ, Turksma AW, Powell DJ Jr, Hooijberg E, de Gruijl TD. IL-21 in cancer immunotherapy: at the right place at the right time. Oncoimmunology. 2013;2:e24522.
104. Nelson PJ, Muenchmeier N. Membrane-anchored chemokine fusion proteins: a novel class of adjuvants for immunotherapy. Oncoimmunology. 2013;2:e26619.
105. Muenchmeier N, Boecker S, Bankel L, Hinz L, Rieth N, Lapa C, Mendler AN, Noessner E, Mocikat R, Nelson PJ. A novel CXCL10-based GPI-anchored fusion protein as adjuvant in NK-based tumor therapy. PLoS One. 2013;8:e72749.
106. Shrimali RK, Yu Z, Theoret MR, Chinnasamy D, Restifo NP, Rosenberg SA. Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. Cancer Res. 2010;70:6171–80.

4 The Role of Tumor Microenvironment in Cancer Immunotherapy

Timothy Frankel, Mirna Perusina Lanfranca, and Weiping Zou

4.1 Introduction

In the past decade, immunotherapy has undergone a metamorphosis, transforming from complex experimental protocols to "off-the-shelf" first line therapy for many previously untreatable malignancies. Unlike traditional chemotherapeutics which target biologic processes of cancer cells, immunotherapy seeks to boost the bodies natural immunologic defense against cancer [1, 2]. This is accomplished by either training resident immune cells to recognize and eliminate cells bearing tumor specific antigens, providing external stimuli to enhance immune mediated tumor cell lysis or abrogating signals directed by tumor cells to dampen immune responsiveness. Both cellular and molecular components of the tumor microenvironment (TME) can serve to impair the efficacy of immunotherapy and strategies to abrogate this are the source of on-going research [3].

The principle components of immune based tumor ablation are T-lymphocytes (T-cells) and natural killer cells (NK cells), which target cells by antigen specific and non-specific means, respectively. The process of antigen specific immune recognition is a complex one that involves orchestrated steps from both the cell presenting the antigen and the lymphocyte which recognizes it. Self and foreign proteins are digested by proteasomes in the cytoplasm to form 8 to 9 amino acid peptides which are transported to the endoplasmic reticulum. They are loaded by chaperones onto major histocompatibility complex (MHC) class I proteins and the pair is transported to the cell surface for display. Once on the surface, a T-cell bearing a receptor specific for a given MHC class I-peptide complex can bind and with the help of co-stimulatory molecules, trigger activation of the immune cell. This physiologic process is often dysfunctional within the TME as cells lose key components of antigen breakdown and processing rendering cancers effectively hidden to immune cells [4]. An example of this is the defect in MHC surface expression and antigen

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display on dendritic cells in the TME of head and neck cancers and other malignancies [5, 6].

In addition to impaired processing and display, production of antigen themselves can be down-regulated by epigenetic silencing in the TME [7]. Alterations in DNA promoter methylation and histone modifications have been implicated in repression of expression of key tumor specific antigens including cancer-testes antigens [8, 9]. Efforts to enhance immunotherapy by altering epigenetic pathways with the goal of enhancing antigen expression have been met with variable success [10–12].

The theory of immune surveillance posits that the body is composed of near countless numbers of T-cells with a vast array of receptors capable of recognizing a wide variety of infectious and cancerous antigens and initiating immune activation [13, 14]. When activated by either a primary transformed cell or via an intermediary cell such as an antigen-presenting cell (APC) within the TME, T-cells begin a cascade of signaling events that results in recruitment of cellular and non-cellular immune components, clonal expansion of antigen specific T-cells and release of stimulatory cytokines. The end result is a local accumulation of pro-inflammatory cells and destruction of the cancerous or infected cell. We now know, however, that despite this complex network of cellular signaling, tumor cells often evade immune detection leading to growth and eventual spread [15–22].

Failure of immune-editing can be attributed to one of five phenomena: (1) lack of recognition by T-cell receptors (TCR) [23, 24], (2) lack of sufficient activation in response to T-cell recognition, (3) failure of clonal expansion of antigen specific T-cells [25], (4) suppression of immune activation by tumor bearing inhibitors of co-stimulation [26], and (5) repression of activation by inhibitory immune cells within the TME [27, 28]. In this chapter we will discuss strategies developed to overcome these failures with the aim of subverting immunosuppression and enhancing the immunologic destruction of cancer cells [29–32].

4.2 History

The birth of modern day immunotherapy traces back to renowned New York surgeon William B. Coley in the early 1890s [33, 34]. He inoculated the unresectable sarcoma of a young man with cultures of erysipelas and noted a dramatic reduction in tumor size [35]. Subsequent to this, he created a mixture of filtered bacteria and lysates composed primarily of *Streptococcus pyogenes and Bacillus prodigiosus* which he termed "Coley's Toxins." In 1893, he published a report of 10 patients treated with his concoction, many of whom experienced tumor reduction [36]. An interesting observation was that, anecdotally, the severity of infectious symptoms seemed to correlate with degree of response. Over the next four decades, Dr. Coley treated close to 1000 patients with his toxin and reported a 10% complete response rate [37]. While his results were unprecedented, they were met with significant skepticism. Many in the scientific and medical communities derided his lack of supporting mechanistic data and noted his therapy was associated with significant toxicity and results difficult to reproduce. These same criticisms would plague immunotherapy research over the next century. Despite this, Dr. Coley is considered by most to be the father of modern immunotherapy [38].

The field of tumor immunology stalled over the next three decades as scientists failed to consistently demonstrate immune specific rejection of transplantable tumors. This led to the statement of Dr. William Woglom in 1929 that "it would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancer." [39] This concept was fortified by the work of Frank Burnet who in 1948 published his theory of self-tolerance and thymic deletion. In it, he described how lymphocytes that were capable of recognizing self antigens were deleted in prenatal life during immunologic development [40].

The field of cancer immunotherapy, which seemed defeated at this point, underwent a resurrection in the 1950s with the discovery that carcinogen induced tumors could effectively

immunize mice against re-challenge with the same syngeneic tumor [41, 42]. In a classic experiment, Prehn and colleagues induced formation of sarcoma in mice by treatment with the carcinogen methyl-cholanthrene (MCA). Tumors were then removed and after recovery, the same tumor cells transplanted back to the mice. Tumors failed to establish in those mice that had previously harbored malignancy. Researchers suggested that there must be antigens present on tumor cells that are not expressed by the host [42]. This ushered in the concept of tumor specific or associated antigens (TAA) that could be recognized by the host immune system.

In the late 1950s, theories emerged that the immune system is not only involved in tumor rejection, but that one of the principle roles of lymphocytes is to troll through the microenvironment deleting transformed cells [43]. This theory of "immune-surveillance" was met by harsh criticism and essentially dismissed, as many pointed to obvious flaws such as the observation that immunodeficient mice were no more prone to develop tumors than their immunocompetent counterparts [44, 45] and emerging data that previously reported tumor immunity may have been virally mediated [46]. It wasn't until the 1980s that that the field experienced a re-birth with the discovery of auto-reactive T-cells in the periphery which had evaded thymic deletion and technologic advancements allowed for the discovery of scores of tumor specific antigens [47, 48]. Thus the modern era of immunotherapy was born with a focus on identifying ways to heighten the capabilities of dormant immune cells to eradicate tumors.

4.3 Active Immunotherapy

Active immunity is defined as immunologic recognition and protection using the body's resident antibodies or lymphocytes. It comes following exposure to antigen and typically takes days to weeks to develop, but lasts a lifetime. *Natural* active immunity is achieved during exposure over the course of ones life to antigens such as viral proteins and confers protection against future infection. For example, once infected with the hepatitis B virus, the active immune response to surface and core antigens allows for clearance of the virus and lifelong immunity to re-infection [49, 50]. Contrary to this, *acquired* active immunity is accomplished by forced exposure to typically non-infective or minimally infective antigens such as the hepatitis B vaccine. After repeated exposure to portions of the hepatitis B surface antigens, active immunity is achieved without the need for systemic infection [50].

There are many factors present in the TME which serve to counteract active immunity [51]. Both natural and acquired immunity can be derailed if "primed" immune cells fail to reach their target or are suppressed by tolerizing cells or molecules. While certain immunogenic tumors such as melanoma and renal cell carcinoma express high levels of lymphocytic homing chemokines such as CCL2 [52] others release signals into the TME which actively suppress immune cell infiltration rendering active immunotherapy futile [53].

If educated antigen specific cytotoxic lymphocytes are able to infiltrate tumors, they face yet another hurdle in the form of immunosuppressive cells within the TME. Whereas some tumors secrete molecules to block lymphocytic infiltration, others attract a specific subset, T regulatory cells, which function to suppress cytotoxicity [20, 54]. Disproportionally high quantities of T-regs with the TME have been identified in multiple tumor types including breast [55], melanoma [56], and ovarian cancer [20]. Regulatory T-cells are directly implicated in suppressing the effects of active immunotherapy [57, 58] and efforts to remove them from the local microenvironment have shown promise in improving the efficacy of treatment [59].

4.3.1 Cancer Vaccines

The principle forms of acquired active immunotherapy for cancer treatment that are currently used or under development are vaccine-based. The premise behind vaccination is that T-cells specific for any one tumor antigen are present in such low numbers within the body that they are

unlikely to encounter tumor cells and trigger an immune response [60, 61]. By providing an antigen in high quantity or more importantly with high affinity for receptor binding, immune activation and expansion can be artificially triggered [62]. Vaccines come in various platforms and can be categorized as peptides, DNA-based or dendritic cell/APC derived. Although they use different mechanisms for T-cell activation, their central premise is display of a TAA which can vary from tumor specific overexpressed self-antigens, mutated antigens or cancer-testis antigens [62].

Peptide vaccines are perhaps the simplest form of active immunization and have been studied extensively since the mid-1990s [63–65]. MHC class 1 restricted peptides are delivered with the hope that they will be displayed on cell surfaces and encountered by cognate TCRs triggering activation. Peptide vaccines are often administered with immune stimulatory compounds such as Freund's adjuvant or cytokines to heighten the immune response [66]. The benefit of this approach is that they are relatively inexpensive to produce in large scale, pose little biologic risk to patients and can be administered with other peptide vaccines increasing likelihood of immune activation. While animal studies showed this strategy to be efficacious, multiple human trials have failed to reveal significant treatment effect [67, 68]. Despite documented ability to expand antigen specific T-cells, peptide vaccination alone leads to only a 2–4% objective response in patients with metastatic melanoma [69, 70]. This phenomenon highlights the complexity of tumor-immune cell interaction and was the impetus for strategies to enhance activation after antigen recognition.

An obvious shortcoming of peptide based vaccine therapy is the assumption that the chosen amino acid sequence is the optimal one for immune activation. DNA vaccines correct this by introducing a plasmid encoding the entire TAA into APCs [71]. The plasmids consist of the gene for the antigen of interest as well as a mammalian promoter that drives its expression. After the plasmid is injected subcutaneously or intramuscularly, it is taken up by resident APCs and the gene is transcribed in the nucleus [72]. The standard cellular machinery then processes the resultant protein and all relevant peptides are displayed on the cell surface for recognition by passing immune surveyors. Despite its ability to process and display multiple epitopes from a single antigen, DNA vaccines suffer from low transduction efficiency and a relative lack of immunogenicity in large mammals such as humans [73].

Use of APCs, particularly dendritic cells (DC), as a vehicle to present antigen has many theoretical advantages. As "professional" antigen presenting cells, they possess the machinery and more importantly the co-stimulatory molecules to produce profound activation of the immune system. DCs can be generated ex vivo from bone marrow stem cells or monocytes using a cocktail of cytokines, pulsed with tumor specific antigens and then re-infused [74]. They can also be transfected with TAA expressing plasmids or tumor genomic DNA to allow for expression of a wider variety of epitopes and antigens. A trial in metastatic prostate cancer demonstrated that pulsation of DCs with a prostatic acid phosphatase-GM-CSF fusion protein followed by re-infusion resulted in a significantly improved 3 year survival, leading to its approval by the FDA in 2010 [75]. Despite its theoretical advantage, most trials of DC based vaccination have been met with disappointing results with few reporting better than 15% overall response rates [76, 77].

Despite countless trials and preparations, there has been only marginal success with active immunotherapy with only three vaccines currently approved by the FDA [78]. Two of these are prophylactic treatments of viruses linked to cancer formation. Vaccination against the hepatitis B virus prevents chronic infection which subsequently reduces the risk of hepatocellular carcinoma [79]. A polyvalent vaccine against the human papilloma virus, has been shown to prevent infection with the most carcinogenic forms of the virus thereby reducing long-term development of cervical cancer [80]. The third, Sipuleucel-T for the treatment of prostate cancer as mentioned above, is the sole vaccine approved for the treatment, not prevention, of cancer [75].

4.3.2 Interleukin-2

The ability of vaccines to produce tumor specific T-cells without significant tumor reduction led many to believe that it was not the lack of recognition, but insufficient activation that was at the heart of failed immuno-editing. Discovered in 1976, interleukin-2 (IL-2) is capable of inducing growth and activation of bone marrow derived T-cells [81]. In the early 1980s, IL-2 was found to be capable of promoting cytotoxic T-cell expansion in vivo and enhancing their tumor lytic abilities transforming dormant cells into lymphokine activated killer cells (LAK) [82, 83]. In a sentinel paper, Rosenberg et al. demonstrated that delivery of high dose IL-2 to mice with metastatic sarcoma to the lungs resulted in profound tumor regression [84]. Immunohistochemical analysis of lung sections 6 days after treatment revealed a massive infiltration of cytotoxic T-cells in the pulmonary interstitium. These promising in vivo findings led to the first clinical trial of IL-2 for the treatment of metastatic cancer. Published in 1986, Lotze and colleagues at the National Cancer Institute treated 10 patients with metastases from various tumor types with increasing doses of IL-2 [85]. Of the ten participants, only those with metastatic melanoma (n = 6) showed tumor response with 50% of patients demonstrating clinically significant tumor reduction. The toxicity of the therapy was dramatic with many patients experiencing renal and respiratory failure, infection and mental status changes harking back to times of Coley's toxin [85]. Like the murine models, biopsies of tumors revealed a profound infiltration with cytotoxic lymphocytes and active tumor necrosis. In follow-up studies of patients with metastatic melanoma and renal cell carcinoma, overall and complete response rates were 17% and 7% and 20% and 7%, respectively. These studies resulted in approval by the FDA of IL-2 for the treatment of renal cell carcinoma in 1992 and melanoma in 1998 introducing the era of non-specific stimulation based immunotherapy [86].

While it remains impossible to predict which patients will respond to IL-2 therapy, important differences observed within the TME after treatment shed light on the potential mechanisms of action of IL-2. Tumors with high prevalence of infiltrating immune cells within the tumor tend to respond better to therapy than those without [87]. It has also been suggested that the presence of T-reg cells within the TME may predict failure of IL-2 therapy [88], but this requires more dedicated research.

4.4 Passive Immunotherapy

While active immunity entails training the body's natural defenses to better recognize pathogens and transformed cells, passive immunity simply delivers the end effectors in the form of antibodies (humoral passive immunity) or cytotoxic cells (adoptive cell transfer). The theoretical advantage of passive immunity is that it avoids potential shortcomings innately present in antigen processing and immune cell recognition to achieve the desired effect. In the case of adoptive cell transfer (ACT) is also allows for ex vivo cellular manipulation and stimulation, decreasing the systemic toxicities such as those experienced following IL-2 administration. The disadvantage of passive immunotherapy is that the effectors are often short-lived limiting their ability to provide long-term remission.

4.4.1 Humoral Immunotherapy

The passive transfer of antibodies for the treatment of disease has existed for over a century with the discovery that "anti-toxins" to diphtheria and tetanus could considerably ameliorate symptoms of the infection. The serum of immunized horses was injected into patients with tetanus, neutralizing the toxin and preventing disease dissemination [89]. Antibody based treatment for cancer can be divided into unconjugated and conjugated groups.

Unconjugated or "naked" antibodies function by binding to cancer cells and either alerting the immune system or interfering with cell signaling. An example of the former is *alemtuzumab*, a monoclonal antibody used to treat chronic lym-

phocytic leukemia (CLL) by binding to CD52 present on lymphocytes targeting them for immune clearance [90]. *Trastuzumab* is a monoclonal antibody which binds the overactive HER2/neu receptor decreasing its signaling and subsequently cell growth. Use of this antibody in overexpressing HER2/neu breast and gastric cancers improves both overall and disease specific survival [91, 92].

Conjugated antibodies utilize the specificity of the variable region to deliver toxic cargo to cancer cells. *Ibritumomab-tiuxetan,* used to treat non-Hodgkin's lymphoma, is a radio-labeled antibody which binds to the B-lymphocyte specific marker CD20 delivering its radioactive payload, inducing cell death [93]. Other antibody-based strategies involve fusing chemotherapeutics to antibodies, better directing their delivery and increasing efficacy while limiting off-target side effects. *Ado-trastuzumab emtansine* fuses the same anti HER2/neu monoclonal antibody mentioned previously to the cytotoxic chemotherapy DM1 which, upon binding, enters the cell disrupting tubulin and promoting cell death [94].

4.4.2 Adoptive Cell Transfer (ACT)

The fundamental principle of ACT is removal of cytotoxic lymphocytes from the body to allow for ex vivo expansion and activation followed by re-infusion [95]. It accomplishes the goals of T-cell immunization without relying on unpredictable factors such as antigen processing and presentation and T-cell recognition and activation. Cells can be manipulated with either cytokines or genetic modification without the need for systemic administration and exposure, thereby limiting off target effects [96].

First proposed in the mid 1950s, Mitchison and colleagues demonstrated in mouse models that "adoptive immunity" could be transferred from one animal to another by transplant of tumor draining lymph node fragments [97]. Until the mid-1970s it was difficult to culture or expand T-cells in vitro, limiting the potential of this finding clinically. The discovery of IL-2 allowed not only for the activation and expansion of lymphocytes ex vivo, but also conferred T-cells with greater cytotoxicity [81, 83]. Murine models in the early 1980s harvested splenocytes from non-tumor bearing mice and activated them by co-culture with IL-2. Reinfusion of these LAK cells in mice with established pulmonary metastases resulted in a dramatic decrease in disease burden and improved overall survival [98]. Unfortunately, these results were not as impressive when translated into human trials. A prospective randomized trial of high-dose IL-2 alone or in conjunction with LAK cells demonstrated only a non-statistically significant trend towards improved survival in patients receiving ACT [85]. Impressively, however, there was a 12% complete response rate in patients receiving LAK therapy, a result that was unprecedented up to that point and sparked considerable interest.

An obvious shortcoming of ACT with LAK cells is that the reactivity is non-specific relying on expansion and activation of lymphocytes indiscriminate of antigen specificity. Over the next decade, strategies to improve ACT were sought by harvesting lymphocytes from resected tumors [95]. This was based in part on the recognition that patients bearing tumors with higher infiltration of cytotoxic cells have improved overall outcomes [99–101]. In theory, tumor-infiltrating lymphocytes (TILs) should inherently possess the chemokine receptors and antigen specific TCRs necessary to hone to and destroy tumors. Animal data reveals that TILs are 50–100 times more potent then LAK cells when adoptively transferred [102]. The cloning and development of techniques to produce therapeutic grade IL-2 allowed lymphocytes to be grown from resected tumors making clinical use of TIL possible.

The procedure for TIL harvest begins with surgical resection of tumors followed by fragmentation and culture in lymphocyte sustaining media supplemented with high dose IL-2 [103]. Over the subsequent days to weeks, non-IL-2 dependent tumor and stromal cells die off leaving only a culture of purified lymphocytes. Classically, these various fragment cultures are assayed for reactivity against autologous tumor

and/or established tumor cell lines. Reactive culture wells are then separated and rapidly expanded using radiated autologous antigen presenting cells as feeders. After one to two rapid expansions, sufficient quantities of cells are present for re-infusion [103].

It was identified through in vivo experiments, that administration of TIL alone was not sufficient for tumor reduction and that preparative chemotherapy with cyclophosphamide was required [104]. This created a state of relative immuno-depletion allowing transferred cells less competition for resources such as nutrients and pro-inflammatory cytokines. Because of these findings, patients receive some form of non-myeloablative therapy prior to adoptive cell transfer. In 1988, Rosenberg and colleagues published a report on 20 patients with metastatic melanoma treated with ACT of TIL followed by high-dose IL-2 and noted a 50% objective response rate and lower toxicity then prior trials of IL-2 alone [105]. An important finding of early trials was that persistence of transferred cells was associated with improved magnitude and duration of treatment response. Fueled by animal data linking persistence to increased levels of lymphodepletion, increasing degrees of preparative immune ablation were studied including the addition of fludarabine and whole body irradiation. Over the next decade, clinical trials revealed that increased intensity of radiation resulted in improved lymphodepletion and cellular persistence leading to improved treatment response, with an objective response rate of 72% in patients with refractory melanoma [106]. This increased intensity was not, however, without consequence as patients occasionally suffered from long-term renal insufficiency secondary to radiation induced thrombotic microangiopathy [107].

There were many translational correlates that emerged from early clinical trials of ACT, which were later studied to improve efficacy of therapy. One such finding was the association of telomere length of the infused TIL with cancer regression signifying that "younger" lymphocytes may be more potent inducers of treatment response [108]. Based on these findings as well as animal data suggesting that naive lymphocytes may be better effectors, a trial was undertaken using minimally cultured TIL [109]. Unlike prior studies, harvested lymphocytes were not tested for reactivity prior to infusion and cells underwent a shorter rapid expansion. Theoretical benefits of this approach include administration of less exhausted lymphocytes as well as simplifying the pre-ACT protocol allowing for more rapid delivery of TIL and wider acceptance into clinical practice. Two trials utilizing young TIL showed similar efficacy to prior approaches with significantly improved ease in cell preparation [110, 111].

There are several important shortcomings to TIL therapy that have limited its widespread acceptance into clinical practice. First, the therapy requires surgical resection of a metastatic lesion, which can often mean a major operation in patients already debilitated by widespread disease. Second, ACT requires ex vivo expansion of lymphocytes which is labor intensive and unpredictable. Finally, and most importantly, until recently, ACT with TIL has been limited to the treatment of melanoma as multiple attempts at harvesting and expanding reactive lymphocytes from other malignancies have failed. The one exception to this is the recent report by Tran et al. of successful treatment of a patient with cholangiocarcinoma using TIL reactive to a mutated cancer specific protein [112].

A recent strategy to overcome these shortcomings has been genetic modification of peripheral lymphocytes to confer tumor reactivity [113]. To accomplish this, TCR genes from lymphocyte clones isolated from TIL which are reactive to shared TAAs are cloned. These genes are then inserted into pheresed non-reactive peripheral lymphocytes via retroviral or lentiviral transduction allowing for expression of the transplanted TCR [114]. Culture of these genetically modified lymphocytes with cell lines expressing the shared tumor antigen confirm transferred reactivity [115]. This technique avoids the need for surgical intervention, produces reliably reactive lymphocytes for infusion and creates an "off-the-self" reagent that could improve accessibility to this therapy. ACT with genetically modified PBL occurs in a similar manner to traditional TIL with

plasma pheresis, viral mediated gene transfer to PBL, preparative chemotherapy and then cellular infusion. The first trial using ACT with genetically modified PBL by Morgan et al. treated metastatic melanoma patients with lymphocytes engineered to express a TCR specific for the melanoma-associated antigen (MAA) MART-1 [114]. They noted significant tumor reduction in two patients but unfortunately no response in a vast majority. Believing the poor efficacy was due to the relative low-affinity of the MART-1 receptor, a second trial was undertaken using PBL transduced to express a much higher affinity receptor to MART-1 or a receptor to the MAA gp-100 [116, 117]. These higher-affinity receptors proved more efficacious, but created significant off target effects attacking melanin-expressing cells in the skin, eyes, and ears [117].

Use of genetically modified lymphocytes has allowed expansion of ACT outside of the realm of melanoma. TCRs specific for multiple cancer testes antigens (CTA) have been cloned conferring lymphocyte specificity for antigens expressed on a wide variety of tumor types. A trial utilizing PBL transduced with a TCR specific for the CTA NY-ESO-1 demonstrated the ability of this therapy to treat sarcomas which have proven refractory to standard treatment [118]. A second trial using a TCR specific for the CTA MAGE-A3 enrolled patients with melanoma, sarcoma as well as esophageal cancer [119].

While genetically engineered PBL show promise, the restriction of TCRs to specific MHC subtypes limit their widespread utility. For example, most TCRs in development are specific for the HLA-A2 haplotype which is expressed by only 50% of Caucasians and 35% of African-Americans [120]. To expand the potential treatment population, chimeric antigen receptors (CAR) have been developed which utilize the antibody binding region joined by a linker to the TCR intracellular signaling domain [121]. Benefits of this strategy are a vast expansion of potential targets and the lack of MHC restriction. Antibodies, however, lack the specificity of TCRs making off target toxicity a concern.

One of the first CARs developed targeted CD19, which is widely expressed in B-cell lymphoma and lymphoblastic leukemia [122]. Patients with medically refractory disease underwent lymphocyte harvest followed by transduction of cells with a CAR composed of an antibody to CD19 and the CD28/CD3ζ intracellular signaling domains. Treatment induced rapid remission in 50% of patients with B-cell lymphomas and up to 100% of patients with acute lymphoblastic leukemia resulting in its approval by the FDA [123–125].

While CAR directed therapy is promising, it is not without its drawbacks. As previously stated, antibodies lack the specificity of TCRs and off target toxicity can be common. A dramatic example of this is the case report by Morgan et al. of a patient receiving a CAR directed to the TAA ERBB2 [126]. Within hours of ACT, the patient suffered multi-organ failure and eventually death due to cytokine storm and pulmonary congestion. Analysis of the CAR transduced lymphocytes showed activity to primary lung tissue lines leading authors to conclude that activation of the cells during first-pass in the lungs led to the subsequent outcome. While results from ACT continue to improve, the complexity of therapy and potential toxicity still limit its use to highly specialized centers.

Despite the numerous advancements in ACT over the past decades, it remains effective in only a small subset of patients. To better determine why the therapy is often ineffective researchers have turned their attention to other components of the TME. There are three principle factors that appear to impair function of transferred cytotoxic T-cells: (1) immunosuppressive cellular elements, (2) local secreted factors, and (3) immune checkpoints.

Tumor infiltrating lymphocyte function can be blocked by various immune cells including T-regs (as discussed in the active immunotherapy section), myeloid derived suppressor cells (MDSC) and type-2 macrophages [127–129]. While difficult to remove from the TME, strategies aimed at suppressing their function are currently being investigated with the goal of enhancing ACT efficacy. Delivery of a cyclooxy-

genase inhibitor can prevent differentiation of MDSCs and enhance immunotherapy in mesothelioma [130]. Type-2 macrophages, which function primarily by releasing arginase have been targeted by attempts to reprogram them to the more tumor destroying type-1 macrophage [131] and inhibition of arginase activity [132].

The cytokine milieu of the TME can tip the balance towards immune mediated destruction or protection. High levels of IL-10 and TFG-B suppress cytotoxic T-cell function and promote expansion of suppressive cellular elements [129]. Contrary to this, cytokines such as IL-12 lead to accumulation of T-cells and enhance efficacy of ACT. Attempts to utilize these pro-inflammatory cytokines clinically has been met with some difficulty as their potency can often lead to undesirable off target effects [133].

The final suppressor of ACT efficacy within the microenvironment is up-regulation of immune regulatory receptors and ligands. As immune excitation occurs, a proportional increase in immunosuppressive signals occurs to prevent the reaction from spiraling out of control. Cells that are expanded for ACT often express high levels of inhibitory receptors and attempts to block these have resulted in increased effector activity [134, 135]. More details regarding the function of checkpoint blockade will be discussed in the following section.

4.5 Checkpoint Blockade

Arguably the greatest advancement in immunotherapy over the past decades has been discovery [31] and clinical introduction of checkpoint inhibitors [26]. As previously stated, lymphocyte mediated immune destruction requires recognition via the TCR and co-stimulation via a variety of cell surface molecules. While these co-stimulatory proteins serve the heighten lymphocyte response to antigen, an assortment of inhibitory cell surface proteins within the TME serve to quell the reaction. This balance ensures adequate immune response without over-activation. Two notable inhibitory proteins cytotoxic T-lymphocyte protein 4 (CTLA-4) [136] and programmed cell-death protein 1 (PD-1) [137] have come to the forefront as pharmacologic strategies to block their activity has yielded impressive anti-tumor response.

4.5.1 CTLA-4

Originally described in the late 1980s and early 1990s, CTLA-4 is a member of the immunoglobulin superfamily and binds to B7-1 and B7-2 on antigen presenting cells. It is similar in structure to the co-stimulatory protein CD28 and functions to suppress T-cell activation [136]. Leach et al. determined that antibody blockade of CTLA-4 resulted in enhanced tumor immunity [138]. In 2010, Hodi et al. published a clinical trial of anti-CTLA-4 in the treatment of refractory metastatic melanoma [139]. Authors demonstrated a response rate of 25% and a long-term disease control rate of 15%. The drug was well tolerated with the principle side effects consisting of autoimmune colitis and hypophysitis. Results led to FDA approval of anti-CTLA-4 (*ipilimumab*) for the treatment of melanoma in 2011. Follow-up studies using *ipilimumab* in patients with less heavily treated disease and in combination with other immune modifying agents have led to improved results [140].

4.5.2 PD-1 and PD-L1

Like CTLA-4, PD-1 is present on lymphocytes and serves to inhibit antigen-mediated reactivity preventing autoimmunity. The principle ligand of PD-1, PD-L1 (B7H1), is often over expressed by tumor cells and APCs [19, 31], and used as a mechanism to evade immune destruction [137]. Blockade of the PD-1 -PD-L1 axis leads to improved tumor recognition and destruction. Unlike *ipilimumab*, the efficacy of PD-1 and PD-L1 inhibitors extends beyond melanoma and has been successfully used to treat non-small cell lung cancer (NSCLC), renal cell carcinoma and ovarian cancer [141]. These promising studies have led to the approval of the PD-1 blockers *pembrolizumab* and *nivolumab* by the FDA [142].

As the expression of PD-L1 and PD-1 is largely in the tumor microenvironment [19, 31], the side effects are less severe and highly manageable.

Tumor immunology and immunotherapy has undergone a renaissance of late. After suffering countless successes and setbacks in the twentieth century, it has now come to the forefront of cancer research and is recognized as an important tool in the anti-tumor armamentarium. Most exciting, is the potential for immunotherapy to not just result in tumor response, but complete and long-term remission. As our understanding of the intricate interactions between T-cells and the tumor microenvironment improves, so to will strategies aimed at derailing tumor mediate immune suppression. While the current focus is on T-cell mediated immunotherapy, emerging literature is suggesting an important role for myeloid derived cells such as macrophages and myeloid derived suppressor cells [143, 144] within the TME. It may be that someday a cocktail of different immune modulators is required to destroy established tumors and achieve cure. Achievements in the last decade have made this dream closer to a reality with more advancements soon to come.

References

1. Gravitz L. Cancer immunotherapy. Nature. 2013;504(7480):S1.
2. Rosenberg SA, Dudley ME, Restifo NP. Cancer immunotherapy. N Engl J Med. 2008;359(10):1072.
3. Gajewski TF, Woo SR, Zha Y, et al. Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. Curr Opin Immunol. 2013;25(2):268–76.
4. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Immunol Today. 2000;21(9):455–64.
5. Whiteside TL, Stanson J, Shurin MR, et al. Antigen-processing machinery in human dendritic cells: up-regulation by maturation and down-regulation by tumor cells. J Immunol. 2004;173(3):1526–34.
6. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27(45):5904–12.
7. Heninger E, Krueger TE, Lang JM. Augmenting antitumor immune responses with epigenetic modifying agents. Front Immunol. 2015;6:29.
8. Yu J, Ni M, Xu J, et al. Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis. BMC Cancer. 2002;2:29.
9. Rao M, Chinnasamy N, Hong JA, et al. Inhibition of histone lysine methylation enhances cancer-testis antigen expression in lung cancer cells: implications for adoptive immunotherapy of cancer. Cancer Res. 2011;71(12):4192–204.
10. Simova J, Pollakova V, Indrova M, et al. Immunotherapy augments the effect of 5-azacytidine on HPV16-associated tumours with different MHC class I-expression status. Br J Cancer. 2011;105(10):1533–41.
11. Wang LX, Mei ZY, Zhou JH, et al. Low dose decitabine treatment induces CD80 expression in cancer cells and stimulates tumor specific cytotoxic T lymphocyte responses. PLoS One. 2013;8(5):e62924.
12. West AC, Smyth MJ, Johnstone RW. The anticancer effects of HDAC inhibitors require the immune system. Oncoimmunology. 2014;3(1):e27414.
13. Iannello A, Raulet DH. Immune surveillance of unhealthy cells by natural killer cells. Cold Spring Harb Symp Quant Biol. 2013;78:249–57.
14. Chow MT, Moller A, Smyth MJ. Inflammation and immune surveillance in cancer. Semin Cancer Biol. 2012;22(1):23–32.
15. Groth A, Kloss S, von Strandmann EP, et al. Mechanisms of tumor and viral immune escape from natural killer cell-mediated surveillance. J Innate Immun. 2011;3(4):344–54.
16. Melchionda F, McKirdy MK, Medeiros F, et al. Escape from immune surveillance does not result in tolerance to tumor-associated antigens. J Immunother. 2004;27(5):329–38.
17. Al-Tameemi M, Chaplain M, d'Onofrio A. Evasion of tumours from the control of the immune system: consequences of brief encounters. Biol Direct. 2012;7:31.
18. Zou W, Machelon V, Coulomb-L'Hermin A, et al. Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. Nat Med. 2001;7(12):1339–46.
19. Curiel TJ, Wei S, Dong H, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. Nat Med. 2003;9(5):562–7.
20. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942–9.
21. Kryczek I, Zou L, Rodriguez P, et al. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. J Exp Med. 2006;203(4):871–81.
22. Cui TX, Kryczek I, Zhao L, et al. Myeloid-derived suppressor cells enhance stemness of cancer cells by inducing microRNA101 and suppressing the corepressor CtBP2. Immunity. 2013;39(3):611–21.

23. Bai XF, Liu J, Li O, et al. Antigenic drift as a mechanism for tumor evasion of destruction by cytolytic T lymphocytes. J Clin Invest. 2003;111(10):1487–96.
24. Bubenik J. MHC class I down-regulation: tumour escape from immune surveillance? (review). Int J Oncol. 2004;25(2):487–91.
25. Topfer K, Kempe S, Muller N, et al. Tumor evasion from T cell surveillance. J Biomed Biotechnol. 2011;2011:918471.
26. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252–64.
27. Singh S, Ross SR, Acena M, et al. Stroma is critical for preventing or permitting immunological destruction of antigenic cancer cells. J Exp Med. 1992;175(1):139–46.
28. Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res. 2007;13(18 Pt 1):5256–61.
29. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer. 2005;5(4):263–74.
30. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol. 2006;6(4):295–307.
31. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol. 2008;8(6):467–77.
32. Zou W, Restifo NP. T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol. 2010;10(4):248–56.
33. Parish CR. Cancer immunotherapy: the past, the present and the future. Immunol Cell Biol. 2003;81(2):106–13.
34. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011;480(7378):480–9.
35. Coley WB II. Contribution to the knowledge of sarcoma. Ann Surg. 1891;14(3):199–220.
36. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. Clin Orthop Relat Res. 1893;1991(262):3–11.
37. Coley WB. The treatment of inoperable sarcoma by bacterial toxins (the mixed toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). Proc R Soc Med. 1910;3(Surg Sect):1–48.
38. American Association for Cancer Research. The 2013 William B. Coley award for distinguished research in basic and tumor immunology. Cancer Immunol Res. 2013;1(6):362–4.
39. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity. 1999;10(3):281–7.
40. Burnet FM, Fenner F. Genetics and immunology. Heredity. 1948;2(Pt. 3):289–324.
41. Foley EJ. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. Cancer Res. 1953;13(12):835–7.
42. Prehn RT, Main JM. Immunity to methylcholanthrene-induced sarcomas. J Natl Cancer Inst. 1957;18(6):769–78.
43. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957;1(5022):779–86.
44. Outzen HC, Custer RP, Eaton GJ, et al. Spontaneous and induced tumor incidence in germfree "nude" mice. J Reticuloendothel Soc. 1975;17(1):1–9.
45. Stutman O. Immunodepression and malignancy. Adv Cancer Res. 1975;22:261–422.
46. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology. 2007;121(1):1–14.
47. Herberman RB, Holden HT. Natural cell-mediated immunity. Adv Cancer Res. 1978;27:305–77.
48. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410(6832):1107–11.
49. Gerlich WH. Medical virology of hepatitis B: how it began and where we are now. Virol J. 2013;10:239.
50. Shepard CW, Simard EP, Finelli L, et al. Hepatitis B virus infection: epidemiology and vaccination. Epidemiol Rev. 2006;28:112–25.
51. Gajewski TF, Meng Y, Blank C, et al. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006;213:131–45.
52. Zhang T, Somasundaram R, Berencsi K, et al. Migration of cytotoxic T lymphocytes toward melanoma cells in three-dimensional organotypic culture is dependent on CCL2 and CCR4. Eur J Immunol. 2006;36(2):457–67.
53. Buckanovich RJ, Facciabene A, Kim S, et al. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med. 2008;14(1):28–36.
54. Turk MJ, Guevara-Patino JA, Rizzuto GA, et al. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med. 2004;200(6):771–82.
55. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol. 2002;169(5):2756–61.
56. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol. 2004;173(2):1444–53.
57. Casares N, Arribillaga L, Sarobe P, et al. CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. J Immunol. 2003;171(11):5931–9.
58. Antony PA, Piccirillo CA, Akpinarli A, et al. CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by

naturally occurring T regulatory cells. J Immunol. 2005;174(5):2591–601.
59. Viehl CT, Moore TT, Liyanage UK, et al. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. Ann Surg Oncol. 2006;13(9):1252–8.
60. Wang RF, Rosenberg SA. Human tumor antigens for cancer vaccine development. Immunol Rev. 1999;170:85–100.
61. Chan AD, Morton DL. Active immunotherapy with allogeneic tumor cell vaccines: present status. Semin Oncol. 1998;25(6):611–22.
62. Rosenberg SA. Cancer vaccines based on the identification of genes encoding cancer regression antigens. Immunol Today. 1997;18(4):175–82.
63. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med. 1998;4(3):321–7.
64. Maeurer MJ, Storkus WJ, Kirkwood JM, et al. New treatment options for patients with melanoma: review of melanoma-derived T-cell epitope-based peptide vaccines. Melanoma Res. 1996;6(1):11–24.
65. Berinstein NL. Carcinoembryonic antigen as a target for therapeutic anticancer vaccines: a review. J Clin Oncol. 2002;20(8):2197–207.
66. Wang F, Bade E, Kuniyoshi C, et al. Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. Clin Cancer Res. 1999;5(10):2756–65.
67. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med. 2011;364(22):2119–27.
68. Eggermont AM. Immunotherapy: vaccine trials in melanoma—time for reflection. Nat Rev Clin Oncol. 2009;6(5):256–8.
69. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol. 2005;175(9):6169–76.
70. Phan GQ, Touloukian CE, Yang JC, et al. Immunization of patients with metastatic melanoma using both class I- and class II-restricted peptides from melanoma-associated antigens. J Immunother. 2003;26(4):349–56.
71. Liu MA. DNA vaccines: a review. J Intern Med. 2003;253(4):402–10.
72. Kutzler MA, Weiner DB. Developing DNA vaccines that call to dendritic cells. J Clin Invest. 2004;114(9):1241–4.
73. Lu S, Wang S, Grimes-Serrano JM. Current progress of DNA vaccine studies in humans. Expert Rev Vaccines. 2008;7(2):175–91.
74. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nat Rev Cancer. 2012;12(4):265–77.
75. Kantoff PW, Higano CS, Shore ND, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 2010;363(5):411–22.
76. Engell-Noerregaard L, Hansen TH, Andersen MH, et al. Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. Cancer Immunol Immunother. 2009;58(1):1–14.
77. Banchereau J, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. Nat Rev Immunol. 2005;5(4):296–306.
78. Bodey B, Bodey B Jr, Siegel SE, et al. Failure of cancer vaccines: the significant limitations of this approach to immunotherapy. Anticancer Res. 2000;20(4):2665–76.
79. Lai CL, Yuen MF. Prevention of hepatitis B virus-related hepatocellular carcinoma with antiviral therapy. Hepatology. 2013;57(1):399–408.
80. Paavonen J, Naud P, Salmeron J, et al. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet. 2009;374(9686):301–14.
81. Welte K, Wang CY, Mertelsmann R, et al. Purification of human interleukin 2 to apparent homogeneity and its molecular heterogeneity. J Exp Med. 1982;156(2):454–64.
82. Donohue JH, Rosenstein M, Chang AE, et al. The systemic administration of purified interleukin 2 enhances the ability of sensitized murine lymphocytes to cure a disseminated syngeneic lymphoma. J Immunol. 1984;132(4):2123–8.
83. Cheever MA, Greenberg PD, Fefer A, et al. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. J Exp Med. 1982;155(4):968–80.
84. Rosenberg SA, Mule JJ, Spiess PJ, et al. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. J Exp Med. 1985;161(5):1169–88.
85. Lotze MT, Rosenberg SA. Results of clinical trials with the administration of interleukin 2 and adoptive immunotherapy with activated cells in patients with cancer. Immunobiology. 1986;172(3–5):420–37.
86. Gaffen SL, Liu KD. Overview of interleukin-2 function, production and clinical applications. Cytokine. 2004;28(3):109–23.
87. Phan GQ, Attia P, Steinberg SM, et al. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J Clin Oncol. 2001;19(15):3477–82.
88. Antony PA, Restifo NP. CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2. J Immunother. 2005;28(2):120–8.

89. Plotkin S. History of vaccination. Proc Natl Acad Sci U S A. 2014;111(34):12283–7.
90. Fraser G, Smith CA, Imrie K, et al. Alemtuzumab in chronic lymphocytic leukemia. Curr Oncol. 2007;14(3):96–109.
91. Hortobagyi GN. Trastuzumab in the treatment of breast cancer. N Engl J Med. 2005;353(16):1734–6.
92. Slamon D, Eiermann W, Robert N, et al. Adjuvant trastuzumab in HER2-positive breast cancer. N Engl J Med. 2011;365(14):1273–83.
93. Witzig TE. Yttrium-90-ibritumomab tiuxetan radioimmunotherapy: a new treatment approach for B-cell non-Hodgkin's lymphoma. Drugs Today (Barc). 2004;40(2):111–9.
94. Amiri-Kordestani L, Blumenthal GM, Xu QC, et al. FDA approval: ado-trastuzumab emtansine for the treatment of patients with HER2-positive metastatic breast cancer. Clin Cancer Res. 2014;20(17):4436–41.
95. Rosenberg SA, Restifo NP, Yang JC, et al. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer. 2008;8(4):299–308.
96. Kalos M, June CH. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. Immunity. 2013;39(1):49–60.
97. Mitchison NA. Adoptive transfer of immune reactions by cells. J Cell Physiol Suppl. 1957;50(Suppl 1):247–64.
98. Mazumder A, Rosenberg SA. Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. J Exp Med. 1984;159(2):495–507.
99. Murray D, Hreno A, Dutton J, et al. Prognosis in colon cancer: a pathologic reassessment. Arch Surg. 1975;110(8):908–13.
100. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102(51):18538–43.
101. Naito Y, Saito K, Shiiba K, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res. 1998;58(16):3491–4.
102. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science. 1986;233(4770):1318–21.
103. Dudley ME, Wunderlich JR, Shelton TE, et al. Generation of tumor-infiltrating lymphocyte cultures for use in adoptive transfer therapy for melanoma patients. J Immunother. 2003;26(4):332–42.
104. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med. 1982;155(4):1063–74.
105. Rosenberg SA, Packard BS, Aebersold PM, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med. 1988;319(25):1676–80.
106. Dudley ME, Yang JC, Sherry R, et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol. 2008;26(32):5233–9.
107. Tseng J, Citrin DE, Waldman M, et al. Thrombotic microangiopathy in metastatic melanoma patients treated with adoptive cell therapy and total body irradiation. Cancer. 2014;120(9):1426–32.
108. Zhou J, Shen X, Huang J, et al. Telomere length of transferred lymphocytes correlates with in vivo persistence and tumor regression in melanoma patients receiving cell transfer therapy. J Immunol. 2005;175(10):7046–52.
109. Tran KQ, Zhou J, Durflinger KH, et al. Minimally cultured tumor-infiltrating lymphocytes display optimal characteristics for adoptive cell therapy. J Immunother. 2008;31(8):742–51.
110. Itzhaki O, Hovav E, Ziporen Y, et al. Establishment and large-scale expansion of minimally cultured "young" tumor infiltrating lymphocytes for adoptive transfer therapy. J Immunother. 2011;34(2):212–20.
111. Dudley ME, Gross CA, Langhan MM, et al. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. Clin Cancer Res. 2010;16(24):6122–31.
112. Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. Science. 2014;344(6184):641–5.
113. Rossig C, Brenner MK. Genetic modification of T lymphocytes for adoptive immunotherapy. Mol Ther. 2004;10(1):5–18.
114. Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science. 2006;314(5796):126–9.
115. Frankel TL, Burns WR, Peng PD, et al. Both CD4 and CD8 T cells mediate equally effective in vivo tumor treatment when engineered with a highly avid TCR targeting tyrosinase. J Immunol. 2010;184(11):5988–98.
116. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood. 2009;114(3):535–46.
117. Seaman BJ, Guardiani EA, Brewer CC, et al. Audiovestibular dysfunction associated with adoptive cell immunotherapy for melanoma. Otolaryngol Head Neck Surg. 2012;147(4):744–9.
118. Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered

lymphocytes reactive with NY-ESO-1. J Clin Oncol. 2011;29(7):917–24.
119. Morgan RA, Chinnasamy N, Abate-Daga D, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother. 2013;36(2):133–51.
120. Ellis JM, Henson V, Slack R, et al. Frequencies of HLA-A2 alleles in five U.S. population groups. Predominance of A*02011 and identification of HLA-A*0231. Hum Immunol. 2000;61(3):334–40.
121. Sadelain M, Brentjens R, Riviere I. The promise and potential pitfalls of chimeric antigen receptors. Curr Opin Immunol. 2009;21(2):215–23.
122. Kochenderfer JN, Feldman SA, Zhao Y, et al. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J Immunother. 2009;32(7):689–702.
123. Kochenderfer JN, Dudley ME, Kassim SH, et al. Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J Clin Oncol. 2015;33(6):540–9.
124. Brentjens RJ, Davila ML, Riviere I, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med. 2013;5(177):177ra38.
125. Grupp SA, Kalos M, Barrett D, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med. 2013;368(16):1509–18.
126. Morgan RA, Yang JC, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18(4):843–51.
127. Marigo I, Dolcetti L, Serafini P, et al. Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells. Immunol Rev. 2008;222:162–79.
128. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. 2009;30(5):636–45.
129. Devaud C, John LB, Westwood JA, et al. Immune modulation of the tumor microenvironment for enhancing cancer immunotherapy. Oncoimmunology. 2013;2(8):e25961.
130. Veltman JD, Lambers ME, van Nimwegen M, et al. COX-2 inhibition improves immunotherapy and is associated with decreased numbers of myeloid-derived suppressor cells in mesothelioma. Celecoxib influences MDSC function. BMC Cancer. 2010;10:464.
131. Buhtoiarov IN, Sondel PM, Wigginton JM, et al. Anti-tumour synergy of cytotoxic chemotherapy and anti-CD40 plus CpG-ODN immunotherapy through repolarization of tumour-associated macrophages. Immunology. 2011;132(2):226–39.
132. Serafini P, Meckel K, Kelso M, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med. 2006;203(12):2691–702.
133. Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol. 1998;16:495–521.
134. Blake SJ, Ching AL, Kenna TJ, et al. Blockade of PD-1/PD-L1 promotes adoptive T-cell immunotherapy in a tolerogenic environment. PLoS One. 2015;10(3):e0119483.
135. Mahvi DA, Meyers JV, Tatar AJ, et al. Ctla-4 blockade plus adoptive T-cell transfer promotes optimal melanoma immunity in mice. J Immunother. 2015;38(2):54–61.
136. Chambers CA, Kuhns MS, Egen JG, et al. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol. 2001;19:565–94.
137. Keir ME, Butte MJ, Freeman GJ, et al. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677–704.
138. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271(5256):1734–6.
139. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):711–23.
140. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369(2):122–33.
141. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443–54.
142. Barbee MS, Ogunniyi A, Horvat TZ, et al. Current status and future directions of the immune checkpoint inhibitors ipilimumab, pembrolizumab, and nivolumab in oncology. Ann Pharmacother. 2015;49(8):907–37.
143. Panni RZ, Linehan DC, DeNardo DG. Targeting tumor-infiltrating macrophages to combat cancer. Immunotherapy. 2013;5(10):1075–87.
144. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162–74.

5 Immunogenic and Non-immunogenic Cell Death in the Tumor Microenvironment

Jonathan M. Pitt, Guido Kroemer, and Laurence Zitvogel

5.1 Introduction

Cell death is essential to the turnover of healthy tissues in the steady state, and in an organism's defense against pathogen infection and malignant cells. In adult humans, approximately 50–70 billion cells die each day during the normal turnover of body tissues [1]. An intricate cross-talk has evolved between cell death circuitries and the vertebrate immune system to allow distinction of potential threats from physiological cell death in healthy tissues. Physiological cell death (better known as apoptosis) is a fundamentally tolerogenic process that prevents autoreactivity to normal host tissues. Cells undergoing physiological apoptosis are cleared by phagocytic cells in a "silent" manner, thus concealing cellular components that might otherwise induce inflammation [2]. In contrast, pathological cell death (often termed necrosis) is an inherently immunogenic and inflammatory process, which alerts the immune system of an abnormality or potential threat. However, this apparent cell death dichotomy may not be as clear-cut as once thought. For example, alternative subtypes of apoptosis that are immunogenic can be initiated under certain circumstances [3, 4].

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The characteristics of an immune response to cell death (e.g., immunogenic vs. tolerogenic responses) are determined by the precise molecular signaling between dying cells and local immune cells. Immune cells recognize pathogen-infected cells through pathogen-associated molecular patterns (PAMPs). PAMPs exposed by infected cells target them for immune cell-mediated killing or for phagocytosis and subsequent antigen presentation by antigen-presenting cells (APCs), which is required to induce antigen-specific immune responses [5–7]. Examples of PAMPs include components of viruses or bacteria, and certain sequences of the nucleic acids that form their genomes [6, 7]. Appropriate immune responses are also required in cases where there is no pathogen involved in the abnormal dying or stressing of a cell, for example in malignant pre-cancerous cells. In 1994, Polly Matzinger proposed the "danger theory," stating that the immune system has evolved an inherent capacity to distinguish between dangerous and innocuous endogenous signals [8]. The theory is supported by evidence that malignant dying cells expose damage-associated molecular patterns (DAMPs), which function as danger signals for the host immune system [9]. PAMPs and DAMPs are recognized by germline-encoded pattern recognition receptors (PRRs) present on immune cells. Toll-like receptors (TLRs) and nucleotide oligodimerization domain (NOD)-like receptors (NLRs) are examples of two important PRR families in immune signaling [5, 10]. PRR recognition of cognate ligand can activate downstream effects such as immune cell differentiation and pro-inflammatory cytokine production, which initiate or propagate immune responses localized around abnormal tissues.

Defining cell death by biomolecular patterns has highlighted many different cell death subroutines, which have extended the original apoptosis and necrosis dichotomy [11]. This greater level of contrast has revealed new opportunities for therapeutic intervention, notably in cancer treatment. In this chapter, we discuss how different cell death modalities impact host immunosurveillance in the tumor microenvironment. We pay particular attention to cell death modalities that occur following administration of certain cancer therapies.

5.2 Tolerogenic Cell Death in the Tumor Microenvironment

Various elements work in unison to determine whether cell death is immunogenic or not. These parameters include the inherent antigenicity of the dying cell, activation or stress encountered by the cell before dying, the properties of the cell death-inducing entity, the specific cell death pathway engaged, and the availability of immune cells [3].

"Physiological" apoptosis evolved to be an immunologically silent event characterized by absence of "don't eat me" signals (e.g., CD31 and CD47), exposure of "eat me" signals (such as phosphatidylserine [PS] upon the outer membrane leaf), and release of "find me" signals and chemokines (e.g., ATP) into the local environment [2] (see Table 5.1). Cancer cells can follow the same physiological pathway to die, or may even begin to mimic aspects of apoptosis (e.g., PS exposure [24]), which would result in concealment of immunostimulatory DAMPs and immune evasion [24]. Indeed (and often forgotten in tumor biology), tumor cell loss plays a significant component in tumor development, with higher levels of apoptosis linked to poorer prognoses [2]. Apoptosis may also actively recruit precursor myeloid cells into the tumor bed through "find me" signals (see Fig. 5.1). Following the uptake of apoptotic tumor material, recruited myeloid cells can differentiate and become polarized toward a tolerogenic (M2) phenotype. These cells are subsequently well positioned to inhibit immunosurveillance [25].

Tumor cell apoptosis may similarly modulate dendritic cell (DCs) function in the tumor microenvironment. DCs are the sentinel APCs of the immune system and act as initiators of antigen-specific T cell responses. When positioned in the proximity of dying cancer cell, DCs can take up tumor-associated antigens (TAAs) and present these to generate T cell-mediated anti-tumor

Table 5.1 "Find me" and "eat me" cell signaling

Signal	Examples	Response to signal	References
"Find me"	ATP	Binds to P2Y2 receptors on myeloid cells to stimulate their chemotactic recruitment	[12]
	UTP	As ATP above	[12]
	Lysophosphatidylcholine	Attracts myeloid cells by binding G2A receptors on their surface	[13]
	CX_3CL1 (fractalkine)	Attracts myeloid cells by binding CXC_3CR1 receptors on their surface	[14]
"Eat me"	Phosphatidylserine	Mediates removal of apoptotic corpses by myeloid cells without activating inflammatory response	[15]
	Calreticulin	Binds CD91 on APCs to stimulate cytokine production	[16, 17]
	Annexin 1	Facilitates apoptotic cell engulfment	[18]
	eIF3a	Externalised on apoptotic cells and binds macrophages to facilitate engulfment	[19]
"Don't eat me"	CD31	Inhibits engulfment by transmitting detachment signals to phagocytes; loses this function during normal apoptosis	[20]
	CD47	Interacts with SIRP-alpha on macrophages to initiate inhibitory signaling and prevent phagocytosis	[16, 21]
	PTX3	Binds late apoptotic cells, inhibits DC uptake to reduce risk of autoimmunity	[22, 23]

ICD immunogenic cell death, *eIF3a* eukaryotic translation initiation factor 3, *DC* dendritic cell

responses. Besides presenting TAAs to T cells, DCs must also receive stimuli such as DAMPs or PAMPs to stimulate their maturation. This in turn allows DCs to upregulate their surface expression of co-stimulatory molecules, required to prime efficient cytotoxic tumor-targeted T cells. However, engulfment of cells undergoing apoptosis may actively prevent this DC maturation [26], leaving DCs in a tolerogenic, immature state [27]. Furthermore, insufficiently activated DCs may cross-present antigens derived from apoptotic material to $CD8^+$ T cells, which can result in immune suppression [28, 29]. This can occur in cases where there is an absence of $CD4^+$ T cell help.

$CD4^+$ T cells program DCs to correctly prime $CD8^+$ cytotoxic T lymphocytes (CTLs) that are resistant to activation-induced cell death on antigen reencounter. This checkpoint against potential CTL-driven autoimmunity is mediated by CD40-CD40 ligand interaction between DCs and $CD4^+$ T cells [3]. Activation-induced cell death is initiated by the death ligand TRAIL (TNF-related apoptosis-inducing ligand), which instigates apoptosis in sub-optimally primed CTLs and other activated T cells. Unlike DCs that engulf necrotic cells, DCs that engulf apoptotic cells have been shown to present antigen to $CD8^+$ T cells, but not to $CD4^+$ T cells [30]. These $CD8^+$ T cells were seen to produce TRAIL on reencounter with the antigen, which blocked cell-mediated immune responses from occurring. Accordingly, TRAIL-deficient mice are resistant to tolerance following injection of apoptotic cells [30].

Apoptosis can also promote tolerance by modifying DAMPs. During apoptosis, naturally produced reactive oxygen species oxidize a key cysteine residue in high-mobility group box 1 (HMGB1), an immunogenic DAMP. This event renders HMGB1 ineffective in promoting immune responses [31]. It is likely that other immunostimulatory molecules are "silenced" by

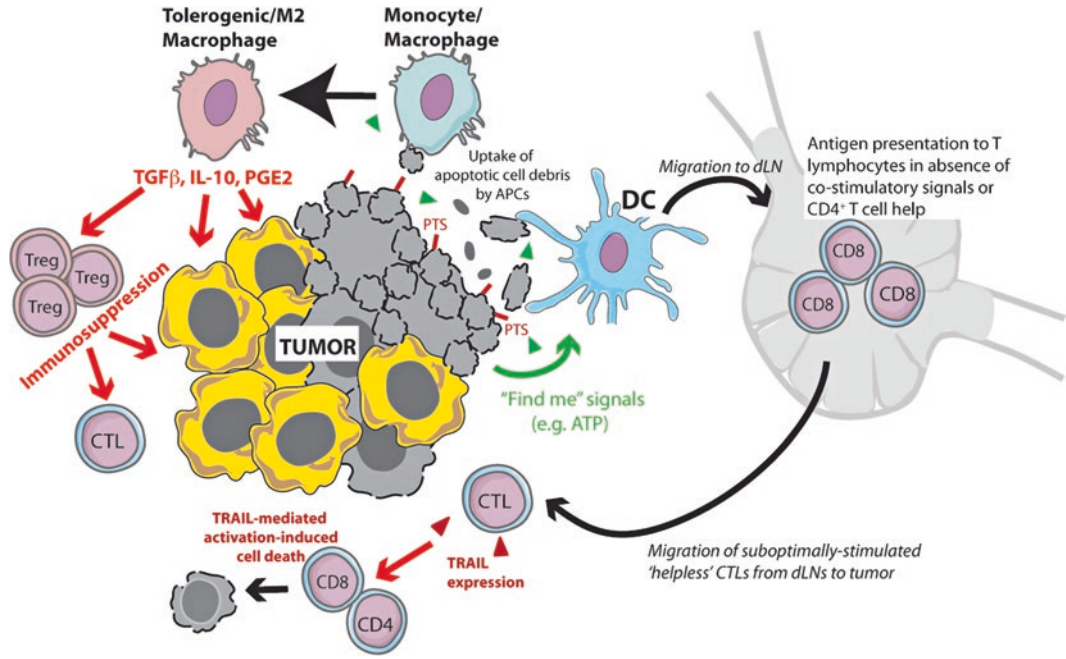

Fig. 5.1 Tolerogenic cell death in the microenvironment of tumors. Apoptosis of cancer cells can induce a local state of tolerance that enables tumors to escape immunosurveillance. Antigen presenting cells (APCs) such as dendritic cells (DCs) are drawn to apoptotic cells in tumor beds via sensing their surface exposure of phosphatidylserine (PTS) or following their release of "find me" signals such as ATP. "Find me" signals cause mobile phagocytic cells to migrate as a whole (chemotaxis) or extend parts of the cell (chemotropism) toward the dying cell. APCs subsequently take up apoptotic debris and associated tumor antigens. Following migration to draining lymph nodes (dLN) DCs can present these antigens to $CD8^+$ T lymphocytes, but in the absence of costimulation. This causes priming of "helpless" cytotoxic lymphocytes that are unable to successfully attack tumors, and which are highly susceptible to TRAIL-mediated activation-induced cell death. Apoptotic debris can also induce the differentiation of myeloid cells into tolerogenic M2 macrophages, which in turn can drive regulatory $CD4^+$ T cell (Treg)-mediated immunosuppression through their production of the cytokines TGF-β and IL-10

ROS, or by other means, as a safeguard against autoimmunity. However, these safety mechanisms could be potentially hijacked by tumor cells.

The release of immunosuppressive mediators by cells undergoing apoptosis, or by cells that have uptaken apoptotic material, is an additional mechanism to ensure a tolerogenic microenvironment. Intravenous infusion of apoptotic cells in vivo induces an expansion of Tregs that appears to be TGF-β-dependent [32, 33]. Lymphocytes dying by apoptosis can produce immunosuppressive cytokines such as IL-10 and TGF-β [34–36], although whether this is true for apoptotic tumor cells is less clear. Nonetheless, immunosuppressive cytokine production following apoptosis is observed from macrophages or DCs that have phagocytozed apoptotic cells. Macrophages have been shown to produce TGFβ, IL-10, and several lipid mediators when they come into contact with apoptotic cells [3, 37–39], whereas proinflammatory cytokine gene expression is inhibited [40]. Moreover, because production of immunosuppressive mediators can induce $Foxp3^+$ regulatory T cells (Treg), positive reinforcement of intratumoral immunosuppression can occur [41–43]. Besides prominent immunosuppressive cytokines, ecto-ATPases exposed or secreted by tumor cells (such as CD39 or CD73) can similarly expand Treg populations. These

enzymes consume immunogenic extracellular ATP, and can increase immunosuppressive adenosine levels in the microenvironment [44–46].

5.3 Immunogenic Death of Cancer Cells

Although apoptosis is considered an immunologically "silent" event, certain stimuli can induce non-classical subtypes of apoptosis that cause immune responses against components of the dying cell. Chemotherapeutic agents and radiotherapy are well-studied inducers of these cell death modalities (Table 5.2). Several studies have revealed responses to chemotherapy are often more efficient in immunocompetent than immunodeficient hosts, this being valid in both mouse [44, 47] and clinical [55] studies. Additionally, *in vivo* injection of murine cancer cells previously treated with anthracyclines, oxaliplatin, or UVC irradiation, confers long-term immune-mediated protection against challenge with live cancer cells of the same type [17, 48]. These findings suggested chemotherapy-induced tumor cell death engages immune cells to propagate anti-tumor immunity.

Studies of the events following the immunogenic cell death (ICD) have identified subtle molecular and metabolic signals (e.g., DAMPs) that stimulate APCs to promote immune

Table 5.2 Inducers of immunogenic and non-immunogenic cell death

Group	Inducer	Mechanism of action	References
ICD induction	Anthracyclines	Exposure causes dying cancer cells to induce protective immune responses in vivo, in absence of adjuvants. Induces exposure of CRT, ATP, HMGB1, type I IFN	[4, 17, 44, 47–49]
	Oxaliplatin	Similar to anthracyclines above, induces bona fide ICD	[4, 47, 50]
	Cardiac glycosides	Induce CRT, ATP, HMGB1 exposure following ER stress and autophagy induction	[51]
	Cyclophosphamide	Cyclophosphamide derivative mafosfamide can promote CRT exposure and HMGB1 release.	[52]
	Irradiation	Ionizing irradiation with UVC light or gamma-rays causes CRT exposure and HMBG1 and ATP release	[4, 17, 47]
Non-ICD inducers	Cisplatin	Unlike oxaliplatin, fails to induce CRT exposure on killing cancer cells, and hence does not induce anticancer immunity. Can be combined with other agents, such as cardiac glycosides or thapsigargin, to induce ICD	[50, 51, 53]
	Mitomycin C	A DNA-damaging anticancer agent that fails to induce ICD. Absorption of recombinant CRT to cells dying by mitomycin C can restore ICD	[4, 17, 54]
	Etoposide	Fails to induce ICD on its own. Similarly to the case with mitomycin C, absorption of recombinant CRT to dying cells can restore ICD	[17]

ICD immunogenic cell death, *CRT* calreticulin, *IFN* interferon, HMGB1 high-mobility group box 1

responses. DCs act as the central immune cell able to sense and transform cell death signals into anti-tumor T cell responses. A population of $CD11c^{+}CD11b^{+}Ly6C^{hi}$ myeloid precursor cells (characteristic of inflammatory monocytes) have been shown to be the precursors to intratumoral DCs following ICD-inducing chemotherapy. This precursor population accumulates in tumor beds following anthracycline treatment, and appears particularly efficient at engulfing dying tumor cells and presenting TAAs to $CD8^{+}$ T cells [56]. Intratumoral accumulation of this myeloid population appears dependent upon the release of ATP from cells dying by ICD, as this process fails to occur in murine tumors engineered to overexpress the ecto-ATPase CD39. Subsequent studies have revealed that intratumoral accumulation of $CD11c^{+}CD11b^{+}Ly6C^{hi}MHCII^{+}$ myeloid cells post anthracycline chemotherapy also requires Ccl2 chemokine signaling, since neoplasms growing in mice deficient for Ccl2 fail to recruit this population post treatment [57]. Interestingly, the same study identified that draining lymph nodes are dispensable for the priming and proliferation of antigen-specific immunity post anthracycline chemotherapy [57]. Intratumoral accumulation of myeloid precursor cells has been corroborated in a taxane-based chemotherapy setting [58].

Antineoplastic chemotherapeutic agents can alter the immune infiltrate of tumors, the characteristics of which often determine therapeutic outcome. A good example is how certain anticancer agents increase the ratio of CTLs to $Foxp3^{+}$ Tregs, which often correlates with favorable therapeutic responses [59]. Of the various T cell populations, CTLs and CD4+ T helper (Th)1 cells, prominent producers of the cytokine interferon (IFN)-γ, are key mediators of tumor eradication. The role that interleukin (IL)-17 producing T cells (e.g., Th17) play post chemotherapy is less well understood. IL-17A production by γδ T cells appears to be a key component for optimal anticancer responses following ICD-inducing chemotherapy or radiotherapy [60, 61]. Tumor infiltration by this innate lymphocyte population was demonstrated as a prerequisite for subsequent accumulation of tumor-killing $CD8^{+}$ αβ T cells. Before triggering this immune response, the γδ T cells first required activation by the pro-inflammatory cytokine IL-1β [49, 60]. The indispensable role of the cytokines mediating this cascade of events following ICD has been confirmed in experimental settings using either neutralizing antibodies, or through knockout of the genes encoding the cytokines (*Il1b*, *Il17a*, *Ifng*) and their receptors (*Il1r*, *Il17r*, *Ifngr*) [17, 44, 49, 56, 60]. TNF-α signaling does not appear to contribute to the antineoplastic effects of anthracycline chemotherapies in murine tumor models [62].

Several subtle biochemical changes in the plasma membrane and microenvironment of dying cells drive anti-tumor immunity through PRRs of innate immune cells. These prominent hallmarks of ICD include ER stress and calreticulin (CRT) exposure, the release of HMGB1, the autophagy-dependent release of ATP, and viral mimicry that induces type I IFN signaling.

5.3.1 ER Stress and Calreticulin Exposure

An early event required for immune stimulation following ICD is the exposure of CRT on dying cells [17]. CRT is present in high concentrations in the endoplasmic reticulum (ER) lumen, as well as other subcellular compartments. Following exposure to certain ICD-inducing chemotherapies (e.g., anthracyclines), malignant cells undergo a rapid phase of intense ER stress. ER stress may be defined as a disturbance in the homeostatic protein processing function of the ER, caused by an imbalance between protein folding load and capacity [9]. To fully activate danger signaling, the overproduction of ROS likely synergizes with the cellular response to ER stress since optimal immunogenicity of ICD requires the combination of these two factors (indeed, ICD is reduced in the presence of antioxidants [54]) [9]. Downstream of these events occurs an increase in the cytoplasmic Ca^{2+} concentration and activation of the protein kinase PERK (protein kinase RNA-like ER kinase). Activated PERK phosphorylates the eukaryotic

translation initiation factor 2α (eIF2α), which in turn results in a halt in protein translation. These processes are necessary for the successful propagation of ICD, since siRNA-mediated downregulation of PERK, knock-in of a non-phosphorylatable variant of eIF2α, or the use of intracellular Ca^{2+} chelators, each prevent the anthracycline-induced exposure of CRT and thus immunogenicity [63]. Subsequent to these events, downstream activation of caspase-8 along with the pro-apoptotic proteins BAX and BAK occurs, these latter two proteins playing a central role in mitochondrial outer membrane permeabilization. Finally, anterograde transport of CRT from the ER to the Golgi apparatus takes place, which allows exocytosis of CRT-containing vesicles to the cell surface membrane [4, 54]. External cell membrane exposure of CRT appears only to occur with ICD-inducing anticancer therapies, although the exact intracellular molecules and signaling pathways required for CRT translocation appear to be heterogeneous and dependent on the particular ICD trigger [4]. Additionally, any inhibition of CRT exposure through blocking antibodies or CRT transcript knockdown abrogates anthracycline immunogenicity, highlighting the key requirement of this process to ICD [17].

Recent studies have shown that the chemokine CXCL8 (better known as IL-8), and its mouse ortholog CXCL2, are also involved in the translocation of CRT to the outer leaflet of the plasma membrane [64]. Treatment with the ICD-inducing agent mitoxantrone stimulates human cancer cells to produce CXCL8 in vitro, and murine tumors to produce CXCL2 *in vivo*. In addition, mitoxantrone-induced CRT exposure is diminished if the receptors of CXCL8 (CXCR1) or CXCL2 (CXCR1 and CXCR2) are knocked down in human or murine cancer cells, respectively. Knockdown of the receptors for CXCL2 was also observed to reduce the immunogenicity of mitoxantrone-treated dying tumor cells in vivo, which could be restored if exogenous CXCL2 was provided [64].

The exposure of CRT on the cell surface of dying tumor cells couples to induction of anti-tumor immune responses by acting as an "eat me" signal to APCs such as DCs (Fig. 5.2). Recognition of CRT and engulfment of CRT-exposing cells can occur through the transmembrane receptor CD91. On binding to CD91, CRT stimulates APCs to produce proinflammatory cytokines, such as TNF-α and IL-6, which facilitate antigen presentation and T cell responses. ICD-induced CRT exposure precedes PS exposure at the cell surface. Although both act as "eat me" signals, whereas CRT is required for immunogenicity of this death process, PS mediates the removal of apoptotic corpses without activating inflammatory or immune responses [17]. Also worth mentioning here is that in addition to CRT exposure, a co-translocation of the ER-sessile disulphide isomerase ERp57 occurs to the cell surface. Unlike CRT, ERp57 is however, per se, unable to exert pro-immunogenic effects [63].

Opposing the "eat me" signals that occur on ICD, other membrane molecules externally co-expressed on cancer cells can inhibit phagocytosis by APCs. An example here is CD47 that acts as a "don't eat me" signal. Indeed, antibody blockade of CD47 increases phagocytosis of tumor cells by APCs, and helps initiate anti-tumor CTL responses [65]. CRT exposure (and the balance between surface expression of CRT and CD47) appears also to correlate favorably with the control of various human cancers, including acute myeloid leukemia [66], non-Hodgkin lymphoma [67], and colorectal cancer [68].

5.3.2 HMGB1 and TLR4

A second hallmark contributor to ICD is the release of HMGB1 from dying cells, and its sensing by TLR4 on APCs (Fig. 5.2). TLR4 is best characterized as a member of the TLR family of PRRs evolutionarily conserved for the detection of lipopolysaccharide (LPS) constructs present in Gram-negative bacteria [5, 10]. TLR4 pathway activation results in a potent immune cell production of proinflammatory cytokines. Three lines of evidence have demonstrated the important role of TLR4 in ICD: (a) expression of TLR4 (and its adaptor MyD88) is required by DCs for immune responses against dying cells in vivo following

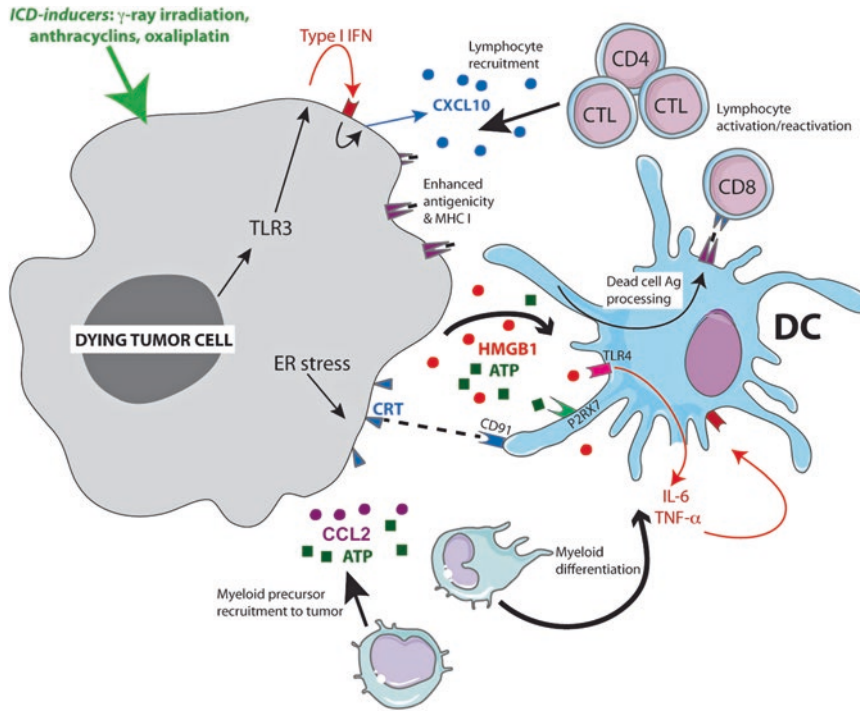

Fig. 5.2 Induced immunogenic cell death in the tumor microenvironment. Exposure of tumor cells to certain forms of chemotherapy or γ-ray irradiation induces a pattern of cell death that stimulates anti-tumor host immune responses. This immunogenic cell death (ICD) is characterized by an ER stress response that results in the exposure of calreticulin (CRT) on the cell surface membrane of the dying cell, the release from dying cells of HMGB1 and ATP. These molecules interact with CD91, TLR4, and P2RX7 receptors, respectively, on antigen-presenting cells such as dendritic cells (DC), which can derive from myeloid precursors recruited by CCL2 and ATP signaling post treatment. This results in maturation of DCs and their secretion of pro-inflammatory cytokines such as IL-6 and TNF-α. This inflammatory environment, alongside the uptake by mature DCs of tumor antigens, helps to drive anti-tumor T cell responses. The resulting T cells can be attracted into tumor beds via tumor cell secretion of the chemokine CXCL10, which results from autocrine and paracrine type I IFN signaling among tumor cells post chemotherapy

treatment with certain chemotherapies or radiotherapy; (b) the nonhistone chromatin protein HMGB1 released by dying tumor cells prompts DC processing and cross-presentation of TAAs through its ligating and triggering of TLR4; (c) node-positive breast cancer patients carrying a *TLR4* loss-of-function allele relapse faster post radiotherapy or chemotherapy compared to patients carrying the normal *TLR4* allele [47].

In support of these findings, a high-potency and exclusive TLR4 agonist has been shown to improve the immunogenicity and efficacy of chemotherapy against tumors with low expression of HMGB1, or in tumors where HMGB1 is depleted by RNA interference [69]. Furthermore, the natural TLR4 ligands deriving from gut commensals can determine the efficacy of chemotherapy, since eliminating this source of bacterial TLR4 ligands through treating mice with antibiotics (or in germ-free settings) inhibits the anti-tumoral efficacy of oxaliplatin as well as the activation and responses of tumor-infiltrating myeloid-derived cells [70]. Finally, some immunotherapies may rely on HMGB1/TLR4 signaling for their efficacy; an example being the therapeutic effect of anti-HER2/neu antibodies, which have been described to occur through host Myd88 and tumor-derived HMGB1 in vivo [71].

5.3.3 Release of ATP by Dying Cells and Autophagy

Besides TLR4 signaling, another PRR family, the inflammasomes, also contribute to ICD. Inflammasomes are an intracellular assembly of activated proteins, enzymes, and adaptor molecules that form a danger-sensing apparatus that may be actuated by PAMPs, or DAMPs such as uric acid and changes in K^+ ion concentration [6, 72]. The outcome of inflammasome assembly and triggering following such stimuli is the downstream activation of caspase-1, which in turn proteolytically matures pro-IL-1β to active IL-1β. Release of IL-1β following its generation enables it to act its role as a potent pro-inflammatory cytokine, and a critical mediator of ICD.

NLRP3 inflammasome activation has been shown to be a key factor in ICD, since mice deficient for the inflammasome component genes *Nlrp3* and *Casp1* fail to generate ICD post oxaliplatin chemotherapy [49]. The mechanism behind this component of ICD was identified to be the release of ATP from dying or stressed cells, which activates the inflammasome within DCs indirectly through P2RX7 receptors present on DC surface membranes. This enabled DC release of IL-1β, necessary for the priming of IFN-γ-producing tumor-specific $CD8^+$ T cells. Notably, it appears probable that activation of other PRRs (e.g., TLR4 activation by HMGB1) is a co-requirement to establish this immune response.

ATP release in response to ICD-inducing chemotherapy was later found to be under the control of autophagy during the dying process [44]. Unlike autophagy-competent cancer cells, those made autophagy-deficient were seen to have reduced release of ATP when undergoing cell death and, when implanted into mice, the resulting tumors failed to attract T lymphocytes and APCs into the tumor bed [44]. These deficiencies could however be reversed by pharmacological inhibition of extracellular ATP-degrading enzymes, which helped to boost ATP concentrations in the tumor microenvironment.

ATP is a potent chemoattractant for DCs and scavenging macrophages, stimulating these myeloid immune cells via their membrane-expressed P2RY2 and P2RX7 purinergic receptors. Indeed, the early tumor infiltration of myeloid precursor cells observed following anthracycline chemotherapy is abolished in the presence of a broad-spectrum purinergic receptor inhibitor or if local extracellular concentrations of ATP are decreased by overexpression of the ecto-ATPase CD39 [56]. Such events following ICD may determine the ensuing microenvironment of tumors, since ATP concentration (and presumably purinergic receptor signaling) might dictate whether myeloid precursors preferentially differentiate towards DCs as opposed to granulocytes (which are hypothesized under some situations to be detrimental to tumor control) [56]. These studies also suggest a potential immunosurveillance-escape strategy for cancer cells, where they may be able to negatively regulate intrinsic autophagic processes (and thus potentially ATP production if this precedes cell death). In accord with this, autophagy is often disabled during early oncogenesis [73], and the expression of ecto-ATPases by triple negative breast cancers promotes poor prognosis [74].

5.3.4 Viral Mimicry and the Release of Type I IFN

The ICD activity of anthracycline and oxaliplatin chemotherapies may also rely on type I IFN signaling. It has been identified that type I IFN signaling takes place in neoplastic cells rather than host cells following ICD-inducing chemotherapy [75]. Anthracycline and oxaliplatin were each seen to stimulate a rapid production of type I IFNs from malignant cells, an effect that was dependent on stimulation of the endosomal PRR TLR3. The precise anthracycline-elicited ligand(s) responsible for TLR3 stimulation in this scenario remains to be determined, though one could postulate that this is a dysregulated structure of self RNA released from stressed or dying cancer cells. Indeed, other DNA-damaging agents have been shown to generate double-stranded RNA molecules that trigger TLR3-dependent cytokine secretion [76]. Subsequent autocrine and paracrine signaling of type I IFNs appears to induce the production of the chemokine CXCL10, a potent chemoattractant that recruits T lymphocytes into the tumor bed. This cascade of events post anthracycline chemotherapy

is supported by findings that tumors deficient for the genes encoding TLR3 or the type I IFN receptor are not controlled unless the subsequent steps in the cascade, type I IFN and CXCL10 respectively, are artificially provided [75]. In line with this experimental setting, the expression of *MX1* by tumor cells (a prominent signature gene downstream of type I IFN signaling) predicts metastasis-free survival in neoadjuvant chemotherapy in breast carcinoma patients with poor prognosis [75].

It has similarly been shown that ionizing radiation-mediated tumor regression depends upon type I IFN signaling [77], this setting requiring the adaptor protein STING, but not MyD88 [78]. STING signaling was required for type I IFN production by DCs following their sensing of irradiated-tumor cells, which occurred through the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS). Addition of exogenous type I IFN was able to rescue DC cross-presentation of TAAs in settings where cGAS- or STING-deficient DCs were used. This signaling cascade in DCs was essential for radiation-induced adaptive immune responses, which could be further enhanced by activating STING with a second messenger cyclic GMP-AMP during radiotherapy [78]. Programmed death ligand-1 (PD-L1) blockade may synergize with radiotherapy in this context to control both local and distant tumors, an effect mediated by $CD8^+$ T cells and a reduced myeloid-derived suppressor cell (MDSC) numbers within the tumor microenvironment [79, 80].

Induced expression of IFN-stimulated genes, and sensing through TLR3, are characteristic of the cellular response to viral infection [81]. Such "viral mimicry" appears to constitute an important attribute of successful chemotherapy and radiotherapy. Indeed, viruses also trigger ER stress and autophagy [73], which, as we have already mentioned, are important for chemotherapy-induced ICD.

5.4 Manipulating Cell Death for Therapeutic Control of Cancer

ICD can be visualized as initiating a cascade of defined biochemical changes and immune/inflammatory signaling pathways (as summarized in Fig. 5.2). However, not many chemotherapeutic treatments are able to induce ICD, instead promoting other cell death modalities such as apoptosis. Screening of 24 distinct cytotoxic chemotherapies revealed that only 4 of these induce protective anticancer immune responses *in vivo*, whereas all agents resulted in equivalent apoptosis of target cells [17]. These immunogenic agents included the three anthracyclines doxorubicin, idarubicin, and mitoxantrone, and the platinum compound oxaliplatin (of note, the structurally-related platinum compound cisplatin does not induce CRT exposure; Table 5.2). Each of these four anti-neoplastic agents induces the key hallmarks of ICD following exposure to tumor cells (i.e., CRT exposure, and release of ATP and HMGB1 during the dying process). Interestingly, by running numerous FDA-approved drugs through a screening platform able to detect ICD-induced biochemical changes, novel compounds have been identified that may prove to be promising adjunctive therapies in cases where standard cancer treatments are inadequately immunogenic [51]. Notably, cardiac glycosides (e.g. digoxin, digitoxin) were found to be a drug class particularly efficient at inducing ICD. Cardiac glycosides may induce ICD by inhibiting surface membrane Na^+,K^+-ATPase pumps, which results in a Ca^{2+} influx into cancer cells that can have proapoptotic and proimmunogenic effects [51].

Theoretically then, it would seem possible, in cases where ICD is absent, to design strategies that make therapeutic tumor cell death immunogenic—for example through administration of adjunctive treatments. Conceivable compensatory strategies might include the intratumoral administration of agents that induce ER stress. Treatment with thapsigargin (an inhibitor of the sarco/ER Ca^{2+} -ATPase), or enforced reduction of ER Ca^{2+} levels through overexpression of the Ca^{2+} channel reticulon 1C, can each restore CRT translocation in cases where CRT is absent following chemotherapy [53, 82]. In addition, inhibitors of the GADD34/PP1 complex (which forms a phosphatase of eIF2α) increase the rate of PERK-dependent eIF2α phosphorylation, thus promoting CRT surface exposure [83]. The proteasome inhibitor bortezomib can also facilitate immunogenic death of human tumors.

Bortezomib induces premortem stress in cancer cells and surface exposure of CRT and Hsp90, which enables phagocytosis via CD91 and potential propagation of anti-tumor immune responses by DCs [84, 85].

Similarly, administration of TLR4 agonists may prove to be a useful adjunctive compensation therapy. Tumors exhibiting weak expression of nuclear HMGB1 respond to chemotherapy more effectively if combined with a local or systemic administration of highly purified TLR4 agonists [69]. Strategies to increase local concentrations of ATP (e.g., through use of ectonucleotidase inhibitors), or replacing this signal through purinergic receptor agonists, might also promote immunogenicity of a non-ICD-inducing agents [44]. Intratumoral therapies of recombinant cytokines, for example IL-1β or IL-17, may be effective if their production by immune cells within the tumor is low or absent. Similarly, patients with molecular defects in any of the molecules involved or downstream of TLR3-induced type I IFN-dependent signaling may benefit from targeted delivery of type I IFN or CXCL10 alongside anthracycline treatment [75, 86]. Finally, with the aforementioned discovery that cardiac glycosides induce ICD, adjunctive administration of cardiac glycosides could prove to be the most reachable future strategy [51].

Blocking the immunosuppressive tumor microenvironment could provide an alternative strategy. Targeting potent immunosuppressive cytokines is likely to be the most effective and tangible approach here, perhaps with intratumoral injection of IL-10- or TGF-β-neutralizing antibodies should these become clinically available. Since the rebooting of anti-cancer T cell responses following ICD induction, combination of ICD inducers with immune checkpoint inhibitors (e.g., blockade of CTLA-4 and the PD-1/PD-L1 axis) may provide another promising intervention.

5.5 Conclusions

The evolution of the immune system to discriminate between physiological and pathological instances of cell death, and to perceive cellular demise as immunogenic or tolerogenic, is pivotal to homeostasis and host defense. In this chapter, we have described how cancer cells can manipulate apoptosis to induce tolerance and evade immunosurveillance. On the other hand, we have discussed how ICD can be used therapeutically to induce durable immune responses that target and eradicate tumors. ICD is defined by set spatiotemporal combinations of DAMPs that are decoded by PRRs on immune cells to (re)activate an antitumor immune response, and to avoid further induction of tolerance. For this process to operate efficiently, certain prerequisites must be met. These include: (i) that cancer cells emit all the signals required for cell death to be interpreted as immunogenic, (ii) that immune cells have or maintain the capacity to properly recognize and decode such signals, and (iii) that the host immune system is able to translate these signals into a robust cell-mediated immune response. The identification and clinical development of agents and strategies that fulfill these criteria could revolutionize how we treat cancer.

References

1. Reed JC. Drug insight: cancer therapy strategies based on restoration of endogenous cell death mechanisms. Nat Clin Pract Oncol. 2006;3(7):388–98.
2. Gregory CD, Pound JD. Cell death in the neighbourhood: direct microenvironmental effects of apoptosis in normal and neoplastic tissues. J Pathol. 2011;223(2):177–94.
3. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nat Rev Immunol. 2009;9(5):353–63.
4. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol. 2013;31:51–72.
5. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197–216.
6. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ. 2007;14(1):10–22.
7. Medzhitov R, Janeway CA Jr. Decoding the patterns of self and nonself by the innate immune system. Science. 2002;296(5566):298–300.
8. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991–1045.
9. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer. 2012;12(12):860–75.

10. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783–801.
11. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piacentini M, Rubinsztein DC, Shi Y, Simon HU, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G, Kroemer G. Molecular definitions of cell death subroutines: recommendations of the nomenclature committee on cell death 2012. Cell Death Differ. 2012;19(1):107–20.
12. Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, Lysiak JJ, Harden TK, Leitinger N, Ravichandran KS. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature. 2009;461(7261):282–6.
13. Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, Xu Y, Autenrieth IB, Schulze-Osthoff K, Belka C, Stuhler G, Wesselborg S. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003;113(6):717–30.
14. Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, Melville L, Melrose LA, Ogden CA, Nibbs R, Graham G, Combadiere C, Gregory CD. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood. 2008;112(13):5026–36.
15. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992;148(7):2207–16.
16. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005;123(2):321–34.
17. Obeid M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med. 2007;13(1):54–61.
18. Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, Mohler W, Han DK. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. Dev Cell. 2003;4(4):587–98.
19. Nakai Y, Shiratsuchi A, Manaka J, Nakayama H, Takio K, Zhang JT, Suganuma T, Nakanishi Y. Externalization and recognition by macrophages of large subunit of eukaryotic translation initiation factor 3 in apoptotic cells. Exp Cell Res. 2005;309(1):137–48.
20. Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. Nature. 2002;418(6894):200–3.
21. Lv Z, Bian Z, Shi L, Niu S, Ha B, Tremblay A, Li L, Zhang X, Paluszynski J, Liu M, Zen K, Liu Y. Loss of cell surface CD47 clustering formation and binding avidity to SIRPalpha facilitate apoptotic cell clearance by macrophages. J Immunol. 2015;195(2):661–71.
22. Rovere P, Peri G, Fazzini F, Bottazzi B, Doni A, Bondanza A, Zimmermann VS, Garlanda C, Fascio U, Sabbadini MG, Rugarli C, Mantovani A, Manfredi AA. The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. Blood. 2000;96(13):4300–6.
23. Jaillon S, Jeannin P, Hamon Y, Fremaux I, Doni A, Bottazzi B, Blanchard S, Subra JF, Chevailler A, Mantovani A, Delneste Y. Endogenous PTX3 translocates at the membrane of late apoptotic human neutrophils and is involved in their engulfment by macrophages. Cell Death Differ. 2009;16(3):465–74.
24. Garg AD, Martin S, Golab J, Agostinis P. Danger signalling during cancer cell death: origins, plasticity and regulation. Cell Death Differ. 2014;21(1):26–38.
25. Weigert A, Tzieply N, von Knethen A, Johann AM, Schmidt H, Geisslinger G, Brune B. Tumor cell apoptosis polarizes macrophages role of sphingosine-1-phosphate. Mol Biol Cell. 2007;18(10):3810–9.
26. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med. 2000;191(3):423–34.
27. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003;21:685–711.
28. Ferguson TA, Herndon J, Elzey B, Griffith TS, Schoenberger S, Green DR. Uptake of apoptotic antigen-coupled cells by lymphoid dendritic cells and cross-priming of CD8(+) T cells produce active immune unresponsiveness. J Immunol. 2002;168(11):5589–95.
29. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol. 2001;19:47–64.
30. Griffith TS, Kazama H, Van Oosten RL, Earle JK Jr, Herndon JM, Green DR, Ferguson TA. Apoptotic cells induce tolerance by generating helpless CD8+ T cells that produce TRAIL. J Immunol. 2007;178(5):2679–87.
31. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity. 2008;29(1):21–32.

32. Kleinclauss F, Perruche S, Masson E, de Carvalho Bittencourt M, Biichle S, Remy-Martin JP, Ferrand C, Martin M, Bittard H, Chalopin JM, Seilles E, Tiberghien P, Saas P. Intravenous apoptotic spleen cell infusion induces a TGF-beta-dependent regulatory T-cell expansion. Cell Death Differ. 2006;13(1):41–52.
33. Maeda A, Schwarz A, Kernebeck K, Gross N, Aragane Y, Peritt D, Schwarz T. Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. J Immunol. 2005;174(10):5968–76.
34. Chen W, Frank ME, Jin W, Wahl SM. TGF-beta released by apoptotic T cells contributes to an immunosuppressive milieu. Immunity. 2001;14(6):715–25.
35. Gao Y, Herndon JM, Zhang H, Griffith TS, Ferguson TA. Antiinflammatory effects of CD95 ligand (FasL)-induced apoptosis. J Exp Med. 1998;188(5):887–96.
36. Tomimori Y, Ikawa Y, Oyaizu N. Ultraviolet-irradiated apoptotic lymphocytes produce interleukin-10 by themselves. Immunol Lett. 2000;71(1):49–54.
37. Chung EY, Liu J, Homma Y, Zhang Y, Brendolan A, Saggese M, Han J, Silverstein R, Selleri L, Ma X. Interleukin-10 expression in macrophages during phagocytosis of apoptotic cells is mediated by homeodomain proteins Pbx1 and Prep-1. Immunity. 2007;27(6):952–64.
38. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998;101(4):890–8.
39. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature. 1997;390(6658):350–1.
40. Kim S, Elkon KB, Ma X. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. Immunity. 2004;21(5):643–53.
41. Ghiringhelli F, Menard C, Terme M, Flament C, Taieb J, Chaput N, Puig PE, Novault S, Escudier B, Vivier E, Lecesne A, Robert C, Blay JY, Bernard J, Caillat-Zucman S, Freitas A, Tursz T, Wagner-Ballon O, Capron C, Vainchencker W, Martin F, Zitvogel L. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. J Exp Med. 2005;202(8):1075–85.
42. Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L. Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med. 2005;202(7):919–29.
43. Wan YY, Flavell RA. The roles for cytokines in the generation and maintenance of regulatory T cells. Immunol Rev. 2006;212:114–30.
44. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L, Kroemer G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science. 2011;334(6062):1573–7.
45. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. Proc Natl Acad Sci U S A. 2010;107(4):1547–52.
46. Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. Oncogene. 2010;29(39):5346–58.
47. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13(9):1050–9.
48. Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, Schmitt E, Hamai A, Hervas-Stubbs S, Obeid M, Coutant F, Metivier D, Pichard E, Aucouturier P, Pierron G, Garrido C, Zitvogel L, Kroemer G. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med. 2005;202(12):1691–701.
49. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini JL, Schlemmer F, Tasdemir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin NM, Haynes NM, Smyth MJ, Kroemer G, Zitvogel L. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med. 2009;15(10):1170–8.
50. Tesniere A, Schlemmer F, Boige V, Kepp O, Martins I, Ghiringhelli F, Aymeric L, Michaud M, Apetoh L, Barault L, Mendiboure J, Pignon JP, Jooste V, van Endert P, Ducreux M, Zitvogel L, Piard F, Kroemer G. Immunogenic death of colon cancer cells treated with oxaliplatin. Oncogene. 2010;29(4):482–91.
51. Menger L, Vacchelli E, Adjemian S, Martins I, Ma Y, Shen S, Yamazaki T, Sukkurwala AQ, Michaud M, Mignot G, Schlemmer F, Sulpice E, Locher C, Gidrol X, Ghiringhelli F, Modjtahedi N, Galluzzi L, Andre F, Zitvogel L, Kepp O, Kroemer G. Cardiac glycosides exert anticancer effects by inducing immunogenic cell death. Sci Transl Med. 2012;4(143):143ra99.
52. Schiavoni G, Sistigu A, Valentini M, Mattei F, Sestili P, Spadaro F, Sanchez M, Lorenzi S, D'Urso MT, Belardelli F, Gabriele L, Proietti E, Bracci L. Cyclophosphamide synergizes with type I interferons through systemic dendritic cell reactivation and induction of immunogenic tumor apoptosis. Cancer Res. 2011;71(3):768–78.
53. Martins I, Kepp O, Schlemmer F, Adjemian S, Tailler M, Shen S, Michaud M, Menger L, Gdoura

A, Tajeddine N, Tesniere A, Zitvogel L, Kroemer G. Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress. Oncogene. 2011;30(10):1147–58.
54. Panaretakis T, Kepp O, Brockmeier U, Tesniere A, Bjorklund AC, Chapman DC, Durchschlag M, Joza N, Pierron G, van Endert P, Yuan J, Zitvogel L, Madeo F, Williams DB, Kroemer G. Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. EMBO J. 2009;28(5):578–90.
55. Ray-Coquard I, Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, Tredan O, Verweij J, Biron P, Labidi I, Guastalla JP, Bachelot T, Perol D, Chabaud S, Hogendoorn PC, Cassier P, Dufresne A, Blay JY, European Organization for R., Treatment of Cancer Soft T., Bone Sarcoma G. Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer Res. 2009;69(13):5383–91.
56. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, Portela Catani JP, Hannani D, Duret H, Steegh K, Martins I, Schlemmer F, Michaud M, Kepp O, Sukkurwala AQ, Menger L, Vacchelli E, Droin N, Galluzzi L, Krzysiek R, Gordon S, Taylor PR, Van Endert P, Solary E, Smyth MJ, Zitvogel L, Kroemer G. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity. 2013;38(4):729–41.
57. Ma Y, Mattarollo SR, Adjemian S, Yang H, Aymeric L, Hannani D, Portela Catani JP, Duret H, Teng MW, Kepp O, Wang Y, Sistigu A, Schultze JL, Stoll G, Galluzzi L, Zitvogel L, Smyth MJ, Kroemer G. CCL2/CCR2-dependent recruitment of functional antigen-presenting cells into tumors upon chemotherapy. Cancer Res. 2014;74(2):436–45.
58. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, Daniel D, Hwang ES, Rugo HS, Coussens LM. Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. Cancer Cell. 2014;26(5):623–37.
59. Zitvogel L, Galluzzi L, Smyth MJ, Kroemer G. Mechanism of action of conventional and targeted anticancer therapies: reinstating immunosurveillance. Immunity. 2013;39(1):74–88.
60. Ma Y, Aymeric L, Locher C, Mattarollo SR, Delahaye NF, Pereira P, Boucontet L, Apetoh L, Ghiringhelli F, Casares N, Lasarte JJ, Matsuzaki G, Ikuta K, Ryffel B, Benlagha K, Tesniere A, Ibrahim N, Dechanet-Merville J, Chaput N, Smyth MJ, Kroemer G, Zitvogel L. Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy. J Exp Med. 2011;208(3):491–503.
61. Mattarollo SR, Loi S, Duret H, Ma Y, Zitvogel L, Smyth MJ. Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. Cancer Res. 2011;71(14):4809–20.
62. Ma Y, Yamazaki T, Yang H, Kepp O, Galluzzi L, Zitvogel L, Smyth MJ, Kroemer G. Tumor necrosis factor is dispensable for the success of immunogenic anticancer chemotherapy. Oncoimmunology. 2013;2(6):e24786.
63. Kepp O, Menger L, Vacchelli E, Locher C, Adjemian S, Yamazaki T, Martins I, Sukkurwala AQ, Michaud M, Senovilla L, Galluzzi L, Kroemer G, Zitvogel L. Crosstalk between ER stress and immunogenic cell death. Cytokine Growth Factor Rev. 2013;24(4):311–8.
64. Sukkurwala AQ, Martins I, Wang Y, Schlemmer F, Ruckenstuhl C, Durchschlag M, Michaud M, Senovilla L, Sistigu A, Ma Y, Vacchelli E, Sulpice E, Gidrol X, Zitvogel L, Madeo F, Galluzzi L, Kepp O, Kroemer G. Immunogenic calreticulin exposure occurs through a phylogenetically conserved stress pathway involving the chemokine CXCL8. Cell Death Differ. 2014;21(1):59–68.
65. Tseng D, Volkmer JP, Willingham SB, Contreras-Trujillo H, Fathman JW, Fernhoff NB, Seita J, Inlay MA, Weiskopf K, Miyanishi M, Weissman IL. Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. Proc Natl Acad Sci U S A. 2013;110(27):11103–8.
66. Wemeau M, Kepp O, Tesniere A, Panaretakis T, Flament C, De Botton S, Zitvogel L, Kroemer G, Chaput N. Calreticulin exposure on malignant blasts predicts a cellular anticancer immune response in patients with acute myeloid leukemia. Cell Death Dis. 2010;1:e104.
67. Zappasodi R, Pupa SM, Ghedini GC, Bongarzone I, Magni M, Cabras AD, Colombo MP, Carlo-Stella C, Gianni AM, Di Nicola M. Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumor cells experiencing immunogenic death. Cancer Res. 2010;70(22):9062–72.
68. Peng RQ, Chen YB, Ding Y, Zhang R, Zhang X, Yu XJ, Zhou ZW, Zeng YX, Zhang XS. Expression of calreticulin is associated with infiltration of T-cells in stage IIIB colon cancer. World J Gastroenterol. 2010;16(19):2428–34.
69. Yamazaki T, Hannani D, Poirier-Colame V, Ladoire S, Locher C, Sistigu A, Prada N, Adjemian S, Catani JP, Freudenberg M, Galanos C, Andre F, Kroemer G, Zitvogel L. Defective immunogenic cell death of HMGB1-deficient tumors: compensatory therapy with TLR4 agonists. Cell Death Differ. 2013;21(1):69–78.
70. Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, Molina DA, Salcedo R, Back T, Cramer S, Dai RM, Kiu H, Cardone M, Naik S, Patri AK, Wang E, Marincola FM, Frank KM, Belkaid Y, Trinchieri G, Goldszmid RS. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science. 2013;342(6161):967–70.
71. Park S, Jiang Z, Mortenson ED, Deng L, Radkevich-Brown O, Yang X, Sattar H, Wang Y, Brown NK, Greene M, Liu Y, Tang J, Wang S, Fu YX. The therapeutic effect of anti-HER2/neu antibody depends

on both innate and adaptive immunity. Cancer Cell. 2010;18(2):160–70.
72. Ogura Y, Sutterwala FS, Flavell RA. The inflammasome: first line of the immune response to cell stress. Cell. 2006;126(4):659–62.
73. Ma Y, Galluzzi L, Zitvogel L, Kroemer G. Autophagy and cellular immune responses. Immunity. 2013;39(2):211–27.
74. Loi S, Pommey S, Haibe-Kains B, Beavis PA, Darcy PK, Smyth MJ, Stagg J. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. Proc Natl Acad Sci U S A. 2013;110(27):11091–6.
75. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, Vitale I, Goubar A, Baracco EE, Remedios C, Fend L, Hannani D, Aymeric L, Ma Y, Niso-Santano M, Kepp O, Schultze JL, Tuting T, Belardelli F, Bracci L, La Sorsa V, Ziccheddu G, Sestili P, Urbani F, Delorenzi M, Lacroix-Triki M, Quidville V, Conforti R, Spano JP, Pusztai L, Poirier-Colame V, Delaloge S, Penault-Llorca F, Ladoire S, Arnould L, Cyrta J, Dessoliers MC, Eggermont A, Bianchi ME, Pittet M, Engblom C, Pfirschke C, Preville X, Uze G, Schreiber RD, Chow MT, Smyth MJ, Proietti E, Andre F, Kroemer G, Zitvogel L. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. Nat Med. 2014;20(11):1301–9.
76. Bernard JJ, Cowing-Zitron C, Nakatsuji T, Muehleisen B, Muto J, Borkowski AW, Martinez L, Greidinger EL, Yu BD, Gallo RL. Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. Nat Med. 2012;18(8):1286–90.
77. Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, Fu YX, Auh SL. The efficacy of radiotherapy relies upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res. 2011;71(7):2488–96.
78. Deng L, Liang H, Xu M, Yang X, Burnette B, Arina A, Li XD, Mauceri H, Beckett M, Darga T, Huang X, Gajewski TF, Chen ZJ, Fu YX, Weichselbaum RR. STING-dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. Immunity. 2014;41(5):843–52.
79. Deng L, Liang H, Burnette B, Beckett M, Darga T, Weichselbaum RR, Fu YX. Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. J Clin Invest. 2014;124(2):687–95.
80. Deng L, Liang H, Burnette B, Weicheslbaum RR, Fu YX. Radiation and anti-PD-L1 antibody combinatorial therapy induces T cell-mediated depletion of myeloid-derived suppressor cells and tumor regression. Oncoimmunology. 2014;3:e28499.
81. MacMicking JD. Interferon-inducible effector mechanisms in cell-autonomous immunity. Nat Rev Immunol. 2012;12(5):367–82.
82. Tufi R, Panaretakis T, Bianchi K, Criollo A, Fazi B, Di Sano F, Tesniere A, Kepp O, Paterlini-Brechot P, Zitvogel L, Piacentini M, Szabadkai G, Kroemer G. Reduction of endoplasmic reticulum Ca2+ levels favors plasma membrane surface exposure of calreticulin. Cell Death Differ. 2008;15(2):274–82.
83. Kepp O, Galluzzi L, Giordanetto F, Tesniere A, Vitale I, Martins I, Schlemmer F, Adjemian S, Zitvogel L, Kroemer G. Disruption of the PP1/GADD34 complex induces calreticulin exposure. Cell Cycle. 2009;8(23):3971–7.
84. Cirone M, Di Renzo L, Lotti LV, Conte V, Trivedi P, Santarelli R, Gonnella R, Frati L, Faggioni A. Primary effusion lymphoma cell death induced by bortezomib and AG 490 activates dendritic cells through CD91. PLoS One. 2012;7(3):e31732.
85. Spisek R, Charalambous A, Mazumder A, Vesole DH, Jagannath S, Dhodapkar MV. Bortezomib enhances dendritic cell (DC)-mediated induction of immunity to human myeloma via exposure of cell surface heat shock protein 90 on dying tumor cells: therapeutic implications. Blood. 2007;109(11):4839–45.
86. Yang X, Zhang X, Fu ML, Weichselbaum RR, Gajewski TF, Guo Y, Fu YX. Targeting the tumor microenvironment with interferon-beta bridges innate and adaptive immune responses. Cancer Cell. 2014;25(1):37–48.

Exosomes in Cancer: Another Mechanism of Tumor-Induced Immune Suppression

6

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6.1 Introduction

Tumor-induced immune suppression has been extensively investigated in the last decade. Indeed, escape of tumors from the host immune system has been considered a major barrier for successful immunotherapy of cancer [1]. Recent unprecedented tumor control that can be achieved by targeting immune checkpoint inhibitors (ICIs) clearly emphasizes the importance of the restoration of anti-tumor immune activity in patients with cancer by the use of antagonistic antibodies to CTLA-4, PD-1, PD-L1 or other ICIs [2]. Used alone or in combination with each other and with conventional therapies, check point inhibition can unleash the power of the immune system in many cancer patients whose anti-tumor responses are compromised. While highly promising, ICIs efficiency in restoring anti-tumor responses varies broadly among patients with cancer [3]. It is not clear why some patients respond to ICIs and others do not, but limitations in responses could be explained by the acknowledged existence of multiple ICI pathways in cancer, only some of which are responsive to ICIs being used for therapy. Based on what is known about various immunosuppressive mechanisms operating in the tumor-microenvironment, some general rules can be formulated as follows: (a) these mechanisms are tumor-induced; (b) they involve one or more cellular and molecular pathways that may differ in primary vs. metastatic tumors, are selectively utilized by different tumor types and vary among patients, even those with the same malignancy; and (c) they are not restricted to the tumor microenvironment (TME) but mediate systemic effects leading to the partial or complete inhibition of anti-tumor immune responses in the entire body.

Among many tumor-derived factors or signals that modulate anti-tumor immunity, exosomes, specifically tumor-derived exosomes or TEX, are emerging as a new and so far not widely appreciated mechanism of immune suppression. This chapter will describe how and why exosomes, which are ubiquitously present in all body fluids of patients with cancer, are currently viewed as conveyors of tumor-derived suppression to the immune cells responsible for surveillance and cancer control.

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6.2 What Are Exosomes?

Exosomes are membrane-bound vesicles which are virus size, are produced by all cells and are released by cells under physiological and pathological conditions. They are the smallest of extracellular vesicles (EVs) released by cells, varying in size from 30 to 150 nm, found in supernatants of cultured cells as well as in all body fluids [4]. Although the nomenclature of EVs is still unclear [5], exosomes are a distinct type of vesicles which differ from larger EVs, such microvesicles (MVs, 200–1000 nm) or apoptotic bodies (1000–5000 nm) not only by their size but also by cellular mechanisms used for their secretion, the molecular content and functional properties [6]. MVs are formed by "blebbing" or "pinching off" from the cellular membrane of the parent cell and contain parts of the cytosol more or less randomly enclosed in vesicular "blebs." Apoptotic bodies are remnants of dead parental cells. The biogenesis of exosomes is unique: they originate from the endocytic compartment and their molecular content reflects, at least in part, that of the parental cell. As tumor cells produce and release masses of exosomes, tumor-derived exosomes (TEX) are ubiquitously present in body fluids of patients with cancer. The ratios of TEX/normal cell-derived exosomes in the plasma of cancer patients varies, but generally TEX represent a substantial proportion of total exosomes recovered from plasma, especially in patients with advanced malignancies [7].

The TEX molecular signature distinguishes them from exosomes derived from normal cells. Further, TEX released by different types of tumor cells have distinct molecular signatures [8]. Exosomes serve as information transfer vehicles, and TEX carry messages from the parent tumor cell to other normal or malignant cells [9]. Upon contacting targeted recipient cells, TEX carrying a cargo consisting of multiple molecular species, including mRNA, miRNA, and DNA, deliver their content to recipient cells and modify functions of these cells [10]. The mechanisms responsible for TEX delivery and processing of their cargo in recipient cells are not entirely understood, but may include the initial ligand-receptor type of binding on the cell surface followed by endocytosis or phagocytosis [11]. Because the TEX cargo is enriched in immunoinhibitory molecules, similar to those present in parental tumor cells, TEX targeting immune cells tend to induce down-stream activation of the inhibitory molecular pathways [12]. It has been shown that TEX isolated from supernatants of cultured tumor cells, which contain only TEX and no other exosomes, effectively mediate suppression of immune cells in *ex vivo* assays and *in vivo* in experimental animals. Thus, immunosuppressive TEX are considered to be able to promote tumor growth and to facilitate tumor escape from the host immune system.

6.3 The Immunosuppressive Cargo of TEX

TEX, which originate from the late endosomal compartment of parent tumor cells, acquire their molecular components through the well-defined series of coordinated inward membrane invaginations taking place in late exosomes and multivesicular bodies (MVBs) [13, 14]. Upon fusion of MVBs with the parent cell surface membrane, TEX are released into the extracellular space. TEX formed by this biogenesis process contain elements derived from endosomes (e.g., TSG101, ALIX) as well as from the cell surface membrane and cytosol of a parent cell [6, 15]. Sorting and packaging of TEX for release from the parent cell is executed by the exosomal sorting complex responsible for transport (ESCRT), which might be parent-cell-specific, directing TEX to a predefined cellular address.

Upon their release from parental cells, TEX carry a broad variety of molecular species, including membrane-associated proteins, glycoproteins, lipids, and glycolipids as well as a rich vesicular content (reviewed in [10]). The surface membrane of TEX is a lipid-protein bilayer that contains cholesterol, ceramides, sphingomyelins, and phospholipids as well as numerous biologically active proteins such as the major histocompatibility complex (MHC) molecules; TAAs; inhibitory ligands such as FasL, TRAIL, PD-L1,

TGF-β/LAP; adhesion molecules, notably ICAM, EPCAM, CD44, integrins; proteases such as MMPS and CD26; ectonucleotidases engaged in adenosine production, CD39/CD73; transmembrane receptors such as CXCR4 and c-Met; heat shock proteins (HSPs); and numerous tetraspanins frequently used as "exosome markers." In the TEX lumen are nucleic acids, including DNA, mRNA, and miRNA; cytosolic proteins including various enzymes; soluble factors, such as PGE_2; cytokines; histones; transport proteins such as ALIX, Rabs, dynamin, LAMPs; cytoskeletal proteins, including actin, tubulin, vimentin, and others; oncoproteins; and a variety of signaling molecules, including MAPK, ERK1/2, Rho, catenin, Wnt, and many others. The TEX molecular and genetic content recapitulates that of the parent cell. However, it is unclear how much of the parent cell content is passed on to exosomes, and the estimates vary widely from 5 to 50%. Nevertheless, It has been convincingly shown that TEX are enriched in some of the key molecules characteristic of the parent cell and thus can serve, at least in part, as surrogates of the parent tumor cells [16].

One intriguing aspect of the cargo TEX carry is that, in addition to a plethora of immunoinhibitory molecules, they also carry tumor-associated antigens (TAA), costimulatory molecules, MHC class I and class II molecules, and intraluminal growth-promoting cytokines [10, 17]. This suggests that TEX are capable of stimulating immune cell responses and that TEX have the dual functional potential. This has led to a controversy regarding TEX and their biological role in cancer, with many investigators viewing TEX as vaccination-promoting vehicles capable of inducing effective anti-tumor immunity [18, 19]. It appears, however, that in the TME, where tumor cells are actively engaged in suppression of anti-tumor immunity and activities of immune cells are blocked, TEX are primarily utilized as an effective mechanism designed to promote tumor progression. It is reasonable to expect that the vesicle-based communication system driven by the tumor is operating to benefit tumor progression and to impair anti-tumor immune responses.

6.4 Communication of TEX with Their Cell Targets

TEX produced by parent cells and released in the extracellular space can interact with local and distant cellular targets. It is unknown whether TEX "carry an address." But their ubiquitous presence in all body fluids suggests that TEX are freely distributed throughout the body and can interact with any recipient cell ready to commit itself and accept the vesicles. In fact, exosomes are admirably equipped to serve as communication vehicles. Their surface is decorated by the parent cell-derived signaling molecules. Their intra-vesicular content of genetic materials, enzymes, and soluble factors, all biologically active and capable of executing functional responses in target cells, is protected by a membrane from potential degradation by extracellular enzymes during transport. Thus, exosome content can be safely delivered to recipient cells and upon exosome up-take can lead to the cell re-programing [14]. Exosomes can interact with target cells utilizing one or more of the following mechanisms: (a) direct signaling via surface molecules to activate intracellular signaling pathways; (b) fusion with the target cell membrane followed by transfer of proteins or genes the cell lumen; (c) phagocytosis of opsonized exosomes and their internalization; (d) receptor-mediated endocytosis [11]. The cargo delivered by exosomes to recipient cells and taken up by phagocytosis or endocytosis may be either directed to the lysosomes for degradation and clearance or directly incorporated into the cellular machinery to initiate functional re-programming of the recipient cells.

The mechanisms through which TEX alter functions of recipient cells are only partly understood and are being intensively investigated. It appears that some of these mechanisms involve the receptor/ligand type signaling and others require up-take and internalization of TEX [11, 20]. In some cases, TEX fusion with the membrane of a recipient cell may be sufficient to generate signals that induce cellular re-programming [11, 20]. It may be that the recipient cell determines the mode of TEX up-take, which in turn

activates downstream molecular/genetic events, culminating in the change of functions. Immune cells differ in their ability to internalize and process TEX. T cells interact with TEX via the receptor/ligand signaling, while other lymphocytes (B cells, NK cells) and monocytes internalize TEX [21]. TEX deliver receptor-mediated signals to T cells that initiate sustained Ca^{2+} flux [20] resulting in subsequent activation of the relevant downstream pathways, alterations in the recipient cell transcriptome and ultimately translate into modified functional responses [21]. Interestingly, TEX deliver negative signals to effector T cells and activating signals to regulatory T cell (Treg) and MDSC, as discussed below.

6.5 Mechanisms Used by TEX to Alter Function of Recipient Cells

All types of immune cells are sensitive to TEX-mediated interference. However, T lymphocytes seem to be especially vulnerable to negative messages delivered by TEX. The two key receptors on T cells are the T-cell receptor (TcR) and interleukin 2 receptor (IL-2R). We and others have reported that TEX negatively regulate functions of these receptors [22, 23]. Specifically, TEX-mediated down-regulation of the TcR zeta chain is consistently seen in T cells co-incubated with TEX [24]. TEX also reduced JAK expression and phosphorylation in activated T cells [22], and since the integrity of the JAK pathway is essential for functions of IL-2, IL-7 and IL-15, the cytokines sharing the y chain of the IL-2R, down-regulation of JAK activity by TEX is detrimental to T-cell proliferation [25]. TEX were shown to inhibit proliferation of CD8+ T cells but promote expansion of CD4+ T cells, specifically of Treg, while exosomes released by normal cells promoted proliferation of all T cells [22]. Consistent with these data, TEX were found to increase STAT5 phosphorylation in activated CD4+ T cells and to inhibit STAT5 phosphorylation in activated CD8+ T cells [25]. These data suggest that TEX modulate functions of transcription factors such as STATs in recipient T cells. In addition, TEX preferentially inhibited proliferation of human melanoma-specific CD8+ T cells generated in cultures of T cells with melanoma peptide-pulsed DC [22] suggesting that TEX can inhibit antigen-specific T-cell responses. There is solid evidence in support of the ability of TEX carrying a membrane form of FasL or PD-L1 to alter functions of immune cells [22, 26]. TEX-mediated signals leading to apoptosis of activated CD8+ T cells were associated with early membrane changes (i.e., Annexin V binding) in recipient cells, caspase3 cleavage, cytochrome C release from mitochondria, loss of mitochondrial membrane potential (MMP) and DNA fragmentation [27]. These data suggest that TEX induce apoptosis in activated CD8+ T cells by engaging extrinsic as well as intrinsic apoptotic cascades. Further, the PI3K/AKT pathway is the key target for TEX in activated CD8+ T cells: dramatic, time-dependent AKT dephosphorylation and concomitant decreases in expression levels of BCL-2, BCL-xL and MCL-1 accompanied by an increase in levels of pro-apoptotic BAX were observed in these cells during co-incubation with TEX [27].

In a recent study, we co-incubated TEX with subsets of human CD4+, CD8+ and CD4+ CD39+ Treg cells isolated from peripheral blood of normal donors [21]. The objective was to study mechanisms used by recipient T cells to translate TEX-delivered signals into transcriptional activity and functional changes. The qRTPCR was used to monitor expression levels of 24 immunoregulatory genes [21]. Interestingly, massive changes in expression levels of multiple immunoinhibitory and immunostimulatory genes in T cells were observed following co-incubation with TEX. We found that the only factors that significantly regulated TEX-induced transcriptional activity in T cells, including changes in expression levels of genes mediating immune suppression or immune activation, were: (a) the presence or absence of exosomes; (b) recipient cell type (CD4+, CD8+ or Treg); and (c) the activation status of the recipient cells. The observed massive changes in mRNA expression levels were equally induced by co-incubation with TEX or

DEX (exosomes produced by human monocyte-derived cultured DC and used as control for TEX). However, TEX and DEX modulated different immunoregulatory genes, and some of the genes were modulated differently in Treg than in CD4+ or CD8+ cells. To show that TEX-mediated signals translated into relevant functions, we concomitantly measured CD69 (an activation marker) expression levels in CD4+ T effector cells by flow cytometry. TEX significantly decreased expression levels of CD69 on the surface of CD4+ T cells, which was consistent with TEX immunosuppressive functions [21]. Also, Treg co-incubated with TEX, which carry both CD39 and CD73 ectonucleotidases [28], significantly up-regulated production of immunosuppressive adenosine in a concentration- and time-dependent manner [21]. This set of data, together with the demonstration that T cells do not readily internalize TEX [20], provided evidence for the hypothesis that TEX signal by engaging surface receptors on recipient T cells and that this signaling negatively modulates T-cell responses.

Our studies of TEX-immune cell interactions have indicated that TEX may exert direct or indirect effects on human immune cells. Directly, TEX induce apoptosis of activated anti-tumor effector T cells [22, 29]; TEX inhibit functions necessary for sustaining anti-tumor responses such as activation, proliferation, and cytotoxicity [22]; TEX interfere with normal differentiation of immune cells [30, 31]; TEX polarize immune cells to tumor-promoting phenotypes and regulate mobilization of immune cells to the tumor [23, 32]. Indirectly, TEX expand proliferation of Treg and myeloid-derived suppressor cells (MDSC) and up-regulate suppressor activity of these cells thus contributing to tumor-induced immune suppression and the tumor immune escape [33, 34]. In addition, TEX can interfere with immune therapies. Antibody-based cancer therapies could be made less effective by TEX carrying TAAs which are targeted by therapeutic antibodies: TEX, ubiquitous in all body fluids, can "soak" therapeutic antibodies diminishing their anti-tumor effects [35]. Adoptively transferred activated T or NK cells may be especially vulnerable to TEX carrying multiple inhibitory ligands [30]. Further, following the delivery of anti-tumor vaccines, newly minted, activated T cells may be highly sensitive to apoptosis by TEX carrying, e.g., FasL among other inhibitory ligands [29]. Emerging evidence clearly points to TEX as a major barrier to successful immunotherapy with antibodies, vaccines or adoptively transferred immune cells in patients with cancer.

6.6 TEX Interactions with Other Immune Cells

T lymphocytes are not the only immune cells targeted by TEX. Activities of human NK cells, B cells, and monocytes are impaired by co-incubation in the presence of TEX. In NK cells, down-regulation in expression of the activating receptors, especially NKG2D, is induced by TEX carrying MICA and MICB ligands [36]. NK-cell activation and cytotoxicity is inhibited by TGF-β, which is prominently displayed on TEX as transforming growth factor-latency associated protein (TGF-LAP), the form necessary for TGF-β activation upon binding to integrins, e.g., α6βV, on the surface of recipient cells [36, 37]. TEX, which are able to make adenosine from ATP by virtue of carrying CD39 and CD73 [28] are implicated in inducing suppressive activity in activated B cells, because adenosine can convert activated B cells into regulatory B cells [38]. TEX have been reported to inhibit normal differentiation of monocytes and to convert monocytes into TGF-β-expressing DCs, which secreted prostaglandin E_2 (PGE_2) and interfered with the generation of cytolytic T cells [34, 39]. In addition, TEX skewed differentiation of myeloid precursor cells toward developing into highly suppressive MDSCs. This function of TEX was dependent on MyD88 signaling in monocytes and the presence of TGF-β and PGE_2 in the TEX cargo [40]. In aggregate, TEX emerge as biologically active vesicles capable of negatively influencing functions of different types of immune cells by mechanisms engaging one or more than one molecular pathway responsible for functional changes in recipient cells.

6.7 Genetic Information Transfer by TEX

Nucleic acids present in the TEX lumen, including DNA, mRNA, and miRNA, play a major role in TEX-mediated delivery of genetic information to recipient cells. To date, relatively little information is available about DNA transfer by TEX [41]. On the other hand, exosomes are known to contain more than 10,000 distinct mRNA species, many of which are known to modulate immune regulation [42]. By far the greatest attention has been directed at miRNA carried by exosomes. MicroRNAs (miRNAs) are small (19–25 nucleotides) non-coding RNAs that suppress the translation of target mRNAs by binding to their 3′ untranslated region. MicroRNAs act as critical regulators of cellular processes such as proliferation, differentiation, apoptosis, and development [43]. MicroRNAs are a prominent component of the TEX cargo [44]. Upon TEX internalization by recipient cells, tumor-derived miRNAs alter gene expression by either repressing protein translation or degradation of multiple targeted mRNA species [45]. TEX are often called "oncomirs," and miRNAs derived from the tumor and transported to recipient cells have been extensively studied because of their potential role as cancer biomarkers and as a mechanism responsible for transcriptional regulation [46]. Numerous studies have shown that expression of individual miRNAs or specific miRNA signatures can be linked to the diagnosis and prognosis of many cancer types [47]. Many tumor-associated miRNAs, such as miR-21, miR-155, miR-146a, or miR-568, which are frequently recognized as components of the TEX cargos, are known to negatively regulate functions of immune cells or induce apoptosis [45, 48]. Current literature is replete with reports of exosomal transfer of miRNA from tumor to recipient immune cells leading to altered expression levels of complementary mRNA and subsequently to alterations in the transcriptional profile of recipient cells.

6.8 Plasma-Derived Exosomes Vs. TEX

While supernatants of cultured tumor cell lines have been widely used as a source of pure TEX, plasma of patients with cancer contains mixtures of exosomes derived from tumor and normal cells. Thus, plasma-derived exosomes are a heterogeneous mix of vesicles. Immune cells are also a rich source of exosomes and, therefore, miRNA or protein signatures of exosomes isolated from plasma of cancer patients probably reflect those of immune cells as well as the tumor and other tissue cells. It follows that to be able to truly understand how TEX modulate functions of immune cells and to define miRNA or protein signatures of TEX, it will be essential to develop methodologies for separation of TEX from immune cell- and other cell-derived exosomes present in patients' plasma. To this end, we and others are experimenting with methods for capture of TEX from patients' plasma and their separation from total plasma exosomes [49]. Meanwhile, total plasma exosome fractions are being used to link the total protein content and molecular as well as genetic exosome profiles to immune dysregulation in patents' with cancer. Remarkably, these studies appear to confirm the enrichment of exosomes bearing the immunosuppressive cargo in plasma of patients with cancer relative to normal donors [26]. Further, these studies confirm the correlations between the exosome immunosuppressive cargo and disease stage, activity and outcome [50].

6.9 Conclusions

Tumor-derived exosomes (TEX) carrying and delivering various inhibitory ligands to recipient immune cells in the TME are emerging as yet another category of CPIs. The available data support the critical role of exosomes in mediating tumor escape. Further, TEX appear to be implicated in down-regulation of effects of immune therapies in cancer. Rapid progress is being made in finding strategies for silencing of their suppressive cargo to protect immune cells from

inhibitory signals TEX deliver and to restore anti-tumor responses. The potential role of TEX as non-invasive biomarkers of cancer diagnosis, progression and outcome is being explored. The future development of TEX as "liquid biopsies" together with measures of TEX impact on functions of immune cells in patients with cancer promises to significantly improve diagnosis and prognosis of human malignancies.

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References

1. Whiteside TL, Demaria S, Rodriguez-Ruiz ME, Zarour HM, Melero I. Emerging opportunities and challenges in cancer immunotherapy. Clin Cancer Res. 2016;22(8):1845–55. https://doi.org/10.1158/1078-0432.CCR-16-0049.
2. Smyth MJ, Ngiow SF, Ribas A, Teng MW. Combination cancer immunotherapies tailored to the tumour microenvironment. Nat Rev Clin Oncol. 2016;13(3):143–58. https://doi.org/10.1038/nrclinonc.2015.209.
3. Beatty GL, Gladney WL. Immune escape mechanisms as a guide for cancer immunotherapy. Clin Cancer Res. 2015;21(4):687–92. https://doi.org/10.1158/1078-0432.CCR-14-1860.
4. Abels ER, Breakefield XO. Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. Cell Mol Neurobiol. 2016;36(3):301–12. https://doi.org/10.1007/s10571-016-0366-z.
5. Gould SJ, Raposo G. As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles. 2013;2. https://doi.org/10.3402/jev.v2i0.20389.
6. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013;200(4):373–83. https://doi.org/10.1083/jcb.201211138.
7. Keller S, Ridinger J, Rupp AK, Janssen JW, Altevogt P. Body fluid derived exosomes as a novel template for clinical diagnostics. J Transl Med. 2011;9:86. https://doi.org/10.1186/1479-5876-9-86.
8. van der Pol E, Boing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. Pharmacol Rev. 2012;64(3):676–705. https://doi.org/10.1124/pr.112.005983.
9. Boyiadzis M, Whiteside TL. Information transfer by exosomes: a new frontier in hematologic malignancies. Blood Rev. 2015;29(5):281–90. https://doi.org/10.1016/j.blre.2015.01.004.
10. Whiteside TL. Exosomes and tumor-mediated immune suppression. J Clin Invest. 2016;126(4):1216–23. https://doi.org/10.1172/JCI81136.
11. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles. 2014;3:24641. https://doi.org/10.3402/jev.v3.24641.
12. Whiteside TL. Tumor-derived exosomes and their role in tumor-induced immune suppression. Vaccine. 2016;4(4):35.
13. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci. 2013;126(Pt 24):5553–65. https://doi.org/10.1242/jcs.128868.
14. Lo Cicero A, Stahl PD, Raposo G. Extracellular vesicles shuffling intercellular messages: for good or for bad. Curr Opin Cell Biol. 2015;35:69–77. https://doi.org/10.1016/j.ceb.2015.04.013.
15. Cocucci E, Meldolesi J. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends Cell Biol. 2015;25(6):364–72. https://doi.org/10.1016/j.tcb.2015.01.004.
16. Atay S, Godwin AK. Tumor-derived exosomes: A message delivery system for tumor progression. Commun Integr Biol. 2014;7(1):e28231. https://doi.org/10.4161/cib.28231.
17. Whiteside TL. Tumor-derived exosomes and their role in cancer progression. Adv Clin Chem. 2016;74:103–41. https://doi.org/10.1016/bs.acc.2015.12.005.
18. Kunigelis KE, Graner MW. The dichotomy of tumor exosomes (TEX) in cancer immunity: is it all in the ConTEXt? Vaccines (Basel). 2015;3(4):1019–51. https://doi.org/10.3390/vaccines3041019.
19. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol. 2014;14(3):195–208. https://doi.org/10.1038/nri3622.
20. Muller L, Simms P, Hong CS, Nishimura MI, Jackson EK, Watkins SC, et al. Human tumor-derived exosomes (TEX) regulate Treg functions via cell surface signaling rather than uptake mechanisms. OncoImmunology. 2016. https://doi.org/10.1080/2162402X.2016.1261243.
21. Muller L, Mitsuhashi M, Simms P, Gooding WE, Whiteside TL. Tumor-derived exosomes regulate expression of immune function-related genes in human T cell subsets. Sci Rep. 2016;6:20254. https://doi.org/10.1038/srep20254.
22. Wieckowski EU, Visus C, Szajnik M, Szczepanski MJ, Storkus WJ, Whiteside TL. Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes. J Immunol. 2009;183(6):3720–30. https://doi.org/10.4049/jimmunol.0900970.
23. Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z. Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Cancer Res. 2007;67(15):7458–66. https://doi.org/10.1158/0008-5472.CAN-06-3456.

24. Taylor DD, Gercel-Taylor C, Lyons KS, Stanson J, Whiteside TL. T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors. Clin Cancer Res. 2003;9(14):5113–9.
25. Whiteside TL. Immune modulation of T-cell and NK (natural killer) cell activities by TEXs (tumour-derived exosomes). Biochem Soc Trans. 2013;41(1):245–51. https://doi.org/10.1042/BST20120265.
26. Hong CS, Funk S, Muller L, Boyiadzis M, Whiteside TL. Isolation of biologically active and morphologically intact exosomes from plasma of patients with cancer. J Extracell Vesicles. 2016;5:29289. https://doi.org/10.3402/jev.v5.29289.
27. Czystowska M, Han J, Szczepanski MJ, Szajnik M, Quadrini K, Brandwein H, et al. IRX-2, a novel immunotherapeutic, protects human T cells from tumor-induced cell death. Cell Death Differ. 2009;16(5):708–18. https://doi.org/10.1038/cdd.2008.197.
28. Schuler PJ, Saze Z, Hong CS, Muller L, Gillespie DG, Cheng D, et al. Human CD4(+) CD39(+) regulatory T cells produce adenosine upon co-expression of surface CD73 or contact with CD73(+) exosomes or CD73(+) cells. Clin Exp Immunol. 2014;177(2):531–43. https://doi.org/10.1111/cei.12354.
29. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clin Cancer Res. 2005;11(3):1010–20.
30. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. Cancer Res. 2007;67(7):2912–5. https://doi.org/10.1158/0008-5472.CAN-07-0520.
31. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, et al. Tumor exosomes inhibit differentiation of bone marrow dendritic cells. J Immunol. 2007;178(11):6867–75.
32. Luga V, Zhang L, Viloria-Petit AM, Ogunjimi AA, Inanlou MR, Chiu E, et al. Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell. 2012;151(7):1542–56. https://doi.org/10.1016/j.cell.2012.11.024.
33. Szajnik M, Czystowska M, Szczepanski MJ, Mandapathil M, Whiteside TL. Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg). PLoS One. 2010;5(7):e11469. https://doi.org/10.1371/journal.pone.0011469.
34. Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, et al. Induction of myeloid-derived suppressor cells by tumor exosomes. Int J Cancer. 2009;124(11):2621–33. https://doi.org/10.1002/ijc.24249.
35. Battke C, Ruiss R, Welsch U, Wimberger P, Lang S, Jochum S, et al. Tumour exosomes inhibit binding of tumour-reactive antibodies to tumour cells and reduce ADCC. Cancer Immunol Immunother. 2011;60(5):639–48. https://doi.org/10.1007/s00262-011-0979-5.
36. Szczepanski MJ, Szajnik M, Welsh A, Whiteside TL, Boyiadzis M. Blast-derived microvesicles in sera from patients with acute myeloid leukemia suppress natural killer cell function via membrane-associated transforming growth factor-beta1. Haematologica. 2011;96(9):1302–9. https://doi.org/10.3324/haematol.2010.039743.
37. Hong CS, Muller L, Whiteside TL, Boyiadzis M. Plasma exosomes as markers of therapeutic response in patients with acute myeloid leukemia. Front Immunol. 2014;5:160. https://doi.org/10.3389/fimmu.2014.00160.
38. Figueiro F, Muller L, Funk S, Jackson EK, Battastini AM, Whiteside TL. Phenotypic and functional characteristics of CD39high human regulatory B cells (Breg). Oncoimmunology. 2016;5(2):e1082703. https://doi.org/10.1080/2162402X.2015.1082703.
39. Bretz NP, Ridinger J, Rupp AK, Rimbach K, Keller S, Rupp C, et al. Body fluid exosomes promote secretion of inflammatory cytokines in monocytic cells via toll-like receptor signaling. J Biol Chem. 2013;288(51):36691–702. https://doi.org/10.1074/jbc.M113.512806.
40. Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, et al. Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. Am J Pathol. 2010;176(5):2490–9. https://doi.org/10.2353/ajpath.2010.090777.
41. Cai J, Wu G, Jose PA, Zeng C. Functional transferred DNA within extracellular vesicles. Exp Cell Res. 2016;349(1):179–83. https://doi.org/10.1016/j.yexcr.2016.10.012.
42. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol. 2008;10(12):1470–6. https://doi.org/10.1038/ncb1800.
43. Ruan K, Fang X, Ouyang G. MicroRNAs: novel regulators in the hallmarks of human cancer. Cancer Lett. 2009;285(2):116–26. https://doi.org/10.1016/j.canlet.2009.04.031.
44. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer. 2006;6(4):259–69. https://doi.org/10.1038/nrc1840.
45. Ye SB, Li ZL, Luo DH, Huang BJ, Chen YS, Zhang XS, et al. Tumor-derived exosomes promote tumor progression and T-cell dysfunction through the regulation of enriched exosomal microRNAs in human nasopharyngeal carcinoma. Oncotarget. 2014;5(14):5439–52.
46. Whiteside TL. The potential of tumor-derived exosomes for noninvasive cancer monitoring. Expert Rev Mol Diagn. 2015;15(10):1293–310.
47. Sato-Kuwabara Y, Melo SA, Soares FA, Calin GA. The fusion of two worlds: non-coding RNAs and extracellular vesicles—diagnostic and therapeutic implications (review). Int J Oncol. 2015;46(1):17–27. https://doi.org/10.3892/ijo.2014.2712.

48. Carissimi C, Carucci N, Colombo T, Piconese S, Azzalin G, Cipolletta E, et al. miR-21 is a negative modulator of T-cell activation. Biochimie. 2014;107 Pt B:319–26. https://doi.org/10.1016/j.biochi.2014.09.021.
49. Hong CS, Muller L, Boyiadzis M, Whiteside TL. Isolation and characterization of CD34+ blast-derived exosomes in acute myeloid leukemia. PLoS One. 2014;9(8):e103310. https://doi.org/10.1371/journal.pone.0103310.
50. Funk S, Floros T, Hong CS, Jackson EK, Lang S, Whiteside TL. Suppression of lymphocyte functions by plasma exosomes correlates with disease activity in patients with head and neck cancer. Clin Cancer Res. 2017;23(16):4843–54.

7 Chemo-Immunotherapy: Role of Indoleamine 2,3-Dioxygenase in Defining Immunogenic Versus Tolerogenic Cell Death in the Tumor Microenvironment

Theodore S. Johnson, Tracy Mcgaha, and David H. Munn

7.1 Introduction

When established tumors are treated with chemotherapy many tumor cells die, and multiple tumor-associated antigens are released. It is becoming increasingly clear that tumors contain many immunogenic antigens [1, 2]; so, ideally, tumor cell death after chemotherapy should be an opportunity for immune activation [3–5]. Unfortunately, under most circumstances, the default response to death of nucleated cells tends to be immunologic tolerance, rather than immune activation. In particular, apoptotic cell death often elicits potent immune suppression, by activating natural tolerogenic mechanisms that normally maintain tolerance to self. Thus, while certain types of chemotherapy, in certain settings, may be spontaneously immunogenic [6], in most cases the immune response following chemotherapy is weak and disappointing. In this chapter we will discuss the possibility that the indoleamine 2,3-dioxygenase (IDO) enzyme may be one tolerogenic pathway that limits the immune response to dying tumor cells.

IDO is one of the regulatory mechanisms that contributes to immune suppression and tolerance in the tumor microenvironment. Like many suppressive pathways that are co-opted by tumors, IDO is a natural mechanism of counter-regulation and tolerance in the immune system. In tumors, IDO can be aberrantly expressed by the tumor cells themselves [7]; or, importantly, IDO can also be naturally induced in host antigen-presenting cells (APCs) by a variety of pro-inflammatory signals. IDO can be induced in response to signals from the adaptive immune system such as IFNγ [8]; or to signals from the innate immune system such as type I interferons [9, 10]; and to pattern-recognition receptors such as TLR4 and TLR9 [11–13]. These IDO-inducing signals may be constitutively present in the inflammatory microenvironment of the tumor [8]; they may be actively up-regulated by the dying cells and release of tumor antigens that occurs after chemotherapy; or they may be actively induced by exogenous immunotherapy (checkpoint blockade, adoptive cellular therapy,

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vaccines or other modalities). In all of these cases, IDO and its related downstream pathways may help create an undesirable tolerogenic milieu, in which the immune system is prevented from responding to antigens released from dying tumor cells.

7.1.1 Natural Role of IDO

IDO is an immunoregulatory enzyme that exerts its biologic effects by degrading the essential amino acid tryptophan [14]. The IDO family includes two closely-related genes, IDO1 and IDO2 [15, 16], both of which catalyze the degradation of tryptophan along the kynurenine pathway. The biologic function of IDO2 is less well studied [17], and in this review we will use the general term "IDO" to include both genes, unless otherwise specified. IDO affects the immune system in two ways: first, by reducing the local concentration of tryptophan; and second, by producing biologically active tryptophan metabolites. Depletion of local tryptophan activates the GCN2 kinase pathway in neighboring cells [18]. GCN2 is a stress-response pathway that is sensitive to depletion of amino acids. Activation of GCN2 inhibits effector cell proliferation and differentiation, and it biases naive $CD4^+$ T cells toward Treg differentiation [18, 19]. In addition, secreted tryptophan metabolites are produced by IDO, comprising kynurenine and its subsequent breakdown products. These metabolites bind to the aryl hydrocarbon receptor (AhR) [20]. Signaling via the AhR can promote Treg differentiation [20], and bias dendtritc cells (DCs) toward an immunosuppressive/tolerogenic phenotype [21, 22]. Thus, IDO acts by multiple pathways to inhibit immune responses.

7.1.1.1 IDO and Acquired Peripheral Tolerance

The IDO pathway is both anti-inflammatory (i.e., it suppresses inflammation from the innate immune system) and tolerogenic (i.e., can create antigen-specific unresponsiveness in T cells). IDO does not participate in central tolerance in the thymus; rather, it acts in the periphery to keep inflammation in check, and to create acquired tolerance to new antigens. Thus, for example, IDO is expressed in the placenta, and pregnant mice treated with an IDO-inhibitor drug spontaneously reject allogeneic fetuses, driven by paternal alloantigens [23–25]. In a variety of experimental models of acquired peripheral tolerance, blocking IDO prevents the induction of mucosal tolerance [26, 27], tolerance created by CTLA-4/B7 or CD40 blockade [28–31], and other forms of acquired peripheral tolerance [32, 33]. Tissue allografts engineered to overexpress the IDO gene are accepted across fully-mismatched MHC barriers without immunosuppression [31, 34, 35]. Conversely, blocking or ablating IDO makes autoimmunity and inflammation markedly worse. Ablating IDO in mouse models of graft-versus-host disease increases lethality [36, 37], and blocking IDO in models of autoimmunity [38–41] or chronic infection [42, 43] markedly increases inflammation and exacerbates disease severity. In all of these models, the role of IDO is narrow and selective. IDO-deficient mice do not have the broad, spontaneous autoimmunity that is seen with mice lacking CTLA-4 or Tregs. But in the settings where IDO is relevant, this pathway can create potent de novo tolerance.

7.1.1.2 Acquired Tolerance to Apoptotic Cells

One striking example of the tolerogenic role of IDO occurs when mice are exposed to apoptotic cells. When apoptotic cells are injected intravenously they are cleared by specialized macrophages and dendritic cells in the spleen. This process normally produces robust antigen-specific tolerance [44, 45]. In this model, apoptotic cells were found to be potent inducers of IDO expression by $CD169^+$ macrophages in the spleen [46]. Blocking or genetic ablation of IDO prevented the immune system from creating the normal tolerance to antigens associated with apoptotic cells, leading to progressive development of a lethal lupus-like autoimmunity after repeated challenge [46]. Importantly, in this model the apoptotic cells were normal, syngeneic thymocytes, and thus contained no mutational neoantigens. Nevertheless, just the normal array

of self antigens associated with apoptotic cells was sufficient to drive rapid breakdown of self-tolerance if the immunosuppressive IDO signal was removed. This natural tolerogenic function of IDO during apoptosis suggests that the IDO pathway might become especially important in tumors during the wave of cell death and antigen release following chemotherapy.

7.1.2 Downstream Mechanisms: IDO-Induced Activation of Tregs

The signals generated by IDO are inherently local and short-range, based on local tryptophan depletion and secretion of bioactive metabolites. Therefore, beyond the immediate vicinity of the IDO-expressing cell these effects would rapidly abate. In tumors and tumor-draining lymph nodes, the number of IDO-expressing host cells is quite small, comprising at most a few percent of total immune cells [47]. Even if the tumor cells themselves express IDO, the distribution is patchy and local. These same observations are also true of IDO expression during infection, autoimmunity, or tolerance to apoptotic cells: in each case, the actual number of IDO-expressing APCs is small. Yet despite this inherently restricted and localized distribution, IDO is able to create robust effects throughout entire lymph nodes, spleen, tumors, and at the systemic (whole-animal) level [9, 12, 13, 18, 31, 46–48]. These widespread and systemic effects appear to rely not upon IDO itself, upon the ability of IDO to activate the potent and mobile regulatory T cell (Treg) population.

IDO can drive naive $CD4^+$ T cells to differentiate into $Foxp3^+$ "inducible" Tregs in vitro [19]. In vivo, IDO expressed by $CD103^+$ DCs in the gut was found to be required for de novo generation of Tregs from naive $CD4^+$ T cells during mucosal tolerance [26]. In human cells, plasmacytoid DCs from peripheral blood up-regulate IDO in vitro in response to CpG oligonucleotides [49] or HIV infection [50], and this can induce differentiation of $CD4^+$ cells into $Foxp3^+$ Treg-like cells. Similar findings have been reported using human monocyte-derived DCs [51, 52]. Thus, IDO can bias $CD4^+$ T cells to differentiate towards a regulatory phenotype.

Tumors are dominated by large numbers of Tregs, with a highly activated phenotype [53, 54]. The role of "inducible" (peripherally-generated) Tregs against unique tumor-specific neo-antigens remains somewhat controversial [55, 56]. However, it is not necessary that tumors create their associated Tregs de novo. Even if most of the Tregs in tumors are thymically-derived, and recognize the same set of self antigens found in normal tissues [57], these Tregs may still be recruited to the tumor in abnormally large numbers. More importantly, tumor-associated Tregs may become potently activated by the conditions of the tumor microenvironment. Consistent with this possibility, highly activated Tregs appear rapidly in growing tumors [58], and Tregs in human tumors have high levels of CTLA-4, PD-1 and other markers of activation [54]. Functionally, Tregs isolated from mouse tumor-draining LNs are constitutively pre-activated for in vitro suppression, without requiring any additional signals [12], and similar constitutive Treg activation seems to occur in human tumors [59].

We have shown that mouse plasmacytoid DCs isolated from tumor-draining LNs express IDO, and potently activate resting Tregs in vitro, in an IDO-dependent fashion [12]. This activation was rapid (occurring within hours) and affected pre-existing, fully mature Tregs. In vivo, Tregs from tumor-draining LNs displayed similar potent, IDO-induced suppressor activity. Tregs activated by IDO acquired a characteristic form of suppressor activity characterized by strict dependence on the PD-1/PD-ligand pathway [12]. While IDO is only one of multiple upstream signals by which Tregs may become activated [60–62], it is a mechanism that is frequently found in the tumor microenvironment.

Finally, IDO appears to stabilize the suppressive phenotype in Tregs so that they do not become destabilized (lose their suppressor activity) during inflammation. It has been somewhat controversial whether mature, thymic-derived Tregs can ever actually lose their suppressive phenotype [63, 64], but a number of

studies now suggest that this may indeed occur in certain biologically-relevant settings of inflammation [65–67]. It is certainly true that artificial genetic ablation of key pathways that maintain Treg stability will cause Tregs to convert into pro-inflammatory effector cells, leading to progressive autoimmunity [67–69]. We have shown that IDO stabilizes the Treg phenotype in the face of inflammation, by maintaining high levels of the Foxp3 co-repressor Eos (*Ikzf4*) and preventing IL-6-driven conversion into "helper-like" pro-inflammatory cells [70–73]. Under normal circumstances, this stabilizing effect of IDO on Tregs is beneficial for maintaining self-tolerance, but in the context of tumors it may instead help maintain the suppressive intra-tumoral milieu, and prevent desirable immune activation during immunotherapy.

In the following sections, we will consider the potential role of IDO in the tumor microenvironment following chemotherapy, during the time that the immune system faces the fundamental decision whether or not to respond to dying tumor cells.

7.2 Tolerance Is a Choice: The Response to Dying Cells Is Dictated by the Local Milieu

In a normal organism, cells are constantly dying and being replaced. Under homeostatic conditions, most of these cells will die by apoptosis, which is classically considered immunologically "silent". But this silence is not because apoptotic cells are invisible or inherently non-immunogenic; rather, it is because apoptotic cells generate specific signals that actively suppress the immune response and create tolerance [74]. IDO is one of these active tolerogenic signals elicited by apoptotic cells [46, 75]. The IDO pathway in turn is closely linked to production of TGFβ, activation of Tregs, and other known immunosuppressive responses to dying cells [12, 46, 75]. This concept of active immunosuppression by apoptotic cells has an important corollary, which is that tolerance to apoptotic cells is not inherent and inevitable—rather, it is a choice. If the suppressive mechanisms that enforce tolerance are blocked, then the same dying cells may now become spontaneously immunogenic. In the following discussion, we will consider primarily the case of chemotherapy, because this modality is widely used. However, similar molecular mechanisms may apply to the dying tumor cells released by immunologic therapy as well; so the discussion may be equally relevant to epitope-spreading after immunotherapy.

7.2.1 Tolerance to Tumor Cells After Chemotherapy Is Not Inevitable

Originally, chemotherapy was assumed to kill tumor cells solely by apoptosis [76]. This implied that cell death after chemotherapy would not be immunogenic. And indeed, in clinical practice this often appears to be the case: e.g., even large chemotherapy-sensitive tumors may melt away without evidence of inflammation or antigen-specific immune response. More recently, however, Drs. Zitvogel, Kroemer and colleagues have shown that, in at least in certain situations, chemotherapy can cause tumor cells to die by much more immunogenic forms of cell death, characterized by exposure of calreticulin and release of HMGB1 or ATP [77–79]. This discovery led to the speculation that the immune system might therefore be a fundamental contributor to the overall efficacy of chemotherapy [80]. While this would be an exciting possibility, the contribution of immunogenic cell death to chemotherapy has not been a universal finding in all tumor models, or with all chemotherapy drugs [81]. Immunogenic cell death has been more evident with anthracyclines or oxaliplatin than with other agents; and it is primarily observed in certain transplantable tumors. In the more refractory autochthonous tumors, which have "co-evolved" throughout their existence with the host immune system to create profound immunosuppression and tolerance, the immune system does not appear to contribute to the effects of chemotherapy [82]. Thus, in many settings, the immune

system does not seem to play the hoped-for role in the response to chemotherapy.

However, from a therapeutic perspective, the key question is not whether the immune system spontaneously contributes to the effect of standard chemotherapy. Indeed, we know that such spontaneous immune activation is probably often suppressed by endogenous counter-regulatory mechanisms. Rather, the relevant question for therapy is whether dying tumor cells would potentially immunogenic, if these endogenous tolerogenic pathways could be blocked. If the relevant endogenous suppressive pathways can be identified and understood, then these pathways present a rich therapeutic opportunity to capitalize upon the wave of antigens released after chemotherapy. By extension, this same opportunity may arise when tumor cells are killed by adoptive transfer of CAR-T cells, or by active immunization or other immunotherapy (although this setting has not been as well studied).

7.2.1.1 After Chemotherapy, both Tolerogenic and Immunogenic Cell Death Can Occur

The classical form of cell death induced by chemotherapy is apoptosis [83]. This should lead to exposure of phosphatidylserine on the outer leaflet of the cell membrane, which triggers production of immunosuppressive TGFβ by the macrophages that phagocytose the debris. The result—at least in theory—is immune suppression and tolerance. However, not all tumor cells die in such a well-behaved fashion. Depending on the type of cytotoxic insult and the nature of the tumor, dying cells may release pro-inflammatory factors such as HMGB1, ATP or free DNA. These can be sensed by cognate receptors (e.g., TLRs, purinergic receptors or STING) leading to inflammation and immune activation. With certain chemotherapy drugs, in certain tumor models, this immunogenic cell death may be quite robust [77, 78]. However, in most tumors the picture is probably mixed, with much immunosuppressive apoptosis occurring side-by-side with more immunogenic forms of cell death. The question therefore becomes which set of signals exerts the dominant effect on the local immune system.

Unfortunately, tumor-cell death takes place in an environment that is already heavily biased toward immune-suppression. Even prior to chemotherapy, the tumor milieu is usually rich in TGFβ and IL-10, and suppressive Tregs dominate over effector T cells. Similarly, the local macrophage population is biased toward an immunosuppressive "M2"-like phenotype, and many of the local myeloid cells are inhibitory myeloid-derived suppressor cells (MDSCs) rather than pro-inflammatory DCs and monocytes. Further, the tumor cells or host APCs may constitutively over-express IDO, and tumor-draining LNs may be dominated by IDO-expressing APCs. Given this extensive pre-existing bias toward suppression, it is not surprising that the degree of immune response following chemotherapy often appears sub-optimal.

7.2.1.2 In the Absence of Inducible Counter-Regulatory Mechanisms, Dying Cells Can be Highly Immunogenic

In the absence of elicited suppressive signals, however, dying cells themselves can be highly inflammatory. Cells that die by either necrosis or necroptosis release multiple pro-inflammatory mediators and danger signals [84]. Even cells that die by apoptosis can be immunogenic if they are phagocytosed by the right APC populations [85]. Indeed, spontaneous cross-presentation of antigens from necroptotic or apoptotic cells can be important in host defense against viral infections [86]. Thus, the underlying (intrinsic) nature of dying cells may actually be immunogenic, and would bias the immune response toward inflammation and immune responses, unless this process is actively suppressed by counter-regulation.

Consistent with this possibility, studies using in vivo challenge with apoptotic cells have revealed a potent regulatory role for IDO in controlling the choice between tolerance and immunity to dying cells [46, 75]. As described above in Sect. 1.1.2, when the IDO pathway was active then challenge with apoptotic cells led to tolerance induction, with high TGFβ and IL-10, and activation of Tregs. In contrast, when IDO was genetically ablated or blocked with indoximod (D-1MT) then apoptotic cells elicited high levels

of IL-6, IL-12 and TNFα, and mice developed lupus autoimmunity. Likewise, genetic ablation of the key IDO-expressing cell type in this system—a population of $CD169^{+}$ macrophages in the splenic marginal zone—resulted in failure to recruit suppressive Tregs, and inability to create acquired systemic tolerance to neo-antigens delivered on apoptotic cells [87]. Thus, IDO acted as a pivotal regulatory "switch" controlling the natural physiologic response to apoptotic cells. If they were allowed to induce IDO then apoptotic cells were tolerogenic, but if IDO was blocked then the same cells were immunogenic.

It is not yet known whether IDO plays a similar controlling role in the response to dying cells after chemotherapy. However, the importance of IDO in the normal physiologic response to apoptotic cells, and the fact that IDO is already either expressed or rapidly inducible in many tumors, suggest that this could be an important regulatory pathway in this setting.

7.2.1.3 Immunologic Contribution to the Effectiveness of Chemotherapy

Exactly how the immunosuppressive milieu in tumors affects responses to dying tumor cells has been difficult to study. Experimental systems using nominal antigens and TCR-transgenic T cells have yielded mixed results, which are sometimes contradictory. Some mouse models suggest that T cell responses to nominal tumor antigens are robust [88], but others suggest that they are poor and difficult to achieve [89]. One confounding factor in many mouse models is that they do not seem to recapitulate the profound degree of immune-suppression associated with actual human tumors. TCR-transgenic T cells often activate and proliferate robustly just by encountering the tumor, even without chemotherapy or other manipulation. It is unclear whether this occurs because the transplantable mouse tumor cells are not suppressive enough, or because the TCR-transgenic T cells are high-affinity and not readily tolerized. But whatever the cause, this does not at all resemble the situation in real human tumors [90]. Thus, results from experimental models that do not recapitulate this baseline level of immune suppression should probably be interpreted with caution.

This is not to say, however, that the immune system does not influence the response to chemotherapy in humans. Patients with large numbers of tumor-infiltrating T cells have a more favorable response to chemotherapy in breast and colon cancer [91, 92]. While this does not necessarily prove a mechanistic link, it is tempting to speculate that the immune system in these patients responds more robustly after chemotherapy, and this improves the outcome. Attempts are being made to exploit the immunogenicity of chemotherapy in the clinic [93]. Nonetheless, with or without a pre-existing immune infiltrate, the tumor milieu in human patients remains dominated by an array of immunosuppressive factors.

7.2.1.4 Breaking Tolerance to Tumor-Associated Antigens

Fortunately, therapeutic tools for reducing tumor-associated immunosuppression are now becoming available. Blocking antibodies against the CTLA-4 pathway and PD-1/PD-L pathway are approved or in development, and IDO-inhibitors are progressing through Phase I and II trials. Other agents are in the pipeline. Thus, the immunosuppressive nature of the tumor microenvironment is no longer an inevitable condition. However, the array of suppressive and counter-regulatory pathways in the tumor is still daunting, and much additional research is needed to understand how these pathways can best be overcome.

One important conceptual breakthrough has been the growing evidence that human tumors inherently possess immunogenic antigens. As genomic sequencing is increasingly used to predict immunogenic mutations, tumors are found to express multiple potential neo-antigens (reviewed in [1]). Importantly, in several studies the number of these putative neo-antigens appears to correlate with the likelihood of response to checkpoint blockade of CTLA-4 or PD-1 [94–96]. This last point is important, because it implies a paradigm shift in how we think about "immunogenic" tumors. In the clinical studies cited, the presence of mutational neo-antigens was not, in and of

itself, associated with an obviously "good-risk" subgroup. All of the patients had progressive disease at study entry; and, left untreated, all would have presumably succumbed. Thus, the presence of neo-antigens was not, by itself, protective against the tumor. The benefit accrued only when the patients received a therapeutic checkpoint inhibitor to help overcome immune suppression. Thus, the *potential* immunogenicity of the mutations was transformed into *actual* benefit only when the tumor-induced immunosuppression was removed. Conceptually, tolerance to these neo-antigens was broken by the therapy.

To extend this paradigm-shift further, it is now clear that tolerance can also be broken even to authentic, unmodified self antigens. This was demonstrated experimentally in the studies described above in Sect. 1.1.2, in which injection of unmodified "self" cells (syngeneic thymocytes) could break tolerance against even ubiquitous self antigens such as histones and DNA, as long as two conditions were met: the cells had to be induced to die, and the IDO pathway had to be blocked at the time of antigen presentation [46]. Thus, while self antigens from dying cells may be tolerogenic under normal circumstances, this apparent tolerance may be only contingent and conditional. The same antigens may become highly immunogenic if the relevant regulatory pathways are blocked.

In the setting of human cancer, it has long been observed that patients with immunogenic tumors such as melanoma often have circulating T cells against self antigens associated with the tumor [97]. The relevance (and potential danger) of such self antigens as therapeutic targets is supported by the occurrence of cross-reactive autoimmunity such as vitiligo and uveitis during immunotherapy for melanoma [98]. But the risk of autoimmunity, while real, does not mean that self antigens are not potentially useful targets in cancers. Tumors are very different from normal tissues: they are often much more chronically inflamed [99]; they may re-express antigens not normally found in the adult host (oncofetal antigens); they may process and present even normal self antigens in aberrant and immunogenic ways [100, 101]; and they have a constant level of cellular stress, autophagy and ongoing apoptosis that may render them more immunogenic than normal tissues [85, 102, 103]. These unique attributes of the tumor may allow certain self antigens to become important tumor-associated targets, with a manageable degree of selectivity for tumor over normal tissue. The relative contribution of mutational neo-antigens versus self antigens in anti-tumor therapy is currently unknown. But the key point for this discussion is that both sets of antigens may potentially be immunogenic, if the suppressive pathways in tumors can be blocked. And, unlike the case with a defined vaccine antigen, the optimally immunogenic antigens do not need to be known in advance. If the tumor milieu can be rendered immunogenic rather than immunosuppressive, then the patient's own immune system will identify the immunogenic antigens.

7.3 IDO as a Clinically Relevant Target

The preceding discussion introduces the concept of dying tumor cells as a rich source of antigens that are potentially immunogenic, but which cannot become actually immunogenic unless the relevant inhibitory pathways in the tumor are blocked. Therefore, it becomes important to identify which are the relevant pathways that control immunity versus tolerance to dying tumor cells. At present, this is incompletely understood.

The tumor microenvironment is filled with multiple immunosuppressive pathways. However, only certain of these mechanisms will be relevant to the uptake and cross-presentation of antigens from dying tumor cells. The CTLA-4 and PD-1/PD-L pathways, which are very important for the control of T cells, are not major direct regulators of antigen-presenting cells, or the innate inflammatory milieu. In contrast, IDO has a major effect on the biology of APCs, and in controlling innate inflammation (see Sect. 1.1.1). Thus, IDO and its associated downstream pathways may represent important therapeutic targets for modulating the key initial immune response to tumor-associated antigens.

7.3.1 The Inflammatory Signals Produced by Dying Cells May Elicit IDO

One of the defining attributes of the IDO gene is that it is highly inducible in response to inflammation. Depending on the context, both IFNγ and type I IFNs can be physiologic inducers of IDO, as can signals via the TLR/MyD88 pathway [14]. The degree to which dying tumor cells drive up-regulation of IDO in the tumor and tumor-draining LNs has not been well studied. However, it is known that tumors can be rich in type I IFNs (IFNα and IFNβ), driven in part by "danger" signals released by dying tumor cells [104]. Likewise, following chemotherapy, dying tumor cells may release HMGB1, a ligand for TLR4 [77], or extracellular DNA, which can be sensed via the pro-inflammatory STING pathway [105]. Like IFNs and TLR ligands, in other settings, STING has been shown to be a potent inducer of IDO [106–108], with consequent suppression of T cell responses. IDO can also be induced by prostaglandins such as PGE_2 [109], which can be produced by stressed cells. Thus, dying tumor cells potentially have multiple pathways by which they might induce IDO.

Any chemotherapy or immunologic therapy will, if successful, kill some fraction of the tumor cells, and thus release an array of tumor antigens. It would be highly desirable if the immune system could generate a productive response against this wave of endogenous tumor antigens. One of the important unanswered questions for the field is the extent to which counter-regulatory IDO may suppress immune responses to these endogenous antigens following conventional chemotherapy or immunotherapy; and how this may be targeted for therapy.

7.3.2 IDO and Counter-Regulation

At present, the extent to which IDO is induced and up-regulated in tumors following chemotherapy or immunotherapy remains unknown. In practice, this has been a difficult question to answer in humans, because it requires on-treatment biopsies of the tumor (or tumor-draining LNs) following therapy. To date, however, all studies of IDO have been in untreated tumors, prior to therapy. This is useful for identifying which tumors constitutively express or elicit IDO as part of their underlying biology, but it gives no information about how much reactive (counter-regulatory) IDO may have been elicited in response to cell death and inflammation. This "reactive" IDO may be a critical and highly relevant target for therapy, but it can only be detected by obtaining on-treatment biopsies. The fact that a patient's tumor cells were initially IDO-negative at diagnosis does not mean that the immunosuppressive host APCs will not subsequently up-regulate IDO in response to therapy.

The role of this reactive or counter-regulatory IDO becomes particularly germane in the case of clinical immunotherapy, such as T cell adoptive-transfer or checkpoint blockade. Indeed, preclinical models suggest that even the spontaneous, low-level endogenous T cell response against the tumor may generate enough inflammation to drive counter-regulatory IDO expression [8]. This level of inducible IDO might be greatly increased by interventions such as T cell adoptive transfer or checkpoint blockade. Not only do such treatments cause tumor cell death, but—as a consequence of their own success—they also create intense inflammation within the tumor. Both the cell death and this local inflammation may induce counter-regulatory IDO, and thus blunt the desired effect of therapy. Counter-regulatory IDO would not abrogate the effect entirely (the treatment would still show some efficacy), but there might be substantially more efficacy potentially available if the counter-regulatory IDO were blocked. Emerging evidence from mouse preclinical models suggests that this hypothetical concern may indeed be the case [110, 111]. In these studies, the efficacy of both CTLA-4 blockade and PD-1 blockade were enhanced by adding an IDO-inhibitor drug (indoximod or INCB23843). How much of this effect was due specifically to reactive (counter-regulatory) IDO was not determined, but the effect was recapitulated by genetic deficiency of IDO1 in the host [110], suggesting that the target was host IDO rather than tumor. Recently, using

a mouse xenograft model, it was shown that human CD19 CAR-T cells were strongly inhibited in vivo by IDO expression in the target B cell malignancies; and inhibition was reversed by administering oral indoximod [112]. Here again, the contribution of reactive versus constitutive IDO was not ascertained, but the study shows that human CAR-T cells are susceptible to the effects of IDO.

7.3.3 IDO-Inhibitor Drugs in the Clinic

A number of drugs targeting the IDO pathway are now in early-phase clinical trials, or in preclinical development. Drugs in trials include indoximod (1-methyl-D-tryptophan) and NLG919 (both from NewLink Genetics, Inc.) and INCB024360 (from Incyte Corp.). Published data currently are limited to interim abstracts from on-going trials, so efficacy data are not yet available. However, toxicity profiles have been generally favorable, which has facilitated combinations with additional agents.

Preclinical mouse models show that IDO-inhibitor drugs are synergistic with a variety of chemotherapeutic agents in a number of different tumor models (transplantable and autochthonous) [24, 113, 114]. Based on this, several of the ongoing trials of indoximod are structured to combine this agent with conventional chemotherapy (docetaxel in breast cancer; temozolomide in brain tumors; or gemcitabine/abraxane in pancreatic cancer). Trials are also open combining either INCB024360 or indoximod with CTLA-4 blocking antibody. Combinations with inhibitors of the PD-1/L pathway are also in progress, and are entering Phase 3 trials.

Open questions in the field of IDO drug-development currently include the relative contribution of IDO1 and IDO2 genes to tumor-induced immunosuppression, and the potential contribution of the unrelated enzyme TDO (tryptophan dioxygenase). IDO2 has been much less extensively studied than IDO1, and its biologic role remains unclear. One study found that tumors grown in IDO1-deficient mice had increased levels of IDO2 [115], suggesting that IDO2 may compensate for lack of IDO1. Therefore, inhibitor drugs with dual specificity for both IDO1 and IDO2 may be of benefit. TDO is an unrelated enzyme that catalyzes the same conversion of tryptophan to N-formyl-kynurenine. TDO is constitutively expressed in liver and brain, and it can also be an autocrine growth pathway for brain tumors [116]. Although there is no physiologic role known for TDO in the immune system (in contrast to IDO), there is concern that some tumors may be able up-regulate TDO as an immunosuppressive pathway (or as an escape pathway when IDO is blocked). Hence, there is interest in TDO-inhibitors, and in dual-specificity inhibitors that could inhibit both IDO and TDO.

7.4 Conclusions

Inducible counter-regulation by IDO may be an important inhibitory pathway during chemotherapy and immunotherapy. IDO can be elicited as a natural tolerogenic pathway in response to signals from dying tumor cells. As such, IDO may bias the immune response toward tolerance rather than immune activation following chemotherapy. IDO can also be elicited as a counter-regulatory response to attempted inflammation and immune activation. This is of concern in settings of active immunotherapy, where desirable immune responses may be inadvertently suppressed because the elicit counter-regulatory IDO. However, these effects of IDO also represent a therapeutic opportunity. IDO is emerging as a mechanism that influences the fundamental choice of whether dying cells will be perceived by the immune system as tolerogenic or immunogenic. Thus, if the tolerogenic IDO pathway can be blocked, then conventional chemotherapy may be more spontaneously immunogenic than previously appreciated. Likewise, active immunotherapy may become able to elicit a more robust immune response, with epitope-spreading to additional endogenous tumor antigens. These areas represent topics for future basic research, and therapeutic opportunities for synergistic combinatorial regimens in the clinic.

References

1. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348:69–74.
2. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat Rev Cancer. 2014;14:135–46.
3. Medler TR, Cotechini T, Coussens LM. Immune response to cancer therapy: mounting an effective antitumor response and mechanisms of resistance. Trends Cancer. 2015;1:66–75.
4. Belvin M, Mellman I. Is all cancer therapy immunotherapy? Sci Transl Med. 2015;7:315fs48.
5. Galluzzi L, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunological effects of conventional chemotherapy and targeted anticancer agents. Cancer Cell. 2015;28:690–714.
6. Bezu L, Gomes-de-Silva LC, Dewitte H, Breckpot K, Fucikova J, Spisek R, Galluzzi L, Kepp O, Kroemer G. Combinatorial strategies for the induction of immunogenic cell death. Front Immunol. 2015;6:187.
7. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van Den Eynde BJ. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003;9:1269–74.
8. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF. Up-regulation of PD-L1, IDO, and Tregs in the melanoma tumor microenvironment is driven by CD8+ T cells. Sci Transl Med. 2013;5:200ra116.
9. Baban B, Hansen A, Chandler P, Manlapat A, Bingaman A, Kahler D, Munn D, Mellor A. A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type 1 interferon-signaling following B7 ligation. Int Immunol. 2005;17:909–19.
10. Manlapat AK, Kahler DJ, Chandler PR, Munn DH, Mellor AL. Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory CD19(+) dendritic cells. Eur J Immunol. 2007;37:1064–71.
11. Hwu P, MX D, Lapointe R, Do M, Taylor MW, Young HA. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol. 2000;164:3596–9.
12. Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagita H, Azuma M, Blazar BR, Mellor AL, Munn DH. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Invest. 2007;117:2570–82.
13. Mellor AL, Baban B, Chandler PR, Manlapat A, Kahler DJ, Munn DH. Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN type 1 signaling. J Immunol. 2005;175:5601–5.
14. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol. 2013;34:137–43.
15. Metz R, Duhadaway JB, Kamasani U, Laury-Kleintop L, Muller AJ, Prendergast GC. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. Cancer Res. 2007;67:7082–7.
16. Ball HJ, Yuasa HJ, Austin CJ, Weiser S, Hunt NH. Indoleamine 2,3-dioxygenase-2; a new enzyme in the kynurenine pathway. Int J Biochem Cell Biol. 2009;41:467–71.
17. Fatokun AA, Hunt NH, Ball HJ. Indoleamine 2,3-dioxygenase 2 (IDO2) and the kynurenine pathway: characteristics and potential roles in health and disease. Amino Acids. 2013;45:1319–29.
18. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, Mellor AL. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity. 2005;22:633–42.
19. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C, Orabona C, Bianchi R, Belladonna ML, Volpi C, Santamaria P, Fioretti MC, Puccetti P. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol. 2006;176:6752–61.
20. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol. 2010;185:3190–8.
21. Quintana FJ, Murugaiyan G, Farez MF, Mitsdoerffer M, Tukpah AM, Burns EJ, Weiner HL. An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2010;107:20768–73.
22. Jaronen M, Quintana FJ. Immunological relevance of the coevolution of IDO1 and AHR. Front Immunol. 2014;5:521.
23. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C, Mellor AL. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281:1191–3.
24. Muller AJ, Duhadaway JB, Donover PS, Sutanto-Ward E, Prendergast GC. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med. 2005;11:312–9.
25. Mellor AL, Sivakumar J, Chandler P, Smith K, Molina H, Mao D, Munn DH. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. Nat Immunol. 2001;2:64–8.
26. Matteoli G, Mazzini E, Iliev ID, Mileti E, Fallarino F, Puccetti P, Chieppa M, Rescigno M. Gut CD103+

dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut. 2010;59:595–604.
27. van der Marel AP, Samsom JN, Greuter M, van Berkel LA, O'Toole T, Kraal G, Mebius RE. Blockade of IDO inhibits nasal tolerance induction. J Immunol. 2007;179:894–900.
28. Sucher R, Fischler K, Oberhuber R, Kronberger I, Margreiter C, Ollinger R, Schneeberger S, Fuchs D, Werner ER, Watschinger K, Zelger B, Tellides G, Pilat N, Pratschke J, Margreiter R, Wekerle T, Brandacher G. IDO and regulatory T cell support are critical for cytotoxic T lymphocyte-associated Ag-4 Ig-mediated long-term solid organ allograft survival. J Immunol. 2012;188:37–46.
29. Grohmann U, Orabona C, Fallarino F, Vacca C, Calcinaro F, Falorni A, Candeloro P, Belladonna ML, Bianchi R, Fioretti MC, Puccetti P. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol. 2002;3:1097–101.
30. Mellor AL, Baban B, Chandler P, Marshall B, Jhaver K, Hansen A, Koni PA, Iwashima M, Munn DH. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. J Immunol. 2003;171:1652–5.
31. Guillonneau C, Hill M, Hubert FX, Chiffoleau E, Herve C, Li XL, Heslan M, Usal C, Tesson L, Menoret S, Saoudi A, Le Mauff B, Josien R, Cuturi MC, Anegon I. CD40Ig treatment results in allograft acceptance mediated by CD8CD45RC T cells, IFN-gamma, and indoleamine 2,3-dioxygenase. J Clin Invest. 2007;117:1096–106.
32. Tsai S, Shameli A, Yamanouchi J, Clemente-Casares X, Wang J, Serra P, Yang Y, Medarova Z, Moore A, Santamaria P. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. Immunity. 2010;32:568–80.
33. Lan Z, Ge W, Arp J, Jiang J, Liu W, Gordon D, Healey D, DeBenedette M, Nicolette C, Garcia B, Wang H. Induction of kidney allograft tolerance by soluble CD83 associated with prevalence of tolerogenic dendritic cells and indoleamine 2,3-dioxygenase. Transplantation. 2010;90:1286–93.
34. Swanson KA, Zheng Y, Heidler KM, Mizobuchi T, Wilkes DS. CDllc+ cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. Am J Respir Cell Mol Biol. 2004;30:311–8.
35. Liu H, Liu L, Fletcher BS, Visner GA. Novel action of indoleamine 2,3-dioxygenase attenuating acute lung allograft injury. Am J Respir Crit Care Med. 2006;173:566–72.
36. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, Taylor PA, Mellor AL, Munn DH, Blazar BR. Indoleamine 2,3-dioxygenase is a critical regulator of acute GVHD lethality. Blood. 2008;111:3257–65.
37. Lu Y, Giver CR, Sharma A, Li JM, Darlak KA, Owens LM, Roback JD, Galipeau J, Waller EK. IFN-gamma and indoleamine 2,3-dioxygenase signaling between donor dendritic cells and T cells regulates graft versus host and graft versus leukemia activity. Blood. 2012;119:1075–85.
38. Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG, Stenson WF. Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. Gastroenterology. 2003;125:1762–73.
39. Szanto S, Koreny T, Mikecz K, Glant TT, Szekanecz Z, Varga J. Inhibition of indoleamine 2,3-dioxygenase-mediated tryptophan catabolism accelerates collagen-induced arthritis in mice. Arthritis Res Ther. 2007;9:R50.
40. Yan Y, Zhang GX, Gran B, Fallarino F, Yu S, Li H, Cullimore ML, Rostami A, Xu H. IDO upregulates regulatory T cells via tryptophan catabolite and suppresses encephalitogenic T cell responses in experimental autoimmune encephalomyelitis. J Immunol. 2010;185:5953–61.
41. Fallarino F, Volpi C, Zelante T, Vacca C, Calvitti M, Fioretti MC, Puccetti P, Romani L, Grohmann U. IDO mediates TLR9-driven protection from experimental autoimmune diabetes. J Immunol. 2009;183:6303–12.
42. Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, Vacca C, Bistoni F, Fioretti MC, Grohmann U, Segal BH, Puccetti P. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. Nature. 2008;451:211–5.
43. Grohmann U, Volpi C, Fallarino F, Bozza S, Bianchi R, Vacca C, Orabona C, Belladonna ML, Ayroldi E, Nocentini G, Boon L, Bistoni F, Fioretti MC, Romani L, Riccardi C, Puccetti P. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. Nat Med. 2007;13:579–86.
44. Miyake Y, Asano K, Kaise H, Uemura M, Nakayama M, Tanaka M. Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. J Clin Invest. 2007;117:2268–78.
45. McGaha TL, Chen Y, Ravishankar B, van Rooijen N, Karlsson MC. Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen. Blood. 2011;117:5403–12.
46. Ravishankar B, Liu H, Shinde R, Chandler P, Baban B, Tanaka M, Munn DH, Mellor AL, Karlsson MC, McGaha TL. Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase. Proc Natl Acad Sci U S A. 2012;109:3909–14.
47. Munn DH, Sharma MD, Hou D, Baban B, Lee JR, Antonia SJ, Messina JL, Chandler P, Koni PA, Mellor A. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest. 2004;114:280–90.
48. Mellor AL, Chandler P, Baban B, Hansen AM, Marshall B, Pihkala J, Waldmann H, Cobbold S, Adams E, Munn DH. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. Int Immunol. 2004;16:1391–401.
49. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is

essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol. 2008;181:5396–404.
50. Manches O, Munn D, Fallahi A, Lifson J, Chaperot L, Plumas J, Bhardwaj N. HIV-activated human plasmacytoid DCs induce Tregs through an indoleamine 2,3-dioxygenase-dependent mechanism. J Clin Invest. 2008;118:3431–9.
51. Chung DJ, Rossi M, Romano E, Ghith J, Yuan J, Munn DH, Young JW. Indoleamine 2,3-dioxygenase-expressing mature human monocyte-derived dendritic cells expand potent autologous regulatory T cells. Blood. 2009;114:555–63.
52. Jurgens B, Hainz U, Fuchs D, Felzmann T, Heitger A. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. Blood. 2009;114:3235–43.
53. Savage PA, Malchow S, Leventhal DS. Basic principles of tumor-associated regulatory T cell biology. Trends Immunol. 2013;34:33–40.
54. Nishikawa H, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Curr Opin Immunol. 2014;27:1–7.
55. Waight JD, Takai S, Marelli B, Qin G, Hance KW, Zhang D, Tighe R, Lan Y, Lo KM, Sabzevari H, Hofmeister R, Wilson NS. Cutting edge: epigenetic regulation of Foxp3 defines a stable population of CD4+ regulatory T cells in tumors from mice and humans. J Immunol. 2015;194:878–82.
56. Huehn J, Floess S, Ohkura N, Sakaguchi S. Comment on "cutting edge: epigenetic regulation of Foxp3 defines a stable population of CD4+ regulatory T cells in tumors from mice and humans". J Immunol. 2015;194:3533.
57. Malchow S, Leventhal DS, Nishi S, Fischer BI, Shen L, Paner GP, Amit AS, Kang C, Geddes JE, Allison JP, Socci ND, Savage PA. Aire-dependent thymic development of tumor-associated regulatory T cells. Science. 2013;339:1219–24.
58. Darrasse-Jeze G, Bergot AS, Durgeau A, Billiard F, Salomon BL, Cohen JL, Bellier B, Podsypanina K, Klatzmann D. Tumor emergence is sensed by self-specific CD44hi memory Tregs that create a dominant tolerogenic environment for tumors in mice. J Clin Invest. 2009;119(9):2648–62.
59. Menetrier-Caux C, Gobert M, Caux C. Differences in tumor regulatory T-cell localization and activation status impact patient outcome. Cancer Res. 2009;69:7895–8.
60. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, Bettini ML, Vogel P, Finkelstein D, Bonnevier J, Workman CJ, Vignali DA. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. Nature. 2013;501:252–6.
61. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature. 2007;450:566–9.
62. Chaudhry A, Rudensky AY. Control of inflammation by integration of environmental cues by regulatory T cells. J Clin Invest. 2013;123:939–44.
63. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol. 2009;10:1000–7.
64. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, Benoist C, Rudensky AY. Stability of the regulatory T cell lineage in vivo. Science. 2010;329:1667–71.
65. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, Fehling HJ, Bluestone JA. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. Immunity. 2013;39:949–62.
66. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, Tanaka S, Bluestone JA, Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med. 2014;20:62–8.
67. Huynh A, DuPage M, Priyadharshini B, Sage PT, Quiros J, Borges CM, Townamchai N, Gerriets VA, Rathmell JC, Sharpe AH, Bluestone JA, Turka LA. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. Nat Immunol. 2015;16:188–96.
68. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain Th1 and Tfh cell responses. Nat Immunol. 2015;16:178–87.
69. Lee Jee H, Elly C, Park Y, Liu Y-C. E3 ubiquitin ligase VHL regulates hypoxia-inducible factor-1α to maintain regulatory T cell stability and suppressive capacity. Immunity. 2015;42:1062–74.
70. Sharma MD, Hou DY, Liu Y, Koni PA, Metz R, Chandler P, Mellor AL, He Y, Munn DH. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. Blood. 2009;113:6102–11.
71. Baban B, Chandler PR, Sharma MD, Pihkala J, Koni PA, Munn DH, Mellor AL. IDO activates regulatory T cells and blocks their conversion into Th17-like T cells. J Immunol. 2009;183:2475–83.
72. Sharma MD, Hou DY, Baban B, Koni PA, He Y, Chandler PR, Blazar BR, Mellor AL, Munn DH. Reprogrammed Foxp3(+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice. Immunity. 2010;33:942–54.
73. Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H, Pan F, Blazar BR, Pardoll DM, Mellor AL, Shi H, Munn DH. An inherently bifunctional subset of Foxp3 T helper cells is controlled by the transcription factor Eos. Immunity. 2013;38:998–1012.
74. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology

and therapeutic potential. Nat Rev Immunol. 2014;14:166–80.
75. Sharma MD, Shinde R, McGaha T, Huang L, Holmgaard RB, Wolchok JD, Mautino MR, Celis E, Sharpe A, Francisco LM, Powell DJ Jr, Yagita H, Mellor AL, Blazar BR, Munn DH. The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment. Sci Adv. 2015;1:e1500845.
76. Hannun YA. Apoptosis and the dilemma of cancer chemotherapy. Blood. 1997;89:1845–53.
77. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13(9):1050.
78. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, Portela Catani JP, Hannani D, Duret H, Steegh K, Martins I, Schlemmer F, Michaud M, Kepp O, Sukkurwala AQ, Menger L, Vacchelli E, Droin N, Galluzzi L, Krzysiek R, Gordon S, Taylor PR, Van Endert P, Solary E, Smyth MJ, Zitvogel L, Kroemer G. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity. 2013;38:729–41.
79. Michaud M, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L, Kroemer G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science. 2011;334:1573–7.
80. Zitvogel L, Apetoh L, Ghiringhelli F, Andre F, Tesniere A, Kroemer G. The anticancer immune response: indispensable for therapeutic success? J Clin Invest. 2008;118:1991–2001.
81. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol. 2013;31:51–72.
82. Ciampricotti M, Hau CS, Doornebal CW, Jonkers J, de Visser KE. Chemotherapy response of spontaneous mammary tumors is independent of the adaptive immune system. Nat Med. 2012;18:344–6. author reply 6
83. Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. 1989;49:5870–8.
84. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity. 2013;38:209–23.
85. Spel L, Boelens JJ, Nierkens S, Boes M. Antitumor immune responses mediated by dendritic cells: how signals derived from dying cancer cells drive antigen cross-presentation. Oncoimmunology. 2013;2:e26403.
86. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol. 2012;12:557–69.
87. Ravishankar B, Shinde R, Liu H, Chaudhary K, Bradley J, Lemos HP, Chandler P, Tanaka M, Munn DH, Mellor AL, McGaha TL. Marginal zone CD169+ macrophages coordinate apoptotic cell-driven cellular recruitment and tolerance. Proc Natl Acad Sci U S A. 2014;111:4215–20.
88. Anyaegbu CC, Lake RA, Heel K, Robinson BW, Fisher SA. Chemotherapy enhances cross-presentation of nuclear tumor antigens. PLoS One. 2014;9:e107894.
89. Zeelenberg IS, van Maren WW, Boissonnas A, Van Hout-Kuijer MA, Den Brok MH, Wagenaars JA, van der Schaaf A, Jansen EJ, Amigorena S, Thery C, Figdor CG, Adema GJ. Antigen localization controls T cell-mediated tumor immunity. J Immunol. 2011;187:1281–8.
90. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348:74–80.
91. Halama N, Michel S, Kloor M, Zoernig I, Benner A, Spille A, Pommerencke T, von Knebel DM, Folprecht G, Luber B, Feyen N, Martens UM, Beckhove P, Gnjatic S, Schirmacher P, Herpel E, Weitz J, Grabe N, Jaeger D. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. Cancer Res. 2011;71:5670–7.
92. Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, Budczies J, Darb-Esfahani S, Kronenwett R, Hanusch C, von Torne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M, von Minckwitz G. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol. 2010;28:105–13.
93. Pol J, Vacchelli E, Aranda F, Castoldi F, Eggermont A, Cremer I, Sautès-Fridman C, Fucikova J, Galon J, Spisek R, Tartour E, Zitvogel L, Kroemer G, Galluzzi L. Trial watch: immunogenic cell death inducers for anticancer chemotherapy. OncoImmunology. 2015;4:e1008866.
94. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, Hollmann TJ, Bruggeman C, Kannan K, Li Y, Elipenahli C, Liu C, Harbison CT, Wang L, Ribas A, Wolchok JD, Chan TA. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014;371:2189–99.
95. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015;348:124–8.
96. Brown SD, Warren RL, Gibb EA, Martin SD, Spinelli JJ, Nelson BH, Holt RA. Neo-antigens predicted by tumor genome meta-analysis corre-

late with increased patient survival. Genome Res. 2014;24:743–50.
97. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, Brasseur F, Lethe B, De Plaen E, Velu T, Boon T, Coulie PG. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. J Exp Med. 2005;201:241–8.
98. Palmer DC, Chan CC, Gattinoni L, Wrzesinski C, Paulos CM, Hinrichs CS, Powell DJ Jr, Klebanoff CA, Finkelstein SE, Fariss RN, Yu Z, Nussenblatt RB, Rosenberg SA, Restifo NP. Effective tumor treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity. Proc Natl Acad Sci U S A. 2008;105:8061–6.
99. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science. 2013;339:286–91.
100. Savage PA, Vosseller K, Kang C, Larimore K, Riedel E, Wojnoonski K, Jungbluth AA, Allison JP. Recognition of a ubiquitous self antigen by prostate cancer-infiltrating CD8+ T lymphocytes. Science. 2008;319:215–20.
101. Vigneron N, Stroobant V, Chapiro J, Ooms A, Degiovanni G, Morel S, van der Bruggen P, Boon T, Van den Eynde BJ. An antigenic peptide produced by peptide splicing in the proteasome. Science. 2004;304:587–90.
102. Senovilla L, Vitale I, Martins I, Tailler M, Pailleret C, Michaud M, Galluzzi L, Adjemian S, Kepp O, Niso-Santano M, Shen S, Marino G, Criollo A, Boileve A, Job B, Ladoire S, Ghiringhelli F, Sistigu A, Yamazaki T, Rello-Varona S, Locher C, Poirier-Colame V, Talbot M, Valent A, Berardinelli F, Antoccia A, Ciccosanti F, Fimia GM, Piacentini M, Fueyo A, Messina NL, Li M, Chan CJ, Sigl V, Pourcher G, Ruckenstuhl C, Carmona-Gutierrez D, Lazar V, Penninger JM, Madeo F, Lopez-Otin C, Smyth MJ, Zitvogel L, Castedo M, Kroemer G. An immunosurveillance mechanism controls cancer cell ploidy. Science. 2012;337:1678–84.
103. Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. Immunity. 2009;31:184–96.
104. Fuertes MB, Woo SR, Burnett B, YX F, Gajewski TF. Type I interferon response and innate immune sensing of cancer. Trends Immunol. 2013;34:67–73.
105. Woo SR, Corrales L, Gajewski TF. The STING pathway and the T cell-inflamed tumor microenvironment. Trends Immunol. 2015;36:250–6.
106. Lemos H, Huang L, Chandler PR, Mohamed E, Souza GR, Li L, Pacholczyk G, Barber GN, Hayakawa Y, Munn DH, Mellor AL. Activation of the STING adaptor attenuates experimental autoimmune encephalitis. J Immunol. 2014;192:5571–8.
107. Huang L, Li L, Lemos H, Chandler PR, Pacholczyk G, Baban B, Barber GN, Hayakawa Y, McGaha TL, Ravishankar B, Munn DH, Mellor AL. Cutting edge: DNA sensing via the STING adaptor in myeloid dendritic cells induces potent tolerogenic responses. J Immunol. 2013;191:3509–13.
108. Lemos H, Mohamed E, Huang L, Ou R, Pacholczyk G, Arbab AS, Munn D, Mellor AL. STING promotes the growth of tumors characterized by low antigenicity via IDO activation. Cancer Res. 2016;76(8):2076–81.
109. von Bergwelt-Baildon MS, Popov A, Saric T, Chemnitz J, Classen S, Stoffel MS, Fiore F, Roth U, Beyer M, Debey S, Wickenhauser C, Hanisch FG, Schultze JL. CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition. Blood. 2006;108:228–37.
110. Holmgaard RB, Zamarin D, Munn DH, Wolchok JD, Allison JP. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med. 2013;210:1389–402.
111. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. J Immunother Cancer. 2014;2:3.
112. Ninomiya S, Narala N, Huye L, Yagyu S, Savoldo B, Dotti G, Heslop HE, Brenner MK, Rooney CM, Ramos CA. Tumor indoleamine 2,3-dioxygenase (IDO) inhibits CD19-CAR T cells and is down-regulated by lymphodepleting drugs. Blood. 2015;125:3905–16.
113. Hou DY, Muller AJ, Sharma MD, Duhadaway JB, Banerjee T, Johnson M, Mellor AL, Prendergast GC, Munn DH. Inhibition of IDO in dendritic cells by stereoisomers of 1-methyl-tryptophan correlates with anti-tumor responses. Cancer Res. 2007;67:792–801.
114. Li M, Bolduc AR, Hoda MN, Gamble DN, Dolisca SB, Bolduc AK, Hoang K, Ashley C, McCall D, Rojiani AM, Maria BL, Rixe O, MacDonald TJ, Heeger PS, Mellor AL, Munn DH, Johnson TS. The indoleamine 2,3-dioxygenase pathway controls complement-dependent enhancement of chemo-radiation therapy against murine glioblastoma. J Immunother Cancer. 2014;2:21.
115. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, Cheng Y, Kim JW, Qiao J, Zhang L, Han Y, Lesniak MS. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. Clin Cancer Res. 2014;20:5290–301.
116. Opitz CA, Litzenburger UM, Sahm F, Ott M, Tritschler I, Trump S, Schumacher T, Jestaedt L, Schrenk D, Weller M, Jugold M, Guillemin GJ, Miller CL, Lutz C, Radlwimmer B, Lehmann I, von Deimling A, Wick W, Platten M. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature. 2011;478:197–203.

Targeting Myeloid-Derived Suppressor Cells in Cancer

8

Waseem Anani and Michael R. Shurin

8.1 Introduction

The bone marrow is comprised of two types of progenitor cells: myeloid stem cells and lymphoid stem cells. Myeloid cells are the most numerous and functionally diverse types of cells that the bone marrow produces [1]. A subset of myeloid cells called immature myeloid cells (IMCs) or myeloid derived suppressor cells (MDSCs) have gained interest, because they play a key role in tumor development, growth, progression, and resistance to therapy [2, 3]. MDSCs affect the local tumor microenvironment by suppressing host immune responses; many studies have identified a correlation between the number of MDSCs in the peripheral blood of cancer patients with the clinical stage and metastatic tumor burden [4–6]. To improve the outcomes and efficacy of current treatment regimens, therapeutic strategies targeting MDSCs are being explored. This chapter will briefly introduce MDSCs to provide context about the current and potential treatment modalities targeting MDSCs in the tumor environment.

8.2 Development of MDSCs

Normally, hematopoietic stem cells mature into lymphoid or myeloid precursor cells depending on the local environment and cytokine/growth factor balance. As a stem cell becomes a mature myeloid cell, the local factors continue to shape the final cell type (e.g., monocyte and granulocyte) [7–9]. IMCs or MDSCs are a less common product of myeloid precursor cells and account for approximately 0.5% of peripheral blood mononuclear cells [5, 10–12]. Traveling from the bone marrow to the peripheral tissue, they quickly differentiate into mature granulocytes, macrophages, and dendritic cells (DCs) based on their environment. However, inflammatory conditions such as cancer, sepsis, and trauma can promote factors that block the differentiation of MDSCs into more mature forms. As much as a 10-fold accumulation can be observed through suppressive feed-back loops including a host of factors: IL-6, IL-1, granulocyte colony stimulating factor, fms-related tyrosine kinase 3 ligand, and transforming growth factor-beta (TGF-β) [5, 10–13].

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8.3 Phenotypes

Although MDSCs were described two decades ago, defining their phenotypic profile has been difficult [14]. The heterogeneous population of cells is characterized by only some of the granulocytic and monocytic surface markers of fully differentiated monocytes, macrophages, and dendritic cells [15]. Additionally, murine MDSC phenotypes studied in cancer models differs from those found in humans, complicating their characterization.

Tumor suppressing cells in mice with cancer were first described as cluster designation (CD) 11b+ and lymphocyte antigen 6 complex (Ly6) locus C positive and were later elucidated as mostly two cell types: monocyte and granulocyte [7]. Monocytic MDSCs (M-MDSCs) express CD11b and Ly6C, whereas granulocytic MDSCs (G-MDSCs) express CD11b and Ly6 locus G [16–19]. Although the two populations can express additional markers, CD11b and Ly6 are the minimum defining criteria for murine MDSCs [19]. The specific roles of each subtype are still being investigated, but the overall goal of MDSCs remains to decrease the immune response to a stimulus [20–22].

In humans, MDSCs are only defined by consensus because identification of specific biomarkers is not as straightforward due to their large heterogeneity. MDSCs often express CD11b+, CD33+, and HLA-DR weak positive/negative and are also subdivided into two populations [23]. Typically, M-MDSCs are CD14+ and G-MDSCs are CD66b+ [11, 12, 24]. The diverse phenotypes arise from the tumor environment milieu favoring the development of unique MDSC subtypes [6]. For example, renal cell carcinoma patients express CD14-, CD15+, CD11b+, and CD66b+ G-MDSCs, while melanoma patients display CD14+ and weakly positive HLA-DR2 M-MDSCs [25, 26].

8.4 Mechanisms of MDSC-Induced Immunosuppression

MDSCs modulate the host environment through a number of mechanisms: depletion of amino acids, oxidative stress, decreased trafficking of antitumor effector cells, and increased regulatory T (Treg) and regulatory DC (regDCs) cell responses [1]. MDSC subgroups exert distinct immunosuppressive functions that favor the proliferation of tumors depending on the local environment. While G-MDSCs account for 70–80% of the MDSC population, they are less immunosuppressive than M-MDSCs [7].

8.4.1 Depletion of Amino Acids and Oxidative Stress

MDSCs can modulate local concentrations of L-arginine that is required for T cell regulation and maturation, suppressing antitumor CD4+ and CD8+ T cells, and promoting tumor formation [7]. Each MDSC subtype disrupts L-arginine, which affects T cell function, through different mechanism(s): arginase-1, inducible nitric oxide synthase (iNOS), or reactive oxygen and nitrogen species (ROS and RNS). M-MDSCs decrease L-arginine directly via arginase-1 to produce L-ornithine, blocking the T cell cycle via decreased expression of T cell receptors [27]. They also halt the T cell cycle by combining iNOS and L-arginine to form nitric oxide (NO) and urea, preventing the upregulation of cyclin D3 and cyclin-dependent kinase 4 [4, 28]. Finally, T cell apoptosis can be induced by a Fas-dependent pathway with M-MDSC nitric oxide formation further reducing T cell numbers [29]. G-MDSCs mainly target antitumor effector CD8+ T cells through ROS that can form peroxynitrite, a powerful nitrating agent. T cell

receptors lose the ability to recognize MHC peptides when nitrated, and subsequently, their antitumor activity [30].

Methionine is another essential amino acid needed for normal T cells function and is supplied by antigen presenting cells [31]. DCs and macrophages import cysteine to create methionine for secretion and also release thioredoxin catalyzing cysteine to methionine. In the tumor microenvironment, MDSCs transport cysteine intracellularly depriving T cells of methionine [32].

8.4.2 Decreased Trafficking of Antitumor T Cells

MDSCs promote uninhibited tumor growth of many cell types by altering the cytokine milieu via the production of CC-chemokine ligand (CCL) 2, CCL3, and CCL4 in G-MDSCs, and CCL2, CCL3, CCL4, IL-6, and IL-8 in M-MDSCs [33]. In the tumor environment, MDSC levels are inversely correlated with L-selectin concentrations, which decrease the T cell migration to lymph nodes [34]. Additionally, MDSCs can indirectly affect the antitumor T cell responses through the production of IL-10 and TGF-β to recruit immunosuppressive regulatory T (Treg) cells [35]. By inhibiting CD8+ T cells, the host cannot mount an antitumor response leading to unrestrained tumor growth.

8.5 Therapeutic Targeting of MDSCs

A number of methods are being devised to favor an immune response that destroys tumors, ranging from experimental studies in murine models to Food and Drug Administration-approved therapies. Specifically, MDSC targeting is an attractive option, because the large network of interactions that MDSCs' influence can cause many downstream effects promoting multifaceted tumor destruction. Early observations in cancer patients demonstrated that the concentration of peripheral blood MDSCs were positively correlated with tumor burden and clinical stage; surgical removal of tumors decreased the concentration of MDSCs in blood [5, 6, 12]. Many of the current studies demonstrating the effectiveness of MDSC therapies are in murine models or early clinical trials. Treatment strategies can be categorized as: inhibiting MDSC development and expansion, inhibiting MDSC function, differentiating MDSCs into more mature cells, and destroying MDSCs (Figs. 8.1 and 8.2).

8.5.1 Inhibiting MDSC Development and Expansion (Fig. 8.3)

8.5.1.1 Stem Cell Factor Blockade

Stem cell factor is highly expressed by tumor cells and can enhance the development and migration of MDSCs to tumors [8]. Decreasing stem cell factor in mice with small interfering RNA and tyrosine kinase inhibitors such as sunitinib, pazopanib, and sorafenib reduced the development of MDSCs in the bone marrow [36, 37]. Murine colon carcinoma and lung cancer treated with stem cell factor blocking agents showed increased anti-tumor responses, tumor shrinkage, and increased survival. Mice with renal cell carcinoma also showed decreased numbers of MDSCs; similar effects were observed in renal cell carcinoma patients [37–40]. Clinical trials evaluating stem cell factor levels with tyrosine kinase therapy are ongoing. Kinase inhibitors will be discussed further in a subsequent section.

8.5.1.2 Modulators of Cell Signaling

JAK2-STAT3 Inhibition

The Janus kinase (JAK) and STAT pathway is one method of intracellular regulation of the immune response via cytokines [41]. When JAK is phosphorylated and subsequently activated, STATs are translocated into the nucleus where they regulate gene transcription. Constitutive activation of the JAK-STAT pathway immortalizes cells and causes uncontrolled cell proliferation. Unregulated JAK-STAT has been implicated

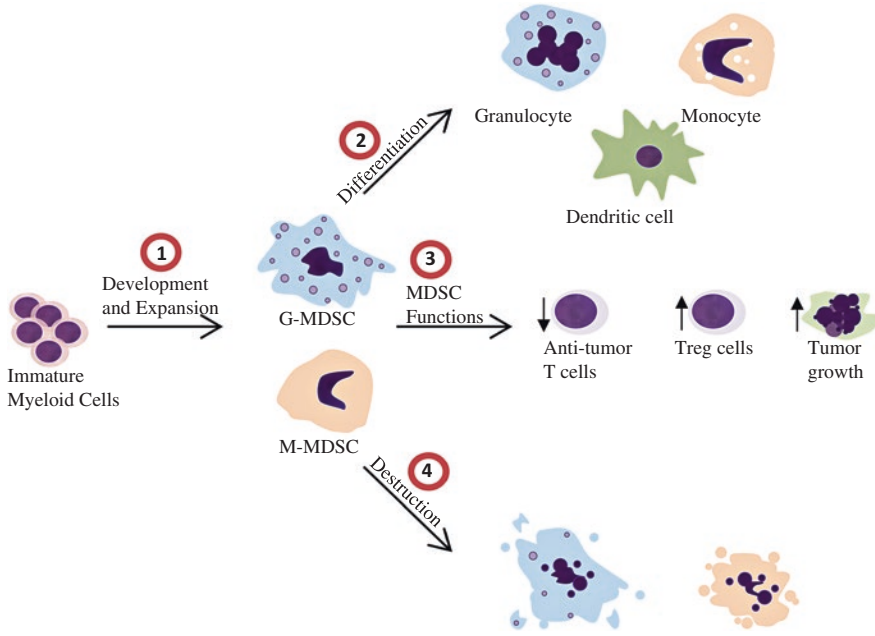

Fig. 8.1 Experimental treatments may target MDSC development and expansion, differentiation, function, and destruction. *MDSC* myeloid derived suppressor cell, *Treg* T regulatory

1. Inhibiting MDSC Development and Expansion	2. Differentiating/Depleting MDSC	3. Inhibiting MDSC Function
Stem Cell Factor Blockade **Modulators of Cell Signaling** • JAK-STAT Inhibitors • VEGF Inhibitors • Multi-kinase Inhibitors **Migration and Recruitment** • Anti-Glycan • Anti-CSF Receptor-1 • Anti-IL-17 • MMP-9 Inhibitors • Vemurafenib • Anti-Prokinecticin • Anti-CCL2 • Anti-IL-6	**Vitamins A and D** **All-Trans Retinoic Acid** **IL-12** **TLR-9 Inhibitor** **Taxanes** **Beta-Glucan Particles** **TDE Inhibitor** **VSSP** **4. Destroying MDSCs** **Cytotoxic Agents** • Gemcitabine • 5-Fluorouracil • Cisplatin **Anti-Ephrin A2 and HSP 90 inhibitor** **Anti-IL-13** **Histamine Blockade**	**NO Inhibitors** • Nitroaspirin • PDE-5 Inhibitors **ROS and RNS Inhibitor** • RNS Inhibitors • Triterpenoids **Arginase Inhibitors** • N-hydroxy-L-arginine Inhibitor • L-NAME Inhibitor **COX-2 Inhibitors** • COX-2 selective inhibitors • COX-1/2 nonselective inhibitors

Fig. 8.2 The current treatment strategies categorized by general mechanism of action. *CCL* CC-chemokine ligand, *CSF* colony stimulating factor, *COX-2* cyclooxygenase-2, *HSP* heat shock protein, *iNOS* inducible nitric oxide synthase, *IL* interleukin, *JAK* Janus kinase, *L-NAME* N^G-nitro-L-arginine methyl ester, *MMP-9* metalloproteinase-9, *MDSC* myeloid derived suppressor cell, *PDE-5* phosphodiesterase-5, *PGE* prostaglandin E, *RNS* reactive nitrogen species, *RAGE* receptor for advanced glycation end products, *ROS* reactive oxygen species, *STAT* signal transducer and activator of transcription, *TLR4* toll like receptor 4, *TDE* tumor-derived exosome, *VEGF* vascular endothelial growth factor, *VSSP* very small size proteoliposomes

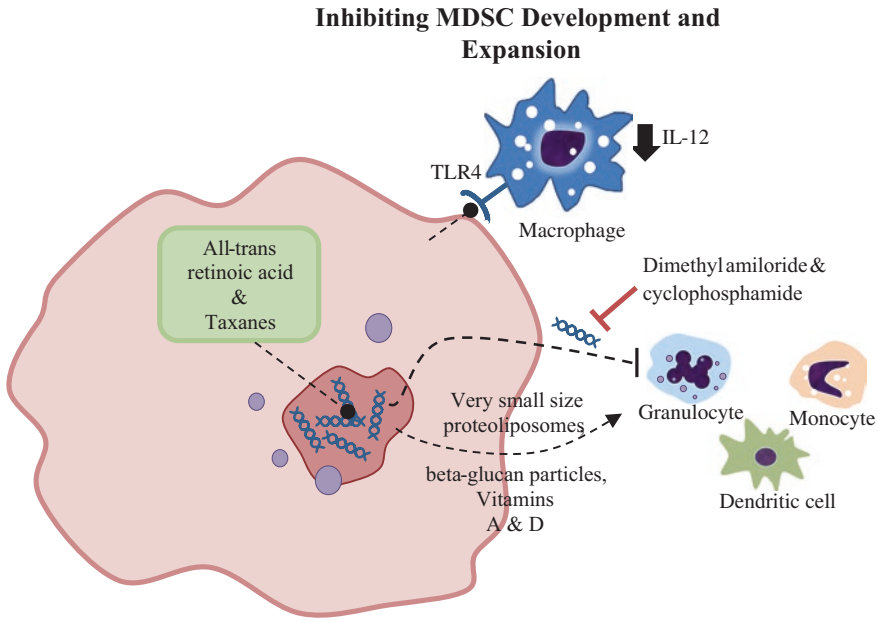

Fig. 8.3 A summary of treatments aimed at inhibiting MDSC development and expansion. *IL* interleukin, *TLR4* toll like receptor 4

in the proliferation of MDSCs via anti-apoptotic and pro-proliferative genes [42]. The arginase-1 and iNOS immunosuppressive activities by M-MDSCs are controlled via STAT1, and proliferation and ROS formation by G-MDSCs are regulated via STAT3 [20, 43].

Inhibition of the JAK-STAT pathway has been accomplished with various inhibitors in ongoing and completed clinical trials [44]. One particular STAT3 inhibitor derived from the herb *Curcuma longa Linn*, a component of the spice turmeric, was tested for an effect on the MDSC population [45, 46]. The herb derivative curcumin prevented STAT3 activation, and as a result, decreased tumor intracellular growth factors [47]. Mice with colon and gastric cancer treated with oral or intraperitoneal curcumin had fewer MDSCs at the tumor site and in their blood [48]. As expected, G-MDSCs decreased in number due to STAT3 inhibition, but unexpectedly, M-MDSCs expressed markers associated with a phenotype that promoted tumor destruction [49].

Clinical trials with *Curcuma longa Linn* derivatives have grappled with poor oral absorption, but oral administration of curcumin down-regulated STAT3 activation by 69% in multiple myeloma patients. A phase IIa clinical trial of curcumin decreased pre-malignant colorectal growths including aberrant crypt foci and adenomas by 40% when treated with 4 g instead of 2 g of curcumin [50]. Histologic improvements in patients with precancerous lesions were also seen in: 50% of those with resected bladder cancer, 29% with oral leukoplakia, 17% with intestinal metaplasia of the stomach, 25% with cervical intraepithelial neoplasia, and 33% with Bowen's disease [51]. Administration of another derivative, curcurbitacin B, demonstrated differentiation of MDSCs to DCs in lung cancer patients *in vitro* [52].

Vascular Endothelial Growth Factor (VEGF) Inhibition

Neovascularization in tumors is fueled in part by high levels of VEGF, but directed targeting of the growth factor has demonstrated limited benefit on MDSCs. Renal cell carcinoma patients with elevated VEGF concentrations were treated with anti-VEGF and had increased numbers of mature circulating DCs [25]. However, the total immune response and the number of MDSCs were unchanged. A phase I clinical trial of a VEGF

trap—a fusion protein attached to the Fc region of a human IgG antibody that can bind all VEGF isomers—also failed to decrease MDSC levels [53, 54]. These studies suggest that a broad treatment approach rather than directed VEGF inhibition may be needed to affect MDSCs.

Multi-Kinase Inhibition

Multi-kinase inhibitors like sunitinib disrupt cell functions by stopping multiple tyrosine kinase signaling pathways including VEGF and stem cell factor receptors [55]. The wide-spread interference causes apoptosis of tumor cells and quantitatively decreases immunosuppressive cells [56, 57]. Mice treated with sunitinib had decreased pro-tumor cytokines, decreased MDSCs, and Treg cells as well as improved survival when combined with a dendritic cell vaccine.

The murine model studies of sunitinib translated into sustained changes in clinical trials. Currently, the Food and Drug Administration has approved sunitinib as a first-line treatment of metastatic renal cell carcinoma and for gastrointestinal stromal tumors that do not respond to imatinib. Renal cell carcinoma patients demonstrated fewer circulating MDSCs and Treg cells; however, tumor explants showed minimal changes in MDSCs and pro-tumor factors like metalloproteinases [38, 40, 58, 59]. Further investigation revealed sunitinib resistance can occur when high levels of granulocyte macrophage-CSF in renal cell carcinoma patients circumvented the inhibition of STAT3 by sunitinib [38, 58, 60]. Combination therapy with cancer vaccines may aid the efficacy of multi-kinase inhibitors.

8.5.1.3 MDSC Migration and Recruitment

Anti-glycan

In the tumor microenvironment, the receptor for advanced glycation end products (RAGE) is modified with glycans and acts on the complementary MDSCs ligand S100A8/9 in mice [61, 62]. This interaction causes MDSCs to migrate and accumulate at the tumor site. Anti-glycans prevent S100A8/9 from binding with RAGE and can inhibit MDSC buildup and tumor growth by decreasing favorable tumor conditions. Murine colon carcinoma treated with chemotherapy and anti-glycan mAbGB3.1 had a reduced colon carcinoma formation and circulating pro-tumor cytokines [63]. Currently, there are no human studies exploring anti-glycan on tumor formation and growth.

Colony Stimulating Factor Receptor-1 Inhibition

Tumors produce a favorable environment for growth through many mechanisms including cytokines such as colony stimulating factor-1 (CSF-1) [64]. Myeloid cells express CSF-1 receptor resulting in MDSC proliferation and myeloid cell differentiation into MDSCs [65, 66]. Murine cancer models show increased secretions of cytokines that can be blocked with CSF-1 receptor inhibitors [67, 68]. Altering the cytokine milieu decreased the MDSC-mediated tumor burden, tumor angiogenesis, and activation of immunosuppressive genes. CSF-1 antibodies also prevented tumor-induced MDSC accumulation as tumor destruction increases CSF-1 release. In irradiated mice with prostate cancer, blocking the cytokine effects decreased tumor growth compared with only irradiated mice [69]. A clinical trial utilizing PLX3397, a colony stimulating factor-1 receptor inhibitor, with adoptive cell therapy of T cells enhanced tumor infiltrating T cells and decreased tumor MDSCs compared with monotherapy alone [64].

Anti-interleukin-17

CD4+ and CD8+ T cells secrete inflammatory cytokine IL-17 attracting leukocytes to areas of inflammation; however in tumors, IL-17 prevents CD8+ T cells infiltrating tumors and boosts MDSC in tumor sites [70]. Mice deficient in IL-17 receptors had greater T cell migration into tumors and lower MDSC levels. The same result was observed when anti-IL-17 was used to treat tumor-bearing mice. There are no clinical trials evaluating IL-17 antibodies at this time.

Metalloproteinase-9 Inhibition

Metalloproteinase-9 initiates osteoclast-mediated bone resorption and post-translational modifica-

tions to proteins via farnesyl-diphosphate synthase [71]. Metalloproteinase-9 also mobilizes MDSCs from the bone marrow and distal sites by cleaving c-kit and increasing VEGF [72]. N-bisphosphonate is a metalloproteinase-9 inhibitor that can decrease the movement of MDSCs to tumors. Mice treated with the inhibitor had reduced levels of metalloproteinase-9 and VEGF as well as a lower tumor burden compared with control mice [73]. The addition of a plasmid vaccine improved the host antitumor response and decreased tumor-induced bone marrow hematopoiesis. Mice with pancreatic cancer treated with zoledronic acid, a N-bisphosphonate, were associated with slower tumor growth, increased survival, and more antitumor T cells at the tumor site [74].

The benefits metalloproteinase-9 inhibition on MDSCs in mice has not been easily translated in the treatment of human malignancies. Premenopausal women with estrogen receptor positive breast cancer treated with endocrine therapy (anastrozole and tamoxifen) had equivalent rates of disease-free survival compared with zoledronic acid alone [75]. The combination of the two therapies increased the disease-free survival rate from 90.8% to 94% at 47.8 months. Another study observed that women with a high risk of breast cancer metastasis treated with a clodronate (another N-bisphosphonate) for 2 years, surgery, and adjuvant therapy had reduced bony and organ metastasis [76]. After 36 months, women treated with clodronate had half as many metastatic bone lesions as the control group.

Although these findings suggest a beneficial effect of N-bisphosphonates, the mechanism of action may be independent of MDSCs. In a phase 1 clinical trial of non-metastatic, resectable pancreatic ductal adenocarcinoma patients treated with zoledronic acid, G-MDSC levels were measured before and after treatment [76]. In addition to no benefit in the overall and progression free survival, blood and bone marrow concentrations of G-MDSCs did not change. Further research is needed to resolve the conflicting reports of N-bisphosphonate therapy in cancer treatment in relation to MDSCs.

Vemurafenib

Melanomas often have constitutive V600E *BRAF* mutations in the mitogen-activated protein kinase pathway and can be targeted with vemurafenib [77, 78]. This agent causes tumor apoptosis and modulates the tumor microenvironment by affecting MDSC recruitment. Vemurafenib treated melanoma had decreased circulating M-MDSC and G-MDSC numbers and *in vitro* studies suggested that MDSC migration was inhibited because melanoma cells were no longer secreting factors that attracted MDSCs. A phase I clinical trial exploring vemurafenib and ipilimumab treatment was discontinued due to severe liver toxicity [79].

Anti-prokinecticin

Prokinecticin facilitates tumor angiogenesis and mobilization of cells from the bone marrow such as MDSCs [80]. Anti-prokinecticin inhibited the growth of tumors and suppressed angiogenesis [80, 81]. When combined with weekly, low-dose continuous (metronomic chemotherapy) gemcitabine, there was a reduction in tumor growth, angiogenesis, and metastasis of murine pancreatic adenocarcinoma [82]. Currently, there are no clinical trials of anti-prokinecticin agents.

Anti-CCL2

Tumor cells and stroma secrete CCL2 promoting angiogenesis, macrophage infiltration, tumor extension, and tumor metastasis [83]. Animals treated with anti-CCL2 and docetaxel inhibited the growth of prostate cancer in bone with lasting effects even after discontinuation of treatment [84]. A phase II clinical trial of patients with metastatic castrate-resistant prostate cancer previously treated with docetaxel were additionally given carlumab, an anti-CCL2 [83]. Although the drug was well tolerated, no antitumor activity was observed.

IL-6 Receptor Inhibition

IL-6 is a cytokine that is associated with suppression of CD8+ T cells and enhancement of MDSCs. Blocking IL-6 receptors with a monoclonal antibody in mice with squamous cell carcinoma reduced tumor growth, decreased MDSC

subtypes, and improved T cell function [85]. The addition of gemcitabine enhanced the suppression of MDSCs and T cell response via IFN-γ production. There are multiple ongoing clinical trials including ovarian cancer patients treated with chemotherapy and tocilizumab, an IL-6 receptor inhibitor.

8.5.2 Inhibition of MDSC Function (Fig. 8.4)

8.5.2.1 Nitric Oxide Inhibition

Nitroaspirin

Nitroaspirin consists of a nitric oxide group covalently linked to aspirin that suppresses the production of ROS and iNOS from restoring L-arginine levels in T cells [86]. A study of AT38, or [3-(aminocarbonyl)furoxan-4-yl]methyl salicylate, found decreased MDSC-induced nitration of T cell receptors, improving CCL2 binding and T cell tumor infiltration in mice [87]. Other nitroaspirins tested in mice with colon carcinoma identified improved T cell proliferation and activity, decreased MDSC tumor infiltration, and slower tumor growth [67]. However, no tumor shrinkage or increased survival was noted until the addition of a DNA vaccine. Mice with colon cancer and mammary cancer cell lines treated for 18 days with a nitroaspirin drug and a DNA vaccine outlived matched mono-therapy mice and had fewer tumors develop at 120 days. A phase I clinical trial (NCT00331786 at clinicaltrials.gov) evaluating the effectiveness of nitroaspirin preventing colorectal carcinoma in high risk patients is currently in progress.

Phosphodiesterase-5 Inhibition

Phosphodiesterase-5 (PDE-5) inhibitors, such as tadalafil and sildenafil, prevent the breakdown of cyclic guanosine monophosphate decreasing arginase-1 and iNOS expression [88]. Although unclear, the mechanism of action may result from high cyclic guanosine monophosphate levels decreasing signal transducer and activator of

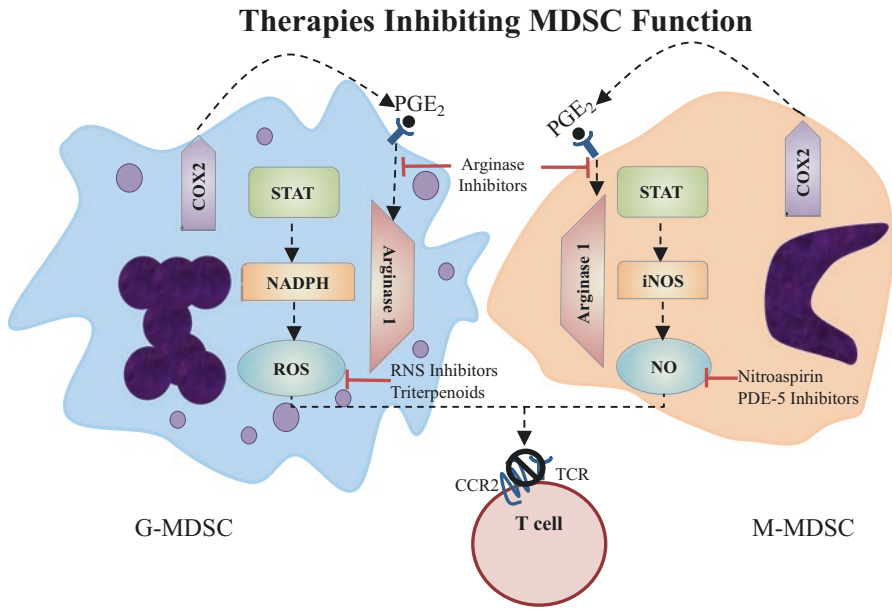

Fig. 8.4 A summary of treatments aimed at inhibiting MDSC function. *COX-2* cyclooxygenase-2, *G-MDSC* granulocyte-myeloid derived suppressor cell, *iNOS* inducible nitric oxide synthase, *M-MDSC* monocyte-myeloid derived suppressor cell, *NADPH* dihydronicotinamide-adenine dinucleotide phosphate, *NO* nitric oxide, *PDE-5* phosphodiesterase-5, *PGE* prostaglandin E, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *STAT* signal transducer and activator of transcription, *TCR* T cell receptor

transcription (STAT) 6 activity and concentrations of iNOS and argininase-1 in MDSCs. Amino acids essential to T cell function such as cysteine and tryptophan may also be increased with PDE-5 inhibitors. Other proposed mechanisms include destabilizing iNOS mRNA and decreasing cytosolic calcium concentrations to reduce MDSC calcium-dependent protein kinase C signal transduction.

Murine models with colon cancer, mammary adenocarcinoma, fibrosarcoma, and melanoma have demonstrated delayed tumor growth treated with PDE-5 inhibitors [88, 89]. Sildenafil decreased iNOS in MDSCs, increased infiltration of CD4+ and CD8+ T cells, and improved the potency of adoptive T cell transfer therapy—an autologous transfer of T cells following *in vitro* treatment. Mice with colon cancer had improved CD8+ T cell activity conferring a stronger antitumor response. PDE-5 inhibitors were also noted to change the local tumor milieu to favor of tumor destruction by decreasing concentrations of IL-1β, VEGF, CSF, IL-6, S100A9, CCL2, CCL3 in mice with metastatic melanoma [89]. Although tumor growth was decreased, murine models failed to demonstrate shrinkage or eradication of tumors.

PDE-5 studies in cancer patients have shown promise as a monotherapy and combined with other treatments. *In vitro* sildenafil administration of head and neck carcinoma and multiple myeloma blood samples demonstrated antitumor T cell expansion. Several ongoing clinical trials are expanding on this work and should provide evidence for the use of PDE-5 inhibitors [88]. Current phase II studies identifying improvement in treatment response include multiple myeloma patients receiving lenalidomide and dexamethasone with and without tadalafil (NCT01374217 at clinicaltrials.gov) and tadalafil following surgical removal of oropharyngeal and oral squamous cell carcinoma (NCT00843635 at clinicaltrials.gov). The efficacy of cytotoxic therapies with PDE-5 inhibitors in untreated non-small cell lung carcinoma patients is also being evaluated in a randomized control trial of sildenafil with and without carboplatin and taxol (NCT00752115 at clinicaltrials.gov). Finally, a clinical trial of pancreatic carcinoma patients receiving tadalafil and a telomerase vaccine as immunotherapy in addition to chemotherapy and radiation will monitor for improved outcomes.

8.5.2.2 Reactive Oxygen and Nitrogen Species Inhibition

Reactive Nitrogen Species

MDSCs and tumor cells produce RNS such as peroxynitrite that are toxic to myeloid and lymphoid cells. They can prevent T cell migration and proper functioning by: inducing apoptosis, decreased protein tyrosine phosphorylation via nitration of tyrosine residues, nitrating voltage-gated anion channels, and nullifying CCL2 [8, 90–92]. Without CCL2 directing T cells to the tumor site, they surround the malignancy without being able to invade. Prostate, colon, liver, and breast cancer in mice and humans produce high concentrations of RNS [90]. Mice with prostate cancer, colon cancer, and thymomas treated with AT38 ([3-(aminocarbonyl) furoxan-4-yl]methyl salicylate) had decreased arginase-1, iNOS, and peroxynitrite leading to a massive influx of CD8+ T cells into the tumors [87]. When combined with adoptive cell therapy, the primed cancer-killing cells could be driven into tumors and lead to tumor eradication [93].

Triterpenoids

Synthetically produced triterpenoids activate multiple antioxidant genes through the transcription factor nuclear factor (erythroid-derived 2)-like 2 [94]. Up-regulation of genes controlling NAPH, thioredoxin, catalase, superoxide dismutase, and heme-oxygenase reduce ROS intracellularly to counteract the effects of MDSCs [95]. Bardoxolone methyl or CDDO-ME is a synthetic triterpenoid based on the scaffold of oleanolic acid that is actively being studied [96].

Mice with colon carcinoma, lung carcinoma, and thymomas treated with bardoxolone methyl had increased activation of NQO1, an antioxidant enzyme. Treatment led to decreased levels of reactive oxygen and nitrogen species, led to decreased tumor growth and improved T cell function [95]. Tumors treated in combination

with a vaccine composed of genetically modified dendritic cells had a synergistic effect. However, MDSC numbers, viability, and levels of NO and arginase-1 were not influenced by triterpenoids.

Many of the triterpenoids clinical trials focused on treatment of chronic kidney disease and pulmonary hypertension but few studies have investigated triterpenoids in cancer patients. In humans, small doses of bardoxolone methyl in renal cell carcinoma were found to nullify MDSC-inhibited T cells *in vivo* [97]. However, a phase I trial of patients with solid tumors and lymphoid malignancies showed biopsy-proven decreases in iNOS, cyclooxygenase-2 (COX-2), and arginase-1 with disease stabilization in some patients [98]. A phase II trial of bardoxolone methyl in patients with unresectable pancreatic cancer will be forthcoming.

Prostaglandin and Arginase Inhibition

Cyclooxygenase-2 Inhibition

MDSCs create an immunosuppressive environment by producing (PGE_2), which is regulated through the enzyme COX-2 [99]. PGE_2 activates prostaglandin E (PGE) receptors on MDSCs and alters the differentiation of MDSCs and macrophages. In the bone marrow, PGE_2 receptors hamper the maturation of MDSCs into antigen presenting cells, while increased PGE_2 changes monocytes into MDSCs via increased expression of indoleamine 2,3- dioxygenase (IDO), IL-4Rα, iNOS, and IL-10 [100, 101].

Blocking the production of PGE_2 with COX-2 inhibitors showed reduced levels in renal cell carcinoma, colon carcinoma, and head and neck tumors [99]. Mice models treated with COX-2 inhibitors led to a 50% reduction in tumor growth rate and decreased levels of MDSCs [102]. PGE_2-producing murine lung carcinoma receiving COX-2 inhibitors had decreased MDSC arginase-1 and slower tumor growth [103]. COX-2 inhibitors may also provide other antitumor effects. Aspirin and celecoxib, COX-2 inhibitors, decreased MDSC recruitment and increased CD8+ T cell tumor infiltration in gliomas and colon carcinoma by decreasing CCL2 and increasing CXC motif chemokine 10 concentrations, respectively [43, 104]. Currently, there are no clinical trials exploring COX-2 inhibition on MDSCs.

N-hydroxy-L-arginine and N^G-nitro-L-arginine Methyl Ester Inhibition

N-hydroxy-L-arginine is an intermediate in the conversion of arginine to citruline and NO by iNOS [4]. They are potent physiologic inhibitors of arginase-1, the major immunosuppressive mechanism of MDSCs. Animals exposed to the intermediates demonstrated inhibition of MDSC function, and mice with B cell lymphoma treated with N-hydroxy-L-arginine had decreased numbers of Treg cells and improved immune responses to the cancer [105].

N^G-nitro-L-arginine methyl ester (L-NAME) is another intermediate that inhibits iNOS and arginase-1 [106, 107]. Mice with colon carcinoma and lymphoma had slower tumor growth and improved anti-tumor responses when exposed to L-NAME [108]. Treatment also improved survival in mice with transplanted prostate cancer, but *in vitro* studies demonstrated the continued dysfunction of CD8+ T cells. Further work on the above intermediates in clinical trials may provide information their efficacy in human malignancies.

8.5.3 Inducing MDSC Differentiation/Depletion of MDSCs (Fig. 8.5)

8.5.3.1 Vitamins A and D

Early work showed that Vitamins A and D may aid in differentiation of MDSCs to more mature cells through an unknown mechanism. Mice that were vitamin A deficient had larger numbers of MDSCs in their bone marrow than Vitamin A replete mice [109]. Similar results were observed in mice with lung cancer and patients with non-small cell lung cancer 3 weeks before surgery [110, 111]. After treatment with 1α, 25-hydroxyvitamin D3, MDSC levels increased and tumors showed a greater number of mature dendritic cells than immature myeloid and dendritic cells. Patients with squa-

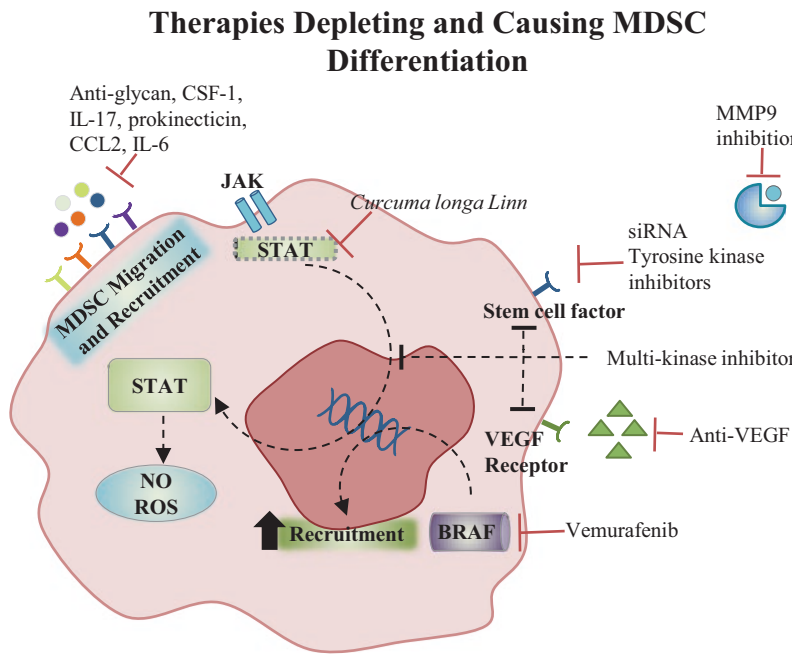

Fig. 8.5 A summary of treatments aimed at depleting and differentiating MDSCs. *CCL* CC-chemokine ligand, *CSF* colony stimulating factor, *IL* interleukin, *JAK* Janus kinase, *MMP-9* metalloproteinase-9, *MDSC* myeloid derived suppressor cell, *NO* nitric oxide, *ROS* reactive oxygen species, *STAT* signal transducer and activator of transcription, *siRNA* small interfering RNA, *VEGF* vascular endothelial growth factor

mous cell carcinoma of the head and neck treated with high dose Vitamin D also showed fewer immature myeloid cells as well as increased IL-12 and interferon-gamma, which defend against tumor development [112].

Clinical trials (NCT01409681 and NCT00794547 at clinicaltrials.gov) investigating Vitamin D treatments include studies on non-small cell lung cancer [113, 114]. Patients with a new diagnosis of non-small cell lung cancer and treated with 1α, 25-hydroxyvitamin D3 before surgical treatment showed increased intratumoral lymphocytes. Tumor recurrence after surgery was extended from 181 to 620 days compared to the untreated patients.

8.5.3.2 All-Trans Retinoic Acid

Vitamin A metabolism produces all-trans retinoic acid, among other metabolites, to act on retinoid–activated transcriptional regulators in DNA modulating transcription [115]. In myeloid cells, all-trans retinoic acid targets genes responsible for cell maturation that are less likely to favor tumor growth; maturing MDSCs into DCs, granulocytes, and monocytes [116, 117]. Mice with fibrosarcomas and mammary adenocarcinomas receiving all-trans retinoic acid showed MDSC maturation [117]. In combination with a DC vaccine, tumors decreased in size and CD4+ T cells regained functionality. Mice treated with a vaccine targeting the human papilloma virus 16 protein E7 and all-trans retinoic acid displayed a three-fold decrease in tumor growth and IFN-γ production, a tumor promoting cytokine. Additionally, antitumor T cell responses could be elicited 4 weeks after tumor inoculation in mice with sarcomas.

Based on the initial findings, clinical trials were started evaluating all-trans retinoic acid in various malignancies. Renal cell carcinoma patients with high serum concentrations of all-trans retinoic acid had less peripheral blood MDSCs, improved T cell responses after 7 days of treatment, and expressed more mature surface markers on MDSCs such as HLA-DR [10, 118]. The addition of a vaccine-based immunotherapy bolstered the effect for a more prolonged response. However, most clinical trials that employed all-trans retinoic acid to modulate MDSCs showed little promise [119, 120]. For instance, a phase II trial of lung adenocarcinoma treated with chemotherapy, tumor vaccine, and all-trans retinoic acid failed to show changes in MDSC levels or radiographic evidence of tumor regression.

8.5.3.3 IL-12

The cytokine environment in tumors can actively improve the immune system and decrease tumor progression and metastasis. In malignancy, MDSCs act on macrophage toll like receptor 4 (TLR4) preventing IL-12 accumulation in the tumor microenvironment [121]. Mice with metastasizing mammary carcinoma cells had suppressed Th1 cells owing to MDSC-mediated IL-12 reduction. The change in tumor cytokine milieu favored Th2 cells allowing tumor growth to go unchecked. However, when mice were treated with IL-12, they showed increased survival, decreased lung metastasis, and decreased NO [122]. Another study using T cells engineered to target VEGF receptor (highly expressed marker in vasculature of tumors) and IL-12 found decreased tumor growth and lower MDSC activity levels [123]. The IL-12 acted on DCs, macrophages, and MDSCs in the bone marrow to improve the efficacy of the transplanted T cells [124].

Many clinical trials of IL-12 therapy have been completed, some showing a benefit to patients. In a phase I study of IL-12 and rituximab in B cell non-Hodgkin lymphoma, patients had a 20-fold increase in IFN-γ [125]. Another phase I study of trastuzumab and IL-12 in metastatic breast cancer patients demonstrated increased *in vitro* destruction of HER2-expresing tumor cells via the expansion of NK cells [126]. As a monotherapy for epithelial, ovarian, fallopian tube, and primary peritoneal cancers, the efficacy was limited, suggesting that the most beneficial outcomes will be achieved when IL-12 is combined with other treatment modalities [127].

8.5.3.4 Toll-like Receptor 9 Acitivation

DNA contains regions of unmethylated deoxycytosine-deoxyguanine dinucleotides that can be inactivated through methylation, and when the immune system is exposed to DNA containing these regions, (e.g., bacteria and viruses) they can stimulate an immune response via TLR 9 [128]. Downstream effects on DCs, monocytes, and NK cells lead to increased IL-12 and interferons [71]. Mice treated synthetic unmethylated deoxycytosine-deoxyguanine dinucleotides decreased the abundance of MDSCs and increased IFN-α causing maturation of MDSCs [129]. Although preclinical models were promising, randomized clinical trials using the synthetic dinucleotides have not shown any benefit. For instance, results from a phase II trial in non-small cell lung cancer led to two phase III trials observing the effect of the synthetic reagent plus chemotherapy. Both were terminated early during an interim analysis due to lacking substantial improvements in treatment [130].

8.5.3.5 Taxanes

Taxanes, such as docetaxel and paclitaxel, are a class of chemotherapeutic drugs that stabilize microtubules during cell division causing cell arrest and death. They also differentiate MDSCs in mice and humans with cancer [131, 132]. Intraperitoneal administration of taxanes to mice with mammary carcinoma showed a decreased number of MDSCs, decreased MDSC activity, and increased CD8+ T cell activity [133]. The maturation of MDSCs was demonstrated by the increased expression of chemokine receptor type 7. MDSCs treated with low-dose paclitaxel and a peptide vaccine in healthy mice also noted MDSC maturation into DCs assessed by the expression of surface markers like CD11c, CD86, and CD40 [134]. When combined with T cell adoptive therapy and irradiation, mice with melanoma had significantly lower MDSCs and increased antitumor T cell activity [135].

Clinical trials utilizing taxanes are abundant, but their influence on MDSCs in human malignancy is still evolving. Women with HER-2 neu negative, stage II-IIa breast cancer treated with doxorubicin and cyclophosphamide followed with docetaxel who had a complete response showed lower circulating MDSCs [136]. A pilot trial in Germany (GCRC, Heidelberg) based on the promising results of low-dose paclitaxel in mice will evaluate the effects on metastatic melanoma patients with unresectable tumors.

8.5.3.6 Beta-Glucan Particles

Yeast, fungi, and bacteria cell walls are comprised of beta-glucans, which are composed of

polymers with a glucose backbone and side chains of varying sizes [137]. These particles are recognized by the immune system without prior exposure and can stimulate tumor destruction [20]. Beta-glucan treatment causes M-MDSCs to maturate *in vitro* and decrease the activity of M-MDSCs on the immune system [137]. Additionally, an influx of intratumor DCs and macrophages led to delayed tumor progression in mice. There are multiple ongoing and completed clinical trials evaluating beta-glucans [138]. The post-operative prognosis of stage Ic, II, or III ovarian cancer patients improved after treatment with chemotherapy and a beta-glucan [139]. Non-small cell lung cancer patients also saw an improvement in five-year survival rates with the addition of a beta-glucan after radiation therapy [140]. Finally, patients with unresectable gastric cancer receiving chemotherapy and a beta-glucan had a higher one-year survival rate than the control group [141]. Of note, some studies failed to show any improvement with beta-glucan therapy, and may be due to the impurity of the source material used to generate the extract [142, 143].

8.5.3.7 Tumor-Derived Exosome Inhibition

Tumor cells release 30–100 nm endosome-derived, small membrane vesicles or exosomes that are enriched in proteins and nucleic acids [144]. Exosomes can contain tumor antigens that can suppress immune responses allowing for tumor growth, metastasis, and drug resistance. They also affect mature and developing myeloid cells in the bone marrow to favor the formation of MDSCs. Tumor-derived exosomes prevent immature myeloid cells and monocytes in the bone marrow from becoming mature DCs. As a result, an overall favorable tumor microenvironment is created as exosomes increase MDSC levels and inflammatory markers such as IL-6, VEGF, and PGE_2 [145].

Tumors in mice produce exosomes with membrane associated heat shock protein 72 that activate MDSCs via the IL-6 receptor and intracellular phosphorylation of STAT3. Dimethyl amiloride, a potassium sparing diuretic, was found to inhibit exosome formation in mice when used with cyclophosphamide, but tumor growth was not changed when dimethyl amiloride was used alone [146]. The work was extended to MDSCs collected from cancer patients *ex vivo* demonstrating dimethyl amiloride suppressed MDSC function. Currently, there are no clinical trials exploring exome suppression in tumors.

8.5.3.8 Very Small Size Proteoliposomes

Very small size proteoliposomes are nanosized particles formed from the outer membrane vesicles of *Neisseria meningitidis* and GM3 ganglioside [147]. They induce DC maturation and an antitumor response from CD8+ T cells [148, 149]. In mice, very small size proteoliposomes increased MDSC numbers but also decreased their immunosuppressive ability [150]. The adjuvant therapy also matures MDSCs into antigen presenting cells that can prime the immune system against tumors. Four clinical trials of cancer vaccines using very small size proteoliposomes are ongoing [151]. Two include phase I trials based on recombinant proteins of epidermal growth factor receptor and VEGF vaccines in murine models [152, 153]. A phase II trial based on promising evidence of grade II/III cervical intraepithelial neoplasia and human papillomavirus regression treated with a human papillomavirus peptide vaccine and very small size proteoliposomes [154]. Finally, a phase II trial will evaluate a gonadotropin releasing hormone peptide vaccine with very small size proteoliposomes in prostate cancer patients based on positive results in rats [155].

8.5.4 MDSC Destruction (Fig. 8.6)

8.5.4.1 Cytotoxic Agents

Chemotherapeutic agents employ a variety of mechanisms to destroy rapidly dividing cells, including MDSCs. Gemcitabine, 5-fluorouracil, and cisplatin have been identified to induce MDSC cell death in addition to tumor cell apoptosis. Pyrimidine analogues such as gemcitabine and 5-fluorouracil abolished G-MDSCs in the spleens and tumors of mice without changes in T cells, B cells, NK cells, or macrophages [156–

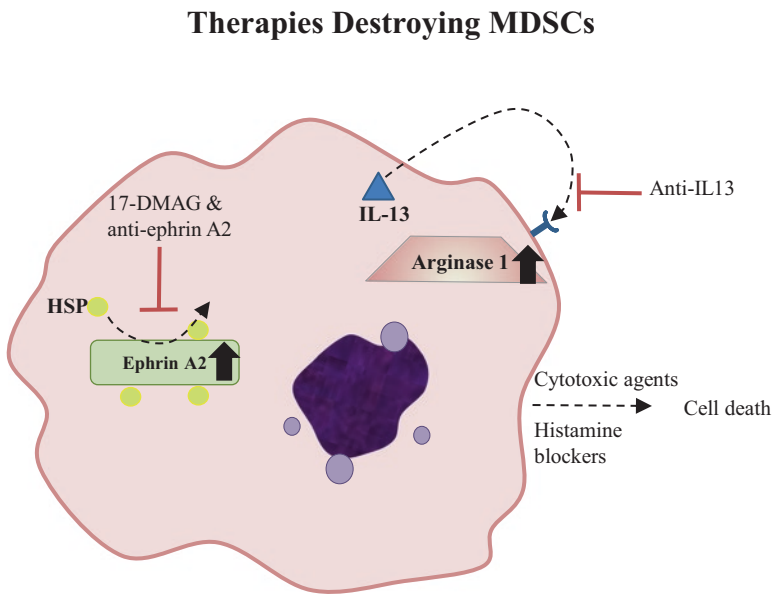

Fig. 8.6 A summary of treatments aimed at destroying MDSCs. *DMAG* dimethylaminoethylamino-17-demethoxygeldanamycin, *IL* interleukin

159]. The removal of G-MDSCs also enhanced IFN-γ production by CD8+ T cells increasing antitumor activity. Combined with other chemotherapy modalities such as cyclophosphamide, rosiglitazone, interferon-gamma, and adenovirus vaccine immunotherapies, the host antitumor response can be enhanced [156, 160–162]. Cisplatin is another chemotherapeutic agent that alkylates DNA and was found to decrease MDSCs and Treg cells in mice with lung cancer [163]. However, murine models have also shown the reverse effect can occur when treated with cytotoxic agents, suggesting drug dose and schedule play an important role [164].

Clinical trials evaluating MDSC levels after cytotoxic chemotherapy are numerous but are usually measured as secondary outcomes. MDSC levels in peripheral blood of breast cancer patients were correlated with the stage of disease; patients with metastatic tumors had the largest discrepancy in MDSC numbers compared with T cell activity [5]. Doxorubicin and cyclophosphamide treatment increased MDSCs in these patients but fell with the addition of paclitaxel. This highlights the complexity of MDSCs in tumor microenvironment and the need for a multi-prong approach in targeting MDSCs.

8.5.4.2 Ephrin A2 Degradation

Heat shock protein 90 stabilizes tyrosine kinase ephrin A2 intracellularly, and overexpression of ephrin A2 in cancer patients is associated with increased metastatic potential [165]. 17-Dimethylaminoethylamino-17-demethoxygeldanamycin is a heat shock protein 90 inhibitor that targets ephrin A2 for proteome degradation and can be combined with ephrin A2 antibodies to cause MDSC destruction. Combination treatment in mice with sarcomas had no residual tumor and reduced MDSCs by FAS-mediated apoptosis, *in vitro* [165, 166]. There are no clinical trials exploring heat shock protein 90 or ephrin A2 inhibition.

8.5.4.3 Anti-IL-13

During inflammation, MDSCs, and mast cells increase arginase-1 via IL-13 secretion that result in greater immunosuppression [167, 168]. Sarcoma-bearing mice treated with an IL-13 *Pseudomonas* exotoxin drug conjugate and a DNA vaccine against the IL-13 receptor had a five-fold decrease in tumor growth compared with vaccination alone [169]. The drug conjugate decreased MDSCs while the DNA vaccine bolstered the effect to cause increased CD8+ T cells.

Clinical trials targeting pediatric glioblastomas expressing IL-13 receptors with drug conjugates have shown some promise, but MDSC levels have not been studied in these patients [170].

8.5.4.4 Histamine Blockers

Histamine type 2 receptor antagonists, usually prescribed for gastrointestinal conditions, can promote an antitumor environment [171, 172]. Although the exact mechanism of action is unknown, histamine blockade may induce Fas-dependent MDSC apoptosis inhibiting tumor growth. In mice with lung cancer, cimetidine (histamine type 2 receptor antagonist) decreased MDSC tumor promotion but failed to impact the proliferation, survival, and invasion of the cancer [171]. Although not currently approved for use in the United States, treatment of acute myelogenous leukemia and solid tumors in Europe includes the antihistamine dihydrochloride [173]. A clinical trial showed better outcomes when histamine blockade was combined with immunotherapy in patients with metastatic melanoma. However, solid tumors and acute myelogenous leukemia lacked the same marked improvements.

8.6 Conclusions

MDSCs play an important role in tumor formation and progression. They influence a number of pathways that can be targeted for improved cancer treatments. The current treatment strategies include: inhibiting MDSC development and expansion, inhibiting MDSC function, differentiating MDSCs into more mature cells, and destroying MDSCs. Although MDSC-directed treatments are in their infancy, these agents may prove vital to future cancer therapies. Further work will unravel the intricacies of MDSC pathways and will aid in the creation of new therapies. Challenges of MDSC-targeted therapy include classifying MDSC heterogeneity for better treatment options, a lack of unique MDSC markers, and overcoming the complexity of the tumor environment. It is clear, however, that MDSC-targeting therapies will play a role in multi-drug treatment strategies.

References

1. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–68. https://doi.org/10.1038/nri3175.
2. Gutkin DW, Shurin MR. Clinical evaluation of systemic and local immune responses in cancer: time for integration. Cancer Immunol Immunother. 2014;63(1):45–57. https://doi.org/10.1007/s00262-013-1480-0.
3. Zhong H, Gutkin DW, Han B, Ma Y, Keskinov AA, Shurin MR, Shurin GV. Origin and pharmacological modulation of tumor-associated regulatory dendritic cells. Int J Cancer. 2014;134(11):2633–45. https://doi.org/10.1002/ijc.28590.
4. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162–74. https://doi.org/10.1038/nri2506.
5. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother. 2009;58(1):49–59. https://doi.org/10.1007/s00262-008-0523-4.
6. Zhang B, Wang Z, Wu L, Zhang M, Li W, Ding J, Zhu J, Wei H, Zhao K. Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma. PLoS One. 2013;8(2):e57114. https://doi.org/10.1371/journal.pone.0057114.
7. Keskinov AA, Shurin MR. Myeloid regulatory cells in tumor spreading and metastasis. Immunobiology. 2015;220(2):236–42. https://doi.org/10.1016/j.imbio.2014.07.017.
8. Albeituni SH, Ding C, Yan J. Hampering immune suppressors: therapeutic targeting of myeloid-derived suppressor cells in cancer. Cancer J. 2013;19(6):490–501. https://doi.org/10.1097/PPO.0000000000000006.
9. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404(6774):193–7. https://doi.org/10.1038/35004599.
10. Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, Lush RM, Antonia S, Gabrilovich DI. All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. Cancer Res. 2006;66(18):9299–307. https://doi.org/10.1158/0008-5472.CAN-06-1690.
11. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. Clin Cancer Res. 2007;13(2 Pt 2):721s–6s. https://doi.org/10.1158/1078-0432.CCR-06-2197.
12. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells

in cancer patients: a mechanism of immunosuppression in cancer. J Immunol. 2001;166(1):678–89.
13. Lechner MG, Russell SM, Bass RS, Epstein AL. Chemokines, costimulatory molecules and fusion proteins for the immunotherapy of solid tumors. Immunotherapy. 2011;3(11):1317–40. https://doi.org/10.2217/imt.11.115.
14. Pak AS, Wright MA, Matthews JP, Collins SL, Petruzzelli GJ, Young MR. Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. Clin Cancer Res. 1995;1(1):95–103.
15. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol. 2008;181(8):5791–802.
16. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol. 2004;172(2):989–99.
17. Kusmartsev S, Gabrilovich DI. Immature myeloid cells and cancer-associated immune suppression. Cancer Immunol Immunother. 2002;51(6):293–8. https://doi.org/10.1007/s00262-002-0280-8.
18. Seung LP, Rowley DA, Dubey P, Schreiber H. Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection. Proc Natl Acad Sci U S A. 1995;92(14):6254–8.
19. Youn JI, Collazo M, Shalova IN, Biswas SK, Gabrilovich DI. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. J Leukoc Biol. 2012;91(1):167–81. https://doi.org/10.1189/jlb.0311177.
20. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood. 2008;111(8):4233–44. https://doi.org/10.1182/blood-2007-07-099226.
21. Dietlin TA, Hofman FM, Lund BT, Gilmore W, Stohlman SA, van der Veen RC. Mycobacteria-induced Gr-1+ subsets from distinct myeloid lineages have opposite effects on T cell expansion. J Leukoc Biol. 2007;81(5):1205–12. https://doi.org/10.1189/jlb.1006640.
22. Youn JI, Gabrilovich DI. The biology of myeloid-derived suppressor cells: the blessing and the curse of morphological and functional heterogeneity. Eur J Immunol. 2010;40(11):2969–75. https://doi.org/10.1002/eji.201040895.
23. Jiang J, Guo W, Liang X. Phenotypes, accumulation, and functions of myeloid-derived suppressor cells and associated treatment strategies in cancer patients. Hum Immunol. 2014;75(11):1128–37. https://doi.org/10.1016/j.humimm.2014.09.025.
24. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. Cancer Res. 2001;61(12):4756–60.
25. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, Ochoa AC. Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res. 2009;69(4):1553–60. https://doi.org/10.1158/0008-5472.CAN-08-1921.
26. Poschke I, Mougiakakos D, Hansson J, Masucci GV, Kiessling R. Immature immunosuppressive CD14+HLA-DR−/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign. Cancer Res. 2010;70(11):4335–45. https://doi.org/10.1158/0008-5472.CAN-09-3767.
27. Rodriguez PC, Zea AH, Ochoa AC. Mechanisms of tumor evasion from the immune response. Cancer Chemother Biol Response Modif. 2003;21:351–64.
28. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. Blood. 2007;109(4):1568–73. https://doi.org/10.1182/blood-2006-06-031856.
29. Mannick JB, Hausladen A, Liu L, Hess DT, Zeng M, Miao QX, Kane LS, Gow AJ, Stamler JS. Fas-induced caspase denitrosylation. Science. 1999;284(5414):651–4.
30. Lu T, Ramakrishnan R, Altiok S, Youn JI, Cheng P, Celis E, Pisarev V, Sherman S, Sporn MB, Gabrilovich D. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. J Clin Invest. 2011;121(10):4015–29. https://doi.org/10.1172/JCI45862.
31. Angelini G, Gardella S, Ardy M, Ciriolo MR, Filomeni G, Di Trapani G, Clarke F, Sitia R, Rubartelli A. Antigen-presenting dendritic cells provide the reducing extracellular microenvironment required for T lymphocyte activation. Proc Natl Acad Sci U S A. 2002;99(3):1491–6. https://doi.org/10.1073/pnas.022630299.
32. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. Cancer Res. 2010;70(1):68–77. https://doi.org/10.1158/0008-5472.CAN-09-2587.
33. Eruslanov E, Neuberger M, Daurkin I, Perrin GQ, Algood C, Dahm P, Rosser C, Vieweg J, Gilbert SM, Kusmartsev S. Circulating and tumor-infiltrating myeloid cell subsets in patients with bladder cancer. Int J Cancer. 2012;130(5):1109–19. https://doi.org/10.1002/ijc.26123.
34. Hanson EM, Clements VK, Sinha P, Ilkovitch D, Ostrand-Rosenberg S. Myeloid-derived suppressor cells down-regulate L-selectin expression on CD4+ and CD8+ T cells. J Immunol. 2009;183(2):937–44. https://doi.org/10.4049/jimmunol.0804253.
35. Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J, Divino CM, Chen SH. Gr-1+CD115+

immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res. 2006;66(2):1123–31. https://doi.org/10.1158/0008-5472.CAN-05-1299.
36. Pan PY, Wang GX, Yin B, Ozao J, Ku T, Divino CM, Chen SH. Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. Blood. 2008;111(1):219–28. https://doi.org/10.1182/blood-2007-04-086835.
37. Sevko A, Umansky V. Myeloid-derived suppressor cells interact with tumors in terms of myelopoiesis, tumorigenesis and immunosuppression: thick as thieves. J Cancer. 2013;4(1):3–11. https://doi.org/10.7150/jca.5047.
38. Finke J, Ko J, Rini B, Rayman P, Ireland J, Cohen P. MDSC as a mechanism of tumor escape from sunitinib mediated anti-angiogenic therapy. Int Immunopharmacol. 2011;11(7):856–61. https://doi.org/10.1016/j.intimp.2011.01.030.
39. Panka DJ, Liu Q, Geissler AK, Mier JW. Effects of HDM2 antagonism on sunitinib resistance, p53 activation, SDF-1 induction, and tumor infiltration by CD11b+/Gr-1+ myeloid derived suppressor cells. Mol Cancer. 2013;12:17. https://doi.org/10.1186/1476-4598-12-17.
40. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res. 2009;15(6):2148–57. https://doi.org/10.1158/1078-0432.CCR-08-1332.
41. Dutta P, Li WX. Role of the JAK-STAT signalling pathway in cancer. In: eLS. Wiley. 2001. doi:https://doi.org/10.1002/9780470015902.a0025214.
42. Ugel S, Delpozzo F, Desantis G, Papalini F, Simonato F, Sonda N, Zilio S, Bronte V. Therapeutic targeting of myeloid-derived suppressor cells. Curr Opin Pharmacol. 2009;9(4):470–81. https://doi.org/10.1016/j.coph.2009.06.014.
43. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol. 2011;32(1):19–25. https://doi.org/10.1016/j.it.2010.10.002.
44. Sansone P, Bromberg J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. J Clin Oncol Off J Am Soc Clin Oncol. 2012;30(9):1005–14. https://doi.org/10.1200/JCO.2010.31.8907.
45. Lin L, Deangelis S, Foust E, Fuchs J, Li C, Li PK, Schwartz EB, Lesinski GB, Benson D, Lu J, Hoyt D, Lin J. A novel small molecule inhibits STAT3 phosphorylation and DNA binding activity and exhibits potent growth suppressive activity in human cancer cells. Mol Cancer. 2010;9:217. https://doi.org/10.1186/1476-4598-9-217.
46. Bill MA, Fuchs JR, Li C, Yui J, Bakan C, Benson DM Jr, Schwartz EB, Abdelhamid D, Lin J, Hoyt DG, Fossey SL, Young GS, Carson WE 3rd, Li PK, Lesinski GB. The small molecule curcumin analog FLLL32 induces apoptosis in melanoma cells via STAT3 inhibition and retains the cellular response to cytokines with anti-tumor activity. Mol Cancer. 2010;9:165. https://doi.org/10.1186/1476-4598-9-165.
47. Dhillon N, Aggarwal BB, Newman RA, Wolff RA, Kunnumakkara AB, Abbruzzese JL, Ng CS, Badmaev V, Kurzrock R. Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res. 2008;14(14):4491–9. https://doi.org/10.1158/1078-0432.CCR-08-0024.
48. SP T, Jin H, Shi JD, Zhu LM, Suo Y, Lu G, Liu A, Wang TC, Yang CS. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. Cancer Prev Res. 2012;5(2):205–15. https://doi.org/10.1158/1940-6207.CAPR-11-0247.
49. Yang WC, Ma G, Chen SH, Pan PY. Polarization and reprogramming of myeloid-derived suppressor cells. J Mol Cell Biol. 2013;5(3):207–9. https://doi.org/10.1093/jmcb/mjt009.
50. Carroll RE, Benya RV, Turgeon DK, Vareed S, Neuman M, Rodriguez L, Kakarala M, Carpenter PM, McLaren C, Meyskens FL Jr, Brenner DE. Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia. Cancer Prev Res. 2011;4(3):354–64. https://doi.org/10.1158/1940-6207.CAPR-10-0098.
51. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 2001;21(4B):2895–900.
52. Lu P, Yu B, Xu J. Cucurbitacin B regulates immature myeloid cell differentiation and enhances antitumor immunity in patients with lung cancer. Cancer Biother Radiopharm. 2012;27(8):495–503. https://doi.org/10.1089/cbr.2012.1219.
53. Stewart MW. Aflibercept (VEGF trap-eye): the newest anti-VEGF drug. Br J Ophthalmol. 2012;96(9):1157–8. https://doi.org/10.1136/bjophthalmol-2011-300654.
54. Fricke I, Mirza N, Dupont J, Lockhart C, Jackson A, Lee JH, Sosman JA, Gabrilovich DI. Vascular endothelial growth factor-trap overcomes defects in dendritic cell differentiation but does not improve antigen-specific immune responses. Clin Cancer Res. 2007;13(16):4840–8. https://doi.org/10.1158/1078-0432.CCR-07-0409.
55. Aparicio-Gallego G, Blanco M, Figueroa A, Garcia-Campelo R, Valladares-Ayerbes M, Grande-Pulido E, Anton-Aparicio L. New insights into molecular mechanisms of sunitinib-associated side effects.

Mol Cancer Ther. 2011;10(12):2215–23. https://doi.org/10.1158/1535-7163.MCT-10-1124.
56. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, Ko JS, Cohen PA, Finke JH, Storkus WJ. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. Int J Cancer. 2011;129(9):2158–70. https://doi.org/10.1002/ijc.25863.
57. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, Schwartz M, Divino CM, Pan PY, Chen SH. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. Cancer Res. 2009;69(6):2514–22. https://doi.org/10.1158/0008-5472.CAN-08-4709.
58. Ko JS, Rayman P, Ireland J, Swaidani S, Li G, Bunting KD, Rini B, Finke JH, Cohen PA. Direct and differential suppression of myeloid-derived suppressor cell subsets by sunitinib is compartmentally constrained. Cancer Res. 2010;70(9):3526–36. https://doi.org/10.1158/0008-5472.CAN-09-3278.
59. van Cruijsen H, van der Veldt AA, Vroling L, Oosterhoff D, Broxterman HJ, Scheper RJ, Giaccone G, Haanen JB, van den Eertwegh AJ, Boven E, Hoekman K, de Gruijl TD. Sunitinib-induced myeloid lineage redistribution in renal cell cancer patients: CD1c+ dendritic cell frequency predicts progression-free survival. Clin Cancer Res. 2008;14(18):5884–92. https://doi.org/10.1158/1078-0432.CCR-08-0656.
60. Xin H, Zhang C, Herrmann A, Du Y, Figlin R, Yu H. Sunitinib inhibition of Stat3 induces renal cell carcinoma tumor cell apoptosis and reduces immunosuppressive cells. Cancer Res. 2009;69(6):2506–13. https://doi.org/10.1158/0008-5472.CAN-08-4323.
61. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. J Immunol. 2008;181(7):4666–75.
62. Srikrishna G. S100A8 and S100A9: new insights into their roles in malignancy. J Innate Immun. 2012;4(1):31–40. https://doi.org/10.1159/000330095.
63. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, Nguyen M, Olsson A, Nawroth PP, Bierhaus A, Varki N, Kronenberg M, Freeze HH, Srikrishna G. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. Carcinogenesis. 2008;29(10):2035–43. https://doi.org/10.1093/carcin/bgn188.
64. Mok S, Koya RC, Tsui C, Xu J, Robert L, Wu L, Graeber TG, West BL, Bollag G, Ribas A. Inhibition of CSF-1 receptor improves the antitumor efficacy of adoptive cell transfer immunotherapy. Cancer Res. 2014;74(1):153–61. https://doi.org/10.1158/0008-5472.CAN-13-1816.
65. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Sylvestre V, Stanley ER. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood. 2002;99(1):111–20.
66. Li J, Chen K, Zhu L, Pollard JW. Conditional deletion of the colony stimulating factor-1 receptor (c-fms proto-oncogene) in mice. Genesis. 2006;44(7):328–35. https://doi.org/10.1002/dvg.20219.
67. De Santo C, Serafini P, Marigo I, Dolcetti L, Bolla M, Del Soldato P, Melani C, Guiducci C, Colombo MP, Iezzi M, Musiani P, Zanovello P, Bronte V. Nitroaspirin corrects immune dysfunction in tumor-bearing hosts and promotes tumor eradication by cancer vaccination. Proc Natl Acad Sci U S A. 2005;102(11):4185–90. https://doi.org/10.1073/pnas.0409783102.
68. Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, Johnson M, Lusis AJ, Cohen DA, Iruela-Arispe ML, Wu L. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of anti-angiogenic therapy. Blood. 2010;115(7):1461–71. https://doi.org/10.1182/blood-2009-08-237412.
69. Xu J, Escamilla J, Mok S, David J, Priceman S, West B, Bollag G, McBride W, Wu L. CSF1R signaling blockade stanches tumor-infiltrating myeloid cells and improves the efficacy of radiotherapy in prostate cancer. Cancer Res. 2013;73(9):2782–94. https://doi.org/10.1158/0008-5472.CAN-12-3981.
70. He D, Li H, Yusuf N, Elmets CA, Li J, Mountz JD, Xu H. IL-17 promotes tumor development through the induction of tumor promoting microenvironments at tumor sites and myeloid-derived suppressor cells. J Immunol. 2010;184(5):2281–8. https://doi.org/10.4049/jimmunol.0902574.
71. Rodan GA, Fleisch HA. Bisphosphonates: mechanisms of action. J Clin Invest. 1996;97(12):2692–6. https://doi.org/10.1172/JCI118722.
72. Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell. 2002;109(5):625–37.
73. Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer Res. 2007;67(23):11438–46. https://doi.org/10.1158/0008-5472.CAN-07-1882.
74. Porembka MR, Mitchem JB, Belt BA, Hsieh CS, Lee HM, Herndon J, Gillanders WE, Linehan DC, Goedegebuure P. Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth. Cancer Immunol Immunother. 2012;61(9):1373–85. https://doi.org/10.1007/s00262-011-1178-0.
75. Gnant M, Mlineritsch B, Schippinger W, Luschin-Ebengreuth G, Postlberger S, Menzel C, Jakesz R,

Seifert M, Hubalek M, Bjelic-Radisic V, Samonigg H, Tausch C, Eidtmann H, Steger G, Kwasny W, Dubsky P, Fridrik M, Fitzal F, Stierer M, Rucklinger E, Greil R, Investigators A-T, Marth C. Endocrine therapy plus zoledronic acid in premenopausal breast cancer. N Engl J Med. 2009;360(7):679–91. https://doi.org/10.1056/NEJMoa0806285.

76. Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M, Bastert G. Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med. 1998;339(6):357–63. https://doi.org/10.1056/NEJM199808063390601.
77. Schilling B, Sucker A, Griewank K, Zhao F, Weide B, Gorgens A, Giebel B, Schadendorf D, Paschen A. Vemurafenib reverses immunosuppression by myeloid derived suppressor cells. Int J Cancer. 2013;133(7):1653–63. https://doi.org/10.1002/ijc.28168.
78. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D, Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353(20):2135–47. https://doi.org/10.1056/NEJMoa050092.
79. Ribas A, Hodi FS, Callahan M, Konto C, Wolchok J. Hepatotoxicity with combination of vemurafenib and ipilimumab. N Engl J Med. 2013;368(14):1365–6. https://doi.org/10.1056/NEJMc1302338.
80. Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale FV, van Bruggen N, Ho C, Ross J, Tan M, Carano RA, Meng YG, Ferrara N. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature. 2007;450(7171):825–31. https://doi.org/10.1038/nature06348.
81. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, Yu L, Ross J, Korsisaari N, Cao T, Bou-Reslan H, Kallop D, Weimer R, Ludlam MJ, Kaminker JS, Modrusan Z, van Bruggen N, Peale FV, Carano R, Meng YG, Ferrara N. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc Natl Acad Sci U S A. 2010;107(50):21248–55. https://doi.org/10.1073/pnas.1015855107.
82. Hasnis E, Alishekevitz D, Gingis-Veltski S, Bril R, Fremder E, Voloshin T, Raviv Z, Karban A, Shaked Y. Anti-Bv8 antibody and metronomic gemcitabine improve pancreatic adenocarcinoma treatment outcome following weekly gemcitabine therapy. Neoplasia. 2014;16(6):501–10. https://doi.org/10.1016/j.neo.2014.05.011.
83. Pienta KJ, Machiels JP, Schrijvers D, Alekseev B, Shkolnik M, Crabb SJ, Li S, Seetharam S, Puchalski TA, Takimoto C, Elsayed Y, Dawkins F, de Bono JS. Phase 2 study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), in metastatic castration-resistant prostate cancer. Investig New Drugs. 2013;31(3):760–8. https://doi.org/10.1007/s10637-012-9869-8.
84. Kirk PS, Koreckij T, Nguyen HM, Brown LG, Snyder LA, Vessella RL, Corey E. Inhibition of CCL2 signaling in combination with docetaxel treatment has profound inhibitory effects on prostate cancer growth in bone. Int J Mol Sci. 2013;14(5):10483–96. https://doi.org/10.3390/ijms140510483.
85. Sumida K, Wakita D, Narita Y, Masuko K, Terada S, Watanabe K, Satoh T, Kitamura H, Nishimura T. Anti-IL-6 receptor mAb eliminates myeloid-derived suppressor cells and inhibits tumor growth by enhancing T-cell responses. Eur J Immunol. 2012;42(8):2060–72. https://doi.org/10.1002/eji.201142335.
86. Fiorucci S, Santucci L, Cirino G, Mencarelli A, Familiari L, Soldato PD, Morelli A. IL-1 beta converting enzyme is a target for nitric oxide-releasing aspirin: new insights in the antiinflammatory mechanism of nitric oxide-releasing nonsteroidal antiinflammatory drugs. J Immunol. 2000;165(9):5245–54.
87. Molon B, Ugel S, Del Pozzo F, Soldani C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, Savino B, Colombo P, Jonjic N, Pecanic S, Lazzarato L, Fruttero R, Gasco A, Bronte V, Viola A. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208(10):1949–62. https://doi.org/10.1084/jem.20101956.
88. Serafini P, Meckel K, Kelso M, Noonan K, Califano J, Koch W, Dolcetti L, Bronte V, Borrello I. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med. 2006;203(12):2691–702. https://doi.org/10.1084/jem.20061104.
89. Meyer C, Sevko A, Ramacher M, Bazhin AV, Falk CS, Osen W, Borrello I, Kato M, Schadendorf D, Baniyash M, Umansky V. Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci U S A. 2011;108(41):17111–6. https://doi.org/10.1073/pnas.1108121108.
90. Kasic T, Colombo P, Soldani C, Wang CM, Miranda E, Roncalli M, Bronte V, Viola A. Modulation of human T-cell functions by reactive nitrogen species. Eur J Immunol. 2011;41(7):1843–9. https://doi.org/10.1002/eji.201040868.
91. Rosenberg SA. Cancer vaccines based on the identification of genes encoding cancer regression antigens. Immunol Today. 1997;18(4):175–82.
92. Maier T, Holda JH, Claman HN. Murine natural suppressor cells in the newborn, in bone marrow, and after cyclophosphamide. Genetic variations and dependence on IFN-gamma. J Immunol. 1989;143(2):491–8.
93. Zhang B, Zhang Y, Bowerman NA, Schietinger A, YX F, Kranz DM, Rowley DA, Schreiber H. Equilibrium between host and cancer caused by effector T cells killing tumor stroma. Cancer Res.

2008;68(5):1563–71. https://doi.org/10.1158/0008-5472.CAN-07-5324.
94. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, Kalchenko V, Geissmann F, Jung S. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. J Exp Med. 2007;204(1):171–80. https://doi.org/10.1084/jem.20061011.
95. Nagaraj S, Youn JI, Weber H, Iclozan C, Lu L, Cotter MJ, Meyer C, Becerra CR, Fishman M, Antonia S, Sporn MB, Liby KT, Rawal B, Lee JH, Gabrilovich DI. Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. Clin Cancer Res. 2010;16(6):1812–23. https://doi.org/10.1158/1078-0432.CCR-09-3272.
96. Thimmulappa RK, Fuchs RJ, Malhotra D, Scollick C, Traore K, Bream JH, Trush MA, Liby KT, Sporn MB, Kensler TW, Biswal S. Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils. Antioxid Redox Signal. 2007;9(11):1963–70. https://doi.org/10.1089/ars.2007.1745.
97. Wesolowski R, Markowitz J, Carson WE 3rd. Myeloid derived suppressor cells—a new therapeutic target in the treatment of cancer. J Immunother Cancer. 2013;1:10. https://doi.org/10.1186/2051-1426-1-10.
98. Hong DSKR, Supko JG, Lawrence DP, Wheler JJ, Meyer CJ, Mier JW, Andreeff M, Shapiro GI, Dezube BJ. Phase I trial with a novel oral NF-κB/STAT3 inhibitor RTA 402 in patients with solid tumors and lymphoid malignancies. J Clin Oncol (Meeting Abstracts). 2008;26:3517.
99. Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB, Gilbert J, Ochoa AC. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med. 2005;202(7):931–9. https://doi.org/10.1084/jem.20050715.
100. Eruslanov E, Daurkin I, Ortiz J, Vieweg J, Kusmartsev S. Pivotal advance: tumor-mediated induction of myeloid-derived suppressor cells and M2-polarized macrophages by altering intracellular PGE(2) catabolism in myeloid cells. J Leukoc Biol. 2010;88(5):839–48. https://doi.org/10.1189/jlb.1209821.
101. Obermajer N, Kalinski P. Generation of myeloid-derived suppressor cells using prostaglandin E2. Transplant Res. 2012;1(1):15. https://doi.org/10.1186/2047-1440-1-15.
102. Raccosta L, Fontana R, Maggioni D, Lanterna C, Villablanca EJ, Paniccia A, Musumeci A, Chiricozzi E, Trincavelli ML, Daniele S, Martini C, Gustafsson JA, Doglioni C, Feo SG, Leiva A, Ciampa MG, Mauri L, Sensi C, Prinetti A, Eberini I, Mora JR, Bordignon C, Steffensen KR, Sonnino S, Sozzani S, Traversari C, Russo V. The oxysterol-CXCR2 axis plays a key role in the recruitment of tumor-promoting neutrophils. J Exp Med. 2013;210(9):1711–28. https://doi.org/10.1084/jem.20130440.
103. Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, Hoft DF, Peng G. Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. J Immunol. 2013;190(5):2403–14. https://doi.org/10.4049/jimmunol.1202369.
104. Fujita M, Kohanbash G, Fellows-Mayle W, Hamilton RL, Komohara Y, Decker SA, Ohlfest JR, Okada H. COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. Cancer Res. 2011;71(7):2664–74. https://doi.org/10.1158/0008-5472.CAN-10-3055.
105. Serafini P, Carbley R, Noonan KA, Tan G, Bronte V, Borrello I. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res. 2004;64(17):6337–43. https://doi.org/10.1158/0008-5472.CAN-04-0757.
106. Yu LB, Dong XS, Sun WZ, Zhao DL, Yang Y. Effect of a nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester on invasion of human colorectal cancer cell line SL-174T. World J Gastroenterol. 2005;11(40):6385–8.
107. Rigamonti N, Capuano G, Ricupito A, Jachetti E, Grioni M, Generoso L, Freschi M, Bellone M. Modulators of arginine metabolism do not impact on peripheral T-cell tolerance and disease progression in a model of spontaneous prostate cancer. Clin Cancer Res. 2011;17(5):1012–23. https://doi.org/10.1158/1078-0432.CCR-10-2547.
108. Capuano G, Rigamonti N, Grioni M, Freschi M, Bellone M. Modulators of arginine metabolism support cancer immunosurveillance. BMC Immunol. 2009;10:1. https://doi.org/10.1186/1471-2172-10-1.
109. Kuwata T, Wang IM, Tamura T, Ponnamperuma RM, Levine R, Holmes KL, Morse HC, De Luca LM, Ozato K. Vitamin A deficiency in mice causes a systemic expansion of myeloid cells. Blood. 2000;95(11):3349–56.
110. Young MR, Wright MA, Vellody K, Lathers DM. Skewed differentiation of bone marrow CD34+ cells of tumor bearers from dendritic toward monocytic cells, and the redirection of differentiation toward dendritic cells by 1alpha,25-dihydroxyvitamin D3. Int J Immunopharmacol. 1999;21(10):675–88.
111. Kulbersh JS, Day TA, Gillespie MB, Young MR. 1alpha,25-Dihydroxyvitamin D(3) to skew intratumoral levels of immune inhibitory CD34(+) progenitor cells into dendritic cells. Otolaryngol Head Neck Surg. 2009;140(2):235–40. https://doi.org/10.1016/j.otohns.2008.11.011.
112. Lathers DM, Clark JI, Achille NJ, Young MR. Phase 1B study to improve immune responses in head

and neck cancer patients using escalating doses of 25-hydroxyvitamin D3. Cancer Immunol Immunother. 2004;53(5):422–30. https://doi.org/10.1007/s00262-003-0459-7.
113. Walsh JE, Clark AM, Day TA, Gillespie MB, Young MR. Use of alpha,25-dihydroxyvitamin D3 treatment to stimulate immune infiltration into head and neck squamous cell carcinoma. Hum Immunol. 2010;71(7):659–65. https://doi.org/10.1016/j.humimm.2010.04.008.
114. Zhang H, Maric I, DiPrima MJ, Khan J, Orentas RJ, Kaplan RN, Mackall CL. Fibrocytes represent a novel MDSC subset circulating in patients with metastatic cancer. Blood. 2013;122(7):1105–13. https://doi.org/10.1182/blood-2012-08-449413.
115. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene. 2004;328:1–16. https://doi.org/10.1016/j.gene.2003.12.005.
116. Hengesbach LM, Hoag KA. Physiological concentrations of retinoic acid favor myeloid dendritic cell development over granulocyte development in cultures of bone marrow cells from mice. J Nutr. 2004;134(10):2653–9.
117. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R, Gabrilovich D. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. Cancer Res. 2003;63(15):4441–9.
118. Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kubler H, Yancey D, Dahm P, Vieweg J. Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. Clin Cancer Res. 2008;14(24):8270–8. https://doi.org/10.1158/1078-0432.CCR-08-0165.
119. Adamson PC, Matthay KK, O'Brien M, Reaman GH, Sato JK, Balis FM. A phase 2 trial of all-trans-retinoic acid in combination with interferon-alpha2a in children with recurrent neuroblastoma or Wilms tumor: a Pediatric Oncology Branch, NCI and Children's Oncology Group Study. Pediatr Blood Cancer. 2007;49(5):661–5. https://doi.org/10.1002/pbc.21011.
120. Pillai RN, Aisner J, Dahlberg SE, Rogers JS, DiPaola RS, Aisner S, Ramalingam SS, Schiller JH. Interferon alpha plus 13-cis-retinoic acid modulation of BCL-2 plus paclitaxel for recurrent small-cell lung cancer (SCLC): an eastern cooperative oncology group study (E6501). Cancer Chemother Pharmacol. 2014;74(1):177–83. https://doi.org/10.1007/s00280-014-2427-7.
121. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. J Immunol. 2007;179(2):977–83.
122. Steding CE, ST W, Zhang Y, Jeng MH, Elzey BD, Kao C. The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis. Immunology. 2011;133(2):221–38. https://doi.org/10.1111/j.1365-2567.2011.03429.x.
123. Chinnasamy D, Yu Z, Kerkar SP, Zhang L, Morgan RA, Restifo NP, Rosenberg SA. Local delivery of interleukin-12 using T cells targeting VEGF receptor-2 eradicates multiple vascularized tumors in mice. Clin Cancer Res. 2012;18(6):1672–83. https://doi.org/10.1158/1078-0432.CCR-11-3050.
124. Kerkar SP, Goldszmid RS, Muranski P, Chinnasamy D, Yu Z, Reger RN, Leonardi AJ, Morgan RA, Wang E, Marincola FM, Trinchieri G, Rosenberg SA, Restifo NP. IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors. J Clin Invest. 2011;121(12):4746–57. https://doi.org/10.1172/JCI58814.
125. Ansell SM, Witzig TE, Kurtin PJ, Sloan JA, Jelinek DF, Howell KG, Markovic SN, Habermann TM, Klee GG, Atherton PJ, Erlichman C. Phase 1 study of interleukin-12 in combination with rituximab in patients with B-cell non-Hodgkin lymphoma. Blood. 2002;99(1):67–74.
126. Repka T, Chiorean EG, Gay J, Herwig KE, Kohl VK, Yee D, Miller JS. Trastuzumab and interleukin-2 in HER2-positive metastatic breast cancer: a pilot study. Clin Cancer Res. 2003;9(7):2440–6.
127. Alvarez RD, Sill MW, Davidson SA, Muller CY, Bender DP, DeBernardo RL, Behbakht K, Huh WK. A phase II trial of intraperitoneal EGEN-001, an IL-12 plasmid formulated with PEG-PEI-cholesterol lipopolymer in the treatment of persistent or recurrent epithelial ovarian, fallopian tube or primary peritoneal cancer: a gynecologic oncology group study. Gynecol Oncol. 2014;133(3):433–8. https://doi.org/10.1016/j.ygyno.2014.03.571.
128. Vollmer J, Weeratna RD, Jurk M, Samulowitz U, McCluskie MJ, Payette P, Davis HL, Schetter C, Krieg AM. Oligodeoxynucleotides lacking CpG dinucleotides mediate toll-like receptor 9 dependent T helper type 2 biased immune stimulation. Immunology. 2004;113(2):212–23. https://doi.org/10.1111/j.1365-2567.2004.01962.x.
129. Zoglmeier C, Bauer H, Norenberg D, Wedekind G, Bittner P, Sandholzer N, Rapp M, Anz D, Endres S, Bourquin C. CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice. Clin Cancer Res. 2011;17(7):1765–75. https://doi.org/10.1158/1078-0432.CCR-10-2672.
130. Jahrsdorfer B, Weiner GJ. CpG oligodeoxynucleotides as immunotherapy in cancer. Update Cancer Ther. 2008;3(1):27–32. https://doi.org/10.1016/j.uct.2007.11.003.
131. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D. Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br J Cancer. 2002;87(1):21–7. https://doi.org/10.1038/sj.bjc.6600347.
132. Young MR, Lathers DM. Combination docetaxel plus vitamin D(3) as an immune therapy in animals

bearing squamous cell carcinomas. Otolaryngol Head Neck Surg. 2005;133(4):611–8. https://doi.org/10.1016/j.otohns.2005.05.658.
133. Kodumudi KN, Woan K, Gilvary DL, Sahakian E, Wei S, Djeu JY. A novel chemoimmunomodulating property of docetaxel: suppression of myeloid-derived suppressor cells in tumor bearers. Clin Cancer Res. 2010;16(18):4583–94. https://doi.org/10.1158/1078-0432.CCR-10-0733.
134. Michels T, Shurin GV, Naiditch H, Sevko A, Umansky V, Shurin MR. Paclitaxel promotes differentiation of myeloid-derived suppressor cells into dendritic cells in vitro in a TLR4-independent manner. J Immunotoxicol. 2012;9(3):292–300. https://doi.org/10.3109/1547691X.2011.642418.
135. Kodumudi KN, Weber A, Sarnaik AA, Pilon-Thomas S. Blockade of myeloid-derived suppressor cells after induction of lymphopenia improves adoptive T cell therapy in a murine model of melanoma. J Immunol. 2012;189(11):5147–54. https://doi.org/10.4049/jimmunol.1200274.
136. Montero AJ, Diaz-Montero CM, Deutsch YE, Hurley J, Koniaris LG, Rumboldt T, Yasir S, Jorda M, Garret-Mayer E, Avisar E, Slingerland J, Silva O, Welsh C, Schuhwerk K, Seo P, Pegram MD, Gluck S. Phase 2 study of neoadjuvant treatment with NOV-002 in combination with doxorubicin and cyclophosphamide followed by docetaxel in patients with HER-2 negative clinical stage II-IIIc breast cancer. Breast Cancer Res Treat. 2012;132(1):215–23. https://doi.org/10.1007/s10549-011-1889-0.
137. Tian J, Ma J, Ma K, Guo H, Baidoo SE, Zhang Y, Yan J, Lu L, Xu H, Wang S. Beta-glucan enhances antitumor immune responses by regulating differentiation and function of monocytic myeloid-derived suppressor cells. Eur J Immunol. 2013;43(5):1220–30. https://doi.org/10.1002/eji.201242841.
138. Chan GC, Chan WK, Sze DM. The effects of beta-glucan on human immune and cancer cells. J Hematol Oncol. 2009;2:25. https://doi.org/10.1186/1756-8722-2-25.
139. Inoue M, Tanaka Y, Sugita N, Yamasaki M, Yamanaka T, Minagawa J, Nakamuro K, Tani T, Okudaira Y, Karita T, et al. Improvement of long-term prognosis in patients with ovarian cancers by adjuvant sizofiran immunotherapy: a prospective randomized controlled study. Biotherapy. 1993;6(1):13–8.
140. Hayakawa K, Mitsuhashi N, Saito Y, Nakayama Y, Furuta M, Nakamoto S, Kawashima M, Niibe H. Effect of Krestin as adjuvant treatment following radical radiotherapy in non-small cell lung cancer patients. Cancer Detect Prev. 1997;21(1):71–7.
141. Nakano H, Namatame K, Nemoto H, Motohashi H, Nishiyama K, Kumada K. A multi-institutional prospective study of lentinan in advanced gastric cancer patients with unresectable and recurrent diseases: effect on prolongation of survival and improvement of quality of life. Kanagawa Lentinan Research Group. Hepato-Gastroenterology. 1999;46(28):2662–8.
142. Gao Y, Tang W, Dai X, Gao H, Chen G, Ye J, Chan E, Koh HL, Li X, Zhou S. Effects of water-soluble Ganoderma lucidum polysaccharides on the immune functions of patients with advanced lung cancer. J Med Food. 2005;8(2):159–68. https://doi.org/10.1089/jmf.2005.8.159.
143. Chen X, ZP H, Yang XX, Huang M, Gao Y, Tang W, Chan SY, Dai X, Ye J, Ho PC, Duan W, Yang HY, Zhu YZ, Zhou SF. Monitoring of immune responses to a herbal immuno-modulator in patients with advanced colorectal cancer. Int Immunopharmacol. 2006;6(3):499–508. https://doi.org/10.1016/j.intimp.2005.08.026.
144. Yang C, Robbins PD. The roles of tumor-derived exosomes in cancer pathogenesis. Clin Dev Immunol. 2011;2011:842849. https://doi.org/10.1155/2011/842849.
145. Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, Cheng Z, Shah SV, Wang GJ, Zhang L, Grizzle WE, Mobley J, Zhang HG. Induction of myeloid-derived suppressor cells by tumor exosomes. Int J Cancer. 2009;124(11):2621–33. https://doi.org/10.1002/ijc.24249.
146. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, Boireau W, Rouleau A, Simon B, Lanneau D, De Thonel A, Multhoff G, Hamman A, Martin F, Chauffert B, Solary E, Zitvogel L, Garrido C, Ryffel B, Borg C, Apetoh L, Rebe C, Ghiringhelli F. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest. 2010;120(2):457–71. https://doi.org/10.1172/JCI40483.
147. Estevez F, Carr A, Solorzano L, Valiente O, Mesa C, Barroso O, Sierra GV, Fernandez LE. Enhancement of the immune response to poorly immunogenic gangliosides after incorporation into very small size proteoliposomes (VSSP). Vaccine. 1999;18(1–2):190–7.
148. Mesa C, de Leon J, Fernandez LE. Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for generation of CTL responses to peptide and protein antigens. Vaccine. 2006;24(14):2692–9. https://doi.org/10.1016/j.vaccine.2005.08.111.
149. Mesa C, de Leon J, Rigley K, Fernandez LE. Very small size proteoliposomes derived from Neisseria meningitidis: an effective adjuvant for Th1 induction and dendritic cell activation. Vaccine. 2004;22(23–24):3045–52. https://doi.org/10.1016/j.vaccine.2004.02.010.
150. Fernandez A, Mesa C, Marigo I, Dolcetti L, Clavell M, Oliver L, Fernandez LE, Bronte V. Inhibition of tumor-induced myeloid-derived suppressor cell function by a nanoparticulated adjuvant. J Immunol. 2011;186(1):264–74. https://doi.org/10.4049/jimmunol.1001465.

151. Fernandez A, Oliver L, Alvarez R, Hernandez A, Raymond J, Fernandez LE, Mesa C. Very small size proteoliposomes abrogate cross-presentation of tumor antigens by myeloid-derived suppressor cells and induce their differentiation to dendritic cells. J Immunother Cancer. 2014;2:5. https://doi.org/10.1186/2051-1426-2-5.
152. Ramirez BS, Pestana ES, Hidalgo GG, Garcia TH, Rodriguez RP, Ullrich A, Fernandez LE. Active anti-metastatic immunotherapy in Lewis lung carcinoma with self EGFR extracellular domain protein in VSSP adjuvant. Int J Cancer. 2006;119(9):2190–9. https://doi.org/10.1002/ijc.22085.
153. Bequet-Romero M, Morera Y, Ayala-Avila M, Ancizar J, Soria Y, Blanco A, Suarez-Alba J, Gavilondo JV. CIGB-247: a VEGF-based therapeutic vaccine that reduces experimental and spontaneous lung metastasis of C57Bl/6 and BALB/c mouse tumors. Vaccine. 2012;30(10):1790–9. https://doi.org/10.1016/j.vaccine.2012.01.006.
154. Solares AM, Baladron I, Ramos T, Valenzuela C, Borbon Z, Fanjull S, Gonzalez L, Castillo D, Esmir J, Granadillo M, Batte A, Cintado A, Ale M, Fernandez de Cossio ME, Ferrer A, Torrens I, Lopez-Saura P. Safety and immunogenicity of a human papillomavirus peptide vaccine (CIGB-228) in women with high-grade cervical intraepithelial Neoplasia: first-in-human, proof-of-concept trial. ISRN Obstet Gynecol. 2011;2011:292951. https://doi.org/10.5402/2011/292951.
155. Aguilar FF, Barranco JJ, Fuentes EB, Aguilera LC, Saez YL, Santana MD, Vazquez EP, Baker RB, Acosta OR, Perez HG, Nieto GG. Very small size proteoliposomes (VSSP) and Montanide combination enhance the humoral immuno response in a GnRH based vaccine directed to prostate cancer. Vaccine. 2012;30(46):6595–9. https://doi.org/10.1016/j.vaccine.2012.08.020.
156. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res. 2005;11(18):6713–21. https://doi.org/10.1158/1078-0432.CCR-05-0883.
157. Vincent J, Mignot G, Chalmin F, Ladoire S, Bruchard M, Chevriaux A, Martin F, Apetoh L, Rebe C, Ghiringhelli F. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res. 2010;70(8):3052–61. https://doi.org/10.1158/0008-5472.CAN-09-3690.
158. Mundy-Bosse BL, Lesinski GB, Jaime-Ramirez AC, Benninger K, Khan M, Kuppusamy P, Guenterberg K, Kondadasula SV, Chaudhury AR, La Perle KM, Kreiner M, Young G, Guttridge DC, Carson WE 3rd. Myeloid-derived suppressor cell inhibition of the IFN response in tumor-bearing mice. Cancer Res. 2011;71(15):5101–10. https://doi.org/10.1158/0008-5472.CAN-10-2670.
159. Le HK, Graham L, Cha E, Morales JK, Manjili MH, Bear HD. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. Int Immunopharmacol. 2009;9(7–8):900–9. https://doi.org/10.1016/j.intimp.2009.03.015.
160. Tongu M, Harashima N, Monma H, Inao T, Yamada T, Kawauchi H, Harada M. Metronomic chemotherapy with low-dose cyclophosphamide plus gemcitabine can induce anti-tumor T cell immunity in vivo. Cancer Immunol Immunother. 2013;62(2):383–91. https://doi.org/10.1007/s00262-012-1343-0.
161. Bunt SK, Mohr AM, Bailey JM, Grandgenett PM, Hollingsworth MA. Rosiglitazone and gemcitabine in combination reduces immune suppression and modulates T cell populations in pancreatic cancer. Cancer Immunol Immunother. 2013;62(2):225–36. https://doi.org/10.1007/s00262-012-1324-3.
162. Fridlender ZG, Sun J, Singhal S, Kapoor V, Cheng G, Suzuki E, Albelda SM. Chemotherapy delivered after viral immunogene therapy augments antitumor efficacy via multiple immune-mediated mechanisms. Mol Ther. 2010;18(11):1947–59. https://doi.org/10.1038/mt.2010.159.
163. Tseng CW, Hung CF, Alvarez RD, Trimble C, Huh WK, Kim D, Chuang CM, Lin CT, Tsai YC, He L, Monie A, TC W. Pretreatment with cisplatin enhances E7-specific CD8+ T-cell-mediated antitumor immunity induced by DNA vaccination. Clin Cancer Res. 2008;14(10):3185–92. https://doi.org/10.1158/1078-0432.CCR-08-0037.
164. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, Boireau W, Simon B, Ryffel B, Connat JL, Kanellopoulos J, Martin F, Rebe C, Apetoh L, Ghiringhelli F. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. Nat Med. 2013;19(1):57–64. https://doi.org/10.1038/nm.2999.
165. Rao A, Taylor JL, Chi-Sabins N, Kawabe M, Gooding WE, Storkus WJ. Combination therapy with HSP90 inhibitor 17-DMAG reconditions the tumor microenvironment to improve recruitment of therapeutic T cells. Cancer Res. 2012;72(13):3196–206. https://doi.org/10.1158/0008-5472.CAN-12-0538.
166. Ostrand-Rosenberg S, Sinha P, Chornoguz O, Ecker C. Regulating the suppressors: apoptosis and inflammation govern the survival of tumor-induced myeloid-derived suppressor cells (MDSC). Cancer Immunol Immunother. 2012;61(8):1319–25. https://doi.org/10.1007/s00262-012-1269-6.
167. Damle S, Martin R, Saleem S, Folgosa L, Zellner H, Nguyen K, Ryan J, Bear H, Irani AM, Conrad D. Mast cells and mast cell-derived IL-13 play an important role in MDSC activation, migration, and accumulation. (TUM4P. 925). J Immunol. 2014;192(1 Supplement):138.126.
168. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, Taylor PA, Panoskaltsis-

Mortari A, Serody JS, Munn DH, Tolar J, Ochoa AC, Blazar BR. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. Blood. 2010;116(25):5738–47. https://doi.org/10.1182/blood-2010-06-287839.

169. Nakashima H, Terabe M, Berzofsky JA, Husain SR, Puri RK. A novel combination immunotherapy for cancer by IL-13Ralpha2-targeted DNA vaccine and immunotoxin in murine tumor models. J Immunol. 2011;187(10):4935–46. https://doi.org/10.4049/jimmunol.1102095.
170. Sengupta S, Thaci B, Crawford AC, Sampath P. Interleukin-13 receptor alpha 2-targeted glioblastoma immunotherapy. Biomed Res Int. 2014;2014:8. https://doi.org/10.1155/2014/952128.
171. Zheng Y, Xu M, Li X, Jia J, Fan K, Lai G. Cimetidine suppresses lung tumor growth in mice through pro-apoptosis of myeloid-derived suppressor cells. Mol Immunol. 2013;54(1):74–83. https://doi.org/10.1016/j.molimm.2012.10.035.
172. Yang XD, Ai W, Asfaha S, Bhagat G, Friedman RA, Jin G, Park H, Shykind B, Diacovo TG, Falus A, Wang TC. Histamine deficiency promotes inflammation-associated carcinogenesis through reduced myeloid maturation and accumulation of CD11b+Ly6G+ immature myeloid cells. Nat Med. 2011;17(1):87–95. https://doi.org/10.1038/nm.2278.
173. Perz JB, Ho AD. Histamine dihydrochloride for the treatment of acute myeloid leukemia, malignant melanoma and renal cell carcinoma. Future Oncol. 2008;4(2):169–77. https://doi.org/10.2217/14796694.4.2.169.

9 Tryptophan Catabolism and Cancer Immunotherapy Targeting IDO Mediated Immune Suppression

Adaobi Amobi, Feng Qian, Amit A. Lugade, and Kunle Odunsi

9.1 Introduction

Over the last decade, tryptophan catabolism has been firmly established as a powerful mechanism of innate and adaptive immune tolerance. The catabolism of tryptophan is a central pathway maintaining homeostasis by preventing autoimmunity or immunopathology that would result from uncontrolled and overreacting immune responses. This is driven by the key and rate-limiting enzymes indoleamine-2,3-dioxygenase 1 (IDO1) and tryptophan-2,3-dioxygenase 2 (TDO), resulting in local depletion of tryptophan, while tryptophan catabolites accumulate, including kynurenine and its derivatives, depending on the presence of downstream enzymes in the kynurenine pathway. These metabolic modifications result in a local microenvironment that is profoundly immunosuppressive, as a result of various mechanisms whose respective role remains incompletely characterized. Drugs targeting this pathway, specifically IDO1, are already in clinical trials with the aim at reverting cancer-induced immunosuppression. Recent studies have demonstrated favorable pharmacokinetics profiles for first-generation (Indoximod NLG8189) and second-generation IDO1 inhibitors (INCB024360 and NLG919). Targeting tryptophan catabolism in combination with additional methods of therapy may improve efficacy of cancer immunotherapy. These methods include, but are not limited to vaccination, adoptive cellular therapy, checkpoint inhibitor blockade, and cyclooxygenase-2 (COX2) inhibition. Over the last decade, there has been a considerable increase in our understanding of the regulation and downstream mediators of tryptophan metabolism. This detailed understanding will expand opportunities to interfere with the pathway therapeutically on multiple levels. The object of this chapter is to highlight current and past key findings that implicate tryptophan catabolism as an important mediator of cancer immunity and discuss the development of multiple therapeutic targets.

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9.2 Tryptophan Catabolism and the Role of IDO

Indoleamine 2,3 dioxygenase has be identified as a major source of immune regulation. The enzymatic activity of IDO is mediated by catabolism of the essential amino L-tryptophan (Trp) into L-kynurenine (Kyn) via the kynurenine pathway metabolic cascade [1, 2] (Fig. 9.1). The amino acid tryptophan is required by all forms of life for protein synthesis and additional significant metabolic functions [2]. The first and rate-limiting step of kynurenine catabolism is facilitated by three distinct enzymes: indoleamine 2,3-dioxygenase (IDO1), indoleamine 2,3-dioxygenase 2 (IDO2), and tryptophan 2,3-dioxygenase 2 (TDO) [1–8]. Of this family of genes, IDO1 is a potent immunoregulatory enzyme. Gene expression of the non-reductant IDO2 is not induced by interferons that regulate IDO1 gene expression [4, 9, 10]. Moreover, the structurally distinct enzyme, TDO is a hepatic enzyme that catabolizes tryptophan selectively expressed in the liver [6, 11]. Stress-related glucocorticoids, as opposed to inflammatory signals induce the TDO gene [12]. These data suggest that unlike IDO1, IDO2 and TDO may have distinct functional roles [13]. Therefore, IDO1 is the major focus of this editorial, and will be referred to as "IDO", unless otherwise specified in this review.

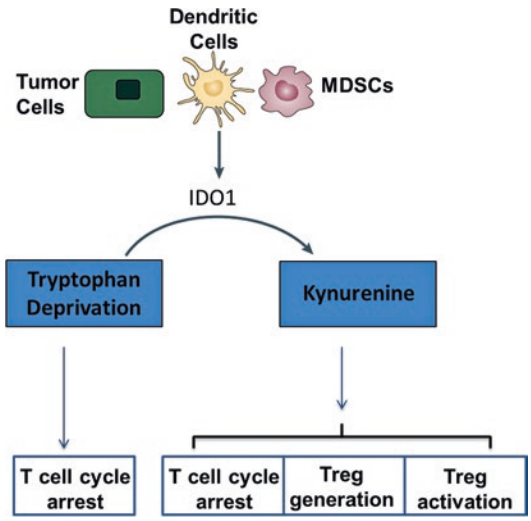

Fig. 9.1 Metabolic modulation result in a local microenvironment becoming profoundly immunosuppressive. Indoleamine 2,3-dioxygenase (IDO1) is an enzyme that catalyzes the essential amino acid, tryptophan, in the initial rate-limiting step along the kynurenine pathway. IDO is expressed by tumor cells, tolerogenic dendritic cells (DCs) and myeloid derived suppresser cells (MDSCs) mediate tryptophan deprivation leading to T cell cycle arrest; and the immunosuppressive catabolite, kynurenine mediates differentiation of $CD4^+$ T cells into regulatory T cells, as well as T cell cycle arrest

IDO is encoded by the INDO gene and has a molecular weight of 45 kDa. Additionally, the gene contains 10 exons and is located on chromosome 8 in human and mice [14, 15]. The IDO gene is tightly regulated by inflammatory mediators such as type I and type II interferons, lipopolysaccharide (LPS), and tumor necrosis factor-alpha (TNF-α). Likewise, the IDO gene promoter region is comprised of the interferon stimulated response element (ISRE) and interferon-gamma activated sequence (GAS) element. Upregulation of the IDO gene is expressed in a wide range of tissues and cellular subsets, such as in the placenta during pregnancy, in a variety of tissues during an infection, transplantation [16], autoimmunity, and cancer [16–19]. In regulatory dendritic cells, IDO expression is upregulated by negative signaling from cytotoxic T lymphocyte protein 4 (CTLA-4) and the glucocorticoid induced tumor necrosis factor receptor (GITR) ligand [20, 21]. This reverse negative signaling was described for the CTLA-4 receptor present on T regulatory cells that binds to its ligands, CD80 and CD86 on dendritic cells and mediated induction of IDO in an interferon-gamma (IFN-γ) dependent manner [20, 22]. Likewise, the toll-like receptor 9 (TLR9) ligand, CpG oligodeoxynucleotides (ODNs) mediate the induction of IDO expression in dendritic cell subsets via type I interferon signaling pathway [23]. Moreover, T regulatory cells are generated by human plasmacytoid dendritic cells via IDO catabolism of tryptophan [24, 25]. These signaling pathways are critical for establishing tolerance to tumor antigens. Additionally, inflammatory stimuli from the signal transducer and activator of transcription 3 (STAT3) stimulated IDO upregulation in myeloid-derived suppressor cells [26]. The initial indication of a role for IDO in immune tol-

erance was demonstrated when it was ascertained that IDO enzyme activity mediated immune privilege and was required to prevent T cell-driven rejection of allogeneic fetuses during pregnancy in mice [27]. Inhibition of the enzymatic activity of IDO by 1-methyl-tryptophan resulted in the rejection of allogeneic, as opposed to syngeneic fetuses. These findings proposed that IDO-medicated depletion of local tryptophan prevented T cell responses to the fetus by limiting the amino acid to proliferating T cells [28]. Likewise, studies performed in the context of IDO genetic knockout mouse models or IDO pharmacological inhibitors have confirmed that the enzyme plays a crucial role in immune tolerance and inflammatory tumorigenesis [27, 29, 30].

IDO modulates of immune responses by two distinct mechanisms. These mechanisms include immune cells deprivation of tryptophan which leads to activation of stress response pathways [19, 28, 31], and through the generation of kynurenine pathway suppressive metabolites [32]. T cells are highly sensitive to tryptophan depletion [33]. A decreased in local tryptophan availability leads to the accumulation of uncharged tryptophan transfer ribonucleic acids (tRNAs). Subsequently, an increase in uncharged tRNAs results in the induction of an integrated stress response kinase general control non-derepressible 2 (GCN2) in T cells. GCN2 kinase acts as a molecular sensor in T cells, and upon activation triggers a stress response program that can result in cell cycle arrest, T cell differentiation, or apoptosis [34]. Amino acid withdrawal modifies the phenotype of dendritic cells and macrophages in GCN2 kinase-dependent manner [35–37]. IDO expressing macrophages acquire the capacity to suppress natural killer (NK) cells and CD8 T cells proliferation. Moreover, GCN2 activation in CD8 effector T cells has been proved to inhibit cell proliferation and induce anergy following T cell receptor stimulation by down regulating the TCR zeta-chain [38, 39]. Likewise, naïve CD4+ T cells GCN2 appears to be important for the differentiation and activation of regulatory T cells (Tregs) [40]. We show that these IDO^+ pDCs directly activate resting $CD4^+CD25^+Foxp3^+$ Tregs for potent suppressor activity. Additionally, data from *in vitro* studies suggest that tryptophan-derived catabolites also inhibit the ability of T cell and natural killer cell proliferation [41].

A second mechanism of IDO-mediated immune suppression is dependent on the accumulation of the tryptophan catabolites, kynurenine. The kynurenine pathway generates several metabolites, including L-kynurenine, kynurenic acid, anthranilic acid, 3 hydroxykynurenine, 3-hydroxyanthranilic acid, quinolinic acid, picolinic acid and nicotinamide adenine dinucleotide [42]. IDO expressing cells release L-kynurenine, 3-hydroxyanthranilic acid (3-HAA) and other tryptophan metabolites into circulation. As a result, innate and adaptive immune responses are modulated in the cells that sense these amino acid catabolites. Moreover, kynurenine is an endogenous ligand of the aryl hydrocarbon receptor (AHR) [43]. The AHR is a ligand-activated member of the basic helix-loop-helix (bHLH) family of transcription factors, originally identified as a receptor for environmental xenobiotic toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Upon ligation, the inactive form of AHR dissociates from cytoplasmic chaperone molecules, associates with the AHR nuclear translocator and binds to the target gene in the nucleus to modulate gene transcription [43, 44]. Tumors select for IDO-mediated tryptophan catabolism that produces kynurenine that binds to AHR and assist the tumor to evade immune surveillance. Moreover, an elevated level of AHR present in tumors is a poor prognostic factor in patients [43, 45]. The role of AHR in immune regulation, inflammation and tumorigenesis genetic studies was defined in genetic mice studies. These experiments confirmed that regulatory T cells (Tregs) generation differentiation from naïve $CD4^+CD25^-$ T cells is dependent on kynurenine activation of the AHR in the present of transforming growth factor beta (TGF-β) [46]. Activation of AHR mediated immune suppression, by promoting FoxP3 differentiation, suppression effector T cell anti-tumor immunity, and decreasing immunogenicity of dendritic cells [11, 43, 47]. However, effect is not seen in AHR null T cells. Specifically, *Mezrich* et al. demonstrated that naïve T cells exposed to KYN leads to mRNA transcription of the downstream targets of AHR

activation, cytochrome P450 family 1 subfamily A polypeptide 1 (CYP1a1), and cytochrome P450 family 1 subfamily B polypeptide 1 (CYP1b1) [46]. AHR plays a role in the generation of Th17 cells in vitro and in vivo [48, 49]. Kynurenine metabolites, via the IDO pathway, also tip the balance of Th17 cell to Treg cells, in favor of Treg cells by suppression of pro-inflammatory Th17 pathway and promoting Treg differentiation [50]. In addition, activation by endogenous AHR ligands, exogenous ligand, 6-formlindolo [3,2-b] carbazole (FICZ), mediates Th17 cell formation upon AHR activation [51]. Further work is needed to understand the mechanisms by which AHR ligation by KYN regulation transcriptional regulation of immune suppressive factors (Fig. 9.2).

IDO is a potent immunoregulatory enzyme [17, 33, 39], and a major source of immune suppression within the tumor microenvironment of ovarian cancer and other tumor types [19, 52] (Fig. 9.3). Constitutive IDO expression in human tumors creates a suppressive microenvironment due to the depletion of typtophan and the synthesis of immune suppressive kynurenine metabolites [53, 54]. Elevated levels of IDO enzyme activity correlates with reduced frequency of tumor infiltrating T lymphocytes in murine cancer models [19]. Studies investigating the role of TILs in ovarian cancer, demonstrated that the presence of intra-epithelial $CD8^{+}$ infiltrating lymphocytes favor overall survival in epithelial ovarian cancer (EOC) patients [52]. A meta-

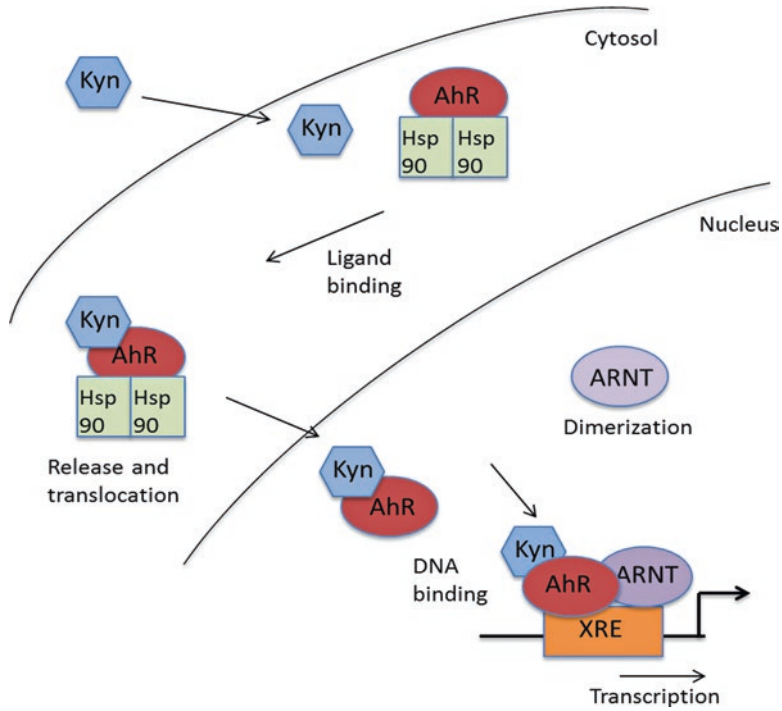

Fig. 9.2 Ligation and activation of the aryl hydrocarbon receptor regulate gene transcription. IDO catabolism of tryptophan leads to the generation of kynurenine (Kyn), an endogenous ligand of the aryl hydrocarbon receptor (AhR). The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that modulates gene transcription. The AhR is present in its inactive form accompanied by Heat Shock Protein 90 chaperones in the cytoplasm of the cell. Following ligation, the cytosolic Kyn-AhR complex undergoes transformation and translocates to the nucleus. In the nucleus the AhR forms a heterodimer with the AhR nuclear translocator (ARNT) which interacts with the core-binding motif of the responsive elements located in regulatory regions of AhR target genes and gene transcription occurs

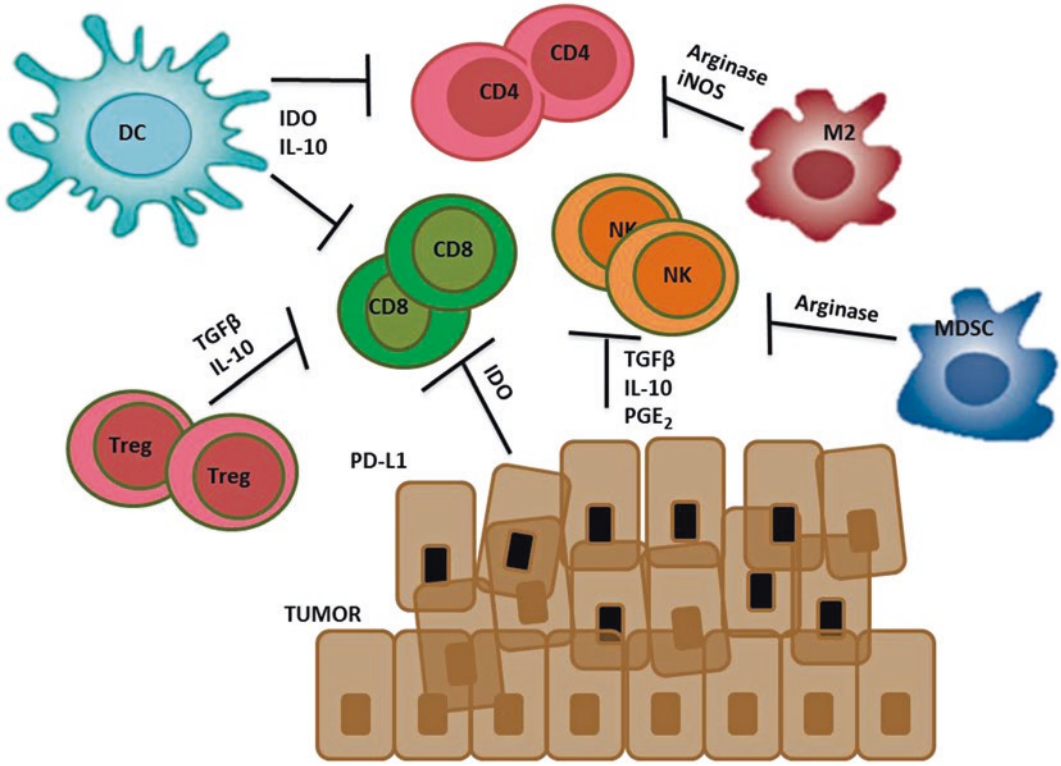

Fig. 9.3 Indoleamine 2,3-dioxyenase is a potent immunoregulatory enzyme within the tumor microenvironment. Immune suppression within the tumor microenvironment is orchestrated by the functions of the cells present that promote tumor growth. Tumors have diverted an immune suppressive role of IDO1 to their own benefit in order to continue to evade immune attack. IDO1 expression by the tumor and tolerogenic dendritic cells (DCs) is associated with a reduced effector T cell function, and an increased frequency of regulatory T cells (Tregs) at the tumor site. Additional immunosuppressive mechanisms within the tumor microenvironment are mediated by: myeloid derived suppressor cells (MDSC), macrophages with an 'M2' phenotype, arginase (Arg1), inducible nitric oxide synthase (iNOS), nitric oxide (NO), prostaglandin E2 (PGE_2), interleukin-10 (IL-10), transforming growth factor βeta (TGF-β), and programmed cell death ligand 1 (PD-L1)

analysis study confirmed a significant survival advantage associated with tumor-infiltrating lymphocytes (TILs) in several tumor types [55–59]. Additionally, these studies indicate that: (1) the beneficial prognostic effect of $CD8^+$ tumor infiltrating lymphocytes in human EOC is negatively affected by $CD4^+CD25^+FOXP3^+$ Treg cells; (2) IDO expression in human ovarian cancer correlates with poor prognosis and decreases overall survival; (3) IDO mediated depletion of tryptophan results in suppression of T cell responses in ovarian cancer patients; and (4) tumor-derived antigen specific $CD8^+$ T lymphocytes from human ovarian cancer demonstrate impaired effector function and enriched expression of the inhibitory molecule, programmed cell death–1 (PD-1), a marker of T cell exhaustion [60]. These results suggest that the regulatory influences of IDO via arrest of T cell proliferation, expansion of $CD4^+$ Tregs, or promotion of T cell exhaustion may dampen the efficacy of tumor reactive effector $CD8^+$ T lymphocytes. Collectively, these findings provide impetus to characterize the molecular and cellular basis of how IDO induced tryptophan catabolism leads to T cell exhaustion; and test novel

strategies for overcoming IDO mediated immune tolerance.

9.3 Cancer Immunotherapy Targeting Tryptophan Catabolism

Several tumor types express IDO, providing them the ability to evade anti-tumor immune surveillance and facilitate immune escape. Consequently, this has motivated the development of IDO inhibitors. Preclinical data demonstrated that a competitive inhibitor of IDO, 1-methyl-tryptophan [61] prevented anergic T cells and suppressed tumor growth in murine carcinoma models. There are multiple approaches targeting tryptophan catabolism in cancer across different levels: (1) blocking IDO expression utilizing strategies which interfere with upstream pathways that regulation IDO transcription and/or translation; (2) enzymatic inhibition by suppressing IDO and/or TDO enzyme activity; (3) and combinatory therapies which target inhibition of tryptophan catabolism with other therapies.

9.4 Inhibitors of IDO Expression

A recent study has linked the therapeutic effects of imatinib, a tyrosine-kinase inhibitor drug, in gastrointestinal stromal tumors (GIST) to inhibition of IDO1 expression, which is driven by the oncogenic KIT signaling. Treatment of experimental tumors with imatinib suppressed IDO expression, resulting in the reversal of IDO1-mediated immunosuppression and thus activation of T effector cells and suppression of Tregs [62]. This finding is striking, because it lends credence to the notion that IDO1 targeting may already be providing a benefit in the context of imatinib treatment of GIST and advocate for conduction preclinical studies in tumor models involving immunocompetent hosts. Based on the association between CTLA-4 and IDO, a rational therapeutic consequence of this observation is to combine KIT blockade with an anti-CTLA-4 approach in GIST, which is currently tested in a clinical trial NCT01643278 (source http://clinicaltrials.gov). Based on COX2-IDO regulatory connection, antitumor implications have been analyzed in a preclinical model of breast cancer treatment with cyclooxygenase-2 (COX2) inhibitors [63], illustrating its therapeutic relevance in principle. T regulatory cell functions disrupted by COX2 inhibition have also been found to be mediated by IDO1 inhibition, possibly contributing to the anticancer properties of COX2 inhibitors [64]. One interesting aspect of IDO is that its enzyme appears to be spontaneously recognized by specific $CD8^+$ T cells that are present in humans [65]. Along this line are preclinical and clinical studies targeting IDO-expressing cells by a peptide vaccine, where early evidence was obtained of long-lasting disease stabilization and a partial response against liver metastasis in metastatic lung cancer patients vaccinated with an IDO-derived peptide, in the absence of notable toxicity [66].

9.5 IDO1 Enzyme Inhibitors

The IDO1 blocking agent 1-methyl-tryptophan (1-MT) has been shown to have significant ability to inhibit IDO1 activity, and co-operate with chemotherapy in mediating regression of established tumors in murine models [30]. In the murine models, the effect of 1-MT was lost in immunodeficient Rag1-knockout (Rag1-KO) hosts, indicating that the antitumor effect of 1-MT was immune mediated. The overall effects of 1-MT include enhanced T cell responses against tumor antigen, allograft antigen, and autoantigens in vivo [67, 68]. Additionally, by blocking IDO, 1-MT inhibits the production of tryptophan catabolites such as kynurenine that have been shown to directly reduce T cell and NK cell proliferation [31, 69–71]. 1-MT is a mixture of the two racemic isoforms 1-methyl-D-tryptophan (D-1-MT) and 1-methyl-L-tryptophan (L-1-MT). In previous studies, while the L stereoisomer of 1-MT was a more potent inhibitor of IDO, the D stereoisomer was shown to be less

active in inhibiting IDO1, but to have superior antitumor activity and to be more effective in inhibiting IDO-expressing tolerogenic DCs in pre-clinical models [72]. In addition, IDO2 was reported to be preferentially targeted by D-1-MT [73]. These findings have been challenged by other groups [7, 74–76]. Nevertheless, D-1-MT is being developed clinically as an IDO-inhibitor (indoximod, NLG8189) for the treatment of several cancers with the aim at reversing cancer-associated immunosuppression.

It is reported that in addition or alternatively to direct IDO1 inhibition, D-1-MT may interfere with transcellular tryptophan transport [77]. Tryptophan transport system L is commonly overexpressed in tumor cells and seems to be the main route for transcellular tryptophan transport in T cells [78]. Myeloid antigen presenting cells have been shown to express an additional high-affinity tryptophan transport mechanism [78] thus being able to take up tryptophan efficiently in a low tryptophan containing microenvironment. Hence, under tryptophan depleting conditions, such as cancer, it seems likely that T cells are more affected by tryptophan starvation and tryptophan is efficiently being shifted toward tryptophan consuming cells. D-1-MT may act as a tryptophan mimetic, provide via mammalian target of rapamycin (mTOR), an intracellular tryptophan sufficiency signal to the cell, maintain mTOR activity also in T cells, thus restore their activity [79]. These findings identify mTOR suppression as an IDO1 effector mechanism and D-1-MT acts as a high-potency tryptophan mimetic in reversing mTOR inhibition and autophagic induction by IDO1 through tryptophan transport interfering. Since L-1-MT is principally capable of exerting the same effects, it is not yet entirely clear why D-1-MT is more effective in restoring T cell activity under physiological conditions [79].

In addition to directly inhibiting IDO enzymatic activity, second-generation IDO1 inhibitors such as INCB024360 and NLG919 have entered clinical trials. These new inhibitors may have a more favorable pharmacokinetic profile. Phase I clinical trials with these orally available compounds have demonstrated safety and indicated biological efficacy based on serum parameters demonstrating reversal of tryptophan depletion and kynurenine accumulation, complementing *in vitro* experiments data [80].

In order to improve the efficacy of cancer immunotherapy, it has become clear that clinical studies targeting tryptophan catabolism should combine with other anti-cancer therapies, based on preclinical animal works. For instance, in spontaneously arising aggressive mammary tumors in the MMTV-neu/HER2 transgenic mouse model of breast cancer, 1-MT had little effect on tumor outgrowth but it could dramatically empower the efficacy of a variety of chemotherapeutic agents, triggering stable regressions of otherwise mainly recalcitrant tumors [30]. As a result, clinical phase I trials have combined indoximod with chemotherapy agents, including the first-in-man phase I trial of indoximod and docetaxel therapy for solid metastaic tumors (NCT02835729; NCT01792050; NCT02077881; NCT01191216; NCT01042535) [81, 82]. A recent preclinical study suggested that IDO1 is a critical resistance mechanism attenuating the efficacy of immunotherapies by antibodies disrupting CTLA-4, PD-1 or GITR, and that 1-MT can safely leverage the antitumor properties of these antibodies [22]. Trials combining indoximod (NCT02073123) or INCB024360 (NCT01604889) with the anti-CTLA-4 antibody ipilimumab in patients with melanoma are underway. Conceptually, and supported by preclinical studies, IDO1 inhibition may enhance the efficacy of active cancer vaccines as it may break cancer-induced tolerance. Two phase II studies are currently evaluating this combination approach (NCT01560923; NCT01042535 and NCT01302821) [83].

Additionally, in an on-going trial by the Cancer Immunotherapy Trials Network (NCT02042430), to determine the magnitude by which INCB024360 alters the frequency of tumor-infiltrating $CD8^+$ T cells once IDO blockade is administered prior to surgery in patients whom are newly diagnosed Stage III-IV with epithelial ovarian, fallopian tube, or primary peritoneal cancer (Fig. 9.4). In this study, ovarian cancer patients receive INCB024360 orally for

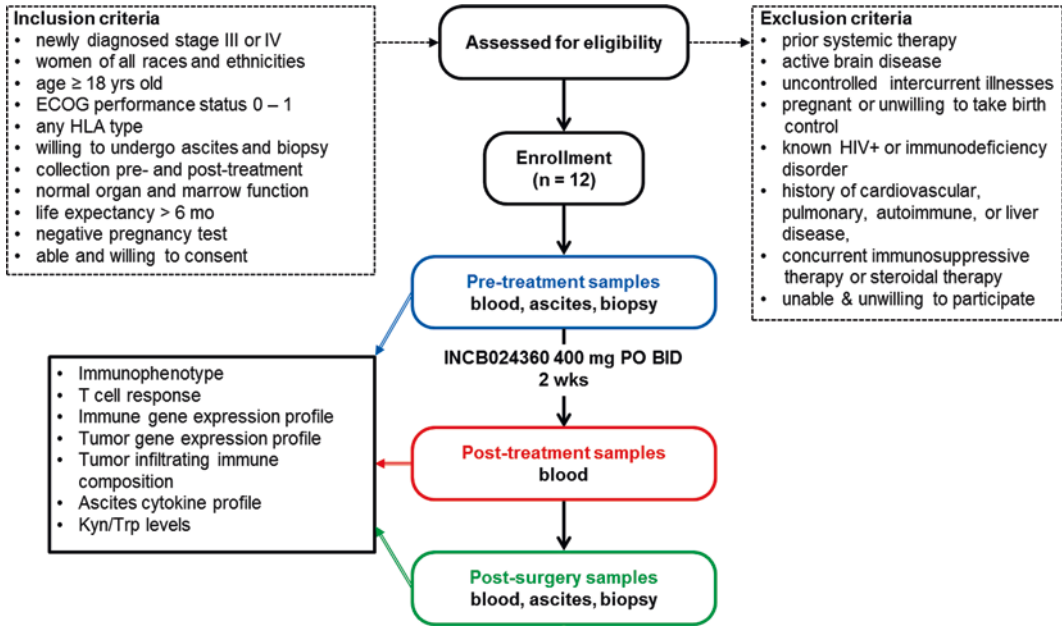

Fig. 9.4 Clinical Trial, NCT02042430, study scheme utilizing an IDO1 inhibitor. This is a pilot clinical trial which studies indoleamine 2,3-dioxygenase (IDO1) inhibitor, INCB024360, before surgery in newly diagnosed stage III-IV epithelial ovarian, fallopian tube, or primary peritoneal cancer patients. Presented here is the clinical trial scheme which includes the patient eligibility criteria, number of enrollment, IDO1 inhibitor treatment dosage and schedule, and tissue samples that are collected pre- and post IDO1 inhibitor treatment, as well as after surgery

up to 3 weeks and undergo surgery at the completion of treatment with the IDO1 inhibitor. In another approach, IDO inhibitors are combined with other immunotherapeutic strategies. In one example, the Roswell Park Cancer Institute-University of Pittsburgh Cancer Institute Ovarian Cancer SPORE investigators testing whether concomitant inhibition of IDO-mediated immune tolerance and vaccination against NY-ESO-1 will enhance the generation of durable anti-tumor $CD8^+$ T cells in patients (Fig. 9.5). The successful completion of these studies will result in the generation of critical data that will bring about further evaluation of IDO blockade to relieve Treg cell-mediated immune tolerance, promote conditions that favor durable host immunity, and prolong disease free survival in ovarian cancer patients (http://trp.cancer.gov/spores/abstracts/roswell_ovarian.htm). An overview of IDO1 inhibitors in clinical trial is described in (Table 9.1).

9.6 Conclusions

The tryptophan catabolism is a central driver of malignant development and progression. It acts in tumor, stromal and immune cells to support

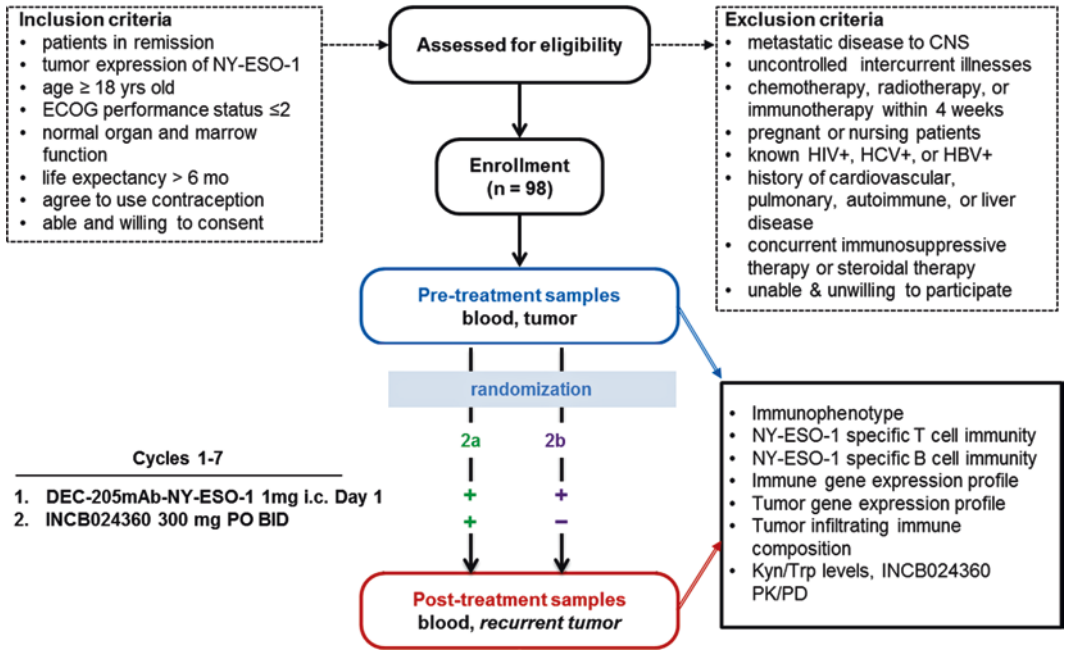

Fig. 9.5 NCT02166905 study scheme. This clinical trial is designed to test whether inhibition of IDO will augment vaccine induced immune responses in patients with ovarian cancer in remission. Presented here is the clinical trial scheme which includes the patient eligibility criteria, the study size, the treatment cycles schedule, and the tissue samples that are collected pre- and post treatment

pathogenic inflammatory processes that engender immune tolerance to tumor antigens. Mechanistic investigations have defined the aryl hydrocarbon receptor, the master metabolic regulator mTOR1 and the stress kinase GCN2 as key effector signaling elements for tryptophan metabolism. The opportunity to interfere with tryptophan metabolism have expanded well beyond inhibiting IDO1 due to the advances in understanding the regulation, as well as the cellular and molecular targets of this pathway. There is an interest in pharmacological targeting of TDO for cancer immunotherapy. Downstream effectors such as AHR and tryptophan transport mechanisms will have to be taken into consideration for future therapeutic strategies. Since it is questionable whether these IDO1/TDO inhibitors will be effective by themselves, rational combinations with already available and/or yet to be identified immunomodulators, such as cancer vaccines or checkpoint inhibitors, deserve thorough basic research and preclinical studies.

Table 9.1 Clinical trials utilizing IDO1 inhibitors

Inhibitor agent	Therapy	Disease sites	Phase	Status	Notes	ClinicalTrials.gov	Sponsor
INCB024360	DEC-205/NY-ESO-1 fusion protein CDX-1401, Poly ICLC, and IDO1 Inhibitor INCB024360 in treating patients with ovarian, fallopian tube, or primary peritoneal cancer in remission	Fallopian tube carcinoma; Ovarian carcinoma; Primary peritoneal carcinoma	I/II	Recruiting	This trial studies the side effects and best dose of IDO1 inhibitor INCB024360 in combination with DEC-205/NY-ESO-1 fusion protein CDX-1401 and poly ICLC and to see how well they work in treating patients with ovarian, fallopian tube, or primary peritoneal cancer who no longer have evidence of disease	NCT02166905	Roswell Park Cancer Institute
	INCB024360 before surgery in treating patients with newly diagnosed Stage III-IV epithelial ovarian, fallopian tube, or primary peritoneal cancer	Newly diagnosed Stage III-IV epithelial ovarian, fallopian tube, or primary peritoneal cancer	0	Recruiting	This pilot clinical trial studies indoleamine 2,3-dioxygenase (IDO1) inhibitor INCB024360 (INCB024360) before surgery in treating patients with newly diagnosed stage III-IV epithelial ovarian, fallopian tube, or primary peritoneal cancer. IDO1 inhibitor INCB024360 may stop the growth of tumor cells by blocking some of the enzymes needed for cell growth.	NCT02042430	National Cancer Institute (NCI)
	INCB024360 and vaccine therapy in treating patients with Stage III-IV Melanoma	Stage III-IV Melanoma	II	Recruiting	This pilot phase II trial studies how well INCB024360 (indoleamine 2,3-dioxygenase 1 [IDO1] inhibitor INCB024360) and vaccine therapy work in treating patients with stage III-IV melanoma.	NCT01961115	Fred Hutchinson Cancer Research Center
	A Phase 2 Study of the IDO Inhibitor INCB024360 versus Tamoxifen for Subjects With Biochemical-recurrent-only EOC, PPC or FTC following complete remission with first-line chemotherapy	Biochemical-recurrent only epithelial ovarian cancer; primary peritoneal carcinoma; fallopian tube cancer	II	Completed	This is an open-label, randomized, phase 2 study of an IDO inhibitor, INCB024360 versus tamoxifen in biochemical recurrent only ovarian cancer patients following complete remission with first-line chemotherapy.	NCT01685255	Incyte Corporation

	Intraperitoneal natural killer cells and INCB024360 for recurrent ovarian, fallopian tube, and primary peritoneal cancer	Ovarian cancer; Fallopian tube carcinoma; Primary peritoneal carcinoma	I	Recruiting	This is a single center phase I trial designed to determine the maximum tolerated dose (MTD) of the oral IDO inhibitor INCB024360 when administered as part of a larger regimen of intraperitoneal (IP) delivery of haploidentical donor NK cells and IL-2 after a non-myeloablative cyclophosphamide/fludarabine (Cy/Flu) preparative regimen for the treatment of recurrent ovarian, fallopian tube, and primary peritoneal cancer.	NCT02118285	Masonic Cancer Center, University of Minnesota
	A Phase 1/2 randomized, blinded, placebo controlled study of Ipilimumab in combination with INCB024360 or placebo in subjects with unresectable or metastatic melanoma	Metastatic Melanoma	I/II	Active, but not recruiting	The study design includes an open-label, dose escalation phase followed by a blinded, randomized phase, which combines INCB024360 (an oral IDO inhibitor) with an approved therapy and compares to approved therapy plus placebo in metastatic melanoma patients.	NCT01604889	Incyte Corporation
	Phase II INCB024360 Study for Patients With Myelodysplastic Syndromes (MDS)	Myelodysplastic Syndromes	II	Completed	The primary purpose of this research study is to assess whether the participant's disease, Myelodysplastic Syndromes (MDS), responds favorably to INCB024360. The study will also evaluate the long-term outcomes of the participant's disease after they have finished taking INCB024360.	NCT01822691	H. Lee Moffitt Cancer Center and Research Institute

(continued)

Table 9.1 (continued)

Inhibitor agent	Therapy	Disease sites	Phase	Status	Notes	ClinicalTrials.gov	Sponsor
	Phase II Study of Sipuleucel-T and Indoximod for Patients With Refractory Metastatic Prostate Cancer	Metastatic Prostate Cancer	II	Recruiting	This is a randomized, double blind, multi-institutional phase II therapeutic study of Indoximod or placebo after the completion of standard of care sipuleucel-T (Provenge®) in men with asymptomatic or minimally symptomatic metastatic prostate cancer that is castration resistant (hormone refractory).	NCT01560923	Masonic Cancer Center, University of Minnesota
	A Dose-escalation Study in Subjects With Advanced Malignancies	Advanced Malignancies	I	Completed	This is an open label, dose escalation study using a 3 + 3 design to determine if INCB024360 (study drug) is safe, well-tolerated and effective in patients with advanced malignancies. Patients will be enrolled and treated in cohorts of three and each observed a minimum of 28 days before the next group is enrolled and may begin to receive study drug.	NCT01195311	Incyte Corporation
Indoximod (NLG-8189)	Study of chemotherapy in combination with IDO inhibitor in metastatic Breast Cancer	Metastatic breast cancer	II	Recruiting	The purpose of this study is to compare the effects, good and/or bad, of standard of care therapy (docetaxel or paclitaxel) with or without the addition of 1-Methyl-D-tryptophan (referred to as indoximod) an experimental drug to find out which treatment is better.	NCT01792050	NewLink Genetics Corporation
	Study of IDO Inhibitor and Temozolomide for adult patients with primary malignant brain tumors	Glioblastoma Multiforme; Glioma; Gliosarcoma; Malignant Brain Tumor	1b/II	Recruiting	In this study, investigators will conduct a phase I/II trial in recurrent (temozolomide resistant) glioma patients. The overall goal of this study is to provide a foundation for future studies with indoximod tested in newly diagnosed glioblastoma patients with radiation and temozolomide, or in combination with vaccine therapies.	NCT02052648	NewLink Genetics Corporation

	Study of IDO inhibitor in combination with Gemcitabine and Nab-Paclitaxel in patients with metastatic pancreatic cancer	Metastatic Pancreatic Adenocarcinoma; Metastatic Pancreatic Cancer	I/II	Recruiting	This phase I/II trial is designed to efficiently identify the regimen limiting toxicity (RLT) and recommended phase 2 dose (RP2D) for the combination of the immunotherapeutic agent indoximod when administered in combination with standard of care chemotherapy gemcitabine plus nab-paclitaxel in subjects with metastatic adenocarcinoma of the pancreas.	NCT02077881	NewLink Genetics Corporation
	IDO Inhibitor Study for Relapsed or Refractory Solid Tumors (D-1MT)	Breast cancer; Lung cancer; Melanoma; Pancreatic cancer; Solid tumors	I	Terminated	This protocol provides an early evaluation of an entirely new class of small molecule agents directed at disruption or elimination of tumor tolerance, a phenomenon now demonstrated to be involved in the growth of many solid tumors. D-1MT, or any other substance targeting this enzymatic pathway indoleamine-(2,3)-dioxygenase (IDO), has not been used previously in humans.	NCT00739609	NewLink Genetics Corporation
	1-Methyl-D-Tryptophan in treating patients with metastatic or refractory solid tumors that cannot be removed by surgery	Unspecified Adult Solid Tumor, Protocol Specific	I	Completed	This phase I trial is studying the side effects and best dose of 1-methyl-D-tryptophan in treating patients with metastatic or refractory solid tumors that cannot be removed by surgery.	NCT00567931	National Cancer Institute (NCI)
	Vaccine Therapy and 1-MT in Treating Patients With Metastatic Breast Cancer	Male breast cancer; Recurrent breast cancer; Stage IV breast cancer; Unspecified adult solid tumor, protocol specific	I/II	Active, but not recruiting	This randomized phase I/II trial studies the side effects and best dose of vaccine therapy and to see how well it works when given together with 1-methyl-D-tryptophan (1-MT) in treating patients with metastatic breast cancer.	NCT01042535	H. Lee Moffitt Cancer Center and Research Institute
NLG919	IDO Inhibitor in Advanced Solid Tumors	Advanced Solid Tumor	I	Not yet open for recruitment	This is an open-label Phase I study to evaluate the safety, tolerability, and pharmacokinetics of escalating oral doses of GDC-0919, an investigational agent intended to inhibit the IDO1 enzyme and help the human immune system attack solid tumor cells more effectively.	NCT02048709	Genentech, Inc.; NewLink Genetics Corporation

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References

1. Lob S, et al. Inhibitors of indoleamine-2,3-dioxygenase for cancer therapy: can we see the wood for the trees? Nat Rev Cancer. 2009;9(6):445–52.
2. Moffett JR, Namboodiri MA. Tryptophan and the immune response. Immunol Cell Biol. 2003;81(4):247–65.
3. Ball HJ, et al. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. Gene. 2007;396(1):203–13.
4. Metz R, et al. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. Cancer Res. 2007;67(15):7082–7.
5. Mehler AH, Knox WE. The conversion of tryptophan to kynurenine in liver. II. The enzymatic hydrolysis of formylkynurenine. J Biol Chem. 1950;187(1):431–8.
6. Knox WE, Mehler AH. The conversion of tryptophan to kynurenine in liver. I. The coupled tryptophan peroxidase-oxidase system forming formylkynurenine. J Biol Chem. 1950;187(1):419–30.
7. Lob S, et al. IDO1 and IDO2 are expressed in human tumors: levo- but not dextro-1-methyl tryptophan inhibits tryptophan catabolism. Cancer Immunol Immunother. 2009;58(1):153–7.
8. Thackray SJ, Mowat CG, Chapman SK. Exploring the mechanism of tryptophan 2,3-dioxygenase. Biochem Soc Trans. 2008;36(Pt 6):1120–3.
9. Ball HJ, et al. Indoleamine 2,3-dioxygenase-2; a new enzyme in the kynurenine pathway. Int J Biochem Cell Biol. 2009;41(3):467–71.
10. Fatokun AA, Hunt NH, Ball HJ. Indoleamine 2,3-dioxygenase 2 (IDO2) and the kynurenine pathway: characteristics and potential roles in health and disease. Amino Acids. 2013;45(6):1319–29.
11. Platten M, et al. Cancer Immunotherapy by Targeting IDO1/TDO and Their Downstream Effectors. Front Immunol. 2014;5:673.
12. Kanai M, et al. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. Mol Brain. 2009;2:8.
13. Croitoru-Lamoury J, et al. Interferon-gamma regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). PLoS One. 2011;6(2):e14698.
14. Kadoya A, et al. Gene structure of human indoleamine 2,3-dioxygenase. Biochem Biophys Res Commun. 1992;189(1):530–6.
15. Najfeld V, et al. Localization of indoleamine 2,3-dioxygenase gene (INDO) to chromosome 8p12→p11 by fluorescent in situ hybridization. Cytogenet Cell Genet. 1993;64(3–4):231–2.
16. Brandacher G, Margreiter R, Fuchs D. Implications of IFN-gamma-mediated tryptophan catabolism on solid organ transplantation. Curr Drug Metab. 2007;8(3):273–82.
17. Mellor AL, Munn DH. Tryptophan catabolism and regulation of adaptive immunity. J Immunol. 2003;170(12):5809–13.
18. Curti A, et al. The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: focus on hematology. Blood. 2009;113(11):2394–401.
19. Uyttenhove C, et al. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003;9(10):1269–74.
20. Grohmann U, et al. CTLA-4-Ig regulates tryptophan catabolism in vivo. Nat Immunol. 2002;3(11):1097–101.
21. Grohmann U, et al. Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. Nat Med. 2007;13(5):579–86.
22. Holmgaard RB, et al. Indoleamine 2,3-dioxygenase is a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4. J Exp Med. 2013;210(7):1389–402.
23. Mellor AL, et al. Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. J Immunol. 2005;175(9):5601–5.
24. Chen W, et al. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol. 2008;181(8):5396–404.
25. Fallarino F, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol. 2003;4(12):1206–12.
26. Yu J, et al. Noncanonical NF-kappaB activation mediates STAT3-stimulated IDO upregulation in myeloid-derived suppressor cells in breast cancer. J Immunol. 2014;193(5):2574–86.
27. Munn DH, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281(5380):1191–3.
28. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? Immunol Today. 1999;20(10):469–73.
29. Mellor AL, et al. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. Nat Immunol. 2001;2(1):64–8.
30. Muller AJ, et al. Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene Bin1, potentiates cancer chemotherapy. Nat Med. 2005;11(3):312–9.
31. Munn DH, et al. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med. 1999;189(9):1363–72.

32. Fallarino F, et al. T cell apoptosis by kynurenines. Adv Exp Med Biol. 2003;527:183–90.
33. Mellor AL, et al. Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses. J Immunol. 2002;168(8):3771–6.
34. McGaha TL, et al. Amino acid catabolism: a pivotal regulator of innate and adaptive immunity. Immunol Rev. 2012;249(1):135–57.
35. Ravishankar B, et al. Tolerance to apoptotic cells is regulated by indoleamine 2,3-dioxygenase. Proc Natl Acad Sci U S A. 2012;109(10):3909–14.
36. Manlapat AK, et al. Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory CD19+ dendritic cells. Eur J Immunol. 2007;37(4):1064–71.
37. Liu H, et al. GCN2-dependent metabolic stress is essential for endotoxemic cytokine induction and pathology. Mol Cell Biol. 2014;34(3):428–38.
38. Fallarino F, et al. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. J Immunol. 2006;176(11):6752–61.
39. Munn DH, et al. GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity. 2005;22(5):633–42.
40. Sharma MD, et al. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Invest. 2007;117(9):2570–82.
41. Frumento G, et al. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. J Exp Med. 2002;196(4):459–68.
42. Hayashi T, et al. 3-Hydroxyanthranilic acid inhibits PDK1 activation and suppresses experimental asthma by inducing T cell apoptosis. Proc Natl Acad Sci U S A. 2007;104(47):18619–24.
43. Opitz CA, et al. An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. Nature. 2011;478(7368):197–203.
44. Mimura J, Fujii-Kuriyama Y. Functional role of AhR in the expression of toxic effects by TCDD. Biochim Biophys Acta. 2003;1619(3):263–8.
45. Prendergast GC. Cancer: Why tumours eat tryptophan. Nature. 2011;478(7368):192–4.
46. Mezrich JD, et al. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol. 2010;185(6):3190–8.
47. Pilotte L, et al. Reversal of tumoral immune resistance by inhibition of tryptophan 2,3-dioxygenase. Proc Natl Acad Sci U S A. 2012;109(7):2497–502.
48. Kimura A, et al. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. Proc Natl Acad Sci U S A. 2008;105(28):9721–6.
49. Veldhoen M, et al. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. J Exp Med. 2009;206(1):43–9.
50. Quintana FJ, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature. 2008;453(7191):65–71.
51. Veldhoen M, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature. 2008;453(7191):106–9.
52. Sato E, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A. 2005;102(51):18538–43.
53. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest. 2007;117(5):1147–54.
54. Platten M, Wick W, Van den Eynde BJ. Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. Cancer Res. 2012;72(21):5435–40.
55. Zhang L, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348(3):203–13.
56. Callahan MJ, et al. Increased HLA-DMB expression in the tumor epithelium is associated with increased CTL infiltration and improved prognosis in advanced-stage serous ovarian cancer. Clin Cancer Res. 2008;14(23):7667–73.
57. Hwang WT, et al. Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. Gynecol Oncol. 2012;124(2):192–8.
58. Hamanishi J, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci U S A. 2007;104(9):3360–5.
59. Tomsova M, et al. Prognostic significance of CD3+ tumor-infiltrating lymphocytes in ovarian carcinoma. Gynecol Oncol. 2008;108(2):415–20.
60. Matsuzaki J, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci U S A. 2010;107(17):7875–80.
61. Cady SG, Sono M. 1-Methyl-DL-tryptophan, beta-(3-benzofuranyl)-DL-alanine (the oxygen analog of tryptophan), and beta-[3-benzo(b)thienyl]-DL-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase. Arch Biochem Biophys. 1991;291(2):326–33.
62. Balachandran VP, et al. Imatinib potentiates antitumor T cell responses in gastrointestinal stromal tumor through the inhibition of Ido. Nat Med. 2011;17(9):1094–100.
63. Basu GD, et al. Cyclooxygenase-2 inhibitor enhances the efficacy of a breast cancer vaccine: role of IDO. J Immunol. 2006;177(4):2391–402.
64. Lee SY, et al. The immune tolerance of cancer is mediated by IDO that is inhibited by COX-2 inhibitors through regulatory T cells. J Immunother. 2009;32(1):22–8.

65. Sorensen RB, et al. The immune system strikes back: cellular immune responses against indoleamine 2,3-dioxygenase. PLoS One. 2009;4(9):e6910.
66. Iversen TZ, et al. Long-lasting disease stabilization in the absence of toxicity in metastatic lung cancer patients vaccinated with an epitope derived from indoleamine 2,3 dioxygenase. Clin Cancer Res. 2014;20(1):221–32.
67. Grohmann U, et al. IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. J Immunol. 2001;167(2):708–14.
68. Grohmann U, et al. CD40 ligation ablates the tolerogenic potential of lymphoid dendritic cells. J Immunol. 2001;166(1):277–83.
69. Hwu P, et al. Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol. 2000;164(7):3596–9.
70. Munn DH, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science. 2002;297(5588):1867–70.
71. Mellor AL, et al. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. J Immunol. 2003;171(4):1652–5.
72. Munn DH. Indoleamine 2,3-dioxygenase, tumor-induced tolerance and counter-regulation. Curr Opin Immunol. 2006;18(2):220–5.
73. Lob S, et al. Levo- but not dextro-1-methyl tryptophan abrogates the IDO activity of human dendritic cells. Blood. 2008;111(4):2152–4.
74. Qian F, et al. Efficacy of levo-1-methyl tryptophan and dextro-1-methyl tryptophan in reversing indoleamine-2,3-dioxygenase-mediated arrest of T-cell proliferation in human epithelial ovarian cancer. Cancer Res. 2009;69(13):5498–504.
75. Qian F, et al. Effects of 1-methyltryptophan stereoisomers on IDO2 enzyme activity and IDO2-mediated arrest of human T cell proliferation. Cancer Immunol Immunother. 2012;61(11):2013–20.
76. Yuasa HJ, et al. 1-L-methyltryptophan is a more effective inhibitor of vertebrate IDO2 enzymes than 1-D-methyltryptophan. Comp Biochem Physiol B Biochem Mol Biol. 2010;157(1):10–5.
77. Travers MT, et al. Indoleamine 2,3-dioxygenase activity and L-tryptophan transport in human breast cancer cells. Biochim Biophys Acta. 2004;1661(1):106–12.
78. Seymour RL, et al. A high-affinity, tryptophan-selective amino acid transport system in human macrophages. J Leukoc Biol. 2006;80(6):1320–7.
79. Metz R, et al. IDO inhibits a tryptophan sufficiency signal that stimulates mTOR: A novel IDO effector pathway targeted by D-1-methyl-tryptophan. Oncoimmunology. 2012;1(9):1460–8.
80. Liu X, et al. Selective inhibition of IDO1 effectively regulates mediators of antitumor immunity. Blood. 2010;115(17):3520–30.
81. Soliman HH, et al. A first in man phase I trial of the oral immunomodulator, indoximod, combined with docetaxel in patients with metastatic solid tumors. Oncotarget. 2014;5(18):8136–46.
82. Soliman HH, et al. A phase I study of indoximod in patients with advanced malignancies. Oncotarget. 2016;7(16):22928–38.
83. Vacchelli E, et al. Trial watch: IDO inhibitors in cancer therapy. Oncoimmunology. 2014;3(10):e957994.

Lipid Inflammatory Mediators in Cancer Progression and Therapy

10

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10.1 Introduction

Leukocyte infiltration of tumors can have either a pro-tumorigenic or tumor-inhibitory functions. As an example tumor-associated macrophages (TAMs) have tumoricidal activity and can induce antitumor T-cells; but, can also suppress cytotoxic T-cell responses capable of inhibiting tumor growth (Fig. 10.1). Myeloid cell infiltration of tumors is associated, in part, with tumor-derived cytokines, GFs, chemokines, and expression of immune checkpoint molecules that regulate the expansion of myeloid progenitors within the marrow and at extramedullary sites and to an extent within the tumor (Fig. 10.2). Numerous studies have demonstrated that activated macrophages can kill tumor cells *in vitro*. However, macrophage infiltration of tumors is predominately, a pro-tumorigenic/tumor-progressive phenotype [1]; although, some human studies have been equivocal [2]. Indeed, most studies have found no relationship between immunogenicity, metastatic propensity and infiltrating TAM frequency [3–5]. Despite this lack of an immune correlation, TAM infiltration is associated with a poor prognosis [6] and rapid tumor progression [7, 8]. Myeloid-derived suppressor cells (MDSCs) have also been identified in the circulation of tumor bearing (TB) hosts and to infiltrate tumors [9–13]. The immunosuppressive activity of MDSCs (both murine and human) occurs through multiple mechanisms including the upregulation of reactive oxygen species (ROS), nitric oxide (NO) production and arginase levels, as well as the secretion of immunosuppressive cytokines [14]. Preclinical studies have shown that MDSCs can control tumor growth [3, 15], while immune augmenting type-1 macrophages (M1) and/or dendritic cells (DC1) cells contribute to the induction of an antitumor T-cell response, although their presence is not sufficient for tumor rejection [16]. M1 macrophage depletion or an increase in infiltrating M2 macrophages, DC2s, and MDSCs are associated with a poor prognosis and increased tumor relapse post resection.

Lymphocytes also infiltrate tumors (Fig. 10.1) and the associated adaptive immune response has a positive prognosis. However, the infiltrating lymphocytes can also be T-cell suppressive. Thus, while T-cells have the potential to kill tumor cells, frequently they are of low frequency and avidity [17], and cannot control tumor growth [18]. Nonetheless, increased T-cell infiltration of

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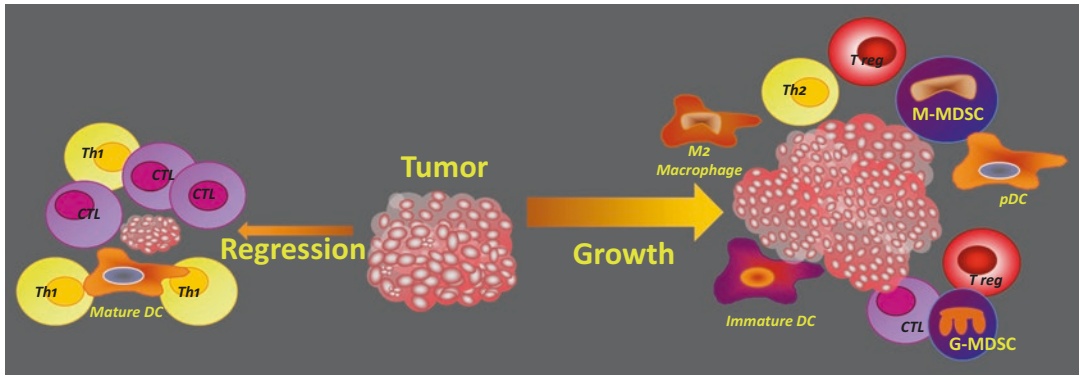

Fig. 10.1 The leukocytes infiltrating tumors regulates their growth and progression. Tumor regression is associated with infiltration by mature dendritic cells (DCs), cytotoxic T cells (CTL) and type 1T-helper cells (Th1). Contrasting with this, tumor growth is facilitated by immune mediated immunosuppression and neoangiogenesis by immature DCs, myeloid-derived suppressor cells, (MDSCs) plasmacytoid DCs, (pDCs) M2 macrophages, as well as T regulatory (T-reg) cells and a low frequency of CD4 and CD8 effector T cells. The expansion of myeloid cell proliferation, including immunosuppressive populations, is regulated by colony stimulating factors (CSFs), chemokines and dietary w-6 PUFA

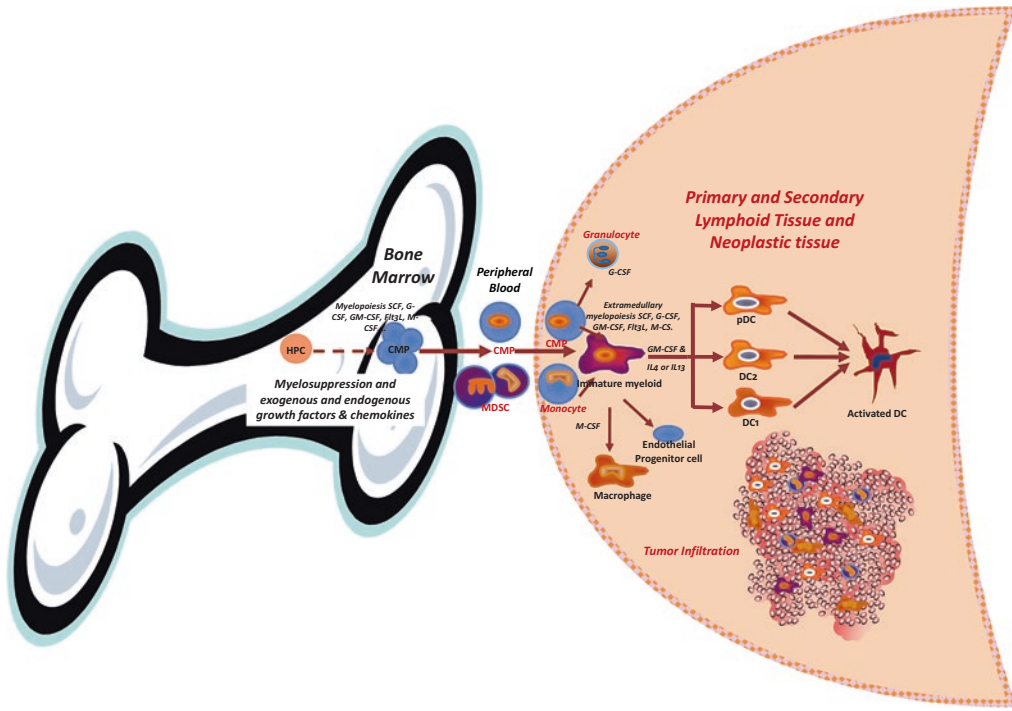

Fig. 10.2 Tumors secrete growth factors that expand and mobilize committed myeloid progenitors (CMP) and hematopoietic progenitor cells (HPC) from the marrow to extramedullary sites of myelopoiesis including the spleen, liver, lungs and primary and metastatic tumor lesions. Diets with increased levels of ω6 polyunsaturated fatty acids (PUFA) can increase myeloplasia largely as an extramedullary process. These CMPs can mature into dendritic cells (DCs), myeloid derived suppressor cells (MDSCs); both monocytic (M) and granulocytic (G), monocytes, endothelial progenitor cells and macrophages including tumor-associated macrophages (TAMs), as well as become activated, or "paralyzed", within the tumor environment. DC1 and DC2 are dendritic cell subsets that are immune augmenting and suppressive respectively. Dependent upon the infiltrating subset and extent of maturation and activation, these cells are critical components and regulators of immune suppression, angiogenesis, vasculogenesis, and tumor regression or growth

tumors is associated with an improved outcome [19–26], and an increased understanding of infiltrating T-cell phenotypes and their functions has resulted in an improved understanding of their prognostic potential. However, some tumor cells express checkpoint molecules that downregulate immune responses. Myeloid cells, including macrophages, PMNs and MDSCs, can also express immunosuppressive checkpoint mediators, such as PD-L1 [27], providing another mechanism to down regulate T-cell proliferation and function. Consequently, although anti-tumor T cells are present in the tumor microenvironment their anti-tumor activity may be limited. However, antibodies that inhibit immune checkpoints are demonstrating efficacy in reactivating anti-tumor T cell responses [28].

10.2 Immune Cell Infiltration of Tumors

The hypothesis that hematological markers of systemic inflammation, in particular the neutrophil–lymphocyte ratio (NLR), can predict survival in tumor bearing patients has recently received much interest. Many groups have investigated the prognostic value of the NLR in a variety of tumors and at different disease stages. To date, over 60 studies (>37,000 patients) have examined the clinical utility of the NLR to predict outcomes [29]. There is also an emerging relationship between proinflammatory cytokines in the plasma of patients with elevated NLR (>5) and the tumor microenvironment. A number of studies have measured circulating cytokines together with the NLR [30, 31] providing insight into the mechanisms underlying the NLR, including one study that documented an elevated NLR associated with an increased peritumoral infiltration of macrophages [30]. Together, these observations suggest that the NLR reflects, at least in part, the up-regulation of innate immunity providing easily measurable biomarkers that can predict OS and PFS in cancer patients.

The interactions between tumor infiltrating immune effector cells takes place primarily around the tumor. Thus, while the NLR may have prognostic significance, specific subsets of infiltrating cells, as discussed above, may prove more informative. Specifically, cytotoxic $CD8^+$ lymphocytes, as a component of tumor-specific adaptive immunity, may constitute a critical mediator. Further, the T-cell suppressive nature of myeloid cells, including MDSCs, M2 macrophages, and DC2s suggests the potential sensitivity and criticality of the myeloid cell-to-$CD8^+$ lymphocyte ratio in tumor tissue. A few studies have undertaken such analyses observing, for example, that $CD66^+$ myeloid cells provide an independent prognostic factor for poor disease free survival (DFS) and overall survival (OS) [32]. This observation has been extended by the analysis of infiltrating NLR (iNLR) as a CD66b:CD8 cell ratio with the observation of a relationship with a cumulative incidence of relapse, OS and tumor stage [33]. As discussed below, a patient's lifestyle, both preceding and following diagnosis, can contribute to not only cancer initiation and progressions but also outcome. Thus, hosts eating a high-fat diet, or one with a high level of saturated fat or ω-6 PUFAs generally have an inflammatory phenotype with neutrophilia, which may contribute to cancer development and poor outcomes. Conversely, and with little data to date, diets with a high ω-3 PUFA content have been associated with decreased inflammation and extramedullary myelopoiesis, and potentially improved clinical outcomes. We posit, herein, that dietary ω3 PUFA may also increase infiltrating T-cells thereby contributing to improved clinical outcomes.

10.3 PUFA Regulation of Inflammatory Cells in Rodents

Several lines of evidence suggest that the dietary PUFA composite can influence inflammatory or anti-inflammatory cellular responses. Fatty acids

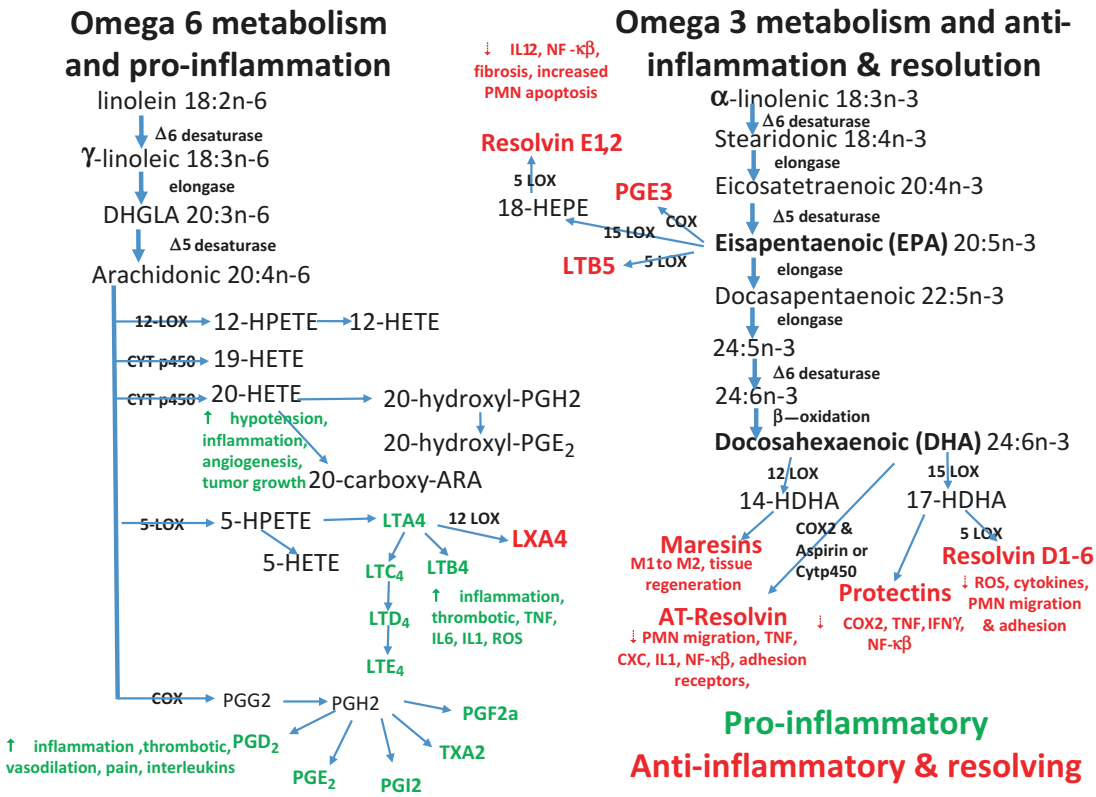

Fig. 10.3 Outline of the eicosanoid and resolvin related mediator synthesis pathways from arachidonic acid (AA) and alpha linolenic acid and their inflammatory and anti-inflammatory activities. *COX* cyclooxygenase, *CYT p450* cytochrome p450, *CXC* CXC chemokines, *HETE* hydroxyeicosatetraenoic acid, *HDHA* hydroxyldocosahexaenoic acid, *HPETE* hydroperoxyeicosatetraenoic acid, *HPDHA,* hydroperoxydocosahexaenoic acid, *HPEPE* hydroperoxyeicosapentaenoic acid, *IL* interleukin, *IFN* interferon, *LOX* lipoxygenase, *LT* leukotriene, *LX* lipoxin, *PG* prostaglandin, *PMN* polymorphonuclear leukocytes, *ROS* reactive oxygen synthetase, *TNF* tumor necrosis factor, *TX* thromboxane

from animal sources, mainly contain saturated fatty acids (SFAs) or ω6 PUFA. In contrast, fatty acids derived from some plant-based oils, and certain types of fatty fish consist mainly of ω3 PUFA. Recent studies have suggested that diets rich in ω6 PUFAs increase the risk of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and asthma [34]. In contrast, diets rich in ω3 PUFAs have anti-inflammatory effects as supported by a decreased risk and control of these diseases [34]. PUFAs can be oxidized to generate either pro-inflammatory or pro-resolving lipid mediators (Fig. 10.3). These mediators have potent immune modulatory capacities and are generated rapidly during an inflammatory response [35]. Pro-inflammatory mediators, including prostaglandin (PG)s and leukotrienes (LTs), are induced in response to "foreign" materials and when they are cleared, pro-resolving lipid mediators restore normal tissue homeostasis [36]. Diets rich in ω3-PUFAs such as α linolenic acid (ALA, **18:3n-3**), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are associated with a decreased incidence and severity of inflammatory diseases [37]. The beneficial effects of these dietary FAs include anti-inflammatory metabolites such as a subset of PGs, LTs, thromboxanes, resolvins and lowered levels of inflammatory cytokines. However, the activities of ω3-PUFA contrast with other FAs that differ mainly in the position of their double bonds in the acyl chain, such as linoleic acid (LA) and arachidonic acid (AA) found with ω6-PUFA containing diets and their corresponding metabolites (Fig. 10.3).

Omega-3 PUFAs are anti-inflammatory in part by modulating the metabolism of inflammatory eicosanoids, cytokines, ROS and the expression of adhesion molecules [38]. EPA and DHA dietary supplementation has proven effective in decreasing intestinal damage and improving gut histology in inflammatory bowel disease [39], as well as, decreased joint pain, number of tender and swollen joints, and duration of morning stiffness in patients with arthritis [40]. Due to these responses, the effects on the immune response in various organs has been the subject of recent review articles [41].

10.4 PUFA and Immune Function

Studies using both ω6 and ω3 PUFA in rodent dietary studies, have documented different effects depending on the type of study (*in vitro* or *in vivo*), and the response measured. *In vitro* studies with ALA have shown an enhanced secretion of superoxides from neutrophils and macrophages [42], resulting in neutrophil adhesion to endothelial cells [43] promoting pro-inflammatory effects. However, ALA has also been shown to inhibit the proliferation of rodent and human lymphocytes following mitogen stimulation [44] suggesting that ALA may also be immunosuppressive. Studies where rodents were fed a high-fat diet, rich in ALA resulted in decreased mitogen-stimulated lymphocyte proliferation and NK cell activity [45].

In vitro studies using the ω6 PUFA; AA, have documented inflammatory properties including enhanced superoxide release [42], neutrophil adhesion to endothelial cells [43], and IL-1β production by macrophages [46]. Feeding mice a diet with high levels of ω-6 PUFA has been shown, in a dose dependant manner, to result in increased levels of LTE_4 and PGE_2 following in vivo stimulation with zymosan [47]. In a recent study, diets high in AA were shown to increase angiotensinogen, IL6 and MCP-1 levels in response to the proinflammatory transcription factor; nuclear factor κβ (NFκβ) stimulation [48].

A number of studies have shown that the ω3 PUFA, ALA inhibits the proliferation of rodent and human lymphocytes in vitro [44, 49, 50]. Studies where rats were fed an oil with a high ALA composition (linseed oil, 100 g/kg diet) for 8 weeks, a decrease in superoxide production by peritoneal macrophages in response to phorbol esters, was observed [51]. However, rodents fed linseed oil also had an increase in TNF secretion by resident macrophages, but no effect on TNF production by inflammatory macrophages [52]. Thus, the precise effect of the w-3 PUFA, ALA on lymphocyte functions appears to depend on the levels of ALA and the total PUFA content of the diet [53].

Because dietary fish oil leads to decreased PGE_2 production, it has been suggested that ω3 PUFAs should have anti-inflammatory activities, enhance the production of Th1-type cytokines, increase MHC II expression, lymphocyte proliferation and NK cell activity, as well as, decrease IgE production. Culture of human neutrophils with EPA or DHA has been shown to inhibit superoxide production and phagocytosis [54]. Similarly, the incubation of murine peritoneal macrophages with EPA or DHA inhibits expression of MHC II [55]. In a study, in which human monocytes were incubated with either EPA or DHA, both were shown to decrease the proportion of HLA-DR or -DP positive monocytes in response to IFN-γ [56] resulting in a reduced ability to present antigen [57]. The addition of fish oil to rodent diets can also decrease superoxide and hydrogen peroxide production by macrophages [58]. As compared to safflower oil, the addition of fish oil to murine diets results in lower peak plasma levels of TNF-α, IL-1β, and IL-6 following endotoxin injection [59]. Furthermore, parenteral nutrition that includes fish oil can decrease serum TNF-α, IL-6, and IL-8 levels in rats with burns compared with animal given ω6 PUFA–rich parenteral nutrition [54]. However, the majority of rodent studies with dietary fish oil use a diet in which EPA plus DHA comprise up to 30% of dietary fatty acids and up to 12% of dietary energy. The conclusions from these studies have been refined by studies in rats and mice that have indicated that relatively low levels of

EPA or DHA at a level of 4.4% of total fatty acids or 1.7% of dietary energy are sufficient to provide anti-inflammatory activities [60].

10.5 Clinical Anti-Inflammatory Activity of ω3 PUFA

There have been a number of clinical trials assessing the benefits of dietary supplementation with fish oil for the treatment of inflammatory diseases in humans, including rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus, and multiple sclerosis [61]. Many of the placebo-controlled, double-blind trials of fish oil in chronic inflammatory diseases have shown significant benefits, including decreased disease activity and a lowered use of anti-inflammatory drugs. The evidence for a beneficial effect of fish oil is strongest in rheumatoid arthritis, where ω3 PUFA has been found to cause a concentration-dependent decrease in enzymes that degrade cartilage, expression of COX-2, but not COX-1, and TNF-α and IL-1β expression in cultured articular cartilage chondrocytes [62]. The mechanisms by which ω3 PUFAs have a beneficial effect in patients with arthritis has been postulated to be a competition with the canonical ω6 substrate AA resulting in eicosanoids that are less potent at inducing inflammation [63]. Recent observations have shown that ω3 PUFAs can be enzymatically converted to novel bioactive lipid mediators, termed resolvins, protectins and maresins, which promote the resolution of inflammation and that are log- orders more potent than their lipid precursors [64]. These observations have generated a paradigm shift documenting that the resolving phase of inflammation is not a passive process, but is actively 'switched-off' via endogenous anti-inflammatory mediators [65]. This contrasts with ω-6 PUFA associated metabolites, PGD_2, LTC_4, LTD_4, and LTE_4, which mediate pulmonary inflammation in asthma and are major mediators of asthmatic bronchoconstriction. AA is a precursor to LTs, which promote allergic inflammation, PGE_2 also regulates macrophage and lymphocyte function. Thus, it has been suggested that increased dietary intake of the w-6 PUFA LA, as the precursor of AA, is causally linked to allergic diseases and suggests a potential treatment focus for ω3 fatty acids [66].

10.6 PUFA Modulated Inflammation and Neoplasia in Rodent Tumor Models

As discussed above, clinically there have been varying associations between PUFA consumption/composition and inflammation; but there are many confounding factors including genetic susceptibility, tissue microenvironments, stress, obesity, age and duration. Murine models have identified a number of mechanisms in the association of dietary PUFA and tumor initiation and progression focused on systemic and tissue inflammation. Inflammation at tumor initiation can be regulated by risk factors, including hormones, obesity and age. However, following tumor initiation, inflammation is modulated by tumor growth in addition to existing risk factors. Thus, inflammatory microenvironments are created by cross talk between tumor-secreted GFs and host immunity.

Using mammary tumors as an example, the cellular microenvironment of mammary glands incorporate hormonal responsive epithelial cells, stromal cells, as well as, immune cells, in association with adipose tissue, that can result in an endocrine as well as an inflammatory organ [67]. The role of inflammation in tumorigenesis is supported by the evidence of a progressive increase in infiltrating inflammatory cells, which include activated macrophages and granulocytes, during the progression from normal tissue to dysplastic cells, which are believed to support tumor initiation [68].

The effect of dietary PUFA in tumor progression and metastasis has been studied in animal, and xenograft models of mammary cancer. In a xenograft model using MDA-MB-435 injected athymic nude mice given diets of either LA, EPA or DHA, significant retardation of tumor growth and metastasis was observed in the mice given EPA or DHA including a reduction in AA levels in tumor membrane phospholipids [69]. Further

when EPA and DHA were given as a neoadjuvant therapy, prior to tumor excision, pulmonary metastases were significantly suppressed compared to mice maintained on a LA diet [70]. Similar immune-augmenting and therapeutic activities were observed in R3230RC and MCF-7 mammary adenocarcinoma models [71, 72]. These anti-inflammatory activities may also include the regulation of MDSCs that can inhibit both non-antigen specific and antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. The mechanisms of MDSC immunosuppression are diverse, including up-regulation of ROS, NO, and L-arginine metabolism, as well as immunosuppressive cytokines. In one tumor survival study, mice were switched from an 8% corn oil (1% ALA) diet to an 8% canola oil (10% ALA) diet, when the mice had an average primary tumor volume of 60 mm^3. In these studies tumor growth was significantly lower in mice fed the ω-3 based canola oil diet compared to the ω-6 based, corn oil cohort [73].

Interventions using ω-3 PUFA in chemically induced mammary tumor models support the results from xenograft tumor models. In a 7, 12-dimethylbenz (α) anthracene (DMBA) induced mammary tumor model, a fish oil diet significantly reduced tumor incidence, growth and metastasis [74, 75]. The effect of an ω-3 diet on tumor induction and growth correlated with reduced AA serum levels, protection against DNA single strand breaks, suppressed tumor cell proliferation; c-Myc and HER-2/neu expression and an increase in the apoptosis markers Bcl-2 and Bax [75–77]. Similarly, in a model of N-methyl-N-nitrosourea (MNU)-induced rat mammary tumors, the activity of dietary fat compositions including, saturated fatty acid (SFA), monounsaturated fat (MUFA), ω−6 PUFA alone or different ratios of ω−6:ω−3 PUFA were studied. It was found that a 1:1 ratio of ω−6:ω−3 PUFA was more effective in the prevention of mammary tumor development as compared to the other dietary cohorts, by decreasing mRNA expressions of fatty acid synthase, cyclooxygenase-2 (COX-2), and 5-lipoxygenase (5-LOX) in mammary tissues and decreasing peroxisome proliferator-activated receptor gamma (PPAR-γ) levels [78]. Together, these studies directly support a role for ω-3 PUFA in modulating an inflammatory tumor microenvironment by the up regulation of PPAR-γ [77, 78]. When the ω-3 PUFA content was significantly increased to a ω−6:ω3 ratio of 1:14.6 compared to 1:0.7, a 60% reduction in tumor growth was observed. This was associated with decreased cyclin-D1 and phospho-retinoblastoma protein expression and increased levels of cyclin-dependent kinase inhibitors, CIP1 (p21) and KIP1 (p27), an increased apoptotic index, reduced inflammation and *mammalian target of rapamycin* (mTOR) activity [79]. In an orthotopic 4 T1 mammary tumor model, 5% fish oil was used as therapy beginning when hosts had primary tumors that were 8–10 mm^3 and documented a significant reduced tumor growth and metastasis, which was correlated with inhibition of cancer cell proliferation [80].

The ability of ω3 PUFA to downregulate inflammatory mediators and increase apoptotic proteins emphasizes the importance of exogenous regulation of the tumor microenvironment. However the mechanism of regulation is not clear. In-vitro studies, have focused on cellular phenotypes and the effect of ω3 PUFA on inflammatory cells in both LPS and tumor induced inflammation. The majority of these studies have focused on inflammatory pathway factors. Although ω3 PUFA has anti-inflammatory effects in inflammatory diseases including cancer, its regulation of MDSCs, which is a critical regulator of the tumor microenvironments is understudied. Further, the majority of murine models, involve diets that are isocaloric but fully equivalent, raising the question of obesity verses dietary constituents. Since obesity itself is an inflammatory disorder, ruling out the effects of obesity associated inflammation as a confounding factor, is crucial to determine the actual effects of dietary components such as fatty acids in tumor initiation, progression and metastasis.

10.7 PUFA Regulation of Immune Cells: Consequences for Clinical Outcomes in Cancer

Epidemiological studies of the incidence and progression of breast cancer in populations of women of Japanese descent in the USA compared to women in Japan, have indicated a significantly higher incidence in the USA compared to Japan [81]. This observation was supported by the finding that offspring of Japanese immigrants to the United States, but not the immigrants themselves, had breast cancer rates similar to the general American population [82]. In the 1990s, dietary components that were implicated in these different incidences were identified [83]. These relatively weak and sometimes contradictory correlative epidemiologic data were considered plausible, given experiments demonstrating ω3 PUFAs had the potential to reduce pro-inflammatory cytokines, inflammation and development of cancer [84]. Similarly, there are indications that high fat diets increase breast cancer risk and are associated with an increased incidence of aggressive prostate cancer [85].

In an epidemiological study of 56,007 French women over 8 years, it was noted that breast cancer risk was not related to dietary PUFA overall, but a significant risk was associated with ω6 vs. ω3 PUFAs that was inversely related to ω3 PUFAs in women with the highest intake of ω6 PUFAs indicating interactions between PUFA consumption [86]. The decreased risk of breast cancer with ω3 PUFA intake (from fish) was confirmed in a case controlled study [87]. A population based study showed all-cause mortality was reduced 16–34% in women with a high intake of ω3 PUFAs [88]. Overall, during the past 20 years, data has accumulated to indicate that a high ω6 PUFA intake is pro-inflammatory, likely involving COX-2 and NFκβ activation leading to increased breast cancer incidence and all-cause mortality whereas high ω3 PUFA intake is protective, against high ω6 PUFA consumption downregulating NFκβ and decreasing breast cancer incidence and all-cause mortality.

Recent studies have shed additional light on the mechanisms involved in these clinical effects, as well as their relationship to the previously discussed innate and adaptive immune cells in the tumor microenvironment. The regulation by ω3 PUFA of macrophage function, has been documented with the use of antagonists to GPR120 (free fatty acid receptor 4 (FFA4R)) which is expressed by some myeloid cell populations [89]. It is noted that ω3 PUFAs mediate anti-inflammatory effects via this receptor. However, the nuclear receptor PPAR-γ is also a receptor for PUFAs and the regulatory mechanisms of ω3 and ω6 PUFA on obesity [90], postmenopausal breast mammary cancer [91] and microenvironmental inflammation [41] require additional study. Changes in the lipid content of cell membranes associated with ω3 and ω6 PUFA intake have effects on oncogenic signalling through modulation of lipid raft profiles and a reduction in cytokine production [92]. In addition, PUFAs contribute to the regulation of hematopoiesis in the BM, at extramedullary sites such as the spleen [93, 94] and have been suggested to induce the expansion of myeloid derived suppressor cells [95].

In summary, dietary intake of PUFAs have shown significant effects on clinical outcomes in cancer patients. In general ω6 PUFAs are associated with increased risk due to both direct effects on the mammary gland and promotion of a pro-inflammatory tumor microenvironment. In contrast, ω3 PUFAs have protective effects and counter tumor and ω6 PUFA associated inflammation. A general recommendation can be made that individuals should decrease dietary ω−6 PUFA intake and increase their ω3 PUFA consumption such that a dietary ratio of no more than 1–3 to 1 is consumed to support cancer prevention. PPAR-γ and GPR120 agonists also have potential use as neoplastic chemopreventive drugs; although both these drugs and dietary PUFA regulation have yet to definitively document anti-cancer activity. In contrast, long-term use of anti-inflammatory drugs has a clearly documented cancer preventive activity associated with inflammatory cell infiltration of tumors [96].

However, these benefits need to be weighed against the risks associated with the long-term use of anti-inflammatory drugs, which highlights the potential for dietary PUFA regulation of inflammation.

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References

1. Eccles SA, Alexander P. Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature. 1974;250:667–9.
2. Gauci GL, Alexander P. The macrophage content of some human tumors. Cancer Lett. 1975;1:29.
3. Key M, Talmadge JE, Fidler IJ. Lack of correlation between the progressive growth of spontaneous metastases and their content of infiltrating macrophages. J Reticuloendothel Soc. 1982;32:387–96.
4. Evans R, Lawler EM. Macrophage content and immunogenicity of C57BL/6J and BALB/cByJ methylcholanthrene-induced sarcomas. Int J Cancer. 1980;26:831–5.
5. Steele RJ, Eremin O, Brown M, Hawkins RA. A high macrophage content in human breast cancer is not associated with favourable prognostic factors. Br J Surg. 1984;71:456–8.
6. Sun XF, Zhang H. Clinicopathological significance of stromal variables: angiogenesis, lymphangiogenesis, inflammatory infiltration. MMP and PINCH in colorectal carcinomas. Mol Cancer. 2006;5:43.
7. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer. 2004;4:71–8.
8. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008;454:436–44.
9. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 2009;27:451–83.
10. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol. 2009;86:1065–73.
11. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005;5:953–64.
12. Goerdt S, Orfanos CE. Other functions, other genes: alternative activation of antigen-presenting cells. Immunity. 1999;10:137–42.
13. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005;23:344–6.
14. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9:162–74.
15. Alexander P, Eccles SA, Gauci CL. The significance of macrophages in human and experimental tumors. Ann N Y Acad Sci. 1976;276:124–33.
16. Sinha P. Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. Cancer Res. 2005;65:11743–51.
17. Karanikas V, Zamanakou M, Soukou F, Kerenidi T, Gourgoulianis KI, Germenis AE. Naturally occurring tumor-specific CD8+ T-cell precursors in individuals with and without cancer. Immunol Cell Biol. 2010;88:575–85.
18. Gervois N, Guilloux Y, Diez E, Jotereau F. Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumor peptide interactions. J Exp Med. 1996;183:2403–7.
19. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A, Kudoh S, Ochiai A. Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer. 2008;113:1387–95.
20. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med. 2003;348:203–13.
21. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med. 2004;10(9):942.
22. Menon AG, Janssen-van Rhijn CM, Morreau H, Putter H, Tollenaar RA, van de Velde CJ, Fleuren GJ, Kuppen PJ. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest. 2004;84:493–501.
23. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, Ohtani H. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res. 1998;58:3491–4.
24. Piersma SJ, Jordanova ES, van Poelgeest MIE, Kwappenberg KMC, van der Hulst JM, Drijfhout JW, Melief CJM, Kenter GG, Fleuren GJ, Offringa R, van der Burg SH. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res. 2007;67:354–61.

25. Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, Suzuki Y, Shintaku I, Nagura H, Ohtani H. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer Res. 2001;61:5132–6.
26. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2009;29:1093–102.
27. Gorgun G, Samur MK, Cowens KB, Paula S, Bianchi G, Anderson JE, White RE, Singh A, Ohguchi H, Suzuki R, Kikuchi S, Harada T, Hideshima T, Tai YT, Laubach JP, Raje N, Magrangeas F, Minvielle S, Avet-Loiseau H, Munshi NC, Dorfman DM, Richardson PG, Anderson KC: Lenalidomide enhances immune checkpoint blockade-induced immune response in multiple myeloma. Clin Cancer Res. 2015;21(20):4607–4618.
28. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? Curr Opin Immunol. 2015;33:23–35.
29. Guthrie GJ, Charles KA, Roxburgh CS, Horgan PG, McMillan DC, Clarke SJ. The systemic inflammation-based neutrophil-lymphocyte ratio: experience in patients with cancer. Crit Rev Oncol Hematol. 2013;88:218–30.
30. Motomura T, Shirabe K, Mano Y, Muto J, Toshima T, Umemoto Y, Fukuhara T, Uchiyama H, Ikegami T, Yoshizumi T, Soejima Y, Maehara Y. Neutrophil-lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment. J Hepatol. 2013;58:58–64.
31. Kantola T, Klintrup K, Vayrynen JP, Vornanen J, Bloigu R, Karhu T, Herzig KH, Napankangas J, Makela J, Karttunen TJ, Tuomisto A, Makinen MJ. Stage-dependent alterations of the serum cytokine pattern in colorectal carcinoma. Br J Cancer. 2012;107:1729–36.
32. Wang J, Jia Y, Wang N, Zhang X, Tan B, Zhang G, Cheng Y. The clinical significance of tumor-infiltrating neutrophils and neutrophil-to-CD8+ lymphocyte ratio in patients with resectable esophageal squamous cell carcinoma. J Transl Med. 2014;12:7.
33. Ilie M, Hofman V, Ortholan C, Bonnetaud C, Coelle C, Mouroux J, Hofman P. Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer. Cancer. 2012;118:1726–37.
34. Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev. 2010;68:280–9.
35. Serhan CN. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol. 2007;25:101–37.
36. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008;8:349–61.
37. Mocellin MC, Camargo CQ, Nunes EA, Fiates GM, Trindade EB. A systematic review and meta-analysis of the n-3 polyunsaturated fatty acids effects on inflammatory markers in colorectal cancer. Clin Nutr. 2016;35(2):359–69.
38. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21:495–505.
39. Wild GE, Drozdowski L, Tartaglia C, Clandinin MT, Thomson AB. Nutritional modulation of the inflammatory response in inflammatory bowel disease—from the molecular to the integrative to the clinical. World J Gastroenterol. 2007;13:1–7.
40. James M, Proudman S, Cleland L. Fish oil and rheumatoid arthritis: past, present and future. Proc Nutr Soc. 2010;69:316–23.
41. Calder PC. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. Biochim Biophys Acta. 2015;1851:469–84.
42. Badwey JA, Curnutte JT, Robinson JM, Berde CB, Karnovsky MJ, Karnovsky ML. Effects of free fatty acids on release of superoxide and on change of shape by human neutrophils. Reversibility by albumin. J Biol Chem. 1984;259:7870–7.
43. Bates EJ, Ferrante A, Smithers L, Poulos A, Robinson BS. Effect of fatty acid structure on neutrophil adhesion, degranulation and damage to endothelial cells. Atherosclerosis. 1995;116:247–59.
44. Soyland E, Nenseter MS, Braathen L, Drevon CA. Very long chain n-3 and n-6 polyunsaturated fatty acids inhibit proliferation of human T-lymphocytes in vitro. Eur J Clin Invest. 1993;23:112–21.
45. Calder PC. Dietary fatty acids and the immune system. Nutr Rev. 1998;56:S70–83.
46. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, Cannon JG, Rogers TS, Klempner MS, Weber PC, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med. 1989;320:265–71.
47. German JB, Lokesh B, Kinsella JE. The effect of dietary fish oils on eicosanoid biosynthesis in peritoneal macrophages is influenced by both dietary N-6 polyunsaturated fats and total dietary fat. Prostaglandins Leukot Essent Fatty Acids. 1988;34:37–45.
48. Siriwardhana N, Kalupahana NS, Fletcher S, Xin W, Claycombe KJ, Quignard-Boulange A, Zhao L, Saxton AM, Moustaid-Moussa N. n-3 and n-6 polyunsaturated fatty acids differentially regulate adipose angiotensinogen and other inflammatory adipokines in part via NF-kappaB-dependent mechanisms. J Nutr Biochem. 2012;23:1661–7.
49. Santoli D, Phillips PD, Colt TL, Zurier RB. Suppression of interleukin 2-dependent human T cell growth in vitro by prostaglandin E (PGE) and their precursor fatty acids. Evidence for a PGE-independent mechanism of inhibition by the fatty acids. J Clin Invest. 1990;85:424–32.

50. Kelly JP, Parker CW. Effects of arachidonic acid and other unsaturated fatty acids on mitogenesis in human lymphocytes. J Immunol. 1979;122:1556–62.
51. Babu US, Bunning VK, Wiesenfeld P, Raybourne RB, O'Donnell M. Effect of dietary flaxseed on fatty acid composition, superoxide, nitric oxide generation and antilisterial activity of peritoneal macrophages from female Sprague-Dawley rats. Life Sci. 1997;60:545–54.
52. Turek JJ, Schoenlein IA, Bottoms GD. The effect of dietary n-3 and n-6 fatty acids on tumor necrosis factor-alpha production and leucine aminopeptidase levels in rat peritoneal macrophages. Prostaglandins Leukot Essent Fatty Acids. 1991;43:141–9.
53. Jeffery NM, Newsholme EA, Calder PC. Level of polyunsaturated fatty acids and the n-6 to n-3 polyunsaturated fatty acid ratio in the rat diet alter serum lipid levels and lymphocyte functions. Prostaglandins Leukot Essent Fatty Acids. 1997;57:149–60.
54. Hayashi N, Tashiro T, Yamamori H, Takagi K, Morishima Y, Otsubo Y, Sugiura T, Furukawa K, Nitta H, Nakajima N, Suzuki N, Ito I. Effects of intravenous omega-3 and omega-6 fat emulsion on cytokine production and delayed type hypersensitivity in burned rats receiving total parenteral nutrition. JPEN J Parenter Enteral Nutr. 1998;22:363–7.
55. Khair-el-Din TA, Sicher SC, Vazquez MA, Wright WJ, Lu CY. Docosahexaenoic acid, a major constituent of fetal serum and fish oil diets, inhibits IFN gamma-induced Ia-expression by murine macrophages in vitro. J Immunol. 1995;154:1296–306.
56. Hughes DA, Southon S, Pinder AC. (n-3) Polyunsaturated fatty acids modulate the expression of functionally associated molecules on human monocytes in vitro. J Nutr. 1996;126:603–10.
57. Hughes DA, Pinder AC. N-3 polyunsaturated fatty acids modulate the expression of functionally associated molecules on human monocytes and inhibit antigen-presentation in vitro. Clin Exp Immunol. 1997;110:516–23.
58. Hubbard NE, Somers SD, Erickson KL. Effect of dietary fish oil on development and selected functions of murine inflammatory macrophages. J Leukoc Biol. 1991;49:592–8.
59. Sadeghi S, Wallace FA, Calder PC. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. Immunology. 1999;96:404–10.
60. Peterson LD, Thies F, Sanderson P, Newsholme EA, Calder PC. Low levels of eicosapentaenoic and docosahexaenoic acids mimic the effects of fish oil upon rat lymphocytes. Life Sci. 1998;62:2209–17.
61. Calder PC. Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. Mol Nutr Food Res. 2008;52:885–97.
62. Curtis CL, Hughes CE, Flannery CR, Little CB, Harwood JL, Caterson B. n-3 fatty acids specifically modulate catabolic factors involved in articular cartilage degradation. J Biol Chem. 2000;275:721–4.
63. Flower RJ, Perretti M. Controlling inflammation: a fat chance? J Exp Med. 2005;201:671–4.
64. Norling LV, Serhan CN. Profiling in resolving inflammatory exudates identifies novel anti-inflammatory and pro-resolving mediators and signals for termination. J Intern Med. 2010;268:15–24.
65. Gilroy DW, Lawrence T, Perretti M, Rossi AG. Inflammatory resolution: new opportunities for drug discovery. Nat Rev Drug Discov. 2004;3:401–16.
66. Rueter K, Haynes A, Prescott SL. Developing primary intervention strategies to prevent allergic disease. Curr Allergy Asthma Rep. 2015;15:537.
67. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. Nat Rev Immunol. 2011;11:85–97.
68. Hussein MR, Hassan HI. Analysis of the mononuclear inflammatory cell infiltrate in the normal breast, benign proliferative breast disease, in situ and infiltrating ductal breast carcinomas: preliminary observations. J Clin Pathol. 2006;59:972–7.
69. Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-kappaB as the matchmaker. Nat Immunol. 2011;12:715–23.
70. Rose DP, Connolly JM, Coleman M. Effect of omega-3 fatty acids on the progression of metastases after the surgical excision of human breast cancer cell solid tumors growing in nude mice. Clin Cancer Res. 1996;2:1751–6.
71. Mandal CC, Ghosh-Choudhury T, Yoneda T, Choudhury GG, Ghosh-Choudhury N. Fish oil prevents breast cancer cell metastasis to bone. Biochem Biophys Res Commun. 2010;402:602–7.
72. Gonzalez MJ, Schemmel RA, Gray JI, Dugan L Jr, Sheffield LG, Welsch CW. Effect of dietary fat on growth of MCF-7 and MDA-MB231 human breast carcinomas in athymic nude mice: relationship between carcinoma growth and lipid peroxidation product levels. Carcinogenesis. 1991;12:1231–5.
73. Hardman WE. Dietary canola oil suppressed growth of implanted MDA-MB 231 human breast tumors in nude mice. Nutr Cancer. 2007;57:177–83.
74. Manna S, Janarthan M, Ghosh B, Rana B, Rana A, Chatterjee M. Fish oil regulates cell proliferation, protect DNA damages and decrease HER-2/neu and c-Myc protein expression in rat mammary carcinogenesis. Clin Nutr. 2010;29:531–7.
75. Noguchi M, Minami M, Yagasaki R, Kinoshita K, Earashi M, Kitagawa H, Taniya T, Miyazaki I. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. Br J Cancer. 1997;75:348–53.
76. Manna S, Chakraborty T, Ghosh B, Chatterjee M, Panda A, Srivastava S, Rana A, Chatterjee M. Dietary fish oil associated with increased apoptosis and modulated expression of Bax and Bcl-2 during 7,12-dimethylbenz(alpha)anthracene-induced mammary carcinogenesis in rats. Prostaglandins Leukot Essent Fatty Acids. 2008;79:5–14.

77. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed Pharmacother. 2002;56:365–79.
78. Wei N, Wang B, Zhang QY, Mi MT, Zhu JD, Yu XP, Yuan JL, Chen K, Wang J, Chang H. Effects of different dietary fatty acids on the fatty acid compositions and the expression of lipid metabolic-related genes in mammary tumor tissues of rats. Nutr Cancer. 2008;60:810–25.
79. Jiang W, Zhu Z, McGinley JN, El Bayoumy K, Manni A, Thompson HJ. Identification of a molecular signature underlying inhibition of mammary carcinoma growth by dietary N-3 fatty acids. Cancer Res. 2012;72:3795–806.
80. Xue M, Wang Q, Zhao J, Dong L, Ge Y, Hou L, Liu Y, Zheng Z. Docosahexaenoic acid inhibited the Wnt/beta-catenin pathway and suppressed breast cancer cells in vitro and in vivo. J Nutr Biochem. 2014;25:104–10.
81. Berg JW. Can nutrition explain the pattern of international epidemiology of hormone-dependent cancers? Cancer Res. 1975;35:3345–50.
82. Tominaga S. Cancer incidence in Japanese in Japan, Hawaii, and western United States. Natl Cancer Inst Monogr. 1985;69:83–92.
83. Goodstine SL, Zheng T, Holford TR, Ward BA, Carter D, Owens PH, Mayne ST. Dietary (n-3)/(n-6) fatty acid ratio: possible relationship to premenopausal but not postmenopausal breast cancer risk in U.S. women. J Nutr. 2003;133:1409–14.
84. Calder PC. N-3 polyunsaturated fatty acids and immune cell function. Adv Enzyme Regul. 1997;37:197–237.
85. Rose DP. Dietary fatty acids and cancer. Am J Clin Nutr. 1997;66:998S–1003S.
86. Thiebaut AC, Chajes V, Gerber M, Boutron-Ruault MC, Joulin V, Lenoir G, Berrino F, Riboli E, Benichou J, Clavel-Chapelon F. Dietary intakes of omega-6 and omega-3 polyunsaturated fatty acids and the risk of breast cancer. Int J Cancer. 2009;124:924–31.
87. Kim J, Lim SY, Shin A, Sung MK, Ro J, Kang HS, Lee KS, Kim SW, Lee ES. Fatty fish and fish omega-3 fatty acid intakes decrease the breast cancer risk: a case-control study. BMC Cancer. 2009;9:216.
88. Khankari NK, Bradshaw PT, Steck SE, He K, Olshan AF, Shen J, Ahn J, Chen Y, Ahsan H, Terry MB, Teitelbaum SL, Neugut AI, Santella RM, Gammon MD. Dietary intake of fish, polyunsaturated fatty acids, and survival after breast cancer: a population-based follow-up study on Long Island. New York: Cancer; 2015.
89. Im DS. Functions of omega-3 fatty acids and FFA4 (GPR120) in macrophages. Eur J Pharmacol. 2016;785:36–43.
90. Bjursell M, Xu X, Admyre T, Bottcher G, Lundin S, Nilsson R, Stone VM, Morgan NG, Lam YY, Storlien LH, Linden D, Smith DM, Bohlooly YM, Oscarsson J. The beneficial effects of n-3 polyunsaturated fatty acids on diet induced obesity and impaired glucose control do not require Gpr120. PLoS One. 2014;9:e114942.
91. Chung H, Lee YS, Mayoral R, Oh DY, Siu JT, Webster NJ, Sears DD, Olefsky JM, Ellies LG. Omega-3 fatty acids reduce obesity-induced tumor progression independent of GPR120 in a mouse model of postmenopausal breast cancer. Oncogene. 2015;34:3504–13.
92. Turk HF, Chapkin RS. Membrane lipid raft organization is uniquely modified by n-3 polyunsaturated fatty acids. Prostaglandins Leukot Essent Fatty Acids. 2013;88:43–7.
93. Xia S, Li XP, Cheng L, Han MT, Zhang MM, Shao QX, Xu HX, Qi L. Fish oil-rich diet promotes hematopoiesis and alters hematopoietic niche. Endocrinology. 2015;156:2821–30.
94. Schumann T, Adhikary T, Wortmann A, Finkernagel F, Lieber S, Schnitzer E, Legrand N, Schober Y, Nockher WA, Toth PM, Diederich WE, Nist A, Stiewe T, Wagner U, Reinartz S, Muller-Brusselbach S, Muller R. Deregulation of PPARbeta/delta target genes in tumor-associated macrophages by fatty acid ligands in the ovarian cancer microenvironment. Oncotarget. 2015;6:13416–33.
95. Yan D, Yang Q, Shi M, Zhong L, Wu C, Meng T, Yin H, Zhou J. Polyunsaturated fatty acids promote the expansion of myeloid-derived suppressor cells by activating the JAK/STAT3 pathway. Eur J Immunol. 2013;43:2943–55.
96. Talmadge JE, Donkor M, Scholar E. Inflammatory cell infiltration of tumors: Jekyll or Hyde. Cancer Metastasis Rev. 2007;26:373–400.

Oncolytic Virotherapy and the Tumor Microenvironment

11

Sara E. Berkey, Steve H. Thorne, and David L. Bartlett

11.1 Introduction

An oncolytic virus is one that specifically infects and/or replicates in cancer cells, while leaving healthy cells relatively unharmed. This viral infection and viral mediated cytotoxicity leads to profound effects on the tumor microenvironment. Perhaps the most obvious is its effect on the immune microenvironment, as all viruses lead to an inflammatory response in the tumor. In addition, oncolytic viruses can affect tumor vasculature, tumor associated fibroblasts, extracellular matrix, and more. These effects can be enhanced or manipulated using targeted deletions within the virus or expression of specific transgenes [1]. Commonly used virus vectors include poxvirus (vaccinia), adenovirus, and herpes simplex virus [2]. The field has evolved from using wild type viruses, to viruses which selectively replicate in cancer cells via targeted deletions, to selective viruses which express therapeutic transgenes. A variety of transgenes have been shown to be effective at inhibiting tumor progression via its effect on the tumor microenvironment, including suicide genes, factors capable of modulating or enhancing the immune system, agents that have anti-angiogenic effects, and products that disrupt the extracellular matrix [3]. The multiple effects oncolytic viruses have on the tumor microenvironment are discussed here.

11.2 Mechanisms of Viral-Induced Cancer Cell Death

It is increasingly apparent that the mechanism of tumor cell death can define the inflammatory response in the tumor microenvironment. Apoptotic cell death can be non-immunogenic if cell membrane integrity is maintained and no damage-associated molecular pattern molecules (DAMP's) are released. Cancer cells dying by necrosis/necroptosis or pyroptosis are immunogenic. Cells dying by these mechanisms secrete pro-inflammatory cytokines and release their cytoplasmic content, including DAMPs (ATP, HMGB1, uric acid, etc.), into the extracellular space. Some DAMPs (such as HMGB1) can also be secreted through non-classical pathways [4].

Oncolytic viruses in general kill with an immunogenic form of cell death (Table 11.1)

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Table 11.1 Oncolytic viruses lead to specific mode of immunogenic cell death and exposure/release of DAMPs/PAMPs [4]

OV	DAMP/PAMP	Receptor	Type of cell death	Immunological functions	Refs.
Ad5/3-D24-GM-CSF; CVB3; vvDD	ATP	P2Y2 and P2X7	Necrosis, autophagic cell death, and immunogenic apoptosis	Function as a "find-me" signal, and cause NLRP3-inflammasome-based IL-1β production	[5, 8, 11]
Ad5/3-D24-GM-CSF; CVB3	Ecto-CRT (calreticulin)	CD91	Immunogenic apoptosis (either pre-apoptotic, early or mid apoptotic surface exposure) or secondary necrosis	Function as an "eat-me" signal and it is a potent mediator of tumor immunogenicity crucial for elicidation of antitumor immunity	[5, 11]
Parvovirus H-1 (H-1PV)	HSPs: (HSP90, HSP70, Hsp72)	CD91, TLR2, TLR4, SREC1, and FEEL1	Immunogenic apoptosis (surface exposure) or necrosis (passively released)	Surfaced-exposed HSP90 can mediate adaptive antitumor immunity, while secreted HSP90 can inhibit TGF-β1 activation; Leads to TAA-specific antitumor immunity	[13–15]
? (Not identified)	Histones	TLR9	Apoptosis (cell surface exposure) or accidental necrosis (passively released)	Released histones can cause initiation of TLR9-MyD88-mediated inflammation	[16]
Many OVs: Ad; HSV; MV; VV; H-1PV	HMGB1	TLR2, TLR4, RAGE, and TIM3	Immunogenic apoptosis; necrosis; autophagic cell death	Activate macrophages and DCs; recruit neutrophils; promote *in vivo* the production of IFN-γ, TNF-α, IL-6, IL-12, and antigen-specific activation of $CD8^+$ T cells	[6–11]
MV-eGFP	IL-6	IL-6R and GP130	Necroptosis	Stimulate the production of inflammatory cytokines such as IL-1, TNF-α, and IL-6 and chemotactic factors for neutrophils such as IL-8/CXCL8 and S100A8/A9	[6]
Telomelysin (Ad)	Uric acid	P2Y6	Autophagic cell death	A cell type-specific endokine DAMP with potent pro-inflammatory activity	[12–17]
Newcastle disease virus (NDV)	dsRNA and other PAMPs	TLR3; and by the cytoplasmic receptors MDA-5 and RIG-I	Immunogenic Apoptosis; autophagy	(1) Upregulation of HLA antigens and ICAM-1; (2) induction of type I IFNs and chemokines (CCL5 and CXCL10); (3) activate DCs and T effector cells but also to block Treg cells; (4) local therapy with oncolytic NDV induces inflammatory immune infiltrates in distant tumors, making them susceptible to systemic therapy	[18–22]
Reovirus	The virus itself (PAMP)	Dendritic cells (DCs)	(Cancer cell independent mechanism)	Induce DC maturation and stimulate the production of the pro-inflammatory cytokines IFN-α, TNF-α, IL-12p70, and IL-6. Reovirus directly activates human DC and that reovirus-activated DCs stimulate innate killing by not only NK cells, but also T cells	[23]

[4]. They can kill cells via either immunogenic apoptosis or necrotic cell death, and can be manipulated to alter the mode of cell death. Different viruses have different ways of inducing cell death. For example, most oncolytic adenoviruses induce autophagic cell death in cancer cells. Coxsackievirus B3 (CVB3) has been reported to induce immunogenic apoptosis in human non-small cell lung cancer cells [5]. Measles virus (MV) infection leads to the release of inflammatory cytokines and HMGB1, and activation of DCs [6]. HMGB1 release often happens in late stage of apoptosis, during autophagy and in necrotic cells infected with OVs [4]. We first reported in 2005 that human cancer cells infected by an oncolytic poxvirus, led to necrotic/apoptotic death pathways and release of HMGB1 [7]. Later studies have confirmed and extended the findings of HMGB1 release in cancer cells infected with Adenovirus (Ads) [24], CVB3 [25], an MV [6], vaccinia viruses (VVs) [8, 9], HSV [25, 26], and parvovirus H-1 (H-1PV) [10].

Genetic engineering and combination strategies (virus plus chemotherapy or radiation therapy) can skew the cancer cell death into modes of immunogenic cell death and autophagy, leading to potent and sustained antitumor immunity, thus enhancing the efficacy of oncolytic immunotherapy. Workenhe et al. demonstrated that the combination of HSV-1 ICP0 null oncolytic virus with mitoxantrone, which induces ICD, was able to break immune tolerance and provide significant survival benefit to Balb/C mice bearing Her2/neu TUBO-derived mammary tumors. Increased infiltration of neutrophils and tumor antigen-specific $CD8^+$ T cells into tumor tissues provide the protection, and depletion studies verified that CD8-, CD4-, and Ly6G-expressing cells are essential for the enhanced efficacy [27]. Which mode of cell death in the context of oncolytic viruses is the most potent way to elicit antitumor immunity needs further investigation.

11.3 Viral Modulation of the Immune Microenvironment

11.3.1 Natural Immune Activation

Selective infection of tumor cells following treatment with an oncolytic virus induces an inflammatory response that promotes tumor destruction [28]. Viral mediated cell lysis and death occurs as a result of infection, which leads to the release of cytokines, danger signals, and virus- and tumor-associated antigens [3, 29]. This in turn causes activation of the innate immune response, including dendritic cells, natural killer cells, macrophages, and neutrophils. These cells then lead to either direct killing of the virally infected cells or to the recruitment of adaptive immune cells, particularly CD4 and CD8 lymphocytes [3, 28]. Not only does the activation of the adaptive immune response result in additional tumor cell death, but it also creates an in situ vaccination effect with cross-presentation of tumor associated antigens (Fig. 11.1) [30]. For example, in pre-clinical models, animals previously treated with an oncolytic virus have the capacity to reject the tumor when they are re-challenged with the same cancer cell line [1].

It is well recognized that the tumor microenvironment is composed of immune cells. However, it is one which predominantly fosters tumor growth and suppresses those processes that would result in tumor cell elimination [28]. Nevertheless, uncontrolled oncolytic viral replication does not occur after treatment with oncolytic viral therapies. This indicates that the virus is ultimately cleared by the immune response, implying that it is capable of transiently overcoming the local immunosuppressive environment within the tumor. While virally infected cells are eliminated in the tumor microenvironment, the surrounding non-infected cells may not be attacked [1]. The development of an immunologic "bystander" effect for non-infected cells can be enhanced with manipulations of the virus for additional therapeutic benefit.

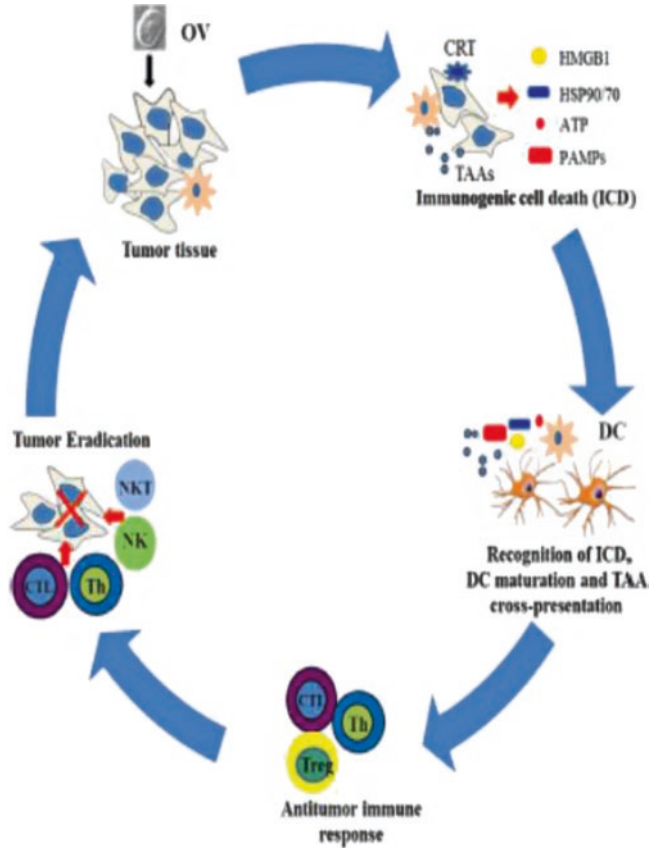

Fig. 11.1 Once an oncolytic virus (OV) reaches the tumor microenvironment, it selectively replicates within tumor and/or stromal cells, which induces death in the infected cells. This leads to the presentation of cell surface signals and the release of danger signals from the necrotic cells. Antigen presenting cells (APCs) engulf apoptotic bodies, from which tumor associated antigens (TAAs) are processed. TAAs are then presented with the MHC complex, as well as the costimulatory molecules, to the naïve T cells. In addition, the DAMPS (and PAMPS) that were released activate and mature dendritic cells. This results in a cytotoxic immune response, involving $CD4^+$ and $CD8^+$ T cells, against the tumor and associated stromal cells, which assists in eradicating the tumor mass. Additional immunotherapies targeting DCs, T cells, and the immunosuppressive tumor microenvironment can further enhance this antitumor immune response [30]

11.3.2 Viral Cytokine Expression

One approach to improve the impact of the oncolytic viruses on the tumor microenvironment is via expression of single cytokines from the viral backbone. Viral-mediated production of cytokines locally within the tumor enhances the immune response, while also reducing the systemic toxicity when compared to treatment with recombinant cytokines. The two most effective cytokines tested are granulocyte-monocyte colony stimulation factor (GM-CSF) [31] and type I interferon (IFN) [33, 64]. GM-CSF is a potent inducer of hematopoietic cell proliferation, including tumor specific cytotoxic T lymphocytes (CTL), while having minimal anti-viral effects. This leads to destruction of tumor cells both by the CTLs directly and secondary by the oncolytic viral infection [31, 32]. There are currently two oncolytic viruses expressing GM-CSF that have reached a primary survival endpoint in randomized clinical testing—a HSV strain (T-Vec, Amgen) and a vaccinia strain (Pexa-vec, Jennerex, part of Sillajen) [1]. However, there is concern

that GM-CSF may also stimulate the production of immune suppressor cells, which have a known tumor promoting effect [32]. Therefore, caution may be needed when using this cytokine. IFN-β has known anti-viral and anti-tumor effects. Its anticancer properties include direct antiproliferative effects and induction of tumor specific CTLs. While in healthy cells, IFN-β normally inhibits viral replication, however, tumor cells are often resistant to IFN-β's antiviral effects. Therefore, in preclinical studies, oncolytic virus replication still occurs within tumor cells and the anti-cancer effects of IFN are still produced [3, 33]. Unfortunately, there is limited data to support the therapeutic effects of IFN-expressing vectors from clinical trials [1].

In addition, interleukin (IL)-2 and tumor necrosis factor (TNF)-α expressing vaccinia vectors have been tested in pre-clinical models. However, despite their anti-tumor properties, these cytokines significantly reduced the amount of viral replication within the tumor cells. Different approaches to express these cytokines are being investigated, which regulate the level or the timing of the transgene expression. This may be done via the choice of the promotor. For example, using a late promoter that is not expressed until after replication of the virus has started. Alternatively, transgene expression can be regulated exogenously using inducible promoters [32]. For example, Grigg et al. created an oncolytic vaccinia virus with inducible expression of IFN-γ under control of a tetracycline regulated promoter [34].

Another approach is to exogenously control protein stability. Chen et al. demonstrated that incorporation of exogenous regulation of cytokine or chemokine transgene function through fusion of a small and externally controllable destabilizing domain to immunogenic proteins allowed for an initial phase of viral replication without cytokine function, permitting enhanced delivery and oncolytic activity before activation of cytokine function and a subsequent phase of enhanced and tumor-targeted immunotherapeutic activity. As a result of this exogenous regulation of cytokine function, both oncolytic and immune-mediated mechanisms of action were optimized, greatly enhancing therapeutic activity, while toxicity was significantly reduced. This creates a period of unhindered oncolytic and replicative viral activity prior to cytokine action [35]. Then as the patient's immune response begins to limit the oncolytic viral activity, an additional phase of activity can be initiated through exogenously controlled immunogenic proteins in the tumor microenvironment.

Chemokines are known to attract a variety of cells that mediate an inflammatory response. CCL5, in particular, enhances tumor targeting of effector T-cells. A vaccina virus expressing CCL5 has been found to increase infiltration of CD4 lymphocytes and dendritic cells into the tumor. Also, a prominent Th2 immune response and increased viral persistence are noted within the tumor after infection with a CCL5 expressing virus. However, if combined with dendritic cell therapy a Th1 response occurs with synergistic anti-tumor effects (Fig. 11.2) [36].

11.3.3 Deletion of Viral Virulence Genes

Many DNA viruses express multiple virulence genes, including those capable of inhibiting certain steps in the immune response. These genes represent attractive targets for deletion or mutation, causing the virus to lose its ability to control that specific step in the immune response. Often these genes interfere with signaling pathways or cytokines involved with activating the innate immune response. The IFN pathway is a common viral target. Indeed, deleting the viral genes that disrupt the IFN pathway have demonstrated a greater therapeutic effects than an IFN-expressing oncolytic virus [37].

11.3.4 Viral Expression of Other Immune Stimulation Molecules

As oncolytic viruses undergo replication within a tumor, they begin to produce molecules which can induce an inflammatory response in the tumor.

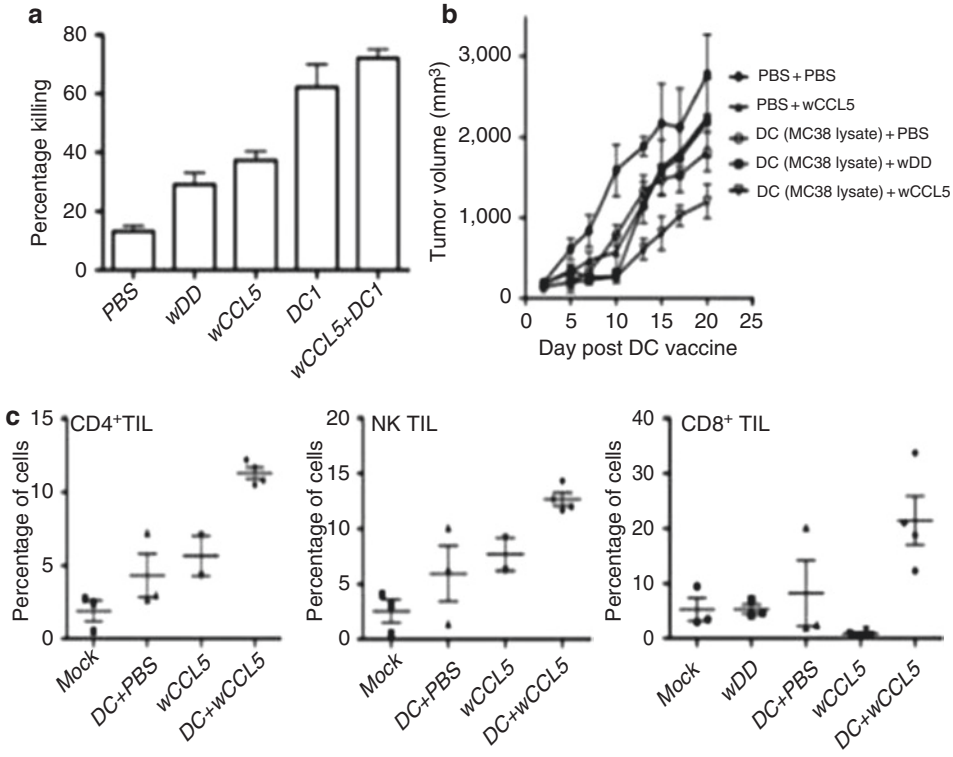

Fig. 11.2 Combining vvCCL5 and dendritic cell (DC) 1 therapies. (**a**) Mice bearing MC38 tumors were treated with either DC1 vaccine loaded with MC38 lysate or phosphate buffered saline (PBS). They were then treated with either with vvCCL5 or PBS 48 h later. 7 days after the different treatments, *in vivo* cytotoxic T lymphocyte (CTL) assays were performed to assess the levels of anti-tumor CTL response produced (n = 3 mice/group). (**b**) Tumor volumes of each treatment group, determined by caliper measurements. Tumor responses of vvCCL5 and DC1 vaccine combination were superior relative to either therapy alone ($P < 0.05$, days 13–20) (n = 10/group). (**c**) Flow cytometry of tumor for each experiment group. Tumors were stained with anti-CD4 antibody, anti-NK1.1 antibody, or anti-CD8 antibody. (n = 3 or 4). *DC* dendritic cell, *NK* natural killer, *TIL* tumor infiltrating lymphocyte [36]

Particular adjuvants are being used to enhance cancer vaccines, such as those that bind to toll-like receptors (TLR). Modification of oncolytic vectors have included creating CpG rich DNA which binds specifically to TLR9. Activation of TLR9 is associated with a favorable immune response. In pre-clinical models, this CpG rich adenovirus activates TLR9 and improves antitumor responses and tumor clearance [38].

Alternatively, expression of tumor associated antigens from the oncolytic virus may also enhance therapeutic effects. The therapeutic benefit occurs by stimulating an adaptive immune response against tumor antigens that may not be induced by the virus alone. Similarly, an oncolytic virus can express bispecific T-cell engagers, which lock the T-cells and tumor cells in close proximity to each other, inducing immune-mediated tumor cell destruction [39].

11.3.5 Targeting of Immunosuppression Within the Tumor

Infection of the tumor with the virus activates the immune response. In addition, these vectors can momentarily overcome immune suppression within the tumor. This is likely only transient as the vectors are removed by the host's immune

response. Some viral vectors have been specifically designed and created to enhance and maintain the virus's capability to overcome the localized immunosuppressive environment [32]. For example, there is a vector that produces a solubilized chemokine receptor (CXCR4) binding domain. Normally, the chemokine CXCL12, which is commonly expressed within tumors, binds to CXCR4. CXCL12 is associated with neovascularization, induction of metastasis, maintenance of cancer stem cells, and the attraction of monocyte derived suppressor cells (MDSC's) into the tumor. The expression of the binding domain CXCR4 by the vector acts as a decoy receptor. This sequesters the chemokine locally and prevents the attraction of MDSC's [40].

In addition, viral vectors expressing antibodies that prevent activation of immune checkpoint inhibitors, including anti-PD-1, anti-PD-L1, and anti-CTLA-4 have been created. This allows for local production of high concentrations of the therapeutic antibody, which can help prevent systemic toxicities [1, 41]. However, some recent data indicates that addition of antibodies to block immune checkpoint inhibition may be more beneficial at later times, such as after initial viral-mediated immune activation [42]. Also, because multiple suppressive immune cells (MDSC, M2 macrophages, and T-reg cells) exist within the tumor, it may be necessary to target multiple cell types in order to ensure a robust adaptive immune response [32].

11.3.6 Combining Oncolytic Viruses with Systemic Therapies

Treatment regimens that combine the capability of the oncolytic virus to transiently modify the tumor microenvironment with a systemic therapy that is able to produce a long lasting anti-tumor response are currently being developed. Many approaches are being explored. Synergistic anti-tumor effects are seen when vaccinia virus is given with chemotherapies such as paclitaxel or irinotecan. Vaccinia virus induces arrest of cell division in the S-phase in order to promote virus replication [43]. This makes the cell more susceptible to the drug therapy. Interesting, after the chemotherapy is given, apoptosis in both virally infected and uninfected cells increases, compared to treatment with either the virus or chemotherapy. The rise in uninfected cell apoptosis is likely because vaccinia virus sensitizes these cells to the effects of either paclitaxel or irinotecan [44, 45]. Cell sensitization to paclitaxel occurs early after viral infection from the release of IFN and later by HMGB1 after viral-mediated cell death [45].

Other combination therapies include the use of the oncolytic virus together with alternative adjuvants or anti-immune checkpoint inhibitors. In addition, oncolytic viruses expressing chemokines known to attract T-cells are being used in combination with dendritic cell vaccination to enhance therapeutic potential. Using CAR T-cells together with oncolytic virus strains that express both chemokines and cytokines, which attract these cells into the tumor and then help to maintain their phenotype, is another possible example [46].

In summary, the immune microenvironment is complex, but we understand much about the mechanisms of immune suppression within that environment that allow tumor progression. Oncolytic viruses are ideally suited to provide a multi-arm attack of immune suppression, and stimulate innate and adaptive immunity against the tumor. Numerous examples of this have been published (Table 11.2) [47]. Many therapeutic options may be available in the future (Fig. 11.3) [78].

11.4 Viral-Mediated Destruction of Tumor Vasculature

11.4.1 Vascular Collapse Within the Tumor

Initial oncolytic viral replication is often restricted to the periphery of the tumor and locations adjacent to the vasculature. However, the virus is still capable of causing cell death in uninfected centrally located cells secondary to viral-mediated tumor-specific vascular collapse. This may occur because of the secretion of pro-inflammatory cytokines by the virus-infected tumor cells and then subsequent recruitment of

Table 11.2 Antitumor immune responses elicited by OVs [47]

OV	Mediator	Mechanism of action	References
HSV-1 (*ICP0* null)	Cytotoxic T Lymphocyte (CTL)	Antiviral and antitumor responses contribute to efficacy in murine breast cancer model	[48]
HSV-1 (*ICP34.5* null)	CTL	Enhanced DC maturation, increased tumor infiltration of INF-γ^+ CTL in murine ovarian cancer model	[49]
HSV-1 (*ICP34.5* null)	Natural Killer (NK) cells/CTL	IT injection results in IFN, MIG, IP-10 production and subsequent infiltration of NK and $CD8^+$ cells	[50]
HSV-1 (*α47* null)	CTL	Enhanced MHCI expression in human cells, enhanced stimulation of matched T cells	[51]
HSV-2	T lymphocytes	Strong T cell responses against primary or metastatic tumors; variety of immune competent murine models	[52–56]
HSV-*GMCSF*	Unspecified	OncoVEX undergoing phase 3 clinical trials for melanoma and squamous cell carcinoma of the head and neck; induction of adaptive antitumor immune responses	[57, 58]
Reovirus	CTL	Generation of antitumor CTL against B16 tumors independent of oncolysis enhanced priming of human CTL against Mel888 cells	[59]
Reovirus	T lymphocyte/NK cells	Expansion of $CD3^+$/ $CD4^+$ and $CD8^+$/$perforin^+$/$granzyme^+$ T cells, and enhanced circulating $CD3^-$/ $CD56^+$ NK cells in patients on phase 1 trial with IV reovirus (T3D)	[60]
Reovirus	NK cells	DCs loaded with reovirus infected melanoma cells results in NK activation and INF-γ secretion	[61]
Reovirus	NK cells/CTL	DCs activated upon reovirus infection; antigen non-restricted tumor cell killing by NK and T cells	[23]
Measles	CTL	Measles virus infected mesothelioma cells activated DCs and primed autologous CTL	[62]
MV-*IFN*-β	$CD68^+$ cells	Infiltration of $CD68^+$ cells innate immune cells in murine mesothelioma improved survival	[63]
Measles	T lymphocytes	Phase 1 trial of cutaneous T cell lymphoma: increased INF-γ^+ and $CD4^+$/$CD8^+$ T cells infiltration; overall expansion of $CD8^+$ T cells	[64]
Adenovirus (Ad-*p*53T)	CTL	Induction of a therapeutically effective tumor-directed CTL response	[65]
Adenovirus (Ad-*GMCSF*)	Neutrophils	Oncolytic adenovirus-*GMCSF* induced neutrophil infiltration and inflammation	[66]
Adenovirus (Ad-*GMCSF*)	CTL	Phase 1 trial: IT treatment with Ad-*GMCSF* led to post-treatment enhancement in circulating antitumor INF-γ secreting CTL	[67]
Vaccinia (JX-594)	Multiple	Melanoma lesions treated IT with JX-594 showed immune infiltration and regression of untreated lesions (phase 1 trial)	[68]
Vaccinia (VV-*ova*)	T lymphocytes	Priming with OVA DNA vaccine and IT treatment with VV-ova enhanced CTL infiltration and killing of OVA-expressing tumors	[69]
Vaccinia	T lymphocytes	Heterologous prime-boost with VV and Semliki forest virus vectors elicits antitumor immunity against murine ovarian surface epithelial carcinomas	[70]
Vaccinia (*B18R* null)	INF-β	Complete tumor response associated with protection from tumor rechallenge in CMT93 murine tumor model	[31]
VSV	CTL	CTL arose against viral and tumor epitopes; antitumor CTL are critical for efficacy of IT VSV	[71]
VSV	NK cells/IL-28	IL-28 induced by VSV sensitized tumors to NK recognition and activation	[72]

(continued)

Table 11.2 (continued)

OV	Mediator	Mechanism of action	References
VSV	CTL/NK cells	Strong correlation between viral gene expression, proinflammatory reaction and therapeutic outcome in B16 ova model	[73]
VSV-*IFN*-β	$CD8^+$ cells	IFN-β potentiated $CD8^+$ T cells generalized reaction in AB12 murine mesothelioma model	[74]
VSV	CTL	Priming with Ad-tumor Ag prior to treatment with VSV-tumor Ag improved survival via antitumor CTL	[75]
VSV	T lymphocytes	Anti-B16 immunity contributes to purging metastases from spleen and lymph nodes and protected from long term metastatic disease	[76]
NDV	T lymphocytes	NDV expressing tumor antigen (+/− *IL-2*) enhanced tumor infiltration by T cells; therapeutic potential was T cell dependent	[77]

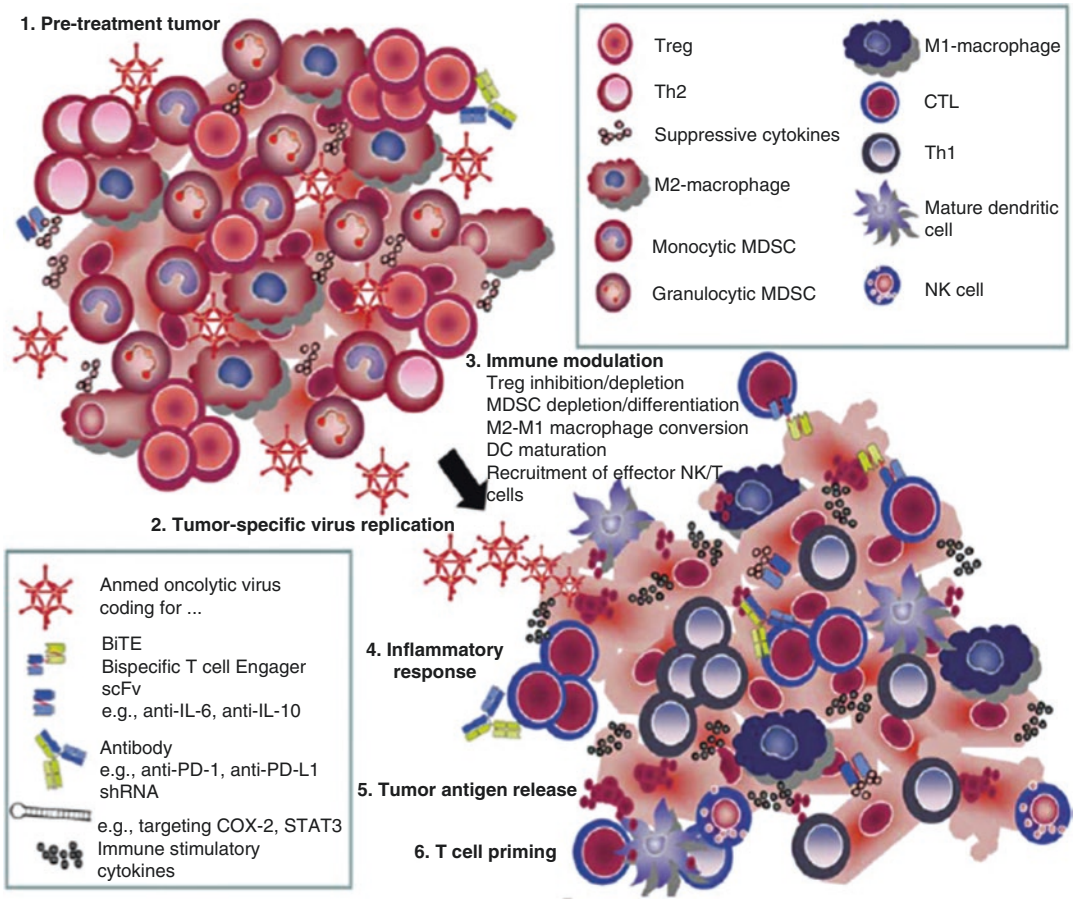

Fig. 11.3 Six steps to induce antitumor T-cell immunity via armed oncolytic viruses. The antitumor efficacy of these viruses depend on successful immune response within the tumor microenvironment and the production of durable antitumor T-cell immunity [78]

inflammatory cells to the tumor. The infiltration of neutrophils leads to thrombosis, acute ischemia, and uninfected tumor cell death [79]. Additionally, there is evidence that vaccinia virus strains are able to infect and replicate within tumor-associated endothelial cells leading directly to their destruction and vascular collapse. Hypoxia and massive necrosis are again seen within the tumor [80]. The hypoxia or ischemia seen, in both of these complimentary and likely simultaneously occurring situations, is likely secondary to the loss of microvascular perfusion following manipulation of the tumor microenvironment by the virus (Fig. 11.4), [79]. It may be as a result of obstruction of small capillaries by neutrophils or the upregulation of tissue factor leading to thrombosis formation within the tumor vasculature [79, 80].

Similar results were seen in clinical studies. In a phase II clinical trial, JX-594 (Pexa-Vec) was given to patients with hepatocellular carcinoma, a hypervascular tumor. JX-594 caused acute tumor vascular disruption and decreased tumor perfusion in these patients, which was maintained for at least 8 weeks. There were no toxicities to normal blood vessels or wound healing noted, but immunohistochemistry demonstrated viral infection of neovasculature within the tumor microenvironment (Fig. 11.5) [80].

11.4.2 Antiangiogenic Effects of the Virus

In addition to promoting vascular collapse within a tumor, oncolytic vaccinia viruses have recently been found to have antiangiogenic properties. Levels of vascular endothelial growth factor (VEGF), a classic mediator of angiogenesis, within the infected tumor were significantly

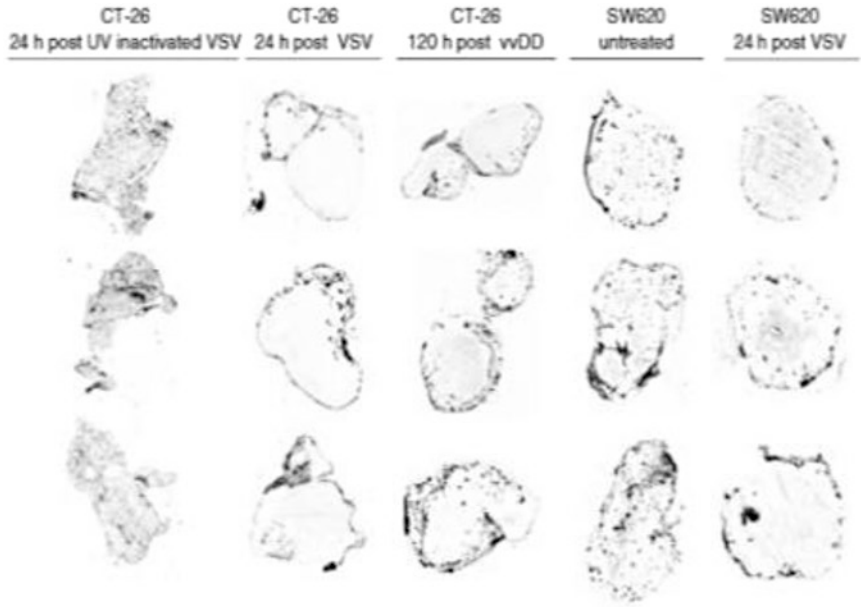

Fig. 11.4 The use of vaccinia virus results in killing of uninfected tumor cells in a xenograft model of human cancer. BALB/c mice with CT-26 tumors were first treated with ultraviolet (UV)-inactivated vesicular stomatitis virus (VSV) particles, then perfused with fluorescent microspheres 24 h after injection, and sacrificed 5 min later. Fluorescence (*black dots*) are was seen uniformly throughout the tumor. VSV expressing green fluorescent protein (GFP) was injected intravenously into BALB/c mice with CT-26 tumors. 24 h later, the tumors were analyzed for perfusion. Another group of BALB/c mice with CT-26 tumors were treated intra-peritoneally with vaccinia virus (vvDD). This time the mice were perfused with fluorescent microspheres 120 h after infection. Similar experiments were conducted using CD1 nude mice with subcutaneous SW620 human colon carcinoma tumors. Both untreated SW620 tumors and SW620 tumors from mice treated intravenously with VSV expressing GFP and perfused with microspheres 24 h are shown. vvDD, double-deleted vaccinia virus [79]

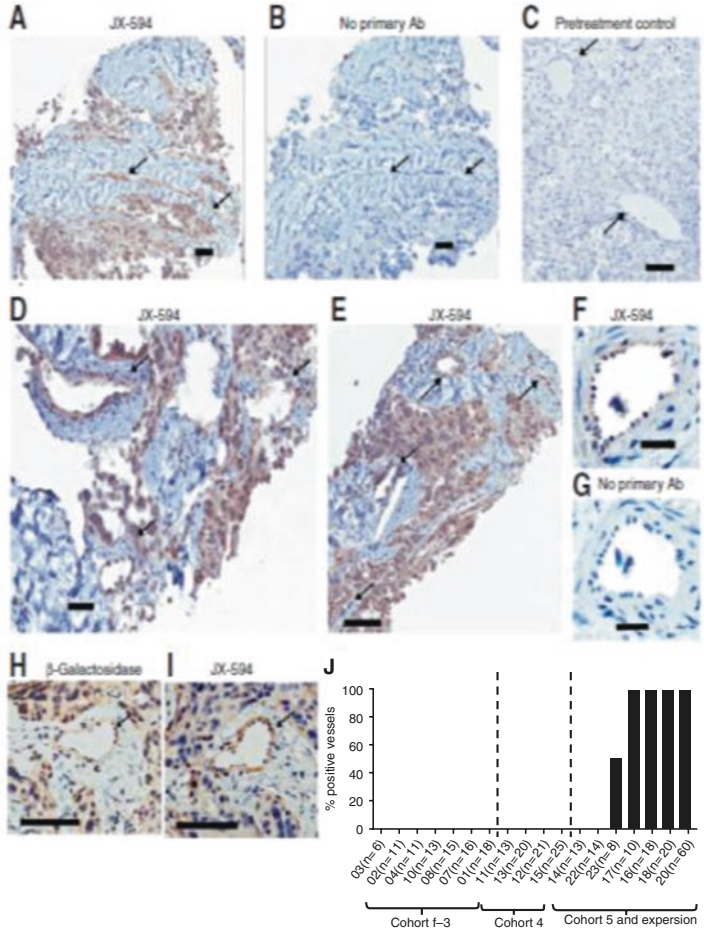

Fig. 11.5 Dose-dependent JX-594 infection of tumor-associated endothelial cells found in patient tumor biopsies after intravenous infusion. Patients received i.v. JX-594 and 7 days lat, tumor biopsies were conducted. The biopsies were evaluated for tumor-associated endothelial cell infection by immunohistochemical (IHC) staining. (**a**) JX-594 infection of tumor-associated endothelial cells (*black arrows*) shown by IHC staining with polyclonal antivaccinia antibody in metastatic ovarian cancer biopsy (lymph node) (scale bar, 50 mm). (**b**) Negative control: corresponding tissue to (**a**). Sample stained with secondary antibody only (scale bar, 50 mm). (**c**) Negative control: tumor biopsies from the patient in (**a**, **b**, **d**, **e**) collected prior to treatment with JX-594. Samples stained with polyclonal anti-vaccinia antibody (scale bar, 50 mm). (**d**) Lower power magnification of the biopsy in (**a**). *Black arrows* indicate infected vessels (scale bar, 100 mm). (**e**) JX-594 infection of another tumor-associated vasculature in an ovarian tumor shown at a higher magnification. *Black arrows* indicate infected vessels (scale bar, 50 mm). (**f**) Tumor biopsy of a patient with metastatic leiomyosarcoma, which shows JX-594 infection of tumor-associated vessels (scale bar, 50 mm). (**g**) Negative control: corresponding serial section to (**f**). Sample stained with secondary antibody only (scale bar, 50 mm). Linear adjustments to brightness and contrast made in (**f**, **g**). (**h**) β-gal in a vessel, detected by IHC, from the patient with ovarian cancer in (**a**). *Black arrow* indicates vessel (scale bars, 100 mm). (**i**) Serial section shows colocalized vaccinia staining (polyclonal antivaccinia antibody) in vessel positive for β-gal (scale bars, 100 mm). Linear adjustments to brightness and contrast made in (**h–j**), Patients cohorts received escalating doses of IV JX-594. Tumor biopsies were collected 7 days later and evaluated for tumor-associated endothelial cell infection in visible vessels. Vessels positive (percentage) for vaccinia IHC in tumor by patient and dose cohort. Number of vessels counted per patient biopsy shown (all vessels in specimen or vessels in five random fields at ×20 magnification). Patients who have received prior antiangiogenic therapy indicated by an *asterisk* [80]

reduced after viral treatment. In fact, the levels remained suppressed during the entirety of the viral infection, even after the induction of hypoxic conditions when levels would be expected to rise. Furthermore, VEGF production was also reduced in neighboring uninfected cells, likely as a result of factors secreted by the infected cells. Therefore, combining oncolytic viruses with additional anti-angiogenic treatments may result in enhanced therapeutic benefit, particularly if the antiangiogenic drug is given after viral clearance when VEGF levels begin to increase again [81].

Antiangiogenic proteins can be expressed by oncolytic viruses for additional anti-angiogenic effects. The soluble VEGF decoy receptor (FP3), due to its high affinity to VEGF, is a highly effective and promising strategy to disrupt VEGF signaling pathway. A novel oncolytic adenovirus (Ad) expressing FP3 (RdB/FP3) was shown to greatly decrease VEGF expression level and vessel density and increase apoptosis in both tumor endothelial and tumor cells, verifying potent suppressive effects of RdB/FP3 on VEGF-mediated tumor angiogenesis in vivo. As a result RdB/FP3 treatment led to a dramatic reduction in tumor growth compared to controls [82].

11.5 Viral Disruption of the Extracellular Matrix

One of the limitations of oncolytic viruses is the inability to efficiently propagate and infect cells distant from the injection site. It appears that the distribution, especially of large viruses, is hindered by the collagen-rich tumor environment. Injecting an oncolytic virus simultaneously with collagenase increases the distribution of the virus by disrupting the collagen network [83]. Additionally, overexpressing matrix metalloproteinase (MMP)-1 and MMP-8 within tumors results in depletion of sulfated glycosaminoglycans, an extracellular matrix component. Possibly as a result of degradation of chondroitin sulfate proteoglycans. When an oncolytic virus is given in conjunction with a MMP-expressing tumor, viral distribution is again noted to be more evenly distributed throughout the entire tumor and not just within the periphery [84]. Both injecting the virus concurrently with collagenase and enhancing the tumor to produce MMP-1 or MMP-8 improves the efficacy of the oncolytic viral therapy [83, 84].

In addition, the concentration of hyaluronan, another extracellular matrix protein, is elevated in several types of cancer and may be correlated with the invasive and metastatic behavior of the tumor. Recent evidence in pre-clinical models reveals that hyaluronidase, the enzyme responsible for hyaluronan degradation, coadministration and hyaluronidase expression by an oncolytic adenovirus enhances viral distribution within the tumor. This results in widespread replication of the virus and overall improved therapeutic outcome [85].

Relaxin-expressing oncolytic viruses were also found to increase viral spread and tumor tissue penetration. Again, this is secondary to degradation of the extracellular matrix within the tumor, likely because relaxin increases the expression of MMPs and pro-collagenase. Furthermore, better distribution of the virus leads to improved antitumor viral activity, including inhibiting tumor growth and metastasis [86]. There are concerns that MMPs may actually increase the metastatic potential of tumors. However, there is emerging evidence that specifically MMP-8 is actually antimetastatic [87].

11.6 Crosstalk Promotes Oncolytic Virus Activity

Another factor that affects the therapeutic potential of oncolytic viruses is the cross talk between different cell types within the tumor microenvironment. The tumor secretes transforming growth factor-β (TGF-β), which coverts normal fibroblasts into cancer-associated fibroblasts. These transformed fibroblasts then produce fibroblast growth factor 2 (FGF-2) that inhibits expression of retinoic acid-inducible gene I in tumor cells. As a result, the ability of the tumor cells to detect and respond to the virus is dampened, increasing

the susceptibility of the tumor cells to viral infection. In a pre-clinical model, a FGF-2 expressing oncolytic virus has been shown to have improved therapeutic efficacy compared to the parental virus [88].

In addition, viral mutations that increase the production of different viral forms, such as the extracellular enveloped form (EEV) of vaccinia, result in oncolytic vectors that are better adapted to spread within a host and can also be incorporated to enhance spread within the tumor, leading to more beneficial therapeutics [89]. This can be taken further through the combination of EEV-enhanced strains with cell based delivery. For example, cytokine-induced killer (CIK) cells pre-infected with an EEV-expressing virus lead to improved CIK cell "homing" to the tumor. This is likely because the EEV is released into the blood stream and seeds the tumor with virus prior to CIK cell infiltration. The virus then modifies the tumor microenvironment to enhance tumor trafficking of the CIK cells towards the virus-infected tumor. In addition, expression of chemokine, CCL5, from the virus could further improve this "homing" effect. This synergistic crosstalk between therapeutic components significantly improves antitumor effects of the oncolytic virus [90].

11.7 Conclusions

As we learn more about the tumor microenvironment and how it promotes tumor growth, we find new targets for treating cancer. Oncolytic viral therapy is a promising new option in cancer treatment, and because they infect and replicate in cancer cells, they are ideally suited to manipulate the tumor microenvironment. Taking advantage of their innate abilities, as well as expressing factors capable of modulating or enhancing the immune system, factors that have anti-angiogenic effects, and that disrupt the extracellular matrix can lead to potent anticancer responses. The viruses have been explored primarily in the pre-clinical models with good results. Hopefully these newer methods described above will be soon translated into clinical therapeutics.

References

1. Sampath P, Thorne SH. Novel therapeutic strategies in human malignancy: combining immunotherapy and oncolytic virotherapy. Oncolytic Virother. 2015;4:75–82.
2. Guo ZH, Thorne SH, Barlett DL. Oncolytic virotherapy: molecular targets in tumor-selective replication and carrier cell-mediated delivery of oncolytic viruses. Biochim Biophys Acta. 2008;1785(2):217–31.
3. Kirn DH, Thorne SH. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. Nat Rev Cancer. 2009;9(1):64–71.
4. Guo ZS, Liu Z, Bartlett DL. Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity. Front Oncol. 2014;4:74.
5. Miyamoto S, Inoue H, Nakamura T, et al. Coxsackievirus B3 is an oncolytic virus with immunostimulatory properties that is active against lung adenocarcinoma. Cancer Res. 2012;72:2609–21.
6. Donnelly OG, Errington-Mais F, Steele L, et al. Measles virus causes immunogenic cell death in human melanoma. Gene Ther. 2013;20:7–15.
7. Guo ZS, Naik A, O'Malley ME, et al. The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2. Cancer Res. 2005;65:9991–8.
8. John LB, Howland LJ, Flynn JK, et al. Oncolytic virus and anti-4-1BB combination therapy elicits strong anti-tumor immunity against established cancer. Cancer Res. 2012;72:1651–60.
9. Whilding LM, Archibald KM, Kulbe H, et al. Vaccinia virus induces programmed necrosis in ovarian cancer cells. Mol Ther. 2013;21:2074–86.
10. Angelova AL, Grekova SP, Heller A, et al. Complementary induction of immunogenic cell death by oncolytic parvovirus H-1PV and gemcitabine in pancreatic cancer. J Virol. 2014;88(10):5263–76.
11. Liikanen I, Ahtiainen L, Hirvinen ML, et al. Oncolytic adenovirus with temozolomide induces autophagy and antitumor immune responses in cancer patients. Mol Ther. 2013;21:1212–23.
12. Endo Y, Sakai R, Ouchi M, et al. Virus-mediated oncolysis induces danger signal and stimulates cytotoxic T-lymphocyte activity via proteasome activator upregulation. Oncogene. 2008;27:2375–81.
13. Moehler M, Zeidler M, Schede J, et al. Oncolytic parvovirus H1 induces release of heat-shock protein HSP72 in susceptible human tumor cells but may not affect primary immune cells. Cancer Gene Ther. 2003;10:477–80.
14. Grekova S, Aprahamian M, Giese N, et al. Immune cells participate in the oncosuppressive activity of parvovirus H-1PV and are activated as a result of their abortive infection with this agent. Cancer Biol Ther. 2010;10:1280–9.
15. Grekova SP, Raykov Z, Zawatzky R, et al. Activation of a glioma-specific immune response by oncolytic parvovirus minute virus of mice infection. Cancer Gene Ther. 2012;19:468–75.

16. Radic M, Marion T, Monestier M. Nucleosomes are exposed at the cell surface in apoptosis. J Immunol. 2004;172:6692–700.
17. Uratsuji H, Tada Y, Kawashima T, et al. P2Y6 receptor signaling pathway mediates inflammatory responses induced by monosodium urate crystals. J Immunol. 2012;188:436–44.
18. Elankumaran S, Rockemann D, Samal SK. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. J Virol. 2006;80:7522–34.
19. Meng C, Zhou Z, Jiang K, Yu S, Jia L, Wu Y, et al. Newcastle disease virus triggers autophagy in U251 glioma cells to enhance virus replication. Arch Virol. 2012;157:1011–8.
20. Washburn B, Schirrmacher V. Human tumor cell infection by Newcastle disease virus leads to upregulation of HLA and cell adhesion molecules and to induction of interferons, chemokines and finally apoptosis. Int J Oncol. 2002;21:85–93.
21. Fournier P, Arnold A, Wilden H, Schirrmacher V. Newcastle disease virus induces pro-inflammatory conditions and type I interferon for counter-acting Treg activity. Int J Oncol. 2012;40:840–50.
22. Zamarin D, Holmgaard RB, Subudhi SK, et al. Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. Sci Transl Med. 2014;6:226–32.
23. Errington F, Steele L, Prestwich R, et al. Reovirus activates human dendritic cells to promote innate antitumor immunity. J Immunol. 2008;180:6018–26.
24. Diaconu I, Cerullo V, Hirvinen ML, et al. Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. Cancer Res. 2012;72:2327–38.
25. Workenhe ST, Simmons G, Pol JG, et al. Immunogenic HSV-mediated oncolysis shapes the antitumor immune response and contributes to therapeutic efficacy. Mol Ther. 2014;22:123–31.
26. Borde C, Barnay-Verdier S, Gaillard C, et al. Stepwise release of biologically active HMGB1 during HSV-2 infection. PLoS One. 2011;6:e16145. https://doi.org/10.1371/journal.pone.0016145.
27. Workenhe ST, Pol JG, Lichty BD, et al. Combining oncolytic HSV-1 with immunogenic cell death-inducing drug mitoxantrone breaks cancer immune tolerance and improves therapeutic efficacy. Cancer Immunol Res. 2013;1:309–19.
28. Worschech A, Haddad D, Stroncek DF, et al. The immunological aspects of poxvirus oncolytic therapy. Cancer Immunol Immunother. 2009;58(9):1355–62.
29. Prestwich RJ, Errington F, Diaz RM, et al. The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon. Hum Gene Ther. 2009;20(10):1119–32.
30. Bartlett DL, Liu Z, Sathaiah M, et al. Oncolytic viruses as therapeutic cancer vaccines. Mol Cancer. 2013;12:103.
31. Kim JH, Oh JY, Park BH, et al. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. Mol Ther. 2006;14(3):361–70.
32. Thorne SH. Immunotherapeutic potential of oncolytic vaccinia virus. Immunol Res. 2011;50(23):286–93.
33. Kirn DH, Wang Y, Le Boeuf F, et al. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. PLoS Med. 2007;4(12):2001–12.
34. Grigg P, Titong A, Jones LA, et al. Safety mechanism assisted by the repressor of tetracycline (SMART) vaccinia virus vectors for vaccines and therapeutics. Proc Natl Acad Sci U S A. 2013;110(38):15407–12.
35. Chen H, Sampath P, Hou W, Thorne SH. Regulating Cytokine Function Enhances Safety and Activity of Genetic Cancer Therapies. Mol Ther. 2013;21(1):167–74.
36. Li J, O'Malley M, Urban J, et al. Chemokine expression from oncolytic vaccinia virus enhances vaccine therapies of cancer. Mol Ther. 2011;19(4):650–7.
37. Wang LC, Lynn RC, Cheng G, et al. Treating tumors with a vaccinia virus expressing IFN-β illustrates the complex relationships between oncolytic ability and immunogenicity. Mol Ther. 2012;20(4):736–48.
38. Cerullo V, Diaconu I, Romano V, et al. An oncolytic adenovirus enhanced for toll-like receptor 9 stimulation increases antitumor immune responses and tumor clearance. Mol Ther. 2012;20(11):2076–86.
39. Albelda SM, Thorne SH. Giving oncolytic vaccinia virus more BiTE. Mol Ther. 2014;22(1):6–8.
40. Gil M, Komorowski MP, Seshadri M, et al. CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer-initiating cells. J Immunol. 2014;193(10):5327–37.
41. Dias JD, Hemminki O, Diaconu I, et al. Targeted cancer immunotherapy with oncolytic adenovirus coding for a fully human monoclonal antibody specific for CTLA-4. Gene Ther. 2012;19(10):988–98.
42. Rojas JJ, Sampath P, Hou W, Thorne SH. Defining effective combinations of immune checkpoint blockade and oncolytic virotherapy. Clin Cancer Res. 2015;21(24):5543–51.
43. Wali A, Strayer DS. Infection with vaccinia virus alters regulation of cell cycle progression. DNA Cell Biol. 1999;18:837–43.
44. Ottolino-Perry, K., Acuna, SA, Angarita, FA, et al. Oncolytic vaccinia virus synergizes with irinotecan in colorectal cancer. Mol Oncol. 2015;9(8):1539–52. https://doi.org/10.1016/j.molonc.2015.04.009.
45. Huang B, Sikorski R, Kirn DH, Thorne SH. Synergistic anti-tumor effects between oncolytic vaccinia virus and paclitaxel are mediated by the IFN response and HMGB1. Gene Ther. 2011;18:164–72.
46. Nishio N, Diaconu I, Liu H, et al. Armed oncolytic virus enhances immune functions of chimeric antigen receptor-modified T cells in solid tumors. Cancer Res. 2014;74(18):5195–205.

47. Melcher A, Paroto K, Rooney CM, Bell JC. Thunder and lightening: Immunotherapy and oncolytic viruses collide. Mol Ther. 2011;19(6):1008–16.
48. Sobol PT, Boudreau JE, Stephenson K, et al. Adaptive antiviral immunity is a determinant of the therapeutic success of oncolytic virotherapy. Mol Ther. 2011;19:335–44.
49. Benencia F, Courrèges MC, Fraser NW, Coukos G. Herpes virus oncolytic therapy reverses tumor immune dysfunction and facilitates tumor antigen presentation. Cancer Biol Ther. 2008;7:1194–205.
50. Benencia F, Courrèges MC, Conejo-García JR, et al. HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. Mol Ther. 2005;12:789–802.
51. Todo T, Martuza RL, Rabkin SD, Johnson PA. Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. Proc Natl Acad Sci U S A. 2001;98:6396–401.
52. Li H, Dutuor A, Tao L, et al. Virotherapy with a type 2 herpes simplex virus-derived oncolytic virus induces potent antitumor immunity against neuroblastoma. Clin Cancer Res. 2007;13:316–22.
53. Li H, Dutuor A, Fu X, Zhang X. Induction of strong antitumor immunity by an HSV-2-based oncolytic virus in a murine mammary tumor model. J Gene Med. 2007;9:161–9.
54. Endo T, Toda M, Watanabe M, Iizuka Y, Kubota T, Kitajima M, et al. In situ cancer vaccination with a replication-conditional HSV for the treatment of liver metastasis of colon cancer. Cancer Gene Ther. 2002;9:142–8.
55. Toda M, Rabkin SD, Kojima H, Martuza RL. Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity. Hum Gene Ther. 1999;10:385–93.
56. Toda M, Iizuka Y, Kawase T, Uyemura K, Kawakami Y. Immuno-viral therapy of brain tumors by combination of viral therapy with cancer vaccination using a replication-conditional HSV. Cancer Gene Ther. 2002;9:356–64.
57. Hu JC, Coffin RS, Davis CJ, et al. A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. Clin Cancer Res. 2006;12:6737–47.
58. Harrington KJ, Hingorani M, Tanay MA, et al. Phase I/II study of oncolytic HSV GM-CSF in combination with radiotherapy and cisplatin in untreated stage III/IV squamous cell cancer of the head and neck. Clin Cancer Res. 2010;16(15):4005.
59. Prestwich RJ, Ilett EJ, Errington F, et al. Immune-mediated antitumor activity of reovirus is required for therapy and is independent of direct viral oncolysis and replication. Clin Cancer Res. 2009;15:4374–81.
60. White CL, Twigger KR, Vidal L, et al. Characterization of the adaptive and innate immune response to intravenous oncolytic reovirus (Dearing type 3) during a phase I clinical trial. Gene Ther. 2008;15:911–20.
61. Prestwich RJ, Errington F, Steele LP, et al. Reciprocal human dendritic cell-natural killer cell interactions induce antitumor activity following tumor cell infection by oncolytic reovirus. J Immunol. 2009;183:4312–21.
62. Gauvrit A, Brandler S, Sapede-Peroz C, et al. Measles virus induces oncolysis of mesothelioma cells and allows dendritic cells to cross-prime tumor-specific CD8 response. Cancer Res. 2008;68:4882–92.
63. Li H, Peng KW, Dingli D, et al. Oncolytic measles viruses encoding interferon beta and the thyroidal sodium iodide symporter gene for mesothelioma virotherapy. Cancer Gene Ther. 2010;17(8):550.
64. Heinzerling L, Künzi V, Oberholzer PA, et al. Oncolytic measles virus in cutaneous T-cell lymphomas mounts antitumor immune responses in vivo and targets interferon-resistant tumor cells. Blood. 2005;106:2287–94.
65. Gürlevik E, Woller N, Strüver N, et al. Selectivity of oncolytic viral replication prevents antiviral immune response and toxicity, but does not improve antitumoral immunity. Mol Ther. 2010;18:1972–82.
66. Bristol JA, Zhu M, Ji H, et al. In vitro and in vivo activities of an oncolytic adenoviral vector designed to express GM-CSF. Mol Ther. 2003;7:755–64.
67. Cerullo V, Pesonen S, Diaconu I, et al. Oncolytic adenovirus coding for granulocyte macrophage colony-stimulating factor induces antitumoral immunity in cancer patients. Cancer Res. 2010;70:4297–309.
68. Mastrangelo MJ, Maguire HC Jr, Eisenlohr LC, et al. Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. Cancer Gene Ther. 1999;6:409–22.
69. Chuang CM, Monie A, Wu A, et al. Combination of viral oncolysis and tumor-specific immunity to control established tumors. Clin Cancer Res. 2009;15:4581–8.
70. Zhang YQ, Tsai YC, Monie A, et al. Enhancing the therapeutic effect against ovarian cancer through a combination of viral oncolysis and antigen-specific immunotherapy. Mol Ther. 2010;18:692–9.
71. Diaz RM, Galivo F, Kottke T, et al. Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. Cancer Res. 2007;67:2840–8.
72. Wongthida P, Diaz RM, Galivo F, et al. Type III IFN interleukin-28 mediates the antitumor efficacy of oncolytic virus VSV in immune-competent mouse models of cancer. Cancer Res. 2010;70:4539–49.
73. Galivo F, Diaz RM, Thanarajasingam U, et al. Interference of CD40L-mediated tumor immunotherapy by oncolytic vesicular stomatitis virus. Hum Gene Ther. 2010;21:439–50.
74. Willmon CL, Saloura V, Fridlender ZG, et al. Expression of IFN-beta enhances both efficacy and safety of oncolytic vesicular stomatitis virus for therapy of mesothelioma. Cancer Res. 2009;69:7713–20.
75. Bridle BW, Hanson S, Lichty BD. Combining oncolytic virotherapy and tumor vaccination. Cytokine Growth Factor Rev. 2010;21:143–8.

76. Qiao J, Kottke T, Willmon C, et al. Purging metastases in lymphoid organs using a combination of antigen-nonspecific adoptive T cell therapy, oncolytic virotherapy and immunotherapy. Nat Med. 2008;14:37–44.
77. Vigil A, Martinez O, Chua MA, García-Sastre A. Recombinant Newcastle disease virus as a vaccine vector for cancer therapy. Mol Ther. 2008;16:1883–90.
78. de Gruijl TD, Janssen AB, van Beusechem VW. Arming oncolytic viruses to leverage antitumor immunity. Expert Opin Biol Ther. 2015;15(7):959–71.
79. Breitbach CJ, Paterson JM, Lemay CG, et al. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. Mol Ther. 2007;15(9):1686–93.
80. Breitbach CJ, Arulanandam R, De Silva N, et al. Oncolytic vaccinia virus disrupts tumor-associated vasculature in humans. Cancer Res. 2013;73(4):1265–75.
81. Hou W, Chen H, Rojas J, et al. Oncolytic vaccinia virus demonstrates antiangiogenic effects mediated by targeting of VEGF. Int J Cancer. 2014;135(5):1238–46.
82. Choi IK, Shin H, Oh E, et al. Potent and long-term antiangiogenic efficacy mediated by FP3-expressing oncolytic adenovirus. Int J Cancer. 2015;137(9):2253–69.
83. McKee TD, Grandi P, Mok W, et al. Degradation of fibrillary collagen in a human melanoma xenograft improves the efficiency of an oncolytic herpes simplex virus vector. Cancer Res. 2006;66(5):2509–13.
84. Mok W, Boucher Y, Rk J. Matrix metalloproteinases-1 and -8 improve the distribution and efficacy of an oncolytic virus. Cancer Res. 2007;67(22):10664–8.
85. Guedan S, Rojas JJ, Gros A, et al. Hyaluronidase expression by an oncolytic adenovirus enhances its intratumoral spread and suppresses tumor growth. Mol Ther. 2010;18(7):1275–83.
86. Kim JH, Lee YS, Kim H, et al. Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy. J Natl Cancer Inst. 2006;98(20):1482–93.
87. Montel V, Kleeman J, Agarwal D, et al. Altered metastatic behavior of human breast cancer cells after experimental manipulation of matrix metalloproteinase 8 gene expression. Cancer Res. 2004;64(5):1687–94.
88. Ilkow CS, Marguerie M, Batenchuk C, et al. Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. Nat Med. 2015;21(5):530–6.
89. Kirn DH, Wang Y, Liang W, et al. Enhancing poxvirus oncolytic effects through increased spread and immune evasion. Cancer Res. 2008;68:2071–5.
90. Sampath P, Li J, Hou W, et al. Crosstalk between immune cell and oncolytic vaccinia therapy enhances tumor trafficking and antitumor effects. Mol Ther. 2013;21(3):620–8.

12 The Impact of Housing Temperature-Induced Chronic Stress on Preclinical Mouse Tumor Models and Therapeutic Responses: An Important Role for the Nervous System

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12.1 Introduction

The TME regulates tumor growth and response to therapy in many ways. Recently, it has been shown that tumors recruit both sympathetic and parasympathetic nerves which produce norepinephrine and acetylcholine in the TME, promoting tumorigeneses, invasion and metastasis [2, 3]. In addition to acting directly on tumor cells, norepinephrine (NE) can regulate the activity of immune cells. The regulation of immune cells is complex; in addition to the cytokines/chemokines released from other cells, they are also responsive to signals from the nervous system. In fact, both primary and secondary immune organs are densely innervated by fibers of the sympathetic nervous system [4] so that the major pathway by which the nervous system controls the immune system is by local release of the neurotransmitter NE from post-ganglionic sympathetic neurons in various immune organs [4, 5]. This pathway is activated during the sympathetic stress response and although in response to an acute stress, sympathetic activation of immune cells is beneficial, when this stress is chronic, there is much evidence that the sympathetic nervous system suppresses immune responses. How is this relevant to pre-clinical mouse models used for research? Lately, concern has been raised that experimental mice in standard housing conditions are "metabolically morbid" [6] and under constant cold stress [7–16]. Our group has observed different biological outcomes in preclinical mouse models of cancer and tumor immunity between mice that are cold-stressed and those in which cold stress is reduced, even though the core body temperature in both groups is the same. An incomplete recognition of these potential differences in experimental outcome could significantly limit the full potential of preclinical models of cancer and other diseases. Here, we will present an overview of this problem with specialfocus on how housing conditions subject laboratory mice to chronic cold stress, resulting in elevated norepinephrine levels, and the suppressive effects of this increased adrenergic signaling on the anti-tumor immune response and tumor response to therapy.

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12.2 Metabolic Effects of "Shoe-Box" Caging on Experimental Mice

Mice have become the most widely used models for studying human/patient biological processes including development, metabolism, normal physiology and disease. "The Guide for the Care and Use of Laboratory Animals" [1] provides detailed guidelines for all aspects of laboratory mouse housing and is followed by research institutions internationally. Comprehensive parameters are provided for all environmental factors including temperature, humidity, ventilation, food, lighting, noise and cage size/ housing density as well as recommendations for enrichment strategies that can reduce stress. It is stated several times in different places that variations in these microenvironmental factors could affect behavior, physiology (reproduction), phenotype and, possibly, experimental outcomes. These recommendations are based on data from publications and experts, being a synthesis of all empirical aspects of operating the animal facilities and are revised periodically (the last edition was in 2011). In practice, animal care personnel handle implementation of these regulations and therefore, the majority of scientists do not take these environmental variables into consideration when designing experiments and analyzing experimental outcomes. They assume the mice are healthy and the outcomes of experiments routinely conducted under these mandated conditions will provide accurate and reproducible baseline data. However, recently, a growing number of investigators have raised significant concerns that this may not be the case.

The first contemporary warning was published by Martin et al., a group at the National Institute of Aging [6]. These investigators raised the alarm by pointing out that, contrary to these presumptions of health, "mice under standard conditions are sedentary, overfed, obese, glucose intolerant" and hypertensive. More importantly, they warned that the biological status of these mice likely "confounds data interpretation on outcomes of human studies". These standard control animals are also at higher risk for developing cancer, diabetes, renal failure and premature death than mice which have reduced food intake, exercise more and have a stimulating environment. In fact, reducing caloric intake can increase life span up to 40% and this is largely due to reduction in these diseases. In a genomic study, this group found significant differences in gene expression when rats on different diets were compared with standard vs lean controls, again emphasizing that the metabolic condition of the control animals has the capability of skewing the results of experiments. Furthermore, they discuss studies suggesting that the efficacy of drugs for treating metabolic, neurological and malignant disease may be more efficacious in mice housed under standard conditions than in more healthy mice, thus contributing to the failure of several drugs to recapitulate the success seen in preclinical models when these drugs are used with patients. They conclude that "The beneficial effects of some drugs in animal models might result from their effects on processes associated with an unhealthy lifestyle (increased oxidative stress, inflammation, insulin resistance, etc.) rather than a specific effect of the drug on the disease process" and propose that experiments should be designed to include both sets of conditions rather than just the one standard one. Other biological concerns about laboratory mice are also being raised. For instance, the immune system which develops in laboratory mice housed under extremely clean conditions is significantly different than that of feral mice which more closely resembles the immune system of humans and this can be altered by exposure to feral mice indicating another environmental variable that can significantly affect experimental outcomes [17].

12.3 Effects of Housing Temperature on Mice: Differences Between Mice at ST and TT

Whereas, Martin et al. [6] were concerned about the metabolic effects of a sedentary, obesogenic lifestyle, a housing parameter which we and others have recently become particularly con-

cerned about is the ambient temperature at which mice are housed. Biologists have studied thermoregulation in mice for years, and are well aware of the unique aspects of their physiology with regard to body temperature control, but it is now becoming clear that the variable of housing temperature has a significant impact on many aspects of mouse physiology which directly affect experimental outcomes [7, 12–14, 18–39]. This is because mice have a large surface to volume ratio and therefore lose heat more quickly in cool temperatures [12, 18]. Of particular concern is the fact that the temperature range recommended by the Guide [1], between 22 and 26 °C, is below the resting metabolic thermoneutral zone of the mouse [18]. This thermoneutral zone is defined as the ambient temperature range at which a stable core temperature is achieved by "adjustments in insulation, posture, and skin blood flow" and is 30–32 °C for mice [18, 40]. In other words, the animal is able to maintain core temperature by basal metabolism alone without activating physiological, thermoregulatory processes for heat production or heat loss which require large amounts of energy. It has been shown that mice, when given a choice, will choose an ambient temperature of 30.9 °C from a range of 18 to 34 °C [41]. Gordon states that the Lower Critical Temperature (LCT) has been extensively studied, is approximately 30 °C and is the point at which mice become susceptible to cold stress. Although mice will select a temperature a few degrees lower during their active, nocturnal period, their core temperature is maintained by heat produced through increased activity. "The Guide" acknowledges that the recommended temperature is lower than thermoneutrality, but it specifically recommends that housing temperatures be kept below the animal's lower critical temperature to avoid heat stress. To compensate, mice often huddle together and although "The Guide" suggests that mice can be given nesting materials and shelters, see also [42, 43], this is often not done and, therefore there is great potential for laboratory mice to be subjected to chronic cold stress. The reason this situation has overall not worried investigators, though, is because mice are able to effectively thermoregulate and the core temperatures at standard temperatures and thermoneutral temperatures are not significantly different [41].

How does this chronic cold stress affect laboratory mice and the outcomes of experiments? There are clear differences in the metabolism of mice housed at standard temperature (ST—22–26 °C) and those housed at thermoneutral temperatures (TT - 30–32 °C) as reviewed by Overton [12]. Although the core body temperatures of mice may vary by 2° during the course of a day, in concert with circadian rhythm and activity level, the core temperature is similar between mice housed at ST and TT [18, 24, 41]. Therefore, the physiological differences are related to increased metabolism and thermogenesis at ST which are required to defend core body temperature. Uchida et al. [34] conducted a study comparing glucose homeostasis in C57BL/6 mice housed at 25 °C vs. 20 °C (instead of the 4 °C which is commonly used to study cold stress). Interestingly, there was no difference in blood glucose or plasma insulin levels in mice, however fasting levels differed significantly with lower insulin and higher glucose levels at the lower temperature. This correlated with an impaired response in a glucose tolerance test. These authors found a significant impairment of glucose-induced insulin secretion (comparable to that seen at 4 °C), which resulted in elevated glucose levels (unlike the response at 4 °C). Additionally, when 20 °C mice were moved back to 25 °C, they reverted to the normal phenotype. They also found that the 20 °C mice had elevated plasma NE but not Epi. NE is known to inhibit insulin secretion from the pancreatic islets [44] and is the stress hormone which drives thermogenesis to maintain body temperature. In measuring NE turnover in various organs, Teramura and colleagues found that the rate of NE turnover and upregulation of UCP-1 in BAT was similar whether the mice were at 4 °C or at 23 °C [45] confirming that physiologically, the degree of cold stress experienced at ST is comparable to that experienced in classic "cold stress" experiments. Comparison of skin temperatures at ST and TT found lower skin temperature in the 20 °C mice while confirming there was no difference between the core temperatures in the two groups.

Lastly, these authors found that changes in the cool mice related to lipid metabolism and fat storage. Clearly, differences in energy metabolism occur at these two sub-thermoneutral temperatures and it would be interesting to compare these results with those from mice housed at TT. These metabolic differences are mirrored by differences in heart rate and blood pressure. Swoap and colleagues have shown that as the ambient housing temperature decreases, heart rate and blood pressure significantly increase [30, 31]. The resting heart rate at 22 °C is 550–600 bpm while at 30 °C, it is reduced to 350–400 bpm [12, 30]. In fact, although it was thought that the autonomic control of heart rate differed between mice and men, these authors concluded that when the autonomic control of heart rate is studied in animals at TT, it is controlled by parasympathetic vagal input in a manner similar to humans, rather than by sympathetic inputs that prevail at ST. These discrepancies call attention to the need to consider ambient temperature when conducting cardiovascular experiments in mice and relating results to humans.

The validity of these warnings about consideration of ambient housing temperature in assessing results from mouse models is clearly demonstrated in experiments with the UCP-1 knock out mouse. UCP-1 is the "uncoupling protein-1" of the mitochondrial inner membrane in brown adipose tissue (BAT) which mediates a thermogenic proton leak, uncoupling oxidative phosphorylation from ATP production and thereby dissipating energy and generating heat by non-shivering thermogenesis in BAT. In one experiment a UCP-1 knock-out mouse developed the expected deficits in non-shivering thermogenesis, but it did not become obese as was expected [46, 47]. This cast doubt on the involvement of UCP-1 in bioenergetics and the usefulness of targeting it to combat obesity. However, these mice were housed at ST and more recently, several groups have shown that these mice do become obese if they are housed at TT [7, 47–49] suggesting that in UCP-1$^{-/-}$ mice, alternative pathways must exist for thermogenesis which burns calories to generate body heat and prevents obesity at ST. A commentary accompanying the Feldman paper reinforced the fact that ambient temperature is a critical variable to consider when assessing the effects of different genotypes in metabolic research [9]. Interestingly, a recent study of the anti-obesity efficacy of 2,4-dinitrophenol (DNP, a chemical uncoupler) concluded that in experiments conducted at TT, DNP treatment decreased body fat by 26% and improved glucose tolerance, but no beneficial effects were observed at ST [50]. This group also tested the β3-adrenergic agonist, CL316243, to determine whether pharmacological activation of brown adipose tissue (which is the major tissue expressing β3-AR) could result in weight loss; again they observed beneficial effects at TT, but not at ST [14]. Ravussin, commenting on the Feldman paper, takes the position that "ambient temperature clearly affects phenotypes related to energy homeostasis in rodents" [51]. Related to the increased metabolism seen in mice at ST vs TT, Jun et al. found that mice at ST had increased lipid uptake in BAT, heart, and lungs and that hypoxia, by suppressing metabolism, caused increased levels of triglycerides in the plasma; however, when mice were exposed to hypoxic conditions at TT, no differences in plasma tryglycerides were detected [52]. One study found differences in the effects of energy restriction on the disease progression of lymphoma over the course of the lives of C57BL/6 mice fed an energy restricted diet at either ST or TT. At ST these mice lived significantly longer than either control mice at ST or mice on an energy restricted diet at TT [53].

12.4 Effects of Housing Temperature on Mouse Models of Infection

There are many studies reporting the deleterious effects of stressors such as restraint and social isolation on the immune response in infection models at standard room temperatures [54, 55]. However, the immune response is also profoundly affected by housing temperature. One hallmark of an effective immune response is the "fever" response in which the set-point of the

core temperature is elevated and the organism recruits thermogenic mechanisms to raise the body temperature. It was thought that mice do not generate fevers as humans do, but it was recently shown that although mice fail to develop a fever following injection of LPS at ST, fevers are generated when they are challenged at thermoneutrality [27]. Are other aspects of the immune response affected by housing temperature? It is known that immune organs are heavily innervated by sympathetic nerve fibers [4] while immune cells express adrenergic receptors, primarily β2-ARs, in a cell type/subset specific pattern [56]. In terms of the overall effect of stress on the immune response, the effect depends on whether the stress is acute (of short duration) or chronic. During the "fight or flight" acute stress response, the immune response is mobilized by sympathetic signaling. This has been hypothesized to be a key evolutionary mechanism by which animals survive stressful challenges which likely would involve injury or exposure to pathogens [57]. Generally speaking, acute stress is "beneficial", mobilizing immune cells to the site and promoting their protective function, while, in contrast, chronic stress is "detrimental" and leads to systemic immunosuppression [54, 57]. As discussed above, laboratory mice housed at ST are chronically cold stressed and thus have elevated NE levels associated with thermogenesis. A relationship between room temperature and the course of pathogenic infections was reported 70 years ago when Moragues noticed that dramatic differences in disease progression, severity and survival following infection with murine typhus rickettsiae correlated with seasonal differences in room temperature, in that all the mice died of disease when the room temperature was approximately 18–23 °C while few deaths occurred when the room was 29–37 °C [58]. Similarly, mice infected with Coe virus had markedly better survival when held at 36 °C vs. 25 °C [59]. A more recent study emphasizes the fact that normal mice housed at 22, 26 or 30 °C all are able to maintain a normal core temperature, which as expected, cycles between 35.5 and 37.5 °C with circadian rhythm [23]. In this study, mice were infected with influenza virus and housed at the three temperatures; the mice at 30 °C showed less "sick behavior" (sleep disturbances, reduced locomotion, inflammatory cytokines) than the mice at the lower temperatures [23]. These studies serve to illustrate the detrimental effects of ST on immune responses to pathogens. Interestingly, there are reports that β-adrenergic blockade (i.e., with propranolol) is able to improve outcomes in viral [60] and parasitic [61, 62] infections in mice housed at ST. This suggests that blocking NE β-adrenergic signaling in these models is the underlying mechanism of the beneficial effect. A study by Grebe et al. [63] in C57/BL6 mice infected with influenza A virus showed that administration of a β2-AR antagonist enhanced the anti-viral responses of CD8+ T-cells (IFNγ expression). Again, it would be interesting to compare the benefit of β2-AR blockade in experiments such as these done at ST with experiments done at TT to determine whether there would be any benefit when NE levels are ameliorated by thermoneutral housing.

12.5 Thermoneutrality Vs. Hyperthermia Treatment (Thermal Therapy)

In another early study, the effect of ambient temperatures of 20–22 °C vs. 35 °C on rabies infected mice was investigated and it was found that the survival rate of mice housed at 35 °C was significantly higher [64]. However, the core temperatures of mice housed at 35 °C were higher than normal (39.5 °C) so that these mice were actually experiencing hyperthermia resulting from the very warm ambient temperatures in which they were housed. Our lab, and many others, has shown that the stress of a short mild hyperthermia treatment can boost immune responses, including anti-tumor activity [65]. The distinction between the thermal/physiological effects of housing mice at thermoneutrality (30–32 °C) vrs. exposing mice to temperatures high enough to raise the core temperature is an important one. At TT, mice are able to maintain a normal body temperature of ~37 °C [23, 41] via basal metabolism and do not need to expend energy to warm or

cool themselves. On the other hand, the goal of many preclinical studies of"thermal therapy" or "hyperthermia" is to expose mice to a temperature high enough to raise the tumor temperature, or core body temperature several degrees, which has been observed to alter the tumor microenvironment, reduce interstitial fluid pressure [66], improve efficacy of radiation and chemotherapy [67], and may trigger various molecular thermostats that are similar to those activated by a fever, helping to boost the immune system [65, 68].

In this active field of hyperthermia research, investigators are well aware of the beneficial effect that short exposures to a warm environmental temperature can have on immune cell activity. However, even in this research field, no studies have examined whether these differences result from the fact that control mice are cold stressed compared to mice in which core temperatures are elevated. It is clear that even research designed to determine the impact of temperature shifts locally or systemically in terms of improving cancer treatment may be (unbeknownst to the investigators) influenced by cold stress in control groups. In this regard, it will be interesting to see the degree of beneficial effects of hyperthermia treatments in mice housed mice at TT.

12.6 Adrenergic Signaling and Tumor Growth at ST

How does the fact that mice at ST are chronically cold-stressed and have elevated NE levels compared to mice at TT impact tumor growth? β-adrenergic receptors (β-ARs) are found on immune cells and are on many tumor cells [69]. Emerging evidence from experiments conducted at ST links catecholamines to tumor progression and this topic has been recently reviewed [70–72]. Evidence for the pro-tumorigenic role of adrenergic signaling comes from both epidemiological studies and experiments with preclinical mouse models. Retrospective analyses by several groups in different tumor types support the idea that patients who were taking β-adrenergic antagonists (β-blockers) for non-cancer indications had reduced disease progression and/or better survival in breast [73–75], ovarian [76, 77], melanoma [78], lung [79], prostate [80], pancreatic [81], cancers. However a few studies have not found benefit [82–85] Interestingly, Lutgendorf et al. found, in ovarian cancer patients, that higher NE levels in the tumors correlated with more advanced disease and the degree of social stress experienced by the patients [86]. Experimental evidence showed that adrenergic signaling induced migratory behavior in tumor cells in vitro (e.g., SW480 human colon carcinoma cells) which could be inhibited by β2-AR blockade [87] and that while treatment of mice with NE increased the development of lymph node metastases (PC-3 prostate cancer cells), this could also be prevented by β2-AR blockade with propranolol [88]. Le et al. [89] have more recently investigated this phenomenon and found that adrenergic signaling recruited inflammatory macrophages to the TME and these induced VEGFC expression by tumors, which leads to remodeling of lymphatics and metastatic spread of breast cancer in a mouse model. In a retrospective patient study, this group found evidence that β-blockers significantly reduced lymph node metastases in patients [89]. In a model of social stress, Hasegawa showed that stress enhanced fibrosarcoma growth promotion could be inhibited with propranolol [90]. β-adrenergic signaling induces tumor cell proliferation [91, 92], invasion [93, 94], protection from anoikis [95], metastasis [94, 96, 97] and changes in the tumor microenvironment such as angiogenesis [98–100]. Thaker et al. used restraint stress or social isolation to show that chronic stress increases catecholamine (NE and epinephrine) levels, increases VEGF and vascularization and increases tumor growth [101]. These effects could be mimicked by treatments with specific β2-AR agonists and reversed by β-AR blockers. Epinephrine also protects prostate cancer cells from apoptosis [102] through phosphorylation of BAD. A role for this anti-apoptotic pathway was demonstrated in prostate cancer models in which restraint stress protected xenografts from apoptosis induced by a PI3K inhibitor by induction of BAD phosphorylation and again, this effect could be blocked by a

β2-AR specific antagonist [103]. There are many other examples of stress induced tumor growth (e.g., [104]). The anatomical basis for adrenergic signaling in the tumor microenvironment was clarified by the work of Magnon and Frenette who were able to visualize both sympathetic and parasympathetic fibers in prostate tumors in mice and show that sympathectomy (preventing the release of NE in the TME) prevented early aspects of cancer development while parasympathetic signaling promoted invasion and metastasis [2]. These authors thus demonstrated that tumors actively recruit autonomic innervation by neurogenesis to support growth in a process akin to angiogenesis (see also [105]). A recent study investigated the possible benefits of combining propranolol and chemotherapy. In a mouse breast cancer model (MDA-MB-231 human cell line in nude mice), Pasquier et al. found that at the very effective doses of chemotherapy used, propranolol did not significantly improve the anti-tumor efficacy, however the median survival was significantly enhanced [106]. These authors also demonstrated that propranolol did, however, enhance the anti-angiogenic effects of chemotherapy in vitro. Together, the epidemiological studies suggesting clinical benefit of β-blockers to cancer patients and the compelling pre-clinical data defining the tumor promoting effects of adrenergic signaling provide enthusiasm and a strong rationale for testing the anti-cancer efficacy of β-blockers in clinical trials in combination with other therapies.

12.7 The Anti-Tumor Immune Response and Response to Therapeutics Are Significantly Improved by Housing at Thermoneutrality or β-Adrenergic Receptor Blockade at Standard Housing Temperatures

In investigating the effects of cold-stress, researchers have taken mice acclimated to standard housing temperatures and subjected them to much lower temperatures (4 °C). However, it is clear from the studies discussed above, that mice at ST are already living with chronic cold stress and the turnover of NE in mice at 4 °C and 22 °C is not significantly different [45]. Therefore, compared to TT, all studies of tumor growth have been conducted under some degree of cold stress and studies of the effect of any stress on tumor growth are actually studies of exacerbated stress.

How is tumor growth affected if adrenergic cold stress in mice is alleviated by housing mice at thermoneutrality? We have previously reported that tumor growth in several syngeneic murine tumor models is significantly reduced when tumor bearing mice are housed at 30 °C instead of 22 °C [24]. In these experiments, mice were acclimated to ST or TT for 1–3 weeks prior to tumor implantation; we also used *moderate numbers of tumor cells* to allow for development of an effective anti-tumor immune response rather than the higher numbers that are often used to insure rapid tumor growth. These models included 4T1 mammary tumors and CT26 colon adenocarcinomas in BALB/c mice and B16.F10 melanoma and Pan02 in C57BL/6 mice, as well as MCA carcinogen induced tumors in BALB/c mice. Additionally, we observed that spontaneous lung metastases of 4T1 to the lungs were also significantly reduced at TT. When these same tumor models were grown in immunodeficient SCID or nude mice, no difference in growth occurred. This points to a critical role for the adaptive immune response in this improved tumor control at TT and this is confirmed by experiments in which depletion of CD8+ T-cells resulted in loss of the improved tumor control at TT. Additional analysis of several immune cell populations involved in the anti-tumor immune response revealed dramatic differences in mice at ST and TT. At TT, significantly greater numbers of CD8+ T-cells were present in 4T1 and Ct26 tumors (as assessed by both IHC and flow cytometry) and staining with pentamers recognizing the H-2L^d/gp70 peptide antigen of ct26 tumors, showed that increased numbers of antigen specific T-cells were found in both the tumor and tumor draining lymph node of mice housed at TT compared to ST. Correlating with their

increased presence, T-cell activation was significantly higher at TT as judged by CD69, IFNγ and Glut-1 expression. Conversely, there were fewer immunosuppressive cells at TT; the numbers of Tregs (FoxP3+ cells) and myeloid derived suppressor cells (MDSC: CD11b+GR-1+) were significantly decreased in the tumor (T-regs) and spleen (MDSC) at TT. It is interesting that others have reported a trend to higher numbers of T-regs in tumors of mice (at ST) subjected to restraint/noise stress [104] . These differences in the anti-tumor immune response at TT vs ST are not the result of differences in body temperature since the core temperatures of these tumor-bearing mice maintained at 22 °C or 30 °C were normothermic for several weeks (~28 days). Only as tumor burden became significantly higher at ST than at TT did the core temperature fall in mice at ST, while mice at TT continued to maintain a normal temperature, reflecting the smaller tumor burden. In addition to $CD8^{+}$T-cells and immune suppressor cells, in a separate study we also examined how housing temperature might impact antigen presenting cells; we investigated the function of dendritic cells (DCs), which are involved in T-cell activation. Results of these experiments suggest that DC's from mice at TT (with 4T1 tumors) are better able to induce T-cell proliferation than are DC's from mice at ST [107] suggesting another aspect of the anti-tumor immune response which is at least partially suppressed by housing mice at ST. Altogether, these findings point out that at ST, DC's are less able to stimulate T-cells, and that the balance of anti-tumor ($CD8^{+}$T-cells) and pro-tumor cells (T-regs, MDSC) is shifted to significantly suppress the anti-tumor immune response. Therefore, these data demonstrate that results from experiments conducted at ST are giving us a biased view of the activity and capabilities of the anti-tumor immune response. Thus, we strongly believe that temperature should always be considered and reported in experiments with an immune component and that investigators could gain important information by repeating selected experiments at TT rather than relying solely on the data from experiments conducted at ST only.

With regard to the direct tumor growth-promoting effects of adrenergic signaling on tumor cells, we have found that at ST (compared to TT) the level of NE is significantly higher in the plasma of non-tumor-bearing and in the plasma and tumors of pancreatic tumor-bearing mice [21]. It has previously been reported that the catecholamine levels are higher in the tissues of tumor-bearing mice subjected to restraint stress in experiments conducted at ST [101]. Interestingly, given the roles of epinephrine and corticosterone in certain types of stress, we found that the levels of these stress hormones are not significantly different at ST and TT. Because it has been reported that adrenergic signaling increases levels of anti-apoptotic molecules (phosphorylated BAD, [102]) and protects tumor cells from apoptosis [95, 102], we investigated the effect of ST vs TT on apoptotic signaling and response to therapy [21]. We found that treatment of murine and human pancreatic adenocarcinoma cell lines in vitro with a β-AR agonist (isoproterenol) increased expression of anti-apoptotic molecules including Bcl-Xl, Bxl-2, Mcl-1 and phosphorylated BAD. The same differences in these anti-apoptotic molecules were seen in vivo in tumors when these cell lines were grown in SCID mice housed at ST vs TT. In SCID mice, as expected in the absence of the adaptive immune response, tumor growth at ST and TT was not significantly different. However, as suggested by the differences in expression of anti-apoptotic molecules, we found that tumors in mice housed at TT were significantly more sensitive to Apo2L/TRAIL, cisplatin and nab-paclitaxel (Abraxane) than tumors in mice at ST. Furthermore, tumors in mice at ST could be sensitized to these therapies by treating the mice with a β-adrenergic receptor antagonist (propranolol) which decreased the expression of these anti-apoptotic molecules [21]. These results show, for the first time, that the degree of stress experienced by mice housed at ST is sufficient to directly impact the outcome of experiments testing the efficacy of therapeutics and, for that reason, it is critical to also conduct these experiments at TT so that the results can be compared.

As these therapeutic studies indicated that giving β-blockers to mice housed at ST can overcome resistance to cytotoxic therapies and achieve responses comparable to those achieved at TT, we wondered whether propranolol could reverse immunosuppression at ST and similarly improve responses to immunotherapy. We recently have found that this is true [38]. Given to mice at ST, propranolol reverses immunosuppression increasing the frequency of $CD8^+$T cells with an effector phenotype and increasing the $CD8^+$ effector/ $CD4^+$ T-reg ratio in the TME. The ability of propranolol to reduce suppressive cells in the TME and increase numbers of cytotoxic T-cells was also recently reported to occur in a spontaneous mouse melanoma model [108]. We have found that these changes in the immune contexture in the tumor (with either housing at TT or propranolol administration at ST) lead to significantly improved response to anti-PD-1 checkpoint inhibition [38]. These results support the development of clinical trials to explore using this combination strategy to benefit those patients who are not currently responding to checkpoint inhibitor therapies.

12.8 Mechanisms by Which Chronic Adrenergic Signaling Suppresses the Cellular Immune Response at ST

Immune cells express adrenergic receptors- primarily β2-AR, although they may express other receptors and the pattern is cell specific [56]. Anti-tumor immunity is primarily dependent on tumor cell killing by cytotoxic CD8+ lymphocytes (CTL), therefore boosting the efficacy of these cells against cancer is the focus of a spectrum of immunotherapies, for example, Chimeric Antigen Receptor T-cells are CD8+ T-cells taken from a cancer patient, engineered to express specific T-cell receptors (CAR-T-cells) which are chimeric in that they have intracellular domains that initiate T-cell activation. These cells are then expanded in vitro, and given back to the patient as adoptive T-cell therapy. Another exciting approach designed to improve T-cell anti-tumor activity is checkpoint inhibition. Checkpoint inhibitors work by modulating the activity of ligands/receptors (e.g., CTLA-4, PD-1/PD-L1) whose natural function is to keep the activity of these cells in check. Given the central, critical role of CTL (cytotoxic T lymphocytes, $CD8^+$T cells) in the anti-tumor response and the growing efforts to maximize their efficacy, how does chronic adrenergic stress contribute to the suppression of these cells? As mentioned above, lymphoid organs are profusely innervated by sympathetic neurons, especially in T-cell areas [5], and Elenkov et al. reported that stress hormones act on antigen presenting cells to promote a Th2 response (favoring B cells/plasma cell maturation and antibody production) to protect against extracellular pathogens [109, 110]. At the same time, in response to β2-AR stimulation, DC production of IL-12 is inhibited and this suppresses Th1 development which would support CTL development [111] while production of anti-inflammatory cytokines Il-10 and Il-6 is upregulated [110]. Another aspect of this skewing to a Th2 response is the fact that β2-AR receptors are expressed on Th1 CD4+ helper cells, but not on Th2 cells. Therefore, adrenergic signaling directly impacts cytokine production by Th1 cells (i.e., IL-12) but not by Th2 cells [112]. In experiments using a novel procedure for inducing stress in mice (exposure to stressful sound) bearing Ct26 tumors, there was a Th1 to Th2 shift as evidenced by decreased levels of IFNγ and increased Il-4 and this correlated with increased tumor growth [113]. In addition to NE production and release by sympathetic post-ganglionic neurons, immune cells also can produce catecholamines; T-cells, macrophage and neutrophils can synthesize and secrete catecholamines that act in an autocrine and paracrine way to modulate an immune response [56]. Nguyen et al. compared the production of catecholamines by adipose tissue associated macrophages at 4 °C, 22 °C and 30 °C and found that macrophage underwent alternative activation at the sub-thermoneutral temperatures. This was IL-4 (a Th2 cytokine) dependent and resulted in the increased production of both Epi and NE [114]. More recently, this

idea has been challenged by Fischer et al. who reported that alternatively activated macrophages do not produce NE [115]. It will be interesting however to determine whether tumor associated macrophages can produce NE and whether this contributes to higher intratumoral levels of NE at ST than at TT as this could be a second source of local NE production suppressing CTL in the tumor microenvironment. As discussed above, our group found that tumor-bearing mice had higher numbers of suppressor cells (Tregs and MDSC) at ST than at TT [24]. CD4+ T-regs express functional β2-ARs and adrenergic signaling increases cAMP and PKA dependent phosphorylation of the transcription factor CREB (cAMP response element binding protein) leading to increased suppressive function, including increased CTLA expression [116]. Jin et al. [117] looked at the effects of restraint stress on MDSC accumulation in bone marrow and found that chronic stress significantly increased the number of MDSC (CD11B+Gr1+; predominantly Ly6C-Ly6G+) and that these were immature neutrophils. This skewing of myelopoiesis by chronic restraint stress could be reversed with propranolol (but not by inhibition of glucocorticoids). Altogether, these data underscore the detrimental effects of chronic adrenergic stress which overall suppresses effector T-cell responses while promoting the development and activities of immune suppressor cells. This potential for mild, housing induced cold stress to inhibit immune responses has been recently reviewed by our group [11].

12.9 How Does Adrenergic Signaling Affect Patient Outcomes?

Going forward, it is important to understand how these observations on the effect(s) of adrenergic stress induced in pre-clinical mouse models can be related to the clinic in terms of treating patients and improving therapeutic outcomes. Patients can be highly stressed by a wide range of stressors (e.g., physical such as pain and psychological such as fear and isolation). One highly relevant study found that ovarian cancer patients who lacked social support had higher levels of NE and epinephrine than patients and that overall this was associated with advanced stage and higher grade tumors [86]. How does this stress affect patient outcome? There are now a number of retrospective, epidemiological reports strongly supporting the idea that patients who are taking β-blockers for hypertension or another indication have better outcomes overall (see Sect. 12.6 above). There are also retrospective reports that β-blockers can reduce the incidence of HCV-associated hepatocellular carcinoma [118] and improve responses to chemotherapy [119]). Thus the potential for these commonly prescribed and comparatively safe β-blockers to be repurposed to treat cancer patients is exciting, but the rationale must be validated in prospective, well-planned clinical trials.

Another way in which the pre-clinical data on cold-stress may have an impact relates to pre-clinical testing of therapies. It is possible, that under a range of conditions, some agents that appear ineffective in models may become effective (or show greater efficacy) when stress is reduced or blocked. These results could pave the way for combination therapies in clinical trials and/or allow lower doses to achieve efficacy thus reducing toxicity. It is also possible that toxicities that did not occur in pre-clinical studies and were therefore not predicted (e.g., autoimmunity with immunotherapies such as checkpoint inhibitors [120], could become apparent if experimental designs included stress reduction which reversed immunosuppression.

12.10 Other Forms of Stress Impacting Mice in Research Facilities

In light of the examples described above, it is a clear that we need to take the effect of stress into account when designing experiments in pre-clinical mouse models and interpreting the results. Our lab has focused on how housing temperature induced cold-stress skews experimental outcomes, but there are many other environmental variables that could also act as stress rheostats,

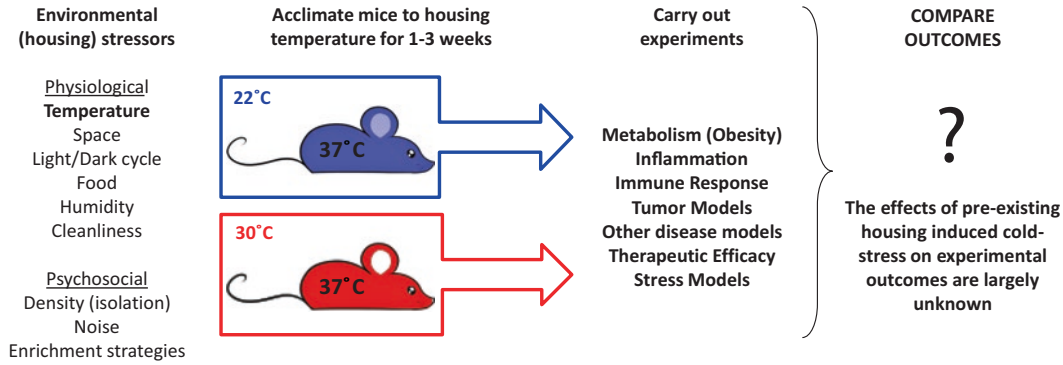

Fig. 12.1 Housing guidelines for experimental mice regulate many environmental factors which affect the physiology of mice used in pre-clinical experiments; variations in these parameters can create differing degrees of stress. In the case of temperature, mice housed at standard subthermoneutral housing temperatures (22 °C) are subjected to chronic cold-stress compared to mice housed at thermoneutral temperatures (30 °C) and, although the body temperatures in both cases are normal, cold-stressed mice have elevated levels of norepinephrine. Thus these mice have a pre-existing level stress which is biologically significant and the effects of this stress on different experimental models is largely unknown

increasing or decreasing the degree of adrenergic stress experienced by mouse disease models (see Fig. 12.1). Because the outcomes of pre-clinical mouse studies form the basis for understanding tumor biology, host responses and determining which therapies to take into clinical trials [121–124], it is critical that researchers are aware of these factors. One major problem that results from variability is irreproducibility [33, 121]. In two major studies by drug companies, Bayer [122] and Amgen [125] investigated the reproducibility of preclinical experiments and found that less than 25% and 11%, respectively, of the studies were able to be duplicated. Furthermore, a landmark study by Landis and colleagues was extremely critical of this lack of reproducibility and pointed to the general dearth of information on the "design, conduct and analysis of the experiments" [126]. These authors asserted that "a core set of research parameters must be defined and should be addressed when reporting the results of animal experiments" and stated that a "*concerted effort by all stakeholders, including funding agencies and journals, will be necessary to disseminate and implement best reporting practices throughout the research community.*"

For decades, institutions have adhered to *The Guide for the Care and Use of Laboratory Animals (The Guide*; [1]*)* which provides guidelines regulating all aspects of the research mouse environment (see Sect. 12.2 above). However, we are beginning to recognize the impact of these variables on the biology of mice and, recently, studies by others on non-tumor bearing mice (e.g., [6, 7, 127]) as well as our own research on cancer models [21, 24, 107], have convinced us that these housing choices have great potential to skew the outcome of experiments (see also Toth review [33]). This viewpoint is echoed in a recent editorial by the editors of Nature Neurobiology who wrote: *"Factors such as animal housing, handling, food, lighting and noise conditions, all of which effect behavior and brain chemistry, can be varied. The key to reproducibility is accurate reporting of these seemingly mundane details, which potentially have large effects"* [128]. Demas and Carlton [129] have reviewed the potential for environmental factors to act on the nervous, immune and endocrine systems, affecting the biology of the mouse. Additionally, experimentally imposed psychosocial stresses such as repeated restraint [101, 104, 130], scream [113], variation in housing density [90] and social isolation [86, 131] have been shown to directly promote tumor cell proliferation, growth, survival and metastasis by increased adrenergic signaling (see recent review by [71]). Two recent studies have demonstrated the striking potential

of environmentally induced stress to affect tumor growth. Li et al. [132] and Garofalo et al. [133] found that when mice were housed in an enriched environment which reduced stress/anxiety, the growth of pancreatic tumors and gliomas was significantly inhibited. These studies are also indicative of how stressful conditions of ST housing are since they show that reducing the stress experienced at ST improves tumor control. Garofalo et al. [133] found that the improved tumor control can involve immune (innate) and non-immune mechanisms, however the role of β-adrenergic signaling was not addressed in these studies and this will be important to compare in the future. Clearly, housing factor induced psychosocial stress is a source of variability between experiments and labs. *However, the degree to which environmental stress caused by housing choices alters the levels of stress hormones and how this potentially impacts preclinical studies of cancer has received very little attention.*

12.11 Conclusions

The tumor-promoting effects of chronic stress are currently the focus of research which provides a rationale for Clinical Trials to test whether β-blockers can be used in combination with chemotherapy and other therapies to improve patient outcome. In analyzing these pre-clinical data, what has not been appreciated is that housing conditions, particularly the sub-thermoneutral ambient temperatures, are subjecting these laboratory mice to a degree of chronic cold stress which is sufficient to raise NE levels, suppress the anti-tumor immune response and induce resistance to therapies tested using these models. Thus increased tumor growth arises from both an increase in the expression of anti-apoptotic molecules in the tumor cells themselves and suppression of the naturally occurring anti-tumor immune response. The implications of these observations are important in assessing how to design preclinical experiments that will maximize our understanding of diseases processes and how the immune response can be regulated to treat diseases, as well as obtaining a broad view of therapeutic responses. We predict that any therapy whose immediate or long-term outcome is even partially dependent on the anti-tumor immune response will be compromised in experiments conducted at ST. In fact, there are now several reports describing experiments whose outcomes are different when they are conducted at ST vs TT [7, 12–14, 19–37]. We believe these studies serve as a caution against accepting the results from experiments conducted under one set of conditions as the "baseline" when in fact, the results may be significanlty different if parameters such as temperature are changed, as in our tumor growth experiments conducted as ST and TT. How can this housing cold-stress be overcome in traditional animal facilities? We have used incubators maintained at 22 °C or 30 °C [21, 24, 25, 107] while others suggest using nesting materials in cages at ST [42, 43]. In any case, going forward, we believe that the housing temperatures and other environmental variables which can impact results, and are a likely source of experimental variability, should be reported in publications. Lastly, we encourage investigators conducting metabolic experiments, immunological investigations and therapeutic efficacy testing to consider comparing outcomes at both ST and TT.

References

1. Animals NRCUCftUotGftCaUoL. Guide for the care and use of laboratory animals. 8th ed. Washington, DC: National Academies; 2011.
2. Magnon C, Hall SJ, Lin J, Xue X, Gerber L, Freedland SJ, Frenette PS. Autonomic nerve development contributes to prostate cancer progression. Science. 2013;341(6142):1236361.
3. Szpunar MJ, Belcher EK, Dawes RP, Madden KS. Sympathetic innervation, norepinephrine content, and norepinephrine turnover in orthotopic and spontaneous models of breast cancer. Brain Behav Immun. 2016;53:223–33.
4. Felten DL, Felten SY, Carlson SL, Olschowka JA, Livnat S. Noradrenergic and peptidergic innervation of lymphoid tissue. J Immunol. 1985;135(2 Suppl):755s–65s.
5. Nance DM, Sanders VM. Autonomic innervation and regulation of the immune system (1987-2007). Brain Behav Immun. 2007;21(6):736–45.
6. Martin B, Ji S, Maudsley S, Mattson MP. "Control" laboratory rodents are metabolically mor-

bid: why it matters. Proc Natl Acad Sci U S A. 2010;107(14):6127–33.
7. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab. 2009;9(2):203–9.
8. Karp CL. Unstressing intemperate models: how cold stress undermines mouse modeling. J Exp Med. 2012;209(6):1069–74.
9. Lodhi IJ, Semenkovich CF. Why we should put clothes on mice. Cell Metab. 2009;9(2):111–2.
10. Maloney SK, Fuller A, Mitchell D, Gordon C, Overton JM. Translating animal model research: does it matter that our rodents are cold? Physiology. 2014;29(6):413–20.
11. Messmer MN, Kokolus KM, Eng JW, Abrams SI, Repasky EA. Mild cold-stress depresses immune responses: implications for cancer models involving laboratory mice. Bioessays. 2014;36(9):884–91.
12. Overton J. Phenotyping small animals as models for the human metabolic syndrome: thermoneutrality matters. Int J Obes. 2010;34(Suppl 2):S53–8.
13. Ravussin Y, LeDuc CA, Watanabe K, Leibel RL. Effects of ambient temperature on adaptive thermogenesis during maintenance of reduced body weight in mice. Am J Physiol Regul Integr Comp Physiol. 2012;303(4):R438–48.
14. Xiao C, Goldgof M, Gavrilova O, Reitman ML. Anti-obesity and metabolic efficacy of the beta3-adrenergic agonist, CL316243, in mice at thermoneutrality compared to 22 degrees C. Obesity (Silver Spring). 2015;23(7):1450–9.
15. David JM, Chatziioannou AF, Taschereau R, Wang H, Stout DB. The hidden cost of housing practices: using noninvasive imaging to quantify the metabolic demands of chronic cold stress of laboratory mice. Comp Med. 2013;63(5):386–91.
16. Hylander BL, Repasky EA. Thermoneutrality, mice and cancer: a heated opinion. Trends Cancer. 2016;2(4):166.
17. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, Thompson EA, Fraser KA, Rosato PC, Filali-Mouhim A, Sekaly RP, Jenkins MK, Vezys V, Haining WN, Jameson SC, Masopust D. Normalizing the environment recapitulates adult human immune traits in laboratory mice. Nature. 2016;532:512.
18. Gordon CJ. Thermal physiology of laboratory mice: defining thermoneutrality. J Therm Biol. 2012;37:654–85.
19. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev. 2004;84:277–359.
20. Cannon B, Nedergaard J. Thermogenesis challenges the adipostat hypothesis for body-weight control. Proc Nutr Soc. 2009;68(4):401–7.
21. Eng JW, Reed CB, Kokolus KM, Pitoniak R, Utley A, Bucsek MJ, Ma WW, Repasky EA, Hylander BL. Housing temperature-induced stress drives therapeutic resistance in murine tumour models through beta-adrenergic receptor activation. Nat Commun. 2015;6:6426.
22. Golozoubova V, Gullberg H, Matthias A, Cannon B, Vennstrom B, Nedergaard J. Depressed thermogenesis but competent brown adipose tissue recruitment in mice devoid of all hormone-binding thyroid hormone receptors. Mol Endocrinol. 2004;18(2):384–401.
23. Jhaveri KA, Trammell RA, Toth LA. Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of C57BL/6J mice. Brain Behav Immun. 2007;21(7):975–87.
24. Kokolus KM, Capitano ML, Lee CT, Eng JW, Waight JD, Hylander BL, Sexton S, Hong CC, Gordon CJ, Abrams SI, Repasky EA. Baseline tumor growth and immune control in laboratory mice are significantly influenced by subthermoneutral housing temperature. Proc Natl Acad Sci U S A. 2013;110(50):20176–81.
25. Leigh ND, Kokolus KM, O'Neill RE, Du W, Eng JW, Qiu J, Chen GL, McCarthy PL, Farrar JD, Cao X, Repasky EA. Housing temperature-induced stress is suppressing murine graft-versus-host disease through beta2-adrenergic receptor signaling. J Immunol. 2015;195(10):5045–54.
26. Povinelli BJ, Kokolus KM, Eng JW, Dougher CW, Curtin L, Capitano ML, Sailsbury-Ruf CT, Repasky EA, Nemeth MJ. Standard sub-thermoneutral caging temperature influences radiosensitivity of hematopoietic stem and progenitor cells. PLoS One. 2015;10(3):e0120078.
27. Rudaya AY, Steiner AA, Robbins JR, Dragic AS, Romanovsky AA. Thermoregulatory responses to lipopolysaccharide in the mouse: dependence on the dose and ambient temperature. Am J Physiol Regul Integr Comp Physiol. 2005;289(5):R1244–52.
28. Smith DL Jr, Yang Y, Hu HH, Zhai G, Nagy TR. Measurement of interscapular brown adipose tissue of mice in differentially housed temperatures by chemical-shift-encoded water-fat MRI. J Magn Reson Imaging. 2013;38(6):1425–33.
29. Stemmer K, Kotzbeck P, Zani F, Bauer M, Neff C, Muller TD, Pfluger PT, Seeley RJ, Divanovic S. Thermoneutral housing is a critical factor for immune function and diet-induced obesity in C57BL/6 nude mice. Int J Obes. 2015;39(5):791–7.
30. Swoap SJ, Li C, Wess J, Parsons AD, Williams TD, Overton JM. Vagal tone dominates autonomic control of mouse heart rate at thermoneutrality. Am J Physiol Heart Circ Physiol. 2008;294(4):H1581–8.
31. Swoap SJ, Overton JM, Garber G. Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach. Am J Physiol Regul Integr Comp Physiol. 2004;287(2):R391–6.
32. Tian Xiao Y, Ganeshan K, Hong C, Nguyen Khoa D, Qiu Y, Kim J, Tangirala Rajendra K, Tonotonoz P, Chawla A. Thermoneutral housing accelerates metabolic inflammation to potentiate athero-

sclerosis but not insulin resistance. Cell Metab. 2015;23(1):165–78.
33. Toth LA. The influence of the cage environment on rodent physiology and behavior: implications for reproducibility of pre-clinical rodent research. Exp Neurol. 2015;270:72.
34. Uchida K, Shiuchi T, Inada H, Minokoshi Y, Tominaga M. Metabolic adaptation of mice in a cool environment. Pflugers Arch. 2010;459(5):765–74.
35. Romanovsky AA, Kulchitsky VA, Simons CT, Sugimoto N. Methodology of fever research: why are polyphasic fevers often thought to be biphasic? Am J Phys. 1998;275(1 Pt 2):R332–8.
36. Hasday JD, Fairchild KD, Shanholtz C. The role of fever in the infected host. Microbes Infect. 2000;2(15):1891–904.
37. Eng JW, Reed CB, Kokolus KM, Repasky EA. Housing temperature influences the pattern of heat shock protein induction in mice following mild whole body hyperthermia. Int J Hyperthermia. 2014;30(8):540–6.
38. Bucsek M, Qiao G, MacDonald C, Giridharan T, Evans L, Niedzwecki B, Liu H, Kokolus KM, Eng JW, Messmer MN, Atwood K, Abrams SI, Hylander BL, Repasky EA. β-adrenergic signaling in mouse models housed at standard temperatures suppresses an effector phenotype in CD8+ T cells and undermines checkpoint inhibitor therapy. Cancer Res. 2017.; in press
39. Ganeshan K, Chawla A. Warming the mouse to model human diseases. Nat Rev Endocrinol. 2017;13(8):458–65.
40. Herrington LP. The heat regulation of small laboratory animals at various environmental temperatures. Am J Physiol. 1940;129:123–39.
41. Gordon CJ. Relationship between autonomic and behavioral thermoregulation in the mouse. Physiol Behav. 1985;34(5):687–90.
42. Gaskill BN, Gordon CJ, Pajor EA, Lucas JR, Davis JK, Garner JP. Heat or insulation: behavioral titration of mouse preference for warmth or access to a nest. PLoS One. 2012;7(3):e32799.
43. Gaskill BN, Gordon CJ, Pajor EA, Lucas JR, Davis JK, Garner JP. Impact of nesting material on mouse body temperature and physiology. Physiol Behav. 2013;110-111:87–95.
44. Gilon P, Henquin JC. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. Endocr Rev. 2001;22(5):565–604.
45. Teramura Y, Terao A, Okada Y, Tomida J, Okamatsu-Ogura Y, Kimura K. Organ-specific changes in norepinephrine turnover against various stress conditions in thermoneutral mice. Jpn J Vet Res. 2014;62(3):117–27.
46. Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP. Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature. 1997;387(6628):90–4.
47. Liu X, Rossmeisl M, McClaine J, Riachi M, Harper ME, Kozak LP. Paradoxical resistance to diet-induced obesity in UCP1-deficient mice. J Clin Invest. 2003;111(3):399–407.
48. Anunciado-Koza R, Ukropec J, Koza RA, Kozak LP. Inactivation of UCP1 and the glycerol phosphate cycle synergistically increases energy expenditure to resist diet-induced obesity. J Biol Chem. 2008;283(41):27688–97.
49. Kozak LP. Brown fat and the myth of diet-induced thermogenesis. Cell Metab. 2010;11(4):263–7.
50. Goldgof M, Xiao C, Chanturiya T, Jou W, Gavrilova O, Reitman ML. The chemical uncoupler 2,4-dinitrophenol (DNP) protects against diet-induced obesity and improves energy homeostasis in mice at thermoneutrality. J Biol Chem. 2014;289(28):19341–50.
51. Ravussin Y. Temperature matters with rodent metabolic studies. Obesity (Silver Spring). 2015;23(7):1330.
52. Jun JC, Shin MK, Yao Q, Devera R, Fonti-Bevans S, Polotsky VY. Thermoneutrality modifies the impact of hypoxia on lipid metabolism. Am J Phys Endocrinol Metab. 2013;304(4):E424–35.
53. Koizumi A, Wada Y, Tuskada M, Kayo T, Naruse M, Horiuchi K, Mogi T, Yoshioka M, Sasaki M, Miyamaura Y, Abe T, Ohtomo K, Walford R. A tumor preventive effect of dietary restriction is antagonized by a high housing temperature through deprivation of torpor. Mech Ageing Dev. 1996;92:67–82.
54. Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. Nat Rev Immunol. 2005;5(3):243–51.
55. Sheridan JF, Dobbs C, Jung J, Chu X, Konstantinos A, Padgett D, Glaser R. Stress-induced neuroendocrine modulation of viral pathogenesis and immunity. Ann N Y Acad Sci. 1998;840:803–8.
56. Marino F, Cosentino M. Adrenergic modulation of immune cells: an update. Amino Acids. 2013;45(1):55–71.
57. Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. Immunol Res. 2014;58(2-3):193–210.
58. Moragues V, Pinkerton H. Variation in morbidity and mortality of murine typhus infection in mice with changes in the environmental temperature. J Exp Med. 1944;79(1):41–3.
59. Underwood GE, Baker CA, Weed SD. Protective effect of elevated temperature on mice infected with Coe virus. J Immunol. 1966;96(6):1006–12.
60. Wang JF, Meissner A, Malek S, Chen Y, Ke Q, Zhang J, Chu V, Hampton TG, Crumpacker CS, Abelmann WH, Amende I, Morgan JP. Propranolol ameliorates and epinephrine exacerbates progression of acute and chronic viral myocarditis. Am J Physiol Heart Circ Physiol. 2005;289(4):H1577–83.
61. Garcia-Miss Mdel R, Mut-Martin MC, Gongora-Alfaro JL. Beta-adrenergic blockade protects BALB/c mice against infection with a small inoculum of Leishmania mexicana mexicana (LV4). Int Immunopharmacol. 2015;24(1):59–67.
62. Montazeri M, Daryani A, Ebrahimzadeh M, Ahmadpour E, Sharif M, Sarvi S. Effect of propran-

olol alone and in combination with Pyrimethamine on acute murine toxoplasmosis. Jundishapur J Microbiol. 2015;8(9):e22572.
63. Grebe KM, Hickman HD, Irvine KR, Takeda K, Bennink JR, Yewdell JW. Sympathetic nervous system control of anti-influenza CD8+ T cell responses. Proc Natl Acad Sci U S A. 2009;106(13):5300–5.
64. Bell JF, Moore GJ. Effects of high ambient temperature on various stages of rabies virus infection in mice. Infect Immun. 1974;10(3):510–5.
65. Repasky EA, Evans SS, Dewhirst MW. Temperature matters! And why it should matter to tumor immunologists. Cancer Immunol Res. 2013;1(4):210–6.
66. Sen A, Capitano ML, Spernyak JA, Schueckler JT, Thomas S, Singh AK, Evans SS, Hylander BL, Repasky EA. Mild elevation of body temperature reduces tumor interstitial fluid pressure and hypoxia and enhances efficacy of radiotherapy in murine tumor models. Cancer Res. 2011;71(11):3872–80.
67. Dewhirst MW, Lee CT, Ashcraft KA. The future of biology in driving the field of hyperthermia. Int J Hyperthermia. 2016;32(1):4–13.
68. Evans SS, Repasky EA, Fisher DT. Fever and the thermal regulation of immunity: the immune system feels the heat. Nat Rev Immunol. 2015;15(6):335–49.
69. Padgett DA, Glaser R. How stress influences the immune response. Trends Immunol. 2003;24(8):444–8.
70. Cole SW, Nagaraja AS, Lutgendorf SK, Green PA, Sood AK. Sympathetic nervous system regulation of the tumour microenvironment. Nat Rev Cancer. 2015;15(9):563–72.
71. Cole SW, Sood AK. Molecular pathways: beta-adrenergic signaling in cancer. Clin Cancer Res. 2012;18(5):1201–6.
72. Eng JW, Kokolus KM, Reed CB, Hylander BL, Ma WW, Repasky EA. A nervous tumor microenvironment: the impact of adrenergic stress on cancer cells, immunosuppression, and immunotherapeutic response. Cancer Immunol Immunother. 2014;63(11):1115–28.
73. Melhem-Bertrandt A, Sood AK. Adrenergic signaling and cancer: deciphering the connections. Cancer Biomark. 2013;13(3):131–2.
74. Powe DG, Voss MJ, Zanker KS, Habashy HO, Green AR, Ellis IO, Entschladen F. Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. Oncotarget. 2010;1(7):628–38.
75. Barron TI, Connolly RM, Sharp L, Bennett K, Visvanathan K. Beta blockers and breast cancer mortality: a population- based study. J Clin Oncol. 2011;29(19):2635–44.
76. Diaz ES, Karlan BY, Li AJ. Impact of beta blockers on epithelial ovarian cancer survival. Gynecol Oncol. 2012;127(2):375–8.
77. Watkins JL, Thaker PH, Nick AM, Ramondetta LM, Kumar S, Urbauer DL, Matsuo K, Squires KC, Coleman RL, Lutgendorf SK, Ramirez PT, Sood AK. Clinical impact of selective and nonselective beta-blockers on survival in patients with ovarian cancer. Cancer. 2015;121(19):3444–51.
78. De Giorgi V, Grazzini M, Gandini S, Benemei S, Lotti T, Marchionni N, Geppetti P. Treatment with beta-blockers and reduced disease progression in patients with thick melanoma. Arch Intern Med. 2011;171(8):779–81.
79. Wang HM, Liao ZX, Komaki R, Welsh JW, O'Reilly MS, Chang JY, Zhuang Y, Levy LB, Lu C, Gomez DR. Improved survival outcomes with the incidental use of beta-blockers among patients with non-small-cell lung cancer treated with definitive radiation therapy. Ann Oncol. 2013;24(5):1312–9.
80. Grytli HH, Fagerland MW, Fossa SD, Tasken KA. Association between use of beta-blockers and prostate cancer-specific survival: a cohort study of 3561 prostate cancer patients with high-risk or metastatic disease. Eur Urol. 2014;65(3):635–41.
81. Udumyan R, Montgomery S, Fang F, Almroth H, Valdimarsdottir U, Ekbom A, Smedby KE, Fall K. Beta-blocker drug use and survival among patients with pancreatic adenocarcinoma. Cancer Res. 2017;77:3700.
82. Kim SA, Moon H, Roh JL, Kim SB, Choi SH, Nam SY, Kim SY. Postdiagnostic use of beta-blockers and other antihypertensive drugs and the risk of recurrence and mortality in head and neck cancer patients: an observational study of 10,414 person-years of follow-up. Clin Transl Oncol. 2017;19:826.
83. Cardwell CR, Coleman HG, Murray LJ, Entschladen F, Powe DG. Beta-blocker usage and breast cancer survival: a nested case-control study within a UK clinical practice research datalink cohort. Int J Epidemiol. 2013;42(6):1852–61.
84. Cardwell CR, Coleman HG, Murray LJ, O'Sullivan JM, Powe DG. Beta-blocker usage and prostate cancer survival: a nested case-control study in the UK clinical practice research datalink cohort. Cancer Epidemiol. 2014;38:279.
85. Hicks BM, Murray LJ, Powe DG, Hughes CM, Cardwell CR. Beta-blocker usage and colorectal cancer mortality: a nested case-control study in the UK clinical practice research datalink cohort. Ann Oncol. 2013;24(12):3100–6.
86. Lutgendorf SK, DeGeest K, Dahmoush L, Farley D, Penedo F, Bender D, Goodheart M, Buekers TE, Mendez L, Krueger G, Clevenger L, Lubaroff DM, Sood AK, Cole SW. Social isolation is associated with elevated tumor norepinephrine in ovarian carcinoma patients. Brain Behav Immun. 2011;25(2):250–5.
87. Masur K, Niggemann B, Zanker KS, Entschladen F. Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. Cancer Res. 2001;61(7):2866–9.
88. Palm D, Lang K, Niggemann B, Drell TL 4th, Masur K, Zaenker KS, Entschladen F. The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int J Cancer. 2006;118(11):2744–9.

89. Le CP, Nowell CJ, Kim-Fuchs C, Botteri E, Hiller JG, Ismail H, Pimentel MA, Chai MG, Karnezis T, Rotmensz N, Renne G, Gandini S, Pouton CW, Ferrari D, Moller A, Stacker SA, Sloan EK. Chronic stress in mice remodels lymph vasculature to promote tumour cell dissemination. Nat Commun. 2016;7:10634.
90. Hasegawa H, Saiki I. Psychosocial stress augments tumor development through beta-adrenergic activation in mice. Jpn J Cancer Res. 2002;93(7):729–35.
91. Al-Wadei HA, Plummer HK 3rd, Ullah MF, Unger B, Brody JR, Schuller HM. Social stress promotes and gamma-aminobutyric acid inhibits tumor growth in mouse models of non-small cell lung cancer. Cancer Prev Res. 2012;5(2):189–96.
92. Lin Q, Wang F, Yang R, Zheng X, Gao H, Zhang P. Effect of chronic restraint stress on human colorectal carcinoma growth in mice. PLoS One. 2013;8(4):e61435.
93. Sood AK, Bhatty R, Kamat AA, Landen CN, Han L, Thaker PH, Li Y, Gershenson DM, Lutgendorf S, Cole SW. Stress hormone-mediated invasion of ovarian cancer cells. Clin Cancer Res. 2006;12(2):369–75.
94. Creed SJ, Le CP, Hassan M, Pon CK, Albold S, Chan KT, Berginski ME, Huang Z, Bear JE, Lane JR, Halls ML, Ferrari D, Nowell CJ, Sloan EK. Beta2-adrenoceptor signaling regulates invadopodia formation to enhance tumor cell invasion. Breast Cancer Res. 2015;17(1):145.
95. Sood AK, Armaiz-Pena GN, Halder J, Nick AM, Stone RL, Hu W, Carroll AR, Spannuth WA, Deavers MT, Allen JK, Han LY, Kamat AA, Shahzad MM, McIntyre BW, Diaz-Montero CM, Jennings NB, Lin YG, Merritt WM, DeGeest K, Vivas-Mejia PE, Lopez-Berestein G, Schaller MD, Cole SW, Lutgendorf SK. Adrenergic modulation of focal adhesion kinase protects human ovarian cancer cells from anoikis. J Clin Invest. 2010;120(5):1515–23.
96. Sloan EK, Priceman SJ, Cox BF, Yu S, Pimentel MA, Tangkanangnukul V, Arevalo JM, Morizono K, Karanikolas BD, Wu L, Sood AK, Cole SW. The sympathetic nervous system induces a metastatic switch in primary breast cancer. Cancer Res. 2010;70(18):7042–52.
97. Nagaraja AS, Dorniak PL, Sadaoui NC, Kang Y, Lin T, Armaiz-Pena G, Wu SY, Rupaimoole R, Allen JK, Gharpure KM, Pradeep S, Zand B, Previs RA, Hansen JM, Ivan C, Rodriguez-Aguayo C, Yang P, Lopez-Berestein G, Lutgendorf SK, Cole SW, Sood AK. Sustained adrenergic signaling leads to increased metastasis in ovarian cancer via increased PGE2 synthesis. Oncogene. 2016;35(18):2390–7.
98. Park SY, Kang JH, Jeong KJ, Lee J, Han JW, Choi WS, Kim YK, Kang J, Park CG, Lee HY. Norepinephrine induces VEGF expression and angiogenesis by a hypoxia-inducible factor-1alpha protein-dependent mechanism. Int J Cancer. 2011;128(10):2306–16.
99. Yang EV, Kim SJ, Donovan EL, Chen M, Gross AC, Webster Marketon JI, Barsky SH, Glaser R. Norepinephrine upregulates VEGF, IL-8, and IL-6 expression in human melanoma tumor cell lines: implications for stress-related enhancement of tumor progression. Brain Behav Immun. 2009;23(2):267–75.
100. Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB, Jewell S, Flavahan NA, Morrison C, Yeh PE, Lemeshow S, Glaser R. Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. Cancer Res. 2006;66(21):10357–64.
101. Thaker PH, Han LY, Kamat AA, Arevalo JM, Takahashi R, Lu C, Jennings NB, Armaiz-Pena G, Bankson JA, Ravoori M, Merritt WM, Lin YG, Mangala LS, Kim TJ, Coleman RL, Landen CN, Li Y, Felix E, Sanguino AM, Newman RA, Lloyd M, Gershenson DM, Kundra V, Lopez-Berestein G, Lutgendorf SK, Cole SW, Sood AK. Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. Nat Med. 2006;12(8):939–44.
102. Sastry KS, Karpova Y, Prokopovich S, Smith AJ, Essau B, Gersappe A, Carson JP, Weber MJ, Register TC, Chen YQ, Penn RB, Kulik G. Epinephrine protects cancer cells from apoptosis via activation of cAMP-dependent protein kinase and BAD phosphorylation. J Biol Chem. 2007;282(19):14094–100.
103. Hassan S, Karpova Y, Baiz D, Yancey D, Pullikuth A, Flores A, Register T, Cline JM, D'Agostino R Jr, Danial N, Datta SR, Kulik G. Behavioral stress accelerates prostate cancer development in mice. J Clin Invest. 2013;123(2):874–86.
104. Partecke LI, Speerforck S, Kading A, Seubert F, Kuhn S, Lorenz E, Schwandke S, Sendler M, Kessler W, Trung DN, Oswald S, Weiss FU, Mayerle J, Henkel C, Menges P, Beyer K, Lerch MM, Heidecke CD, von Bernstorff W. Chronic stress increases experimental pancreatic cancer growth, reduces survival and can be antagonised by beta-adrenergic receptor blockade. Pancreatology. 2016;16(3):423–33.
105. He D, Manzoni A, Florentin D, Fisher W, Ding Y, Lee M, Ayala G. Biologic effect of neurogenesis in pancreatic cancer. Hum Pathol. 2016;52:182–9.
106. Pasquier E, Ciccolini J, Carre M, Giacometti S, Fanciullino R, Pouchy C, Montero MP, Serdjebi C, Kavallaris M, Andre N. Propranolol potentiates the anti-angiogenic effects and anti-tumor efficacy of chemotherapy agents: implication in breast cancer treatment. Oncotarget. 2011;2(10):797–809.
107. Kokolus KM, Spangler HM, Povinelli BJ, Farren MR, Lee KP, Repasky EA. Stressful presentations: mild cold stress in laboratory mice influences phenotype of dendritic cells in naive and tumor-bearing mice. Front Immunol. 2014;5:23.
108. Wrobel LJ, Bod L, Lengagne R, Kato M, Prevost-Blondel A, Le Gal FA. Propranolol induces a favourable shift of anti-tumor immunity in a murine spontaneous model of melanoma. Oncotarget. 2016;7:77825–37.

109. Elenkov IJ, Chrousos GP. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends Endocrinol Metab. 1999;10(9):359–68.
110. Elenkov IJ, Chrousos GP. Stress system—organization, physiology and immunoregulation. Neuroimmunomodulation. 2006;13(5-6):257–67.
111. Panina-Bordignon P, Mazzeo D, Lucia PD, D'Ambrosio D, Lang R, Fabbri L, Self C, Sinigaglia F. Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. J Clin Invest. 1997;100(6):1513–9.
112. Sanders VM, Baker RA, Ramer-Quinn DS, Kasprowicz DJ, Fuchs BA, Street NE. Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. J Immunol. 1997;158(9):4200–10.
113. Hou N, Zhang X, Zhao L, Zhao X, Li Z, Song T, Huang C. A novel chronic stress-induced shift in the Th1 to Th2 response promotes colon cancer growth. Biochem Biophys Res Commun. 2013;439(4):471–6.
114. Nguyen KD, Qiu Y, Cui X, Goh YP, Mwangi J, David T, Mukundan L, Brombacher F, Locksley RM, Chawla A. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. Nature. 2011;480(7375):104–8.
115. Fischer K, Ruiz HH, Jhun K, Finan B, Oberlin DJ, van der Heide V, Kalinovich AV, Petrovic N, Wolf Y, Clemmensen C, Shin AC, Divanovic S, Brombacher F, Glasmacher E, Keipert S, Jastroch M, Nagler J, Schramm KW, Medrikova D, Collden G, Woods SC, Herzig S, Homann D, Jung S, Nedergaard J, Cannon B, Tschop MH, Muller TD, Buettner C. Alternatively activated macrophages do not synthesize catecholamines or contribute to adipose tissue adaptive thermogenesis. Nat Med. 2017;23(5):623–30.
116. Guereschi MG, Araujo LP, Maricato JT, Takenaka MC, Nascimento VM, Vivanco BC, Reis VO, Keller AC, Brum PC, Basso AS. Beta2-adrenergic receptor signaling in CD4+ Foxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner. Eur J Immunol. 2013;43(4):1001–12.
117. Jin J, Wang X, Wang Q, Guo X, Cao J, Zhang X, Zhu T, Zhang D, Wang W, Wang J, Shen B, Gao X, Shi Y, Zhang J. Chronic psychological stress induces the accumulation of myeloid-derived suppressor cells in mice. PLoS One. 2013;8(9):e74497.
118. Nkontchou G, Aout M, Mahmoudi A, Roulot D, Bourcier V, Grando-Lemaire V, Ganne-Carrie N, Trinchet JC, Vicaut E, Beaugrand M. Effect of long-term propranolol treatment on hepatocellular carcinoma incidence in patients with HCV-associated cirrhosis. *Cancer Prev Res*. 2012;5(8):1007–14.
119. Giampieri R, Scartozzi M, Del Prete M, Faloppi L, Bianconi M, Ridolfi F, Cascinu S. Prognostic value for incidental antihypertensive therapy with beta-blockers in metastatic colorectal cancer. Medicine. 2015;94(24):e719.
120. Liu J, Blake SJ, Smyth MJ, Teng MW. Improved mouse models to assess tumour immunity and irAEs after combination cancer immunotherapies. Clin Transl Immunol. 2014;3(8):e22.
121. Freedman L, Gibson M. The impact of preclinical irreproducibility on drug development. Clin Pharmacol Ther. 2015;97(1):16–8.
122. Prinz F, Schlange T, Asadullah K. Believe it or not: how much can we rely on published data on potential drug targets? Nat Rev Drug Discov. 2011;10(9):712.
123. Schein PS, Scheffler B. Barriers to efficient development of cancer therapeutics. Clin Cancer Res. 2006;12(11 Pt 1):3243–8.
124. Talmadge JE, Singh RK, Fidler IJ, Raz A. Murine models to evaluate novel and conventional therapeutic strategies for cancer. Am J Pathol. 2007;170(3):793–804.
125. Begley CG, Ellis LM. Drug development: raise standards for preclinical cancer research. Nature. 2012;483(7391):531–3.
126. Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, Crystal RG, Darnell RB, Ferrante RJ, Fillit H, Finkelstein R, Fisher M, Gendelman HE, Golub RM, Goudreau JL, Gross RA, Gubitz AK, Hesterlee SE, Howells DW, Huguenard J, Kelner K, Koroshetz W, Krainc D, Lazic SE, Levine MS, Macleod MR, McCall JM, Moxley RT 3rd, Narasimhan K, Noble LJ, Perrin S, Porter JD, Steward O, Unger E, Utz U, Silberberg SD. A call for transparent reporting to optimize the predictive value of preclinical research. Nature. 2012;490(7419):187–91.
127. Macri S, Ceci C, Altabella L, Canese R, Laviola G. The directive 2010/63/EU on animal experimentation may skew the conclusions of pharmacological and behavioural studies. Sci Rep. 2013;3:2380.
128. Troublesome variability in mouse studies. Nat Neurosci. 2009;12(9):1075.
129. Demas GE, Carlton ED. Ecoimmunology for psychoneuroimmunologists: considering context in neuroendocrine-immune-behavior interactions. Brain Behav Immun. 2015;44:9–16.
130. Lin CS, Lin WS, Lin CL, Kao CH. Carvedilol use is associated with reduced cancer risk: a nationwide population-based cohort study. Int J Cardiol. 2015;184:9–13.
131. Madden KS, Szpunar MJ, Brown EB. Early impact of social isolation and breast tumor progression in mice. Brain Behav Immun. 2013;30(Suppl):S135–41.
132. Li G, Gan Y, Fan Y, Wu Y, Lin H, Song Y, Cai X, Yu X, Pan W, Yao M, Gu J, Tu H. Enriched environment inhibits mouse pancreatic cancer growth and down-regulates the expression of mitochondria-related genes in cancer cells. Sci Rep. 2015;5:7856.
133. Garofalo S, D'Alessandro G, Chece G, Brau F, Maggi L, Rosa A, Porzia A, Mainiero F, Esposito V, Lauro C, Benigni G, Bernardini G, Santoni A, Limatola C. Enriched environment reduces glioma growth through immune and non-immune mechanisms in mice. Nat Commun. 2015;6:6623.

Immunotherapeutic Targeting of Tumor-Associated Blood Vessels

13

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13.1 The Abnormal Tumor Vasculature

Pathological angiogenesis is a hallmark of cancer [1]. Solid tumors, like normal tissues, require nutrients and oxygen as well as a mechanism to expel waste and carbon dioxide. These requirements necessitate the formation of neovascular networks for tumor growth and maintenance. In adult normal tissues, angiogenesis is quiescent, occurring only in specific physiological events such as wound healing. Notably, in such physiological events, new vessels mature and stabilize rapidly because of the tightly regulated balance of pro- and anti-angiogenic factors. On the other hand, in tumors, the production of pro-angiogenic factors is favored over anti-angiogenic factors, turning on the "angiogenic switch." Tumorigenic conditions such as hypoxia [2], oncogene activation and tumor-suppressor mutation [3] contribute to the skewing of the balance towards the expression of pro-angiogenic factors. This imbalance leads to the sustained growth of tumor blood vessels that are very distinct from their normal counterparts.

Tumors employ several methods to grow blood vessels, the most common and most studied of which is sprouting angiogenesis, in which new capillaries grow from pre-existing ones. During sprouting angiogenesis, vascular endothelial growth factor (VEGF) induces pre-existing capillaries or post-capillary venules to dilate and become leaky [4], allowing plasma proteins to extravasate and form a provisional matrix for activated vascular endothelial cells (VECs). Angiotensin-2 (Ang-2) loosens the association between VECs and abluminal pericytes and, along with proteinases, dissolves the extracellular matrix [5]. Various factors, including VEGF and fibroblast growth factor (FGF), promote the proliferation, migration, and assembly of VECs [6, 7] into tubular structures, followed by the recruitment of perivascular cells and then the production of basement membrane around the new blood vessel [8, 9]. During tumor

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angiogenesis, pericytes atypically remain only loosely-associated with the blood vessels [10] and the basement membrane is abnormally thick or thin [11]. Aside from sprouting angiogenesis, other mechanisms of tumor vessel growth include: [12] vasculogenesis, in which endothelial progenitor cells are recruited from bone marrow or peripheral blood into the TME to form blood vessels, [13] intussusception, in which VECs re-organize causing blood vessels to split and give rise to daughter vessels, [14] vessel co-option, in which tumor cells grow along existing blood vessels and [15] vasculogenic mimicry, in which tumor cells may de-differentiate into endothelial-like cells and form tubes [16].

When compared to blood vessels in normal somatic tissues, the tumor vasculature is architecturally and functionally abnormal. The tumor vascular network is highly tortuous, disorganized and lacks the normal hierarchical arrangement of arterioles, capillaries and venules [17, 18]. The tumor vessels are also morphologically dilated and leaky with chaotic and variable blood flow, resulting in regions of tumor that are hypoxic and acidic [17]. In conjunction, the cellular components of the tumor blood vessels are also abnormal in their phenotype/function. VECs and pericytes exhibit altered gene expression profiles and elicit a defective basement membrane [11, 13, 19]. These alterations in angiogenesis and vascular components in progressor tumors may be targeted therapeutically in order to treat patients with cancer.

13.2 Vascular Endothelial Growth Factor (VEGF)

The VEGF gene family is comprised of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). VEGF-A, usually referred to as VEGF, is the main member of the VEGF family and is a major driver of angiogenesis. The importance of VEGF in the development and differentiation of the vascular system has been demonstrated in a study wherein the inactivation of a single VEGF allele led to embryonic lethality [20]. Alternative splicing of VEGF mRNA leads to the generation of soluble isoforms and ECM- or cell membrane-bound isoforms of the protein [21]. Various metalloproteinases (MMPs) cleave bound VEGF to its diffusible form [22]. The angiogenic functions of VEGF are primarily mediated via VEGF receptor 2 (VEGFR2)-dependent signaling in endothelial cells. In this regard, mice deficient in VEGFR2 fail to develop blood islands and organized blood vessels [23].

VEGF is intrinsically overexpressed in a number of tumors [24]. Transcription of VEGF mRNA is driven by hypoxia but may also be induced by transforming events [21]. Mutations in K-ras are associated with the upregulation of VEGF, with disruption of the mutant K-ras allele shown to decrease VEGF activity [25]. Mutations in the Wnt signaling pathway in colonic adenomas also lead to an increase in VEGF expression [26]. In tumors, VEGF induces neoangiogenesis, increases vascular permeability, induces VEC migration and division, maintains the endothelium and reprograms VEC gene expression profiles [24].

13.3 Components of the Tumor Vasculature

13.3.1 Vascular Endothelial Cells (VECs)

VECs arise from hemangioblast precursors that are derived from the ventral floor of the dorsal aorta in the aorta-gonad-mesonephros region [27, 28]. During *de novo* organization of VECs into vessels (also known as vasculogenesis or angiogenesis), newly formed VECs express growth factor receptors like VEGFR1 and VEGFR2 that allow VECs to proliferate, migrate and form tubal structures in response VEGF and FGF. Once the formed vessels mature, VECs down-regulate their expression of these growth factor receptors. In developing tumor blood vessels, however, VEGFR-1, VEGFR-2 and even VEGFR-3 (which is usually restricted to lymphatic vessels in adults) are re-activated and elevated in the VECs, allowing for the unrestricted proliferation

and formation of blood vessels in the VEGF-rich TME [29–31]. Aside from growth factor receptors, tumor VECs also upregulate other genes that are typically not expressed on normal VECs [32, 33]. Most of these genes play a role in the angiogenic process but some are induced by hypoxia or under conditions of abnormal blood flow [32, 34].

VECs are held together by cell-to-cell junctions to form a continuous monolayer of cells that lines the blood vessel. The normal endothelium acts as a selective barrier that tightly controls the exchange of substances between the blood and the surrounding tissue [28, 35]. Different membrane bound receptors present in the endothelium allow the active transport of large molecules such as proteins, metabolites and hormones from blood to tissue [36]. The endothelial cells have junctional sections that are very similar to adherens and tight junctions in epithelial cells. The tight junctions are localized in the apical regions to seal the clefts between cells in the luminal surface, therefore, functionally restricting paracellular permeability. On the other hand, adherens junctions occupy the basal position and function to limit paracellular permeability, as well as, to control vessel morphogenesis and stability [37–39]. The adherens and tight junctions also transfer intracellular signals that mediate contact inhibition of cell growth, cell polarity and VEC-pericyte interaction and, hence, allow VECs to adapt quickly to evolving changes in their microenvironment, such as release of growth factors, angiogenic cues and inflammatory conditions [39, 40]. Gap junctions are also present in the endothelial cells [38].

The endothelium in tumors is structurally defective, composed of irregularly-shaped endothelial cells that have long, fragile cytoplasmic projections that sometimes extend across the vessel lumen [41] and is characterized by intercellular gaps between endothelial cells, transendothelial holes and endothelial fenestrae (pores) [42–44]. Increased production of VEGF in the TME dissolves the VE-cadherins and other adherens junction complexes, disrupting VEC cell-to-cell interactions [45]. These abnormalities make the tumor vessels unusually leaky and highly-permeable to plasma proteins, and even erythrocytes. The leakiness and uncontrolled extravasation of blood and plasma results in increased interstitial (fluid) pressure within the tumor lesion [46]. Structural abnormalities and high interstitial pressure contribute to irregular blood flow resulting in the uneven and impeded distribution of nutrients, oxygen, and (systemically administered) chemotherapeutic drugs within tumors. This also leads to areas in the tumor that are highly-acidic and hypoxic. The leaky tumor vessel can also promote the traffic of tumor cells into the blood stream and the eventual formation of distal metastases [43].

13.3.2 Pericytes

Pericytes and vascular smooth muscle cells (vSMCs), collectively called the mural cells, cover and support the endothelial tubules to maintain vascular integrity. Pericytes are usually found as single cells or a discontinuous single-cell layer around arterioles, capillaries, and post-capillary venules [47]. On the other hand, vSMCs are generally found around arteries and veins as multiple concentric layers of cells to mediate vascular tone and contraction [14, 47]. However, classification between pericytes and vSMCs are not always clear-cut and it has been suggested that there is a continuum of phenotypes ranging from the canonical vSMCs and classical pericytes instead of just two distinct populations of mural cells [14].

Pericytes have a number of different developmental origins such that with a single region of a given blood vessel may identify pericytes that have arisen from a range of precursors [48]. Furthermore, no single universal pericyte marker has been identified to date. As such, pericytes are typically identified using a combination of different characteristics including location (i.e. cells embedded in a basement membrane that is shared with the endothelium [49]), in addition to morphology and gene expression profile.

During physiological angiogenesis, pericytes are recruited to the developing vessel via signals such as platelet-derived growth factor β (PDGFβ),

sphingosine 1-phospate (S1P), Ang-1 and transforming growth factor-β1 (TGFβ1). The recruited pericytes form tight connections with the underlying endothelial cells with one pericyte usually in contact with multiple endothelial cells. Such pericyte interaction is very important in maintaining the integrity of the endothelium, playing a central role in regulating endothelial proliferation, differentiation, contractility, tone, stability, and permeability [14, 15, 50–53]. However, the pericyte-endothelial cell interaction is perturbed in the TME, with poor pericyte investment usually observed in a large proportion of tumor-associated blood vessels. Tumor pericytes have cytoplasmic processes that extend into the tumor tissue [10] and are loosely associated with the endothelium [8, 10]. This abnormal pericyte-endothelial cell association contributes to increased vascular permeability [43], which results in poor perfusion and hypoxia, and modified basement membrane [54]. Vessels are prone to be more hemorrhagic, with a disrupted basement membrane [54]. The pericyte coverage of the endothelium is also very variable, depending on the type of tumor. For example, islet carcinomas have dense pericyte coverage while glioblastomas have reduced numbers of pericytes per unit venule area [8]. Variable coverage can also occur within the same tumor [54]. The pericyte cover is thought to help maintain and stabilize the tumor vessels [55], with "naked" endothelium seemingly more dependent on VEGF for VEC survival [56]. Moreover, the tumor-associated pericytes exhibit an altered marker and protein expression profile, making them attractive targets for therapeutic intervention. Indeed, in tumors that overexpress PDGFRβ and thus have high pericyte density, blocking the PDGFRβ signaling results in detachment of pericytes from the endothelium and results in restricted tumor growth [57–59].

13.3.3 Fibroblasts

Cancer-associated fibroblasts (CAFs) are spindle-shaped cells embedded within the extracellular matrix (ECM) that originate from resident fibroblasts and bone marrow-derived mesenchymal precursors [60, 61]. They are phenotypically and functionally distinct from normal fibroblasts and comprise a significant component of the tumor stroma [62].

Although technically not part of the blood vessels, cancer-associated fibroblasts still play a major role in angiogenesis and in the promotion of the tumor blood vessel formation, mostly by producing pro-angiogenic factors. Although cancer cells themselves can release VEGF, the principal source of VEGF in the tumor microenvironment is the CAFs [63]. In cancers such as pancreatic cancer and breast cancer, CAFs produce stromal-derived factor 1 (SDF-1) or CXCL12, which contribute to tumor vascularization by recruiting endothelial precursor cells from the bone marrow [64–66]. CAFs could also indirectly promote tumor angiogenesis by secreting chemokines like CXCL1 and CXCL2 to recruit pro-angiogenic macrophages and neutrophils into the tumor microenvironment [67]. Furthermore, CAFs release matrix metalloproteinases that degrade the ECM, thus spatially accommodating the growing blood vessel and, at the same time, releasing VEGF previously sequestered in the ECM [68].

13.3.4 Basement Membrane

The basement membrane (BM) is a complex of proteins, glycoproteins and proteoglycans. The BM is similar to the ECM but has a different density and is always in contact with cells [11, 69]. The general role of the BM is to serve as boundary between tissues compartments, provide structural support, and regulate cell behavior [70].

The vascular basement membrane envelops the VECs and pericytes and is primarily composed of Type IV collagen, laminin, fibronectin and heparin sulfate proteoglycan [69]. The BM is integral in the initiation and resolution of angiogenesis, as it possesses both pro- and anti-angiogenic activities. Quiescent endothelial cells are bound to BM, indicating that the primary signals from BM inhibit VEC proliferation [71]. During active angiogenesis, the BM is degraded

leading to the detachment of VECs and pericytes and the release of sequestered growth factors. Pro-angiogenic factors induce VEC to produce and embed in a provisional matrix composed of vitronectin, fibronectin, type I collagen and thrombin. Cryptic domains of Type IV collagen in partially degraded BM and the provisional matrix provides proliferative cues to the VECs [69, 72].

Deposition of basement membrane is dependent on VEC-pericyte interaction and disrupting this interaction by blocking pericyte recruitment, for example, results in perturbed or reduced basement membrane deposition [73–75]. Therefore, the abnormalities in tumor basement membrane can be partially explained by the disrupted VEC-pericyte interaction in the tumor. It was previously believed that BM was absent in tumor vasculature. However, more recent studies show that BM is present and can even fully cover tumor vessels albeit in an abnormal manner. Tumor vascular BM has variable thickness and multiple layers, suggesting that the BM has undergone several rounds of remodeling. In addition, it has loose association with VECs and pericytes, which is characteristic of degenerating or forming blood vessels [11].

13.4 Tumor Vasculature in Cancer Progression

Several classical studies have shown that tumor blood vessels and the process of angiogenesis are necessary for tumor progression. When tumor cells were implanted in avascular tissues, tumor growth was inhibited and limited until sprouting vessels were able to access an existing blood supply [76, 77]. Since the importance of the tumor blood vasculature was demonstrated, numerous studies have shown that it not only supports the tumor by delivering nutrients, it also promotes tumor cell survival, confers drug resistance, helps to limit immune system surveillance, aids in metastasis and promotes stemness (Fig. 13.1).

13.4.1 Tumor Vasculature Supports Tumor Growth and Survival

The tumor vasculature actively supports tumor growth by secreting factors that promote tumor survival. For example, the CAFs in the tumor microenvironment produce epidermal growth factor (EGF), hepatocyte growth factor (HGF) and heregulin that promote tumor proliferation and vitality [78]. VECs in head and neck squamous cell carcinomas (HNSCC) overexpress the anti-apoptotic molecule Bcl-2, which induces the production of VEGF, IL-6, CXCL8/IL-8, and EGF [79, 80]. VEC-derived VEGF binds the VEGFR1 expressed by tumor cells, triggering the production of Bcl-2 in the HNSCC cells and enhancing their survival. IL-6, CXCL8, and epidermal growth factor (EGF) derived from VECs also activate STAT3, Akt, and extracellular signal-regulated kinase (ERK) in the tumor cells, enhancing migration and preventing apoptosis. Interruption of these signaling cascades by silencing Bcl-2 or the factors secreted by the VECs inhibits tumor growth [80].

13.4.2 Aberrant Tumor-Associated Vasculature Confers Drug Resistance

The tumor vasculature intrinsically confers therapy resistance by impeding drug delivery into the tumor lesion. The structural aberrations of the vessels cause excessive leakiness that leads to abnormal plasma accumulation and retention. This results in heterogeneous blood flow and high-interstitial fluid pressure in tumor tissue, preventing uniform distribution of systemically-administered anti-cancer drugs. Indeed, the erratic blood flow in the tumor promotes the concentrated delivery of drugs to the perfused outer (cortical) regions of the tumor while delivery to the poorly-perfused medullary tumor regions requires efficient perfusion/diffusion [81]. Furthermore, extravasation of therapeutic drugs

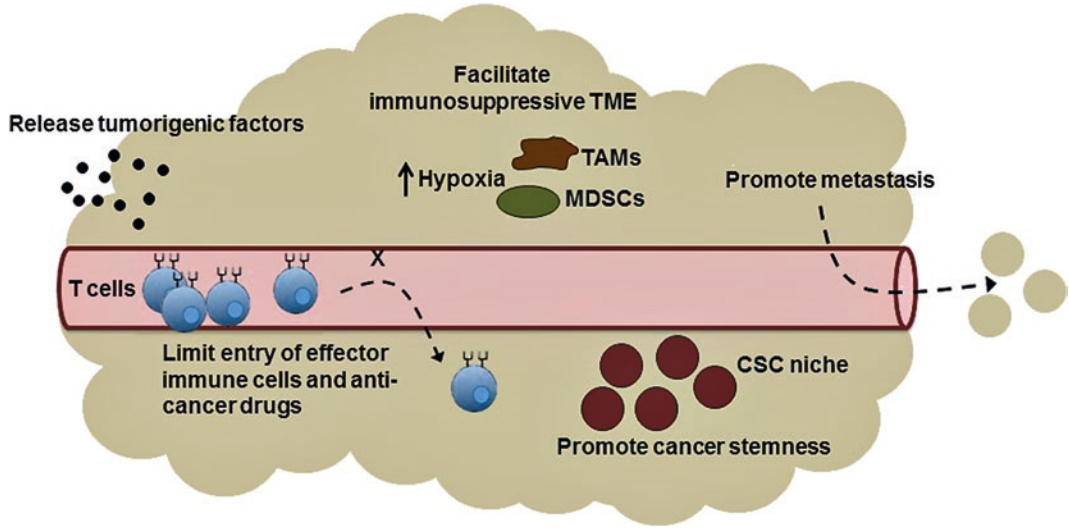

Fig. 13.1 The aberrant tumor vascular system promotes cancer progression. Tumor vasculogenesis is driven by the release of pro-angiogenic factors within the tumor microenvironment (TME) that fosters a state of locoregional hypoxia, acidosis and high interstitial pressure that is contraindicated for the delivery of protective T cells, but which fosters recruitment and immunosuppression mediated by regulatory cell populations including Treg and MDSCs. Cancer stem cells may also be stabilized in the perivascular niche, with metastatic tumor seeding into the blood as a consequence of "leaky" tumor blood vessels

is dependent on fluid movement across the vessel wall, which is influenced by proper pressure gradient between the vessels and the interstitial space. The high interstitial fluid pressure in the tumor disrupts the development of an optimal gradient, thus hindering drug delivery to the tumor core [82]. The tumor-associated vasculature may also actively prevent drug delivery by overexpressing efflux pumps that promote drug removal/detoxification [83, 84].

13.4.3 Tumor-Associated Vasculature Contributes to Immune Escape

The abnormal tumor vasculature not only impedes drug delivery, it also obstructs anti-cancer immune response by limiting entry of effector cells while simultaneously contributing to an immunosuppressive TME. The adaptive T cell response is critical to protective anti-tumor immunity and the endothelium plays an important role in regulating the trafficking of T cells and other leukocytes into peripheral (inflamed) tissues. In normal tissues, resting VECs express little to no adhesion molecules on their luminal surface interfacing with the blood supply. However, during infection or under conditions of tissue injury, pro-inflammatory signals such as lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α and interleukin (IL)-1β induce the expression of adhesion molecules to allow the extravasation of leukocytes in the blood stream [85, 86]. E-selectin and P-selectin on the endothelial cells bind with their ligands Sialyl Lewis^X and P-selectin glycoprotein ligand 1 on the activated T cells and other leukocytes [87, 88]. This binding event is a weak affinity interaction that induces the tethering and rolling of the T cells on the inflamed endothelium. The inflamed endothelium also expresses chemokines that induce surface integrins, such as lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), on rolling T cells to spread and cluster. The LFA-1 and VLA-4 bind strongly to intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) on activated VECs and, hence, mediate the arrest of interactive T cells on the inflamed

endothelium, thus facilitating the extravasation of T cells into tissue. Expression of ICAM-1 and VCAM-1 are down-regulated by an overabundance of angiogenic factors and other VEC-produced proteins (i.e. Egfl7) in the TME [89], resulting in the reduced flux of rolling leukocytes and density of adhering leukocytes in tumor-associated blood vessels [90].

Most studies on leukocyte trafficking into tissues focus on VECs, however, a growing literature suggests that pericytes also play significant roles in the extravasation and activation of effector immune cells. During inflammation, pericytes upregulate adhesion molecules, chemokines and cytokines [91–94] and may also display characteristics that are usually associated with antigen-presenting cells [95, 96]. Innate immune cells, specifically neutrophils, have been observed to crawl on pericytes [97]. It was later demonstrated that the pericyte-expressed molecule NG2 (aka chondroitin-sulfate glycoprotein-4; CSGP4) interacts with neutrophils and macrophages, instructing them to migrate into inflammatory tissue sites [93]. It is currently unknown whether pericytes provide the same homing signals to transmigrating adaptive immune cells in the TME. Moreover, a recent study indicates that pericytes have a direct role in tumor immune escape by inhibiting CD4 T cell activation and by promoting T cell anergy through Regulator of G-protein Signaling-5 (RGS-5)- and IL-6-dependent pathways [98].

13.4.4 Tumor-Associated Vasculature Promotes the evelopment of Immunosuppressive TMEs

Poor blood perfusion in the tumor leads to hypoxia, which has been shown to promote an immunosuppressive tumor microenvironment. Hypoxia promotes the differentiation of tumor-infiltrating myeloid cells to M2-like tumor-associated macrophages (TAMs). These M2-like TAMs adapt to the hypoxic environment by expressing hypoxia inducible factor (HIF)-1. HIFs are transcription factors that are stabilized under hypoxic conditions and are responsible for the expression of factors that allow cells to survive in a low oxygen environment. M2-like TAMs express high levels of IL-10, arginase I (Arg1) and TGFβ, which are factors that suppress protective type-1 T cell-mediated immunity, and VEGF, which exerts pro-angiogenic effects [99, 100]. Furthermore, M2-like TAMs preferentially localize within hypoxic regions and TAMs that are situated near functional blood vessels express lower levels of the aforementioned M2-associated immunosuppressive gene products [100].

Hypoxic conditions also promote tumor cells to produce immunosuppressive molecules that inhibit effector T cell and NK cell survival/function, while coordinately supporting the T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [101, 102]. Hypoxia decreases the survival and activity of T cells in the TME, partly through inhibition of IL-2 in a HIF-1-dependent manner [103, 104]. HIF-1 also drives expression of FoxP3 associated with Treg differentiation [105].

13.4.5 Tumor-Associated Vasculature Contributes to Metastasis

Metastasis is a multistep process that involves dissociation of tumor cells from the primary tumor mass, invasion of surrounding stroma, intravasation to the vasculature, survival in circulation, extravasation to a distal site and subsequent colonization of new organs [106]. The tumor vasculature is primarily involved in intravasation of tumor cells but can also contribute to other metastatic steps [107, 108].

Hypoxia, due to poor blood perfusion brought about by the structural abnormalities in the tumor-associated vasculature, is a contributing factor to the acquisition of malignant phenotype [109]. During hypoxic stress, HIF-1a together with TGFβ triggers epithelial-to-mesenchymal transition (EMT) in tumor cells. EMT is the process wherein epithelial markers are down-regulated and mesenchymal markers are up-regulated, leading to the dissociation of epithelial cell-to-cell interactions and the facilitation of cell motility and invasiveness [110].

Invasion of surrounding stroma by tumor cells requires a remodeling of the ECM and the migration of cancer cells through it. CAFs "lead" the metastatic tumor cells and utilize protease- and force-mediated activities to remodel the ECM [111]. Specifically, fibroblasts implement RhoA and Rho-associated protein kinase 1 to remodel the ECM in an MMP14-dependent manner [112, 113]. Cancer cells then follow these CAF-instructed microtracks in a CDC42-dependent manner [112].

The lack of an intact basement membrane and the disorganized VEC and pericyte interactions make the tumor blood vessels more accessible to motile tumor cells, easing the intravasation process [114]. Studies have shown that a high prevalence of abluminal pericytes limits tumor metastasis [115, 116]. In some tumor models, deficient pericyte coverage of blood vessels, due to a deficiency in pericyte recruitment, can be associated with increased metastatic potential [117]. Conversely, tumor cell intravasation is decreased when pericyte coverage of blood vessels is high within the TME [118].

Cancer cells actively disrupt tumor blood vessel cell interactions in order to facilitate the intravasation process. For example, breast cancer cells express MMP17 that disturbs blood vessel integrity, leading to enhanced tumor cell migration and intravasation [119, 120]. Malignant cells also secrete TGFβ1, which causes endothelial junction retraction [121]. Some cancer cells also express Notch receptors that bind to Notch ligands displayed on VECs, assisting their transmigration through the endothelial junctions [122].

In a complementary manner VECs in the TME also express factors that assist cancer cell intravasation. Breast cancer-associated VECs express the enzyme A Disintegrin And Metalloproteinase 12 (ADAM12) that cleaves vascular endothelial cadherin (VE-cadherin) and TIE2, both of which are also expressed on VECs [123, 124]. VE-cadherin is a component of cell-to-cell adherens junctions that maintains the integrity of the endothelium, hence the ADAM12-induced shedding of this molecule promotes enhanced vascular instability/permeability [124]. TIE2 is the receptor for Ang1 and Ang1/TIE2 ligation is important for pericyte recruitment to nascent blood vessels. ADAM12-mediated disruption of this interaction contributes to vessel destabilization, which is a prerequisite for intravasation [123].

13.4.6 Tumor-Associated Vasculature Promotes Cancer Stemness

Although the existence of cancer stem cells (CSC) remains a subject of active debate, some research suggests that within a tumor mass, a minority of cells retain the ability to give rise to all cell types found in the heterogeneous tumor lesion, much like a normal stem cell has the potential to differentiate into many different types of cells. CSCs usually localize in certain specialized areas in the TME. These niches, composed of cells and matrix components, promote the maintenance of CSCs via direct cell contacts and secreted factors [125]. In HNSCC and glioblastoma, such CSC niches are commonly located in close proximity to tumor blood vessels, defining a perivascular niche [126, 127]. In these cancers, tumor-associated VECs appear directly involved in the maintenance of the CSC population through the release of soluble factors that support CSC self-renewal [128–130].

13.5 Therapies Targeting the Tumor Vasculature

Targeting the tumor vasculature rather than tumor cells themselves has several conceptual advantages. First, vascular cells are more readily accessible to circulating effector cells, antibodies and other therapeutic molecules than tumor cells. Also, each vessel supplies hundreds of tumor cells, thus amplifying the tumoricidal efficiency of vascular targeting strategies. When compared to tumor cells, tumor-associated vascular cells are genetically (antigenically) more stable and they exhibit more consistent expression of major histocompatibility complex (MHC) class I (and class II) molecules required for specific T cell recognition. Lastly, anti-angiogenic treatment is not restricted to a specific tumor type, making the general

approach applicable to all solid (vascularized) forms for cancer. Since Folkman first proposed anti-angiogenesis as a potential therapeutic strategy in a landmark paper in 1971, numerous antiangiogenic drugs have since been developed and applied clinically (Fig. 13.2; [131, 132]).

13.5.1 Anti-VEGF

Given the major role of VEGF in angiogenesis, an array of anti-angiogenic therapies have been developed to antagonize VEGF/VEGFR-mediated signaling. Bevacizumab (Avastin, Genetech), a humanized anti-VEGF-A monoclonal antibody [133], was the first anti-angiogenic drug approved by the US Food and Drug Administration (FDA). It binds all VEGF isoforms within the TME, therefore blocking VEGF-VEGFR interaction [134–136]. In preclinical studies of bevacizumab, it was shown that tumor blood vessels that survived this anti-angiogenic treatment exhibited a more mature phenotype, resulting in a more functional vasculature that improved blood perfusion and reduced hypoxia in the TME. However, bevacizumab-induced vascular "normalization" was determined to be transient in nature, hence, necessitating the co-administration of conventional chemotherapy agents to provide enhanced therapeutic efficacy [137, 138]. Bevacizumab has been approved as first- or second-line therapy in the setting of metastatic colorectal cancer, non-small cell lung cancer (NSCLC), recurrent glioblastoma and metastatic renal cell carcinoma, usually in combination with chemotherapeutic drugs [139].

Aflibercept (Zaltrap, Regeneron and Sanofi Aventis) is a recombinant fusion protein consisting of the extracellular domains of VEGFR1 and VEGFR2 and the Fc portion of human IgG. Aflibercept, also known also VEGF-trap, is

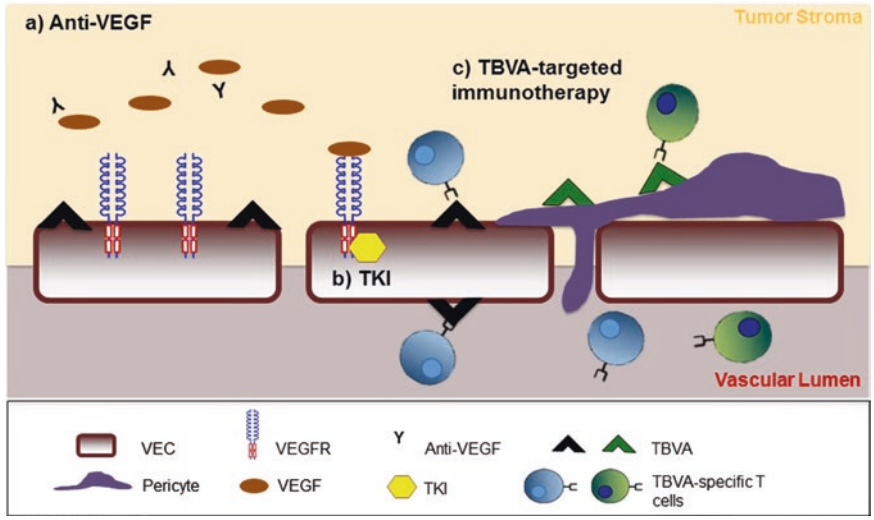

Fig. 13.2 Therapeutic intervention strategies to target tumor-associated blood vessels. The development of tumor-associated blood vessels is heavily VEGF-dependent, supporting the evolution and then clinical application of VEGFR-signaling antagonists, including (**a**) neutralizing anti-VEGF antibodies (such as bevacizumab) and (**b**) small molecule tyrosine kinase inhibitors (TKI) that block VEGFR-mediated signals (such as sunitinib and pazopanib, among others). TKI have also been developed that block signaling pathways important for pericyte survival/function, such as PDGFR-signaling antagonists (such as dasatinib, among others). It has also recently been appreciated that the TME alters the epigenetic programming of host stromal cell populations, including tumor-associated vascular endothelial cells and pericytes, leading to changes in protein expression that allow for specific T cell targeting (vs. blood vessel cells found in normal tissues). Such anti-tumor blood vessel T cells can be elicited by active vaccination or be adoptively transferred as a therapeutic modality after *ex vivo* expansion (i.e. as an adoptive cellular therapy; ACT)

a decoy receptor that binds VEGF-A, VEGF-B and PIGF. It has been approved for second-line treatment for metastatic colorectal carcinoma in combination with chemotherapy [140]. Both bevacizumab and aflibercept inhibit the pro-angiogenic effects of VEGF, resulting in reduced VEC proliferation, vessel permeability, and blood vessel density, and inhibited tumor growth [135, 141–143].

13.5.2 Tyrosine Kinase Inhibitors

Most angiogenic factors (VEGF, PDGF and FGF) contribute pro-tumor signals through specific receptor-type tyrosine kinase (RTKs). In mammals, there are three VEGFR genes *VEGFR1*, *VEGFR2, and VEGFR3* that produce four proteins VEGFR1, VEGFR2, VEGFR3 and sFlt-1 (sVEGFR) [144]. PDGFR has two subtypes (α and β) that bind the four PDGF types (A, B, C, and D) [145]. The FGFR family consists of FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3c, and FGFR4 [146]. RTKs generally consist of extracellular ligand-binding region, a transmembrane helix, and a cytoplasmic tyrosine kinase (TK) domain. Ligand binding activates RTK by inducing receptor dimerization/oligomerization. One receptor in the complex then phosphorylates one or more tyrosines in adjacent RTK then the phosphorylated receptor serves as a site for assembly and activation of intracellular signaling proteins [147]. Tyrosine kinase inhibitors (TKI) are small molecules that interrupt ATP binding to the tyrosine kinase catalytic domain through competition, thereby, inhibiting the downstream receptor signaling [148].

Sunitinib (SU11248, Sutent, Pfizer) targets VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β, FLT3, stem cell factor receptor (c-Kit), RET and CSF-1R [149, 150]. *In vitro*, sunitinib inhibits VEGF-dependent migration and capillary-tube formation by cultured human umbilical vein endothelial cells (HUVECs), while simultaneously promoting VEC apoptosis [151, 152]. *In vivo* efficacy studies performed in mouse xenograft models demonstrated that administration of sunitinib inhibits tumor growth, suppresses angiogenesis and decreases microvessel density [153–155]. Sunitinib has been approved by the FDA as a first-line therapy in the setting of renal cell carcinoma (RCC) and unresectable pancreatic neuroendocrine tumors (PNET), and as a second-line therapy for patients with gastrointestinal stromal tumors (GIST) as a single agent or in combination with chemotherapy or radiation [149, 156]. Although RCC patient outcome has been reported to be improved after treatment with sunitinib, the best observed clinical responses have tended to be temporary disease stabilization. Furthermore, a treatment refractory RCC usually develops, that may be overcome in some instances by increasing the dosage of sunitinib being administered to patients [12, 157]. Objective clinical response to sunitinib has also been reported for other types of cancer, such as small cell lung carcinoma (SCLC), breast cancer, thyroid cancer, and chondrosarcoma. However, sunitinib has reportedly failed as a therapy in the setting of NSCLC, metastatic breast cancer, and advanced-stage esophageal cancer [149].

Sorafenib (BAY439006, Nexavar, Bayer, Inc.) inhibits Raf-1, wild type BRAF, V599E mutant BRAF, VEGFR-2, VEGFR-3, PDGFR-β, FLT3, and c-Kit [150, 158]. In several murine tumor models, sorafenib treatment resulted in decreased microvessel density, decreased vessel arborization and increased pericyte coverage of blood vessels in the TME. Treatment also blocked neovascularization and ERK activation in VECs [159]. Sorafenib may also target tumor cells directly via disruption of the ERK pathway, since ERK phosphorylation and the proliferation of some tumor cell lines could be effectively inhibited by this TKI. Tumor growth inhibition was observed in some but not all colon, breast, ovarian, thyroid, melanoma, RCC, hepatocellular carcinoma (HCC) and NSCLC xenograft models. These results suggest that the anti-proliferative action of sorafenib is dependent on blocking oncogenic signaling pathways driving tumor proliferation [158, 160, 161]. Sorafenib has been approved for the treatment of patients with advanced RCC, unresectable HCC, and recurrent or metastatic differentiated thyroid carcinoma [162, 163].

Pazopanib (GW786034, Votrient, GlaxoSmithKline) is a second-generation TKI that inhibits VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-α, PDGFR-β and c-Kit. Pazopanib also moderately binds FGFR1, FGFR3 and c-fms receptor tyrosine kinases [164]. *In vitro,* pazopanib blocked the VEGF-induced phosphorylation of VEGFR2, and thus prevented the proliferation of HUVECs [165]. FGF-induced proliferation of HUVECs was also blocked by pazopanib [164]. Pazopanib was shown to have anti-tumor efficacy in a range of Hu-SCID tumor xenograft models including those using RCC, NSCLC, colorectal cancer, and multiple myeloma cell lines. However, it is reportedly less effective in inhibiting the progression of melanoma, breast cancer, and prostate cancer growth in xenograft models [166]. Pazopanib has been approved by the FDA as a therapeutic agent for patients with RCC or soft tissue sarcoma after standard-of-care chemotherapy [167, 168].

Axitinib (Inlyta, AG-013736, Pfizer) is a second generation TKI that targets VEGFR1, VEGFR2 and VEGFR3 [169]. Cell assays showed that axitinib inhibited VEGF-mediated survival, tube formation and ERK, NOS and Akt signaling in HUVECs. Axitinib also inhibited tumor growth in orthotopic and xenograft tumor models when applied as a single agent or in combination with chemotherapy [170]. Preclinical data in RCC models strongly support the anti-angiogenic action of axitinib, which however, has proven to be reversible upon withdrawal of this drug [171]. Axitinib has been FDA-approved as a second-line treatment option for patients with RCC [169].

Drug dosage and schedule are two critical variables when administering anti-angiogenic small molecule inhibitors and antibodies as therapeutic agents. High doses of such agents may promote excessive pruning of the blood vessels in the TME, leading to tumor necrosis and delayed tumor growth, however, they may also promote a local state of hypoxia, that could in turn lead to a compensatory activation of alternative pro-angiogenic signaling pathways that promote tumorigenicity and metastasis. Conversely, lower doses of anti-angiogenic agents could prune some abnormal vessels while remodeling others, leading to a more normalized vasculature that reduces hypoxia and IFP within the TME, resulting in an improvement in the delivery and efficacy of co-therapies [100, 172, 173].

13.5.3 Immunotherapeutic Targeting of Tumor-Associated Vascular Cells

As previously mentioned, tumor vascular cells are considered genetically stable and highly-accessible to circulating immune cell populations, making them highly-visible for immune surveillance. Based on altered epigenetic programming instigated by cellular stressors (i.e. hypoxia, acidosis, high-IFP), tumor-associated vascular cells exhibit molecular phenotypes that distinguish them from their counterparts in normal tissues [174, 175]. Such molecular differences may be targeted immunologically via active vaccination to develop specific T cells capable of selectively reacting against and normalizing the vasculature in the TME.

Peptide-based and recombinant vaccines based on the VEGFR1 and VEGFR2 gene products have been shown to limit tumor-associated angiogenesis and disease progression, via elicitation of antigen-specific $CD8^+$ T cell responses in pre-clinical animal models [176–179]. Phase I clinical trials of vaccines that included peptides derived from the VEGFR1 and VEGFR2 proteins demonstrated safety and some degree of efficacy (i.e. they were capable of inducing immune responses that restrict tumor angiogenesis [180–183]. However, VEGFR2 peptide-based vaccines, when applied in combination with the chemotherapeutic drug gemcitabine, did not improve overall patient survival when compared to a placebo control in a phase II/III trial in the setting of advanced-stage pancreatic cancer [184].

Although early tumor vascular vaccines targeted VEGFRs, a range of additional tumor blood vessel-associated antigens (TBVA) are important to vascular formation and maintenance in the TME, and hence, represent cogent vaccine

targets. In this regard, Regulator of G-protein signaling-5 (RGS-5) is overexpressed by tumor-associated (immature) pericytes in the RIP1-Tag5 model of pancreatic islet cell cancer ([185, 186]. Genetic deletion of RGS-5 resulted in pericyte maturation, vasculature normalization and enhancement of adoptive T cell therapy benefits [186]. Expression of the Notch antagonist, Delta-like homolog-1 (DLK-1), is upregulated in pericytes isolated from murine melanoma, colorectal carcinoma and renal cell carcinomas (unpublished). Notably, peptide- and gene-based vaccines targeting DLK1 resulted in delayed tumor progression and the enhancement of CD8+ T cell infiltration in the TME of murine renal carcinomas. Tumors in DLK1-vaccinated mice displayed decreased vascular permeability and hypoxia, reduced expression of hypoxia-inducible proteins in the stroma, and enhanced levels of tumor cell apoptosis *in vivo* [187]. Tumor endothelial marker 1 (TEM-1; aka CD248) is a TBVA upregulated in VECs, pericytes and other stromal cell populations isolated from a range of tumor histotypes. A DNA vaccine composed of the TEM1 gene fused with the C fragment of tetanus toxoid was shown to increase $CD3^+$ T cell infiltrates in the tumors of treated animals in association with slowed tumor growth [188]. Furthermore, dendritic cell (DC)-based vaccines integrating HLA-A2 class I-presented peptides derived from TBVA including DLK1, EphA2, hemoglobin-β (HBB), NG2, neuropilin (NRP)-1, PDGFRβ, RGS5 and TEM1 were shown to be effective in providing protective or therapeutic immunity against HLA-$A2^{neg}$ melanoma and colon carcinomas in HLA-A2 transgenic mice. Specific vaccination induced the generation of HLA-A2-restricted $CD8^+$ T effector cells that recognized relevant antigen-expressing pericytes and/or VECs isolated from tumors, but not normal tissue [189].

Vaccination against TBVAs may also potentially promote the broadening of a therapeutic immune response via a progressive process termed "epitope spreading". In such a paradigm, anti-TBVA T cells would promote vascular normalization and tumor apoptosis, leading to tumor antigen uptake by recruited dendritic cells that could subsequently activate (i.e. crossprime) secondary waves of tumor-reactive T cells yielding added therapeutic benefit based on combined immune targeting of both $TBVA^+$ vascular cells and tumor cells [188].

13.5.4 Combination (Immune) therapies Targeting Tumor-Associated Blood Vessels

Anti-angiogenic antibodies and small molecules have been shown to be efficacious in pre-clinical tumor models and a growing number of human cancers. However, the therapeutic benefits of these agents are commonly transient in nature, with the subsequent progression of (same) treatment-refractory disease. Developed drug-resistance may be attributed to tumor adaptation events and the adoption of alternative pro-angiogenic/pro-survival pathways [Reviewed in [190]]. As a consequence, extensive research is being directed at developing novel second-line or co-first-line (combination) therapeutic regimens that will complement angiogenesis inhibition, leading to improved treatment outcomes.

Notably, many anti-angiogenic agents/treatments create a transient state of vascular normalization in the TME, during which tumor are more receptive to delivery of chemotherapeutic drugs and immune cell populations [191]. This has prompted logical second-set clinical protocols integrating anti-angiogenic drugs such as bevacizumab, along with chemotherapeutic drugs, such as paclitaxel, cisplatin, and carboplatin, amongst others [139]. In an ovarian carcinoma xenograft model, combined treatment with pazopanib and low doses of topotecan exhibited superior anti-tumor efficacy when compared with either single modality [192]. In another example, the combination of sunitinib and gemcitabine inhibited the growth and metastasis of murine pancreatic carcinoma in an orthotopic model to a greater degree than individual treatment with either sunitinib or gemcitabine [193].

However, there are some reports that under some conditions, anti-angiogenic drugs might actually impede the delivery of chemotherapeutic

drugs into the TME. Drug uptake and retention of docetaxel was evaluated in patients with advanced NSCLC treated with bevacizumab and it was shown that VEGF inhibition decreased the penetration of water and the chemotherapeutic agent into tumor sites [194]. This strongly suggests that temporal interval wherein a given angiogenesis inhibitor normalizes the tumor vasculature must first be determined in order to optimize the delivery benefit to the co-therapeutic agent. Unfortunately, there appears to be a high-degree of variation in the temporal window of vascular normalization for various anti-angiogenic modalities likely necessitating the need to develop non-invasive imaging techniques to monitor tumor blood perfusion in patients to define an optimal combination treatment schedule [195, 196].

Tumor hypoxia is known to limit the efficacy of radiation therapy in the cancer setting, hence, the application of angiogenesis inhibitors with radiation has the potential to improve treatment outcomes. Indeed, the combination of VEGF inhibitors with irradiation has consistently demonstrated superior therapeutic benefits (i.e. prolonged delay in tumor growth) despite differences in the combinations of radiation schedules and drugs and doses used [197].

Anti-angiogenic agents may also improve the efficacy of cancer vaccines, that have typically proven to be immunogenic but of limited clinical benefit in the vast majority of trials thus far evaluated [198, 199]. A major limitation in vaccine efficacy likely involves the inefficient delivery of vaccine-induced T cells into the TME [200]. Since angiogenesis inhibitors can normalize blood vessels in the TME and promote preferred changes in T cell recruiting chemokine gradients and vascular adhesion molecules, in addition to reducing hypoxia and immunosuppressive MDSC and Treg content populations [200, 201], anti-angiogenic agents should serve as an effective adjuvant for cancer vaccines. Indeed, DC-based vaccines against the $OVA_{257–264}$ peptide epitope in combination with the TKIs axitinib, sunitinib or dasatinib (a BCR-ABL, SRC, c-KIT, PDGFR, and ephrin tyrosine kinases inhibitor), each demonstrated superior therapeutic benefit in the murine M05 (B16.OVA) melanoma model when compared to treatment with vaccine alone or TKI alone. In vaccine + TKI treated mice, the expression of VCAM-1 and CXCR3 ligand chemokines in the tumor endothelium were augmented in association with robust type-1 T cell infiltration and extended overall survival [201–203].

13.6 Conclusions and Future Directions

The tumor vasculature is an excellent target for developing improved cancer therapeutics. The cells of the tumor vasculature are more genetically stable than tumor cells, express antigens that are not found in their normal cell counterparts, and are more accessible to therapeutic molecules and immune cells. Indeed, anti-angiogenic agents that target tumor blood vessels have proven capable of pruning immature vessels and normalizing the vasculature, resulting in reduced hypoxia and IFP, and increased tumor cell apoptosis. Furthermore, these approaches facilitate the delivery of drugs and immune effector cells into the therapeutic TME. However, since anti-angiogenic agent-induced vascular remodeling is only transient and subject to the development of drug-refractory progressive disease, it fully expected that these modalities will prove most clinically effective only when provided in combination (chemo-, radio-, immuno-therapy) approaches that will require careful optimization of each modality's dose and schedule. In this regard, it will also likely be very important to identify biomarkers that could be used to predict clinical outcome of patients treated with anti-angiogenic drugs as monotherapies and in combination protocols [173].

Therapeutic vaccines promoting immune targeting of tumor vascular cells are an emerging anti-cancer strategy. Ideally, these vaccines should target antigens that are exclusively expressed (or grossly overexpressed) by cells in tumor blood vessels to minimize concerns for negative consequences in normal tissues (including the eye, blood-brain barrier) or upon normal

angiogenic processes (i.e. wound-healing, pregnancy). A number of studies have shown that vaccination based on TBVAs is effective in normalizing the vasculature, inducing a T-cell effector response, and inhibiting tumor growth in murine models, without affecting the wound healing process or aspects of animal husbandry [177]. The continued search for novel TBVAs that are broadly shared across solid cancers is clearly of great interest for prospective clinical targeting. As with any vaccine involving the targeting of non-mutated "self" antigens, adjuvants might have to be administered with TBVA-based vaccines in order to effectively break operational immune tolerance in patients. Furthermore, the ability of vaccine-induced anti-TBVA T cells to be recruited into the TME will require implementation of conditioning regimens that may be accommodated via the use of TKIs that both improve T effector cell delivery into tumor sites and coordinately mitigate the immunosuppressive influence of MDSC and Treg regulatory cell populations. Such potentiation of anti-TBVA T cell vasculocidal/tumoricidal action might also be evidenced in combination regimens integrating immune checkpoint inhibitors, such as anti-CTLA-4 and/or anti-PD-1/PD-L1, which mediate profound anti-tumor clinical activity in the setting of multiple forms of human cancer [204].

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
2. Dor Y, Porat R, Keshet E. Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. Am J Physiol Cell Physiol. 2001;280:C1367–74.
3. Bottos A, Bardelli A. Oncogenes and angiogenesis: a way to personalize anti-angiogenic therapy? Cell Mol Life Sci. 2013;70:4131–40.
4. Bates D, Hillman N, Williams B, Neal C, Pocock T. Regulation of microvascular permeability by vascular endothelial growth factors. J Anat. 2002; 200:581–97.
5. Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science. 1999;284:1994–8.
6. Cross MJ, Claesson-Welsh L. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. Trends Pharmacol Sci. 2001;22:201–7.
7. Gavalas NG, Liontos M, Trachana SP, Bagratuni T, Arapinis C, Liacos C, Dimopoulos MA, Bamias A. Angiogenesis-related pathways in the pathogenesis of ovarian cancer. Int J Mol Sci. 2013;14:15885–909.
8. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol. 2005;7:452–64.
9. Jain RK. Molecular regulation of vessel maturation. Nat Med. 2003;9:685–93.
10. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol. 2002;160:985–1000.
11. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am J Pathol. 2003;163:1801–15.
12. Adelaiye R, Ciamporcero E, Miles KM, Sotomayor P, Bard J, Tsompana M, Conroy D, Shen L, Ramakrishnan S, Ku SY, et al. Sunitinib dose escalation overcomes transient resistance in clear cell renal cell carcinoma and is associated with epigenetic modifications. Mol Cancer Ther. 2015;14:513–22.
13. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012;74:13–40.
14. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res. 2005;97:512–23.
15. Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21:193–215.
16. Plate KH, Scholz A, Dumont DJ. Tumor angiogenesis and anti-angiogenic therapy in malignant gliomas revisited. Acta Neuropathol. 2012;124:763–75.
17. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev. 2005;15:102–11.
18. McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. Nat Med. 2003;9:713–25.
19. Wragg JW, Durant S, McGettrick HM, Sample KM, Egginton S, Bicknell R. Shear stress regulated gene expression and angiogenesis in vascular endothelium. Microcirculation. 2014;21:290–300.
20. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev. 1997;18:4–25.

21. Ferrara N, Mass RD, Campa C, Kim R. Targeting VEGF-A to treat cancer and age-related macular degeneration. Annu Rev Med. 2007;58:491–504.
22. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. J Cell Biol. 2005;169:681–91.
23. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376:62–6.
24. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol Off J Am Soc Clin Oncol. 2002;20:4368–80.
25. Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, Kerbel RS. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. Cancer Res. 1995;55:4575–80.
26. Zhang X, Gaspard JP, Chung DC. Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res. 2001;61:6050–4.
27. Marshall CJ, Moore RL, Thorogood P, Brickell PM, Kinnon C, Thrasher AJ. Detailed characterization of the human aorta-gonad-mesonephros region reveals morphological polarity resembling a hematopoietic stromal layer. Dev Dyn. 1999;215:139–47.
28. Sumpio BE, Riley JT, Dardik A. Cells in focus: endothelial cell. Int J Biochem Cell Biol. 2002;34:1508–12.
29. Ruoslahti E. Specialization of tumour vasculature. Nat Rev Cancer. 2002;2:83–90.
30. Schwartz JD, Rowinsky EK, Youssoufian H, Pytowski B, Wu Y. Vascular endothelial growth factor receptor-1 in human cancer: concise review and rationale for development of IMC-18F1 (human antibody targeting vascular endothelial growth factor receptor-1). Cancer. 2010;116:1027–32.
31. Smith NR, Baker D, James NH, Ratcliffe K, Jenkins M, Ashton SE, Sproat G, Swann R, Gray N, Ryan A, et al. Vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are localized primarily to the vasculature in human primary solid cancers. Clin Cancer Res. 2010;16:3548–61.
32. Otsubo T, Hida Y, Ohga N, Sato H, Kai T, Matsuki Y, Takasu H, Akiyama K, Maishi N, Kawamoto T, et al. Identification of novel targets for antiangiogenic therapy by comparing the gene expressions of tumor and normal endothelial cells. Cancer Sci. 2014;105:560–7.
33. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, et al. Genes expressed in human tumor endothelium. Science. 2000;289:1197–202.
34. Nanda A, St Croix B. Tumor endothelial markers: new targets for cancer therapy. Curr Opin Oncol. 2004;16:44–9.
35. Michiels C. Endothelial cell functions. J Cell Physiol. 2003;196:430–43.
36. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood. 1998;91:3527–61.
37. Dejana E, Orsenigo F, Molendini C, Baluk P, McDonald DM. Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees. Cell Tissue Res. 2009a;335:17–25.
38. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell. 2009b;16:209–21.
39. Lampugnani MG. Endothelial cell-to-cell junctions: adhesion and signaling in physiology and pathology. Cold Spring Harb Perspect Med. 2012;2(10):a006528.
40. Wallez Y, Huber P. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. Biochim Biophys Acta. 2008;1778:794–809.
41. Dudley AC. Tumor endothelial cells. Cold Spring Harb Perspect Med. 2012;2(10):a006536.
42. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am J Pathol. 1988;133:95–109.
43. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol. 2000;156:1363–80.
44. Roberts WG, Delaat J, Nagane M, Huang S, Cavenee WK, Palade GE. Host microvasculature influence on tumor vascular morphology and endothelial gene expression. Am J Pathol. 1998;153:1239–48.
45. Claesson-Welsh L. Blood vessels as targets in tumor therapy. Ups J Med Sci. 2012;117:178–86.
46. Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. Nat Rev Cancer. 2002;2:266–76.
47. Barlow KD, Sanders AM, Soker S, Ergun S, Metheny-Barlow LJ. Pericytes on the tumor vasculature: jekyll or hyde? Cancer microenvironment: official journal of the international cancer microenvironment. Society. 2013;6:1–17.
48. Birbrair A, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes at the intersection between tissue regeneration and pathology. Clin Sci (Lond). 2015;128:81–93.
49. Sims DE. The pericyte—a review. Tissue Cell. 1986;18(2):153–74. PMID 3085281

50. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. 2003;314:15–23.
51. Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg H, Betsholtz C. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol. 2001;153:543–53.
52. Lindahl P, Hellstrom M, Kalen M, Betsholtz C. Endothelial-perivascular cell signaling in vascular development: lessons from knockout mice. Curr Opin Lipidol. 1998;9:407–11.
53. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. Exp Cell Res. 2006;312:623–9.
54. Diaz-Flores L, Gutierrez R, Madrid JF, Varela H, Valladares F, Acosta E, Martin-Vasallo P, Diaz-Flores L Jr. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. Histol Histopathol. 2009;24:909–69.
55. Hall AP. Review of the pericyte during angiogenesis and its role in cancer and diabetic retinopathy. Toxicol Pathol. 2006;34:763–75.
56. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest. 1999;103:159–65.
57. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest. 2003;111:1287–95.
58. Reinmuth N, Liu W, Jung YD, Ahmad SA, Shaheen RM, Fan F, Bucana CD, McMahon G, Gallick GE, Ellis LM. Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. FASEB J. 2001;15:1239–41.
59. Shaheen RM, Tseng WW, Davis DW, Liu W, Reinmuth N, Vellagas R, Wieczorek AA, Ogura Y, McConkey DJ, Drazan KE, et al. Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms. Cancer Res. 2001;61:1464–8.
60. Kharaishvili G, Simkova D, Bouchalova K, Gachechiladze M, Narsia N, Bouchal J. The role of cancer-associated fibroblasts, solid stress and other microenvironmental factors in tumor progression and therapy resistance. Cancer Cell Int. 2014;14:41.
61. Ohlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. J Exp Med. 2014;211:1503–23.
62. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6:392–401.
63. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N, Selig M, Nielsen G, Taksir T, Jain RK, et al. Tumor induction of VEGF promoter activity in stromal cells. Cell. 1998;94:715–25.
64. Cirri P, Chiarugi P. Cancer associated fibroblasts: the dark side of the coin. Am J Cancer Res. 2011;1:482–97.
65. Kryczek I, Wei S, Keller E, Liu R, Zou W. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. Am J Physiol Cell Physiol. 2007;292:C987–95.
66. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335–48.
67. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient Neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell. 2010;17:135–47.
68. Lederle W, Hartenstein B, Meides A, Kunzelmann H, Werb Z, Angel P, Mueller MM. MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. Carcinogenesis. 2010;31:1175–84.
69. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer. 2003;3:422–33.
70. Paulsson M. Basement membrane proteins: structure, assembly, and cellular interactions. Crit Rev Biochem Mol Biol. 1992;27:93–127.
71. Form DM, Pratt BM, Madri JA. Endothelial cell proliferation during angiogenesis. In vitro modulation by basement membrane components. Lab Invest. 1986;55:521–30.
72. Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC. Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. J Cell Biol. 2001;154:1069–79.
73. Geevarghese A, Herman IM. Pericyte-endothelial crosstalk: implications and opportunities for advanced cellular therapies. Translational research. J Lab Clin Med. 2014;163:296–306.
74. Stratman AN, Davis GE. Endothelial cell-pericyte interactions stimulate basement membrane matrix assembly: influence on vascular tube remodeling, maturation, and stabilization. Microsc Microanal. 2012;18:68–80.
75. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. Blood. 2009;114:5091–101.
76. Ausprunk DH, Knighton DR, Folkman J. Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels. Am J Pathol. 1975;79:597–618.
77. Gimbrone MA Jr, Cotran RS, Leapman SB, Folkman J. Tumor growth and neovascularization: an experimental model using the rabbit cornea. J Natl Cancer Inst. 1974;52:413–27.

78. Micke P, Ostman A. Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets. 2005;9:1217–33.
79. Kaneko T, Zhang Z, Mantellini MG, Karl E, Zeitlin B, Verhaegen M, Soengas MS, Lingen M, Strieter RM, Nunez G, et al. Bcl-2 orchestrates a cross-talk between endothelial and tumor cells that promotes tumor growth. Cancer Res. 2007;67:9685–93.
80. Neiva KG, Zhang Z, Miyazawa M, Warner KA, Karl E, Nor JE. Cross talk initiated by endothelial cells enhances migration and inhibits anoikis of squamous cell carcinoma cells through STAT3/Akt/ERK signaling. Neoplasia. 2009;11:583–93.
81. Narang AS, Varia S. Role of tumor vascular architecture in drug delivery. Adv Drug Deliv Rev. 2011;63:640–58.
82. Boucher Y, Baxter LT, Jain RK. Interstitial pressure gradients in tissue-isolated and subcutaneous tumors: implications for therapy. Cancer Res. 1990;50:4478–84.
83. Azzi S, Hebda JK, Gavard J. Vascular permeability and drug delivery in cancers. Front Oncol. 2013;3:211.
84. Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. The journal of histochemistry and cytochemistry: official journal of the Histochemistry. Society. 1990;38:1277–87.
85. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010;10:826–37.
86. Scott DW, Patel RP. Endothelial heterogeneity and adhesion molecules N-glycosylation: implications in leukocyte trafficking in inflammation. Glycobiology. 2013;23:622–33.
87. Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol. 1993;11:767–804.
88. von Andrian UH, Mackay CR. T-cell function and migration. Two sides of the same coin. N Engl J Med. 2000;343:1020–34.
89. Delfortrie S, Pinte S, Mattot V, Samson C, Villain G, Caetano B, Lauridant-Philippin G, Baranzelli MC, Bonneterre J, Trottein F, et al. Egfl7 promotes tumor escape from immunity by repressing endothelial cell activation. Cancer Res. 2011;71:7176–86.
90. Wu NZ, Klitzman B, Dodge R, Dewhirst MW. Diminished leukocyte-endothelium interaction in tumor microvessels. Cancer Res. 1992;52:4265–8.
91. Balabanov R, Washington R, Wagnerova J, Dore-Duffy P. CNS microvascular pericytes express macrophage-like function, cell surface integrin alpha M, and macrophage marker ED-2. Microvasc Res. 1996;52:127–42.
92. Edelman DA, Jiang Y, Tyburski JG, Wilson RF, Steffes CP. Cytokine production in lipopolysaccharide-exposed rat lung pericytes. J Trauma. 2007;62:89–93.
93. Stark K, Eckart A, Haidari S, Tirniceriu A, Lorenz M, von Bruhl ML, Gartner F, Khandoga AG, Legate KR, Pless R, et al. Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and 'instruct' them with pattern-recognition and motility programs. Nat Immunol. 2013;14:41–51.
94. Verbeek MM, Westphal JR, Ruiter DJ, de Waal RM. T lymphocyte adhesion to human brain pericytes is mediated via very late antigen-4/vascular cell adhesion molecule-1 interactions. J Immunol. 1995;154:5876–84.
95. Balabanov R, Beaumont T, Dore-Duffy P. Role of central nervous system microvascular pericytes in activation of antigen-primed splenic T-lymphocytes. J Neurosci Res. 1999;55:578–87.
96. Maier CL, Pober JS. Human placental pericytes poorly stimulate and actively regulate allogeneic CD4 T cell responses. Arterioscler Thromb Vasc Biol. 2011;31:183–9.
97. Proebstl D, Voisin MB, Woodfin A, Whiteford J, D'Acquisto F, Jones GE, Rowe D, Nourshargh S. Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J Exp Med. 2012;209:1219–34.
98. Bose A, Barik S, Banerjee S, Ghosh T, Mallick A, Bhattacharyya Majumdar S, Goswami KK, Bhuniya A, Banerjee S, Baral R, et al. Tumor-derived vascular pericytes anergize Th cells. J Immunol. 2013;191:971–81.
99. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. Cancers (Basel). 2014;6:1670–90.
100. Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. Cancer Res. 2013;73:2943–8.
101. Barsoum IB, Koti M, Siemens DR, Graham CH. Mechanisms of hypoxia-mediated immune escape in cancer. Cancer Res. 2014;74:7185–90.
102. Chouaib S, Messai Y, Couve S, Escudier B, Hasmim M, Noman MZ. Hypoxia promotes tumor growth in linking angiogenesis to immune escape. Front Immunol. 2012;3:21.
103. Lukashev D, Klebanov B, Kojima H, Grinberg A, Ohta A, Berenfeld L, Wenger RH, Ohta A, Sitkovsky M. Cutting edge: hypoxia-inducible factor 1alpha and its activation-inducible short isoform I.1 negatively regulate functions of CD4+ and CD8+ T lymphocytes. J Immunol. 2006;177:4962–5.
104. Zuckerberg AL, Goldberg LI, Lederman HM. Effects of hypoxia on interleukin-2 mRNA expression by T lymphocytes. Crit Care Med. 1994;22:197–203.
105. Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, de Zoeten EF, Cambier JC, Stenmark KR, Colgan SP, et al. Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa. Proc Natl Acad Sci U S A. 2012;109:E2784–93.

106. Jin X, Zhu Z, Shi Y. Metastasis mechanism and gene/protein expression in gastric cancer with distant organs metastasis. Bull Cancer. 2014;101:E1–12.
107. Cao Z, Shang B, Zhang G, Miele L, Sarkar FH, Wang Z, Zhou Q. Tumor cell-mediated neovascularization and lymphangiogenesis contrive tumor progression and cancer metastasis. Biochim Biophys Acta. 2013;1836:273–86.
108. Moserle L, Casanovas O. Anti-angiogenesis and metastasis: a tumour and stromal cell alliance. J Intern Med. 2013;273:128–37.
109. Tsai YP, Wu KJ. Hypoxia-regulated target genes implicated in tumor metastasis. J Biomed Sci. 2012;19:102.
110. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2:442–54.
111. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol. 2009;10:445–57.
112. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, Sahai E. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol. 2007;9:1392–400.
113. Wolf K, Wu YI, Liu Y, Geiger J, Tam E, Overall C, Stack MS, Friedl P. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat Cell Biol. 2007;9:893–904.
114. Beerling E, Ritsma L, Vrisekoop N, Derksen PW, van Rheenen J. Intravital microscopy: new insights into metastasis of tumors. J Cell Sci. 2011;124:299–310.
115. Gerhardt H, Semb H. Pericytes: gatekeepers in tumour cell metastasis? J Mol Med (Berl). 2008;86:135–44.
116. Xian X, Hakansson J, Stahlberg A, Lindblom P, Betsholtz C, Gerhardt H, Semb H. Pericytes limit tumor cell metastasis. J Clin Invest. 2006;116:642–51.
117. Yonenaga Y, Mori A, Onodera H, Yasuda S, Oe H, Fujimoto A, Tachibana T, Imamura M. Absence of smooth muscle actin-positive pericyte coverage of tumor vessels correlates with hematogenous metastasis and prognosis of colorectal cancer patients. Oncology. 2005;69:159–66.
118. Welen K, Jennbacken K, Tesan T, Damber JE. Pericyte coverage decreases invasion of tumour cells into blood vessels in prostate cancer xenografts. Prostate Cancer Prostatic Dis. 2009;12:41–6.
119. Chabottaux V, Ricaud S, Host L, Blacher S, Paye A, Thiry M, Garofalakis A, Pestourie C, Gombert K, Bruyere F, et al. Membrane-type 4 matrix metalloproteinase (MT4-MMP) induces lung metastasis by alteration of primary breast tumour vascular architecture. J Cell Mol Med. 2009;13:4002–13.
120. Sohail A, Marco M, Zhao H, Shi Q, Merriman S, Mobashery S, Fridman R. Characterization of the dimerization interface of membrane type 4 (MT4)-matrix metalloproteinase. J Biol Chem. 2011;286:33178–89.
121. Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. Nat Rev Cancer. 2013;13:858–70.
122. Garcia A, Kandel JJ. Notch: a key regulator of tumor angiogenesis and metastasis. Histol Histopathol. 2012;27:151–6.
123. Frohlich C, Klitgaard M, Noer JB, Kotzsch A, Nehammer C, Kronqvist P, Berthelsen J, Blobel C, Kveiborg M, Albrechtsen R, et al. ADAM12 is expressed in the tumour vasculature and mediates ectodomain shedding of several membrane-anchored endothelial proteins. Biochem J. 2013;452:97–109.
124. Giannotta M, Trani M, Dejana E. VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. Dev Cell. 2013;26:441–54.
125. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell. 2008;132:598–611.
126. Charles N, Holland EC. The perivascular niche microenvironment in brain tumor progression. Cell Cycle. 2010;9:3012–21.
127. Ritchie KE, Nor JE. Perivascular stem cell niche in head and neck cancer. Cancer Lett. 2013;338:41–6.
128. Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, Oh EY, Gaber MW, Finklestein D, Allen M, et al. A perivascular niche for brain tumor stem cells. Cancer Cell. 2007;11:69–82.
129. Krishnamurthy S, Dong Z, Vodopyanov D, Imai A, Helman JI, Prince ME, Wicha MS, Nor JE. Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. Cancer Res. 2010;70:9969–78.
130. Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science. 2004;304:1338–40.
131. Fens MH, Storm G, Schiffelers RM. Tumor vasculature as target for therapeutic intervention. Expert Opin Investig Drugs. 2010;19:1321–38.
132. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182–6.
133. Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 1997;57:4593–9.
134. Rosen LS. Angiogenesis inhibition in solid tumors. Cancer J. 2001;7(suppl 3):S120–8.
135. Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther. 2006;28:1779–802.
136. Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. Angiogenesis. 2004;7:335–45.

137. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. Mol Cancer Ther. 2008;7:3670–84.
138. Zhou Q, Guo P, Gallo JM. Impact of angiogenesis inhibition by sunitinib on tumor distribution of temozolomide. Clin Cancer Res. 2008;14:1540–9.
139. De Falco S. Antiangiogenesis therapy: an update after the first decade. Korean J Intern Med. 2014;29:1–11.
140. Ciombor KK, Berlin J, Chan E. Aflibercept. Clin Cancer Res. 2013;19:1920–5.
141. Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, Yancopoulos GD, Jaffe RB. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res. 2003;9:5721–8.
142. Rosen LS. VEGF-targeted therapy: therapeutic potential and recent advances. Oncologist. 2005;10:382–91.
143. Verheul HM, Hammers H, van Erp K, Wei Y, Sanni T, Salumbides B, Qian DZ, Yancopoulos GD, Pili R. Vascular endothelial growth factor trap blocks tumor growth, metastasis formation, and vascular leakage in an orthotopic murine renal cell cancer model. Clin Cancer Res. 2007;13:4201–8.
144. Shibuya M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. J Biochem. 2013;153:13–9.
145. Chen PH, Chen X, He X. Platelet-derived growth factors and their receptors: structural and functional perspectives. Biochim Biophys Acta. 2013;1834:2176–86.
146. Katoh M, Nakagama H. FGF receptors: cancer biology and therapeutics. Med Res Rev. 2014;34:280–300.
147. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141:1117–34.
148. Hojjat-Farsangi M. Small-molecule inhibitors of the receptor tyrosine kinases: promising tools for targeted cancer therapies. Int J Mol Sci. 2014;15:13768–801.
149. Kim S, Ding W, Zhang L, Tian W, Chen S. Clinical response to sunitinib as a multitargeted tyrosine-kinase inhibitor (TKI) in solid cancers: a review of clinical trials. Onco Targets Ther. 2014;7:719–28.
150. Nguewa PA, Calvo A, Pullamsetti SS, Banat GA, Grimminger F, Savai R. Tyrosine kinase inhibitors with antiangiogenic properties for the treatment of non-small cell lung cancer. Expert Opin Investig Drugs. 2011;20:61–74.
151. Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, Vandenbeldt K, Qian CN, Teh BT. Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. Cancer Res. 2010;70:1053–62.
152. Osusky KL, Hallahan DE, Fu A, Ye F, Shyr Y, Geng L. The receptor tyrosine kinase inhibitor SU11248 impedes endothelial cell migration, tubule formation, and blood vessel formation in vivo, but has little effect on existing tumor vessels. Angiogenesis. 2004;7:225–33.
153. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res. 2003;9:327–37.
154. O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, Yee KW, Wong LM, Hong W, Lee LB, Town A, et al. SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood. 2003;101:3597–605.
155. Young E, Miele L, Tucker KB, Huang M, Wells J, Gu JW. SU11248, a selective tyrosine kinases inhibitor suppresses breast tumor angiogenesis and growth via targeting both tumor vasculature and breast cancer cells. Cancer Biol Ther. 2010;10:703–11.
156. Vinik AI, Raymond E. Pancreatic neuroendocrine tumors: approach to treatment with focus on sunitinib. Ther Adv Gastroenterol. 2013;6:396–411.
157. Joosten SC, Hamming L, Soetekouw PM, Aarts MJ, Veeck J, van Engeland M, Tjan-Heijnen VC. Resistance to sunitinib in renal cell carcinoma: from molecular mechanisms to predictive markers and future perspectives. Biochim Biophys Acta. 2015;1855:1–16.
158. Iyer R, Fetterly G, Lugade A, Thanavala Y. Sorafenib: a clinical and pharmacologic review. Expert Opin Pharmacother. 2010;11:1943–55.
159. Murphy DA, Makonnen S, Lassoued W, Feldman MD, Carter C, Lee WM. Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). Am J Pathol. 2006;169:1875–85.
160. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res. 2006;66:11851–8.
161. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. 2004;64:7099–109.
162. Ibrahim N, Yu Y, Walsh WR, Yang JL. Molecular targeted therapies for cancer: sorafenib mono-therapy and its combination with other therapies (review). Oncol Rep. 2012;27:1303–11.
163. Thomas L, Lai SY, Dong W, Feng L, Dadu R, Regone RM, Cabanillas ME. Sorafenib in metastatic thyroid cancer: a systematic review. Oncologist. 2014;19:251–8.
164. Sonpavde G, Hutson TE, Sternberg CN. Pazopanib, a potent orally administered small-molecule multi-

targeted tyrosine kinase inhibitor for renal cell carcinoma. Expert Opin Investig Drugs. 2008;17:253–61.
165. Harris PA, Boloor A, Cheung M, Kumar R, Crosby RM, Davis-Ward RG, Epperly AH, Hinkle KW, Hunter RN 3rd, Johnson JH, et al. Discovery of 5-[[4-[(2,3-dimethyl-2H-indazol-6-yl) methylamino]-2-pyrimidinyl]amino]-2-methyl-benzenesulfonamide (Pazopanib), a novel and potent vascular endothelial growth factor receptor inhibitor. J Med Chem. 2008;51:4632–40.
166. Hamberg P, Verweij J, Sleijfer S. (Pre-)clinical pharmacology and activity of pazopanib, a novel multikinase angiogenesis inhibitor. Oncologist. 2010;15:539–47.
167. Deeks ED. Pazopanib: in advanced soft tissue sarcoma. Drugs. 2012;72:2129–40.
168. Drabkin HA. Pazopanib and anti-VEGF therapy. Open Access J Urol. 2010;2:35–40.
169. Dabney R, Devine R, Sein N, George B. New agents in renal cell carcinoma. Target Oncol. 2014;9:183–93.
170. Hu-Lowe DD, Zou HY, Grazzini ML, Hallin ME, Wickman GR, Amundson K, Chen JH, Rewolinski DA, Yamazaki S, Wu EY, et al. Nonclinical antiangiogenesis and antitumor activities of axitinib (AG-013736), an oral, potent, and selective inhibitor of vascular endothelial growth factor receptor tyrosine kinases 1, 2, 3. Clin Cancer Res. 2008;14:7272–83.
171. Rixe O, Bukowski RM, Michaelson MD, Wilding G, Hudes GR, Bolte O, Motzer RJ, Bycott P, Liau KF, Freddo J, et al. Axitinib treatment in patients with cytokine-refractory metastatic renal-cell cancer: a phase II study. Lancet Oncol. 2007;8:975–84.
172. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005;307:58–62.
173. Jain RK. Normalizing tumor microenvironment to treat cancer: bench to bedside to biomarkers. J Clin Oncol Off J Am Soc Clin Oncol. 2013;31:2205–18.
174. Ghilardi C, Chiorino G, Dossi R, Nagy Z, Giavazzi R, Bani M. Identification of novel vascular markers through gene expression profiling of tumor-derived endothelium. BMC Genomics. 2008;9:201.
175. Hofmeister V, Schrama D, Becker JC. Anti-cancer therapies targeting the tumor stroma. Cancer Immunol Immunother. 2008;57:1–17.
176. Dong Y, Qian J, Ibrahim R, Berzofsky JA, Khleif SN. Identification of H-2Db-specific CD8+ T-cell epitopes from mouse VEGFR2 that can inhibit angiogenesis and tumor growth. J Immunother. 2006;29:32–40.
177. Ishizaki H, Tsunoda T, Wada S, Yamauchi M, Shibuya M, Tahara H. Inhibition of tumor growth with antiangiogenic cancer vaccine using epitope peptides derived from human vascular endothelial growth factor receptor 1. Clin Cancer Res. 2006;12:5841–9.
178. Nair S, Boczkowski D, Moeller B, Dewhirst M, Vieweg J, Gilboa E. Synergy between tumor immunotherapy and antiangiogenic therapy. Blood. 2003;102:964–71.
179. Wada S, Tsunoda T, Baba T, Primus FJ, Kuwano H, Shibuya M, Tahara H. Rationale for antiangiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2. Cancer Res. 2005;65:4939–46.
180. Hazama S, Nakamura Y, Takenouchi H, Suzuki N, Tsunedomi R, Inoue Y, Tokuhisa Y, Iizuka N, Yoshino S, Takeda K, et al. A phase I study of combination vaccine treatment of five therapeutic epitope-peptides for metastatic colorectal cancer; safety, immunological response, and clinical outcome. J Transl Med. 2014;12:63.
181. Okamoto I, Arao T, Miyazaki M, Satoh T, Okamoto K, Tsunoda T, Nishio K, Nakagawa K. Clinical phase I study of elpamotide, a peptide vaccine for vascular endothelial growth factor receptor 2, in patients with advanced solid tumors. Cancer Sci. 2012;103:2135–8.
182. Okuyama R, Aruga A, Hatori T, Takeda K, Yamamoto M. Immunological responses to a multipeptide vaccine targeting cancer-testis antigens and VEGFRs in advanced pancreatic cancer patients. Oncoimmunology. 2013;2:e27010.
183. Suzuki H, Fukuhara M, Yamaura T, Mutoh S, Okabe N, Yaginuma H, Hasegawa T, Yonechi A, Osugi J, Hoshino M, et al. Multiple therapeutic peptide vaccines consisting of combined novel cancer testis antigens and anti-angiogenic peptides for patients with non-small cell lung cancer. J Transl Med. 2013;11:97.
184. Yamaue H, Tsunoda T, Tani M, Miyazawa M, Yamao K, Mizuno N, Okusaka T, Ueno H, Boku N, Fukutomi A, et al. Randomized phase II/III clinical trial of elpamotide for patients with advanced pancreatic cancer; PEGASUS-PC study. In:Cancer Sci; 2015.
185. Berger M, Bergers G, Arnold B, Hammerling GJ, Ganss R. Regulator of G-protein signaling-5 induction in pericytes coincides with active vessel remodeling during neovascularization. Blood. 2005;105:1094–101.
186. Hamzah J, Jugold M, Kiessling F, Rigby P, Manzur M, Marti HH, Rabie T, Kaden S, Grone HJ, Hammerling GJ, et al. Vascular normalization in Rgs5-deficient tumours promotes immune destruction. Nature. 2008;453:410–4.
187. Chi Sabins N, Taylor JL, Fabian KP, Appleman LJ, Maranchie JK, Stolz DB, Storkus WJ. DLK1: a novel target for immunotherapeutic remodeling of the tumor blood vasculature. Mol Ther. 2013;21:1958–68.
188. Facciponte JG, Ugel S, De Sanctis F, Li C, Wang L, Nair G, Sehgal S, Raj A, Matthaiou E, Coukos G, et al. Tumor endothelial marker 1-specific DNA vaccination targets tumor vasculature. J Clin Invest. 2014;124:1497–511.

189. Zhao X, Bose A, Komita H, Taylor JL, Chi N, Lowe DB, Okada H, Cao Y, Mukhopadhyay D, Cohen PA, et al. Vaccines targeting tumor blood vessel antigens promote CD8(+) T cell-dependent tumor eradication or dormancy in HLA-A2 transgenic mice. J Immunol. 2012;188:1782–8.
190. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008;8:592–603.
191. Griffioen AW, Weiss A, Berndsen RH, Abdul UK, te Winkel MT, Nowak-Sliwinska P. The emerging quest for the optimal angiostatic combination therapy. Biochem Soc Trans. 2014;42:1608–15.
192. Hashimoto K, Man S, Xu P, Cruz-Munoz W, Tang T, Kumar R, Kerbel RS. Potent preclinical impact of metronomic low-dose oral topotecan combined with the antiangiogenic drug pazopanib for the treatment of ovarian cancer. Mol Cancer Ther. 2010;9:996–1006.
193. Tran Cao HS, Bouvet M, Kaushal S, Keleman A, Romney E, Kim G, Fruehauf J, Imagawa DK, Hoffman RM, Katz MH. Metronomic gemcitabine in combination with sunitinib inhibits multisite metastasis and increases survival in an orthotopic model of pancreatic cancer. Mol Cancer Ther. 2010;9:2068–78.
194. Van der Veldt AA, Lubberink M, Bahce I, Walraven M, de Boer MP, Greuter HN, Hendrikse NH, Eriksson J, Windhorst AD, Postmus PE, et al. Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. Cancer Cell. 2012;21:82–91.
195. Franco M, Man S, Chen L, Emmenegger U, Shaked Y, Cheung AM, Brown AS, Hicklin DJ, Foster FS, Kerbel RS. Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. Cancer Res. 2006;66:3639–48.
196. Moserle L, Jimenez-Valerio G, Casanovas O. Antiangiogenic therapies: going beyond their limits. Cancer Discov. 2014;4:31–41.
197. Nieder C, Wiedenmann N, Andratschke N, Molls M. Current status of angiogenesis inhibitors combined with radiation therapy. Cancer Treat Rev. 2006;32:348–64.
198. Vujanovic L, Butterfield LH. Melanoma cancer vaccines and anti-tumor T cell responses. J Cell Biochem. 2007;102:301–10.
199. Yu Z, Restifo NP. Cancer vaccines: progress reveals new complexities. J Clin Invest. 2002;110:289–94.
200. Shi S, Chen L, Huang G. Antiangiogenic therapy improves the antitumor effect of adoptive cell immunotherapy by normalizing tumor vasculature. Med Oncol. 2013;30:698.
201. Bose A, Lowe DB, Rao A, Storkus WJ. Combined vaccine+axitinib therapy yields superior antitumor efficacy in a murine melanoma model. Melanoma Res. 2012;22:236–43.
202. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, Ko JS, Cohen PA, Finke JH, Storkus WJ. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumor immunity in concert with specific vaccination. Int J Cancer. 2011;129:2158–70.
203. Lowe DB, Bose A, Taylor JL, Tawbi H, Lin Y, Kirkwood JM, Storkus WJ. Dasatinib promotes the expansion of a therapeutically superior T-cell repertoire in response to dendritic cell vaccination against melanoma. Oncoimmunology. 2014;3:e27589.
204. Baksh K, Weber J. Immune checkpoint protein inhibition for cancer: preclinical justification for CTLA-4 and PD-1 blockade and new combinations. Semin Oncol. 2015;42:363–77.

Adaptive Resistance to Cancer Immunotherapy

14

A.J. Robert McGray and Jonathan Bramson

14.1 Introduction

Adoptive transfer of tumor-specific T cells or T cells engineered to express tumor-specific receptors (TCRs, CARs) has provided proof that T cells mediate tumor destruction in humans [1–4]. Pre-clinical studies [5–7], as well as the adoptive transfer of enriched CD8+ T cells into patients [8] firmly establish that CD8+ T cells can provide robust anti-tumor immunity. CD4+ T cells have been shown to support CD8+ T cell responses [9, 10], possess cytotoxic function [11], and can promote tumor destruction through multiple mechanisms, including cytokine production and recruitment/activation of innate immune cells [12–15].

These data confirming the anti-tumor properties of T cells notwithstanding, tumors emerge when they acquire the capacity to evade T cell attack. The immunoediting hypothesis argues that tumors must develop mechanisms to evade host immunity. Indeed, there is considerable evidence from pre-clinical models and clinical observations that tumors emerge more readily in the absence of an immune response [16]. Further, tumors that emerge in immune competent hosts often display a wide range of mechanisms to bypass the host immune response. The local immunosuppression within tumors is typically considered in static terms. However, emerging data argue that the immunosuppressive tumor environment is actually a direct response to ongoing immune attack and, thus, reflects a dynamic response that adapts to the nature and magnitude of the T cell attack. This ability of the tumor microenvironment to adapt to immune attack represents a significant barrier to the development of effective and durable immunotherapies. The following review will discuss the current knowledge in this emerging area and potential implications for the design of future immunotherapeutic strategies.

14.2 Immune Surveillance and the Immunoediting Hypothesis

The idea that the immune system can effectively control the growth of cancer dates back to the early 1900s, when Paul Ehrlich proposed the idea

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of host immune protection from cancer [17, 18]. The 'immune surveillance' hypothesis, however was not officially proposed until 1957 by Macfarlane Burnet [19] who proposed that an accumulation of tumor cells possessing novel target antigens could elicit an effective immune response, leading to tumor clearance in the absence of clinical detection [19]. Studies designed to investigate whether immunocompromised mice displayed greater tumor incidence were largely inconclusive. The immune surveillance hypothesis was eventually abandoned when it was observed that immune deficient athymic nude mice developed similar frequencies of chemically induced tumors as wild type mice [20]. However, subsequent investigation revealed that the nude mice used for that research, although immune compromised, were not completely immune deficient and did have detectable populations of functional T cells [21].

The concept of immune surveillance returned in the early 1990s, when more sophisticated mouse models allowed for the direct assessment of immune-mediated cancer control. Indeed, the increased frequency of chemically induced tumors observed in the absence of IFN-γ signaling [22–24], perforin [25], or RAG-2, which is required for T and B cell maturation [24], strongly supported a role for the immune system in preventing tumor growth. Pivotal studies revealed that the immune system cannot only act to eliminate tumors but can also shape their immunogenicity [24], leading to the evolution of the tumor immune surveillance hypothesis towards the concept of cancer immunoediting.

The cancer immunoediting theory comprises three distinct phases: Elimination, Equilibrium, and Escape [18]. In the elimination phase, developing tumors are destroyed by combined innate and adaptive immune responses. Given the genomic instability of tumor cells, daughter cells may emerge that acquire resistance to the antitumor immune attack. When such resistant subclones arise, the tumor enters a state of equilibrium where the overall size may stabilize as the immunogenic tumor cells are eradicated while the resistant subclones continue to proliferate. As such, the tumor is "edited" by the immune system to be comprised of cells with resistance to immune attack. Ultimately, immunoediting will select for tumor cells that are resistant to eradication by host immune cells, allowing the tumor to escapes immune control and grow out uncontrollably [26, 27].

While many of the observations regarding cancer immunoediting have come from studies using laboratory mice, increasing evidence suggests that the same principles apply to human cancers. The elimination phase is exemplified by the increased risk of developing both virally and non-virally induced malignancies among those with immune deficiencies [16, 26], as in the case of individuals with AIDS or those receiving immunosuppressants following organ transplant [16, 28]. Tumor equilibrium may help to explain the improved prognosis for patients exhibiting strong T cell infiltrate and local production of cytokines, such as IFN-γ and TNF-α [16, 26]. Tumor equilibrium is also consistent with reports of cancer patients entering phases of progression free survival or stable disease following treatment with cancer vaccines, [29–32], checkpoint blockade antibodies [33–36], or adoptive T cell transfer-based therapies [4]. Additionally, the report of two kidney transplant recipients developing malignant melanoma after both receiving organ transplants from a woman who had been successfully treated for melanoma 16 years previously [37], suggests that the melanoma metastases had been held in equilibrium within the kidneys for a prolonged period prior to transplant and only emerged in the transplant recipients because they received immunosuppressive drugs. Lastly, clinically detectable tumors are poorly immunogenic and possess intrinsic mechanisms of circumventing or suppressing host immune responses (as will be discussed), suggesting these tumors have effectively escaped immune control.

14.3 Mechanisms of Immune Escape by Tumors

In accordance with the immune editing theory, growing tumors develop an immune refractory microenvironment that limits attack by infiltrat-

ing immune cells, presenting a major hurdle to successful cancer immunotherapy. This section will focus on underlying mechanisms of immune evasion and immune suppression within the local tumor environment, which contribute to limited anti-tumor immunity against growing tumors.

14.3.1 Defects in Tumor Antigen Presentation

Abnormalities in MHC I antigen presentation have been documented in a diverse set of solid and hematological tumors [38], representing an important mechanism through which tumors can escape recognition by CD8+ T cells. Loss of MHC Class I expression has been reported in both murine and human tumors [39, 40], ranging from down regulation to complete absence of protein expression [41–43] and has been associated with poor survival prognosis and disease progression [43, 44]. Indeed, MHC class I expression has been observed to correlate with tumor regression or progression within individual metastatic lesions [45], suggesting that restoring antigen presentation is likely an important determinant in the success of immunotherapy. Defective antigen presentation by tumor cells often results from impaired expression of proteins associated with antigen processing. In some cases, the defect in antigen presentation is irreversible due to genetic alterations. As examples, reports have described mutations in β2-microglobulin [46] or components of the antigen processing machinery [42], as well as loss of heterozygosity at MHC I loci [42, 47–49]. In other cases, the impairment in antigen presentation is reversible as epigenetic alterations have been shown to result in diminished gene transcription and MHC Class I presentation. Such reversible impairments may be therapeutic targets as MHC expression and immunogenicity may be restored in tumor cells through the use of DNA de-methylating agents [50], HDAC inhibitors [51], or through treatment with immunostimulatory cytokines, such as IFN-γ [40, 50, 52].

14.3.2 Immunosuppression Within the Tumor Environment

Growing tumors secrete chemokines that promote tumor infiltration by cell populations that suppress T cell immunity, including regulatory T cells (Tregs; recruited by CCL22) and tumor associated macrophages (recruited by CCL2, CCL5, CCL7, CCL8, CXCL12) [53, 54]. Both of these immune cell subsets play important roles in promoting tumor growth and suppressing anti-tumor immune responses *in situ.* In this regard, both tumor cells and tumor infiltrating immune cells can secrete a range of factors that suppress the anti-tumor activity of infiltrating immune effector cells including T cells [55]. Specifically, IL-10 and TGF-β are often found within the tumor environment [56] and act to suppress T cell immunity by preventing T cell proliferation, cytotoxicity, and cytokine release, promoting T_{regs} function, and inhibiting the pro-inflammatory function of APCs [57–59]. PGE_2 is also often present at high levels in tumor tissue [60] and acts to inhibit DC maturation, limit T cell proliferation and function, increase immunosupression by myeloid cells, and enhance the suppressive effects of T_{regs} [56, 61]. VEGF, an important angiogenic factor required for tumor growth, has been reported to promote recruitment of myeloid derived suppressor cells (MDSCs) and M2 (healer) macrophages to the tumor [56] and prevents immunostimulatory function of APCs [62]. Adenosine, a purine nucleoside derived through the catabolism of adenine nucleotides by the enzymatic activity of CD39 and CD73, is often present at high levels within the tumor [63]. Produced through the activity of both the tumor [64] and T_{regs} [65], adenosine has both pro-angiogenic as well as immunosuppressive functions and limits the function of T cells [63, 65]. Lastly, local production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) within the tumor can contribute to T cell suppression and tolerance by preventing TCR/MHC interactions, impairing T cell responsiveness [66–68], and limiting tumor infiltration by T cells [69].

The availability of essential amino acids are often reduced by local catabolism within the tumor, consequently reducing the proliferative and functional capacity of the infiltrating immune cells. L-arginine is catabolized by arginase and NOS, enzymes often implicated in tumor-induced immune suppression and expressed at high levels within the tumor [56, 67]. Lack of L-arginine results in downregulation of the TCR ζ chain and inhibits the activity of T cells [70, 71]. Similarly, the enzyme IDO, which is also expressed at high levels by tumor cells, stromal cells, and infiltrating immune cells [56], acts to degrade the essential amino acid tryptophan, thereby limiting local immune activity. Additionally, kynurenine and other metabolites resulting from tryptophan breakdown have been shown to have suppressive/toxic effects on T cells, as well as additional immune cell populations including B cells and NK cells [72].

Together, the localized activity mediated by these factors acts to impair local anti-tumor immune responses. While effective inhibitors for many of these factors have been identified and observed to correlate with improved treatment outcome in pre-clinical cancer studies [56], the breadth of immunosuppressive processes within the tumor represents a major hurdle to the production of effective and prolonged anti-tumor immunity.

14.3.3 Immunosuppressive Ligands and Receptors in the Tumor Environment

In addition to locally produced immunosuppressive factors within the tumor, numerous receptor/ligand interactions can also act to promote immune evasion and tumor progression. The finding that apoptosis-inducing FasL and TRAIL are expressed in tumors [73–75] suggests a mechanism by which the tumor can eliminate infiltrating immune cells expressing cognate receptors and underscores active measures by the tumor to evade host immune attack. Similarly, while ICOS-L is expressed on tumor cells [76] and could provide a source of co-stimulation for activated tumor-specific T cells, stimulation of ICOS-expressing regulatory T cells results in increased expansion of IL-10-producing T_{regs} [76], which may suppress local immune activity within the tumor. While CD80 and CD86 are often considered to promote T cell function, binding of CD80 or CD86 to CTLA-4, a T cell suppressive receptor (commonly known as a *checkpoint receptor*) that is upregulated following T cell activation [77], leads to suppression of T cell function. CTLA-4 has a higher affinity for CD80 and CD86 than CD28. As a result, the negative signal from the CTLA-4 receptor on activated T cells will supercede any positive signals from CD28 [78]. Moreover, ligation of CTLA-4 by T_{regs} can promote upregulation of IDO by APCs [79, 80], which produces indirect T cell inhibition by Tregs [81]. PD-1, another member of the CD28 receptor family, is upregulated following T cell activation. Similar to CTLA-4, ligation of the PD-1 checkpoint receptor via the ligands PD-L1 and PD-L2, impairs TCR signaling, cytokine production, and cell survival [82]. Unlike the ligands for CTLA-4, PD-L1 and PD-L2 are often expressed on tumor cells providing a direct mechanism by which tumors can suppress T cell function [83–86]. PD-1 expression has been shown to correlate with reduced functionality of TIL [87]. Additional inhibitory or checkpoint receptors, have been identified that contribute to immune evasion within the tumor. TIM3, a receptor expressed on CD4+ and CD8+ T cells, as well as DCs, monocytes, and other lymphocyte populations [88], has been shown to negatively impact T cell responses through interaction with its ligand galectin-9 [89]. LAG-3 interacts with MHC II [90, 91] and negatively regulates TCR signaling, leading to functional impairment [92]. Recent evidence suggests that LAG-3 can also initiate reverse signaling via MHC II that can protect MHC II-positive tumor cells from apoptosis [93], while LAG-3 expressed on T_{regs} can also interact with MHC II and suppress DC function [94]. Inhibition of TIM3 activity has been shown to improve T cell proliferation and cytokine production [95] and antibody-mediated TIM-3 blockade can enhance T cell-dependent anti-tumor immunity [96–98]. Similarly, LAG-3 blockade enhances cytokine

production by T cells and shows a synergistic improvement in anti-tumor immunity when combined with PD-1 blockade [99], suggesting that blocking multiple checkpoint pathways simultaneously may further improve anti-tumor immunity.

14.4 Immune Suppression in the Tumor Microenvironment Occurs in Response to Immune Attack

As stated previously, the immunosuppressive tumor microenvironment is a critical barrier to successful cancer immunotherapy. While the suppressive mechanisms employed by individual tumors can be varied, these processes are often thought to be intrinsic properties of the tumor. However, emerging evidence suggests that the immunosuppressive microenvironment of the tumor may actually reflect an adaptive response by the tumor to immune attack that has been termed *adaptive resistance* [100].

A study by Taube *et al.* [83] reported that B7-H1 (PD-L1) expression in human melanoma lesions was strongly associated with T cell infiltration and the expression of IFN-γ, indicating that the PD-L1 expression was elevated in direct response to immune attack. A follow up study by the same group further emphasized the intricacies of the inflammatory tumor microenvironment in PD-L1+ tumors of human melanoma patients compared to PD-L1- tumors [101]. In this study, the authors identified a number of markers consistent with T cell activation (CD8A, PRF1, IL-18, IL-21), as well as increased expression of IFN-γ, PD-1, LAG-3, IL-10, and IL-32-γ in PD-L1+ tumors, further emphasizing the concept that PD-L1 is elevated as a consequence of T cell attack. Using *in vitro* studies, the authors further identified IL-10 and IL-32-γ as factors that could enhance expression of PD-1 ligands on monocytes but not tumor cells, suggesting a complex mechanism by which both tumor cells and infiltrating immune cells may act to regulate inflammatory attack within the tumor. An independent study by Kluger *et al.* [102] adds further support to these findings, where the authors reported strong association between PD-L1 expression and the presence of TILs in biopsies collected from different anatomical sites from patients with advanced metastatic melanoma. High expression of PD-L1 was associated with increased TIL density (Total CD3+ and CD8+) and improved patient survival, presenting an apparent paradox where the levels of PD-L1, an immunosuppressive ligand, actually correlate with improved outcome. In truth, there is no paradox as PD-L1 expression is simply a direct measure of local immune activity in melanoma.

Similarly, analysis of tumors from HPV-associated head and neck cancer patients revealed that TILs co-localized with PD-L1-expressing tumor cells [103]. Expression of both CD8 and IFN-γ was higher in PD-L1-positive tumors than PD-L1-negative tumors, reinforcing the concept that TIL activity directly contributes to induction of PD-L1, and, in turn, immune suppression, within the tumor microenvironment. In line with this, the authors noted that PD-1+ CD8+ TILs were functionally impaired compared to PD-1+ peripheral CD8+ T cells. Furthermore, the presence of PD-L1+CD68+ macrophages within the analyzed tumors suggests that infiltrating hematopoietic cells can also contribute to the adaptive resistance within the tumor.

Using an *in vitro* co-culture system to model T cell/tumor cell interactions, Dolen and Esendagli [104] observed that myeloid leukemia cells could provide effective co-stimulation to CD4+ T cells, enhancing T cell activation and proliferation. In turn, the activated CD4+ T cells triggered up regulation of PD-L1 and B7-DC (PD-L2) on the leukemia cells. When these "T cell-conditioned" leukemia cells were used in subsequent co-culture assays with naive CD4+ T cells, the T cells displayed poor proliferative capacity, diminished expression of activation markers (CD25, CD154), and reduced capacity for cytokine production, providing direct evidence that the tumor cells adapt to T cell attack and augment their immunosuppressive properties. Importantly, the blockade of PD-1 in this co-culture system led to reversal of the immunosuppressed CD4+ T

cell phenotype, restoring T cell proliferative and functional capacities confirming a key role for PD-1 ligands in adaptive resistance.

Additional support for adaptive resistance was reported by Spranger *et al.* [105], who observed a strong correlation between CD8+ T cell infiltration in human melanoma and the expression of PD-L1 and IDO, and the infiltration of FoxP3+ T_{regs}. Using mouse models, the authors observed that the induction of IDO and PD-L1 was mediated by CD8+ T cells and IFN-γ. Further, CD8+ T cells reacting to the tumor triggered both *in situ* proliferation and increased tumor infiltration of T_{regs} through a CCL22/CCR4 dependent chemokine axis. Similarly, Hosoi *et al.* [106] observed that tumor infiltration of suppressive myeloid populations, in particular $CD11b^{+}Gr\text{-}1^{int}Ly6C^{+}$ monocytic MDSCs, was driven by tumor-specific CD8+ T cells and the production of IFN-γ. The tumor infiltrating MDSCs were observed to suppress T cells through a variety of mechanisms including iNOS and Arginase I activity, as well as the production of reactive oxygen species (ROS) [106]. In line with this, by analyzing immune cell populations isolated from the ascites of ovarian cancer patients, Wong *et al.* reported that activated type-1 immune cells (NK cells and CD8+ T cells), via secretion of IFN-γ and TNF-α, could promote the production of suppressive factors by MDSC's including IDO1, NOS2, IL-10, and COX2 [107]. Importantly, this "counter-regulatory" immune suppression could be largely reversed through treatment with the COX2 inhibitor celecoxib and resulted in reduced production of these immunosuppressive factors by MDSC's, leading to increased production of IFN-γ and TNF-α by NK cells and restored proliferation of Granzyme B+ CD8+ T cells following MDSC co-culture, suggesting that the COX2/PGE_2 axis functions in multiple ways to promote MDSC-mediated immune suppression in response to local inflammatory events. These results demonstrate that adaptive resistance is more complex than simple up regulation of PD-1 ligands and the adaptation includes both tumor cell intrinsic effects (ex. up regulation of PD-L1) and tumor extrinsic effects (ex. infiltration of Tregs and MDSCs) [105, 106, 108].

We observed that the adaptive response is a key hurdle that limits the therapeutic effect of cancer vaccines. Using the B16F10 murine melanoma model, we noted that as soon as the vaccine-induced T cells infiltrated the tumor, a broad adaptation occurred with up regulation of a multitude of suppressive pathways, including checkpoint receptors/ligands (PD-1, LAG-3, TIM-3 and their corresponding ligands), Arginase and iNOS [7] [and unpublished data]. Expression of these suppressive factors was driven by CD8+ T cells and, to a large extent, the production of IFN-γ. Strikingly, these pathways were upregulated as soon as the vaccine-induced T cells infiltrated the tumor demonstrating the rapidity of the adaptive resistance. Unlike previous reports, we noted a temporal relationship between the adaptive response and T cell immunity within the tumor. Whereas the vaccine-induced T cells were initially highly functional within the tumor, over time the functionality of the intratumoral, vaccine-induced T cells waned while the adaptive response gained in magnitude, resulting in a very transient growth suppression. These findings are of particular clinical interest, as immunotherapies, including vaccines, often require long treatment intervals or multiple immunizations to generate high numbers anti-tumor T cells. In turn, such therapies may instigate suppressive events in the tumor early in the course of treatment and long before maximal immune reactivity against the tumor is achieved.

Using the same model, we determined that tumor regression could be achieved by combining vaccination with either administration of immunomodulatory antibodies (anti-CD137 + anti-PD-1) [109] or adoptive transfer of tumor-specific CD8+ T cells [7]. Tumor regression did not result from the absence of an adaptive response following those therapeutic strategies. In contrast, we observed that the effective therapies produced a heightened anti-tumor T cell response, which actually resulted in an elevated magnitude of the adaptive immunosuppressive response. In fact, throughout our studies, we observed that the magnitude of the adaptive response was directly related to the magnitude of the therapy-induced immune attack. Thus, adap-

tive resistance may be a barrier, but it is not absolute and can be overcome when sufficient numbers of T cells are present in the tumor, despite impairments in T cell function that arise from the adaptive immune suppression.

Since adaptive immune suppression is evidence of immune attack within the tumor, measures of the adaptation may provide prognostic value. Tumeh *et al.* [108] and Taube et al. [83] observed co-localization of tumor infiltrating CD8+ T cells with expression of immune-inhibitory PD-1/PD-L1 markers, consistent with the theory that immune attack is responsible for adaptive immune resistance within the tumor microenvironment. Taube *et al.* observed that metastatic melanoma patients with elevated PD-L1 expression survived longer than those with low PD-L1 expression. Tumeh *et al.* went further and determined that patients with a high density of TILs and markers of adaptive resistance (PD-1/PD-L1) at both the invasive margin and in the tumor were more likely to respond to PD-1 blocking therapy (pembrolizumab) when compared to patients with poor TIL infiltration or PD-1/PD-L1 expression. The authors employed this information to develop a model that predicted which patients would respond or progress on PD-1 blocking therapy. Furthermore, Gajewski has emphasized that patients showing favorable clinical outcome often had pre-treatment tumor transcriptional profiles consistent with T cell infiltration and an inflamed tumor microenvironment, but that these same tumors also had the highest expression of genes associated with inhibitory mechanisms, including IDO, PD-L1, and a profile consistent with FoxP3+ T_{regs} infiltration [110–115].

Conceptually, the existing experimental data support induction of multiple adaptive resistance mechanisms in tumors in response to immune attack and it is likely that additional pathways/mechanisms will be identified. Currently, two possible scenarios have emerged whereby the adaptive response has the potential to be overcome therapeutically to promote anti-tumor immunity with curative potential. As depicted in Fig. 14.1a, inflamed tumors, which can arise either spontaneously or in response to immunotherapy, induce only low level immune attack on the tumor, resulting in adaptive resistance, localized immune suppression, and tumor growth. In this case, adaptive resistance mechanisms can be partially overcome through interventions aimed at disrupting these immunosuppressive pathways (including co-stimulatory agents, checkpoint blockade, as well as chemical inhibitors) to promote re-invigoration of local immune attack and tumor regressions, whether complete or transient. In contrast, delivery of more robust immunotherapies, such as ACT, can initiate rapid immune attack and result in tumor regressions despite the presence of the same adaptive resistance mechanisms [7], suggesting that increasing the magnitude and/or rate of immune attack on the tumor may also improve the likelihood of overcoming adaptive immunosuppressive mechanisms and achieving therapeutic benefit (Fig. 14.1b).

14.5 Adaptive Immune Suppression in the Tumor: Does the Tumor Benefit from Conventional Homeostatic Mechanisms of Immune Tolerance?

Chronic inflammation had been implicated in driving immunosuppressive mechanisms within the tumor, thereby limiting anti-tumor immune responses [116–120]. However, as described above, the emerging concept of an adaptive immune resistance argues that the broad network of suppressive factors within the tumor microenvironment may actually be instigated as a consequence of immune attack on the tumor. Adaptive immune resistance does not appear to be unique to tumor tissue, as many of the same suppressive mechanisms have also been implicated in the maintenance of immune tolerance under normal homeostatic conditions and in the control of autoimmune pathology under chronic inflammatory conditions.

PD-L1 expression has been observed to increase with pancreatic inflammation in a mouse model of diabetes [121]. In the womb, PD-L1 is expressed in the placenta [122] and by T_{regs} [123]

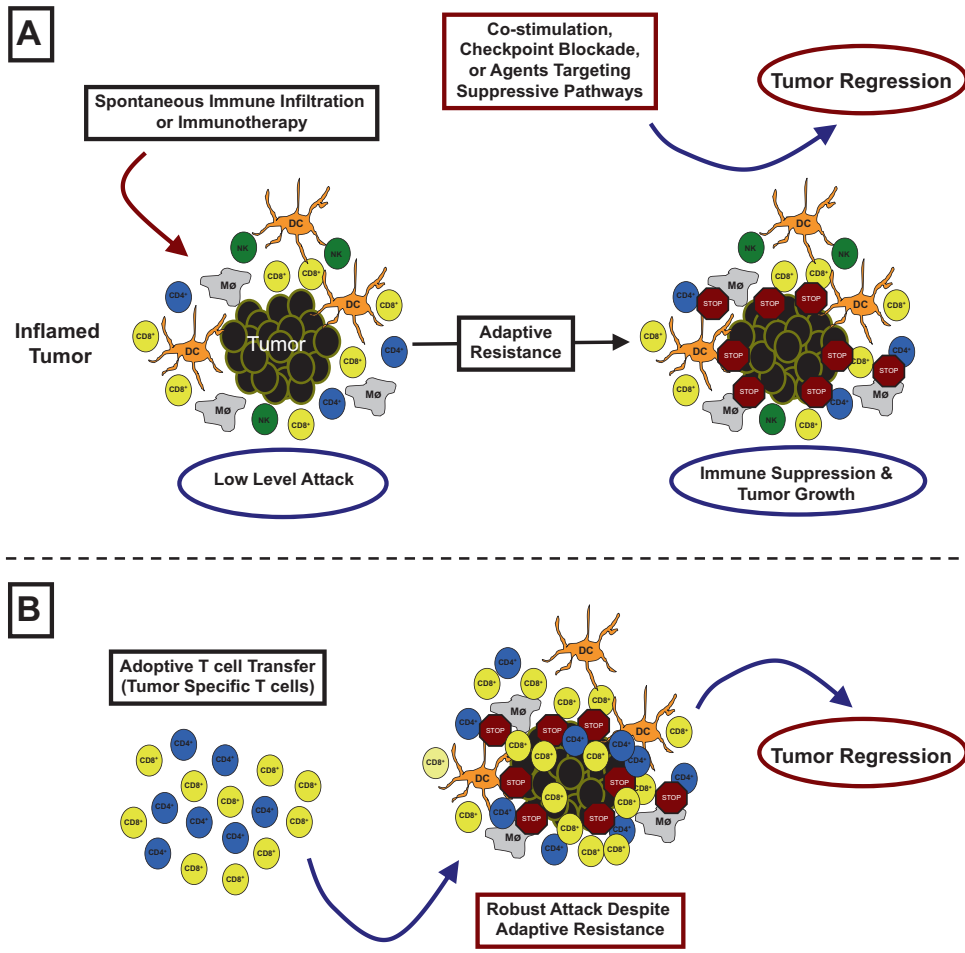

Fig. 14.1 Adaptive resistance occurs in direct response to immune attack on tumors but can be overcome therapeutically. (**a**) Spontaneous immune infiltration or delivery of immunotherapy results in an inflamed tumor microenvironment but only low level immune attack, resulting in adaptive resistance, immune suppression, and continued tumor growth. Intervention(s) with agents that act to enhance or restore local immune attack (co-stimulatory molecules, checkpoint inhibitors, or agents targeting immunosuppressive pathways) can partially overcome adaptive resistance mechanisms, leading to tumor regression. (**b**) Delivery of more robust immunotherapies (such as ACT) results in vigorous immune attack on tumors despite induction of adaptive resistance mechanisms and leads to tumor regression

to prevent immune attack on the semi-allogeneic fetus. PD-L2 has been implicated in the maintenance of oral tolerance to ingested antigens [124] and has been shown to aid in controlling airway asthmatic responses [125]. Not surprisingly, the checkpoint receptors PD-1, LAG-3, and TIM-3 play a role in the suppression of inflammatory processes to control autoimmune pathologies or tissue homeostasis [126–128], as do arginase [71], iNOS [129], TGF-β1 [130, 131], IDO [132, 133], and Galectin-9 [89]. These observations argue that tumor tissue is not unique in its ability to evade inflammatory attack, but instead exploits natural homeostatic mechanisms that limit unwanted auto-immune destruction of healthy tissues. IFN-γ is particularly interesting in this regard as it has been implicated as critical effector molecule promoting anti-tumor immunity, while also playing a key role in the induction of the many immune suppressive pathways within the tumor. The contribution of IFN-γ to tissue homeostasis is underscored by the emergence of severe autoimmune pathology in mice deficient in IFN-γ or the IFN-γ receptor [134–138]. IFN-γ,

therefore, acts as a double-edged sword, regulating both inflammation and immune destruction [139]. Developing strategies to mitigate the regulatory properties of IFN-γ while maximizing tumor destruction will greatly enhance the effectiveness of cancer immunotherapy.

14.6 Adaptive Resistance: Knowledge to Practice

Blockade of checkpoint receptors (i.e. CTLA-4, PD-1) and their ligands (i.e. PD-L1) has confirmed therapeutic benefit in multiple clinical trials across a broad range of tumors [34–36, 140–149]. These important successes notwithstanding, single-agent checkpoint blockade only achieved therapeutic effects in a fraction of patients and data emerging from both mouse and human studies has demonstrated additional therapeutic benefit through blockade of multiple immunosuppressive pathways simultaneously [7, 34, 99, 149–152]. As expected, given the complexity of adaptive resistance, the combination of anti-CTLA-4 and anti-PD-1 produced more objective responses in melanoma than either agent alone [153]. The requirement to overcome multiple resistance mechanisms is further supported by analysis of tumor-specific T cells isolated from tumor-positive lymph nodes of patients, which revealed that these T cells upregulate a host of suppressive receptors including LAG-3, TIM-3, PD-1, BTLA, 2B4, and CTLA-4 [154], further demonstrating that multiple pathways contribute to T cell impairment. Indeed, the effects of these pathways are additive as T cells receiving multiple suppressive signals possess greater functional impairments [96, 155–157]. Based on these observations, it is clear that a combinatorial approach will be required to effectively block local immunosuppressive processes and achieve more consistent objective responses in a larger number of patients. Given the high cost associated with each of the checkpoint blockade antibodies, however, it is unlikely that payers will accept using all blockade strategies with all patients. In this regard, it is imperative that we develop effective predictive tools that can determine which checkpoint blockade strategies will be most effective with individual patients. Adding further complexity to this challenge, a recent report investigating combination radiotherapy and CTLA-4 therapy revealed that PD-L1 was upregulated in therapy-resistant tumors [158], suggesting that re-invigoration of tumor attack by overcoming a single immunosuppressive pathway may, in fact, lead to induction of additional non-redundant mechanisms of adaptive resistance. Interestingly, adoptive T cell therapy can produce tumor regression despite clear evidence of adaptive resistance by the tumor [7]. Thus, it is possible to overcome the adaptive immune resistance when the level of immune attack is high enough. Of course, adoptive T cell therapy is imperfect and will likely require some aspect of checkpoint blockade to maximize clinical activity. Nevertheless, the impressive clinical outcomes with checkpoint blockade and adoptive T cell therapy support further research to identify not only mechanisms leading to the induction of adaptive resistance in tumors, but also to understand potential cross talk, interplay, as well as differences in the expression kinetics/upregulation of well-characterized and emerging immunoregulatory mechanisms that function to limit immune attack on tumors.

Collectively, these studies demonstrate that immune attack on the tumor triggers a complex and dynamic feedback mechanism through which cells present within the tumor actively respond to the attack by upregulating immunosuppressive pathways that limit the durability of the therapeutic anti-tumor effects. Understanding the triggers of these responses will be key in the development of strategies to suppress the adaptive resistance and enhance clinical outcomes with immunotherapy.

References

1. Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-Cell transfer immunotherapy. Clin Cancer Res. 2011;17:4550–7. https://doi.org/10.1158/1078-0432.CCR-11-0116.
2. Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor

effects and can establish memory in patients with advanced leukemia. Sci Transl Med. 2011;3:95ra73. https://doi.org/10.1126/scitranslmed.3002842.
3. Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348:62–8. https://doi.org/10.1126/science.aaa4967.
4. Robbins PF, Kassim SH, Tran TLN, et al. A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. Clin Cancer Res. 2015;21:1019–27. https://doi.org/10.1158/1078-0432.CCR-14-2708.
5. Klebanoff CA, Gattinoni L, Palmer DC, et al. Determinants of successful CD8+ T-cell adoptive immunotherapy for large established tumors in mice. Clin Cancer Res. 2011;17:5343–52. https://doi.org/10.1158/1078-0432.CCR-11-0503.
6. Overwijk WWW, Tsung AA, Irvine KRK, et al. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self-"reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med. 1998;188:277–86.
7. McGray AJR, Hallett R, Bernard D, et al. Immunotherapy-induced CD8+ T cells instigate immune suppression in the tumor. Mol Ther. 2014;22:206–18. https://doi.org/10.1038/mt.2013.255.
8. Dudley ME, Gross CA, Langhan MM, et al. CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. Clin Cancer Res. 2010;16:6122–31. https://doi.org/10.1158/1078-0432.CCR-10-1297.
9. Wong SBJ, Bos R, Sherman LA. Tumor-specific CD4+ T cells render the tumor environment permissive for infiltration by low-avidity CD8+ T cells. J Immunol. 2008;180:3122–31.
10. Bos R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer Res. 2010;70:8368–77. https://doi.org/10.1158/0008-5472.CAN-10-1322.
11. Ray S, Chhabra A, Chakraborty NG, et al. MHC-I-restricted melanoma antigen specific TCR-engineered human CD4+ T cells exhibit multifunctional effector and helper responses, in vitro. Clin Immunol. 2010;136:338–47. https://doi.org/10.1016/j.clim.2010.04.013.
12. Ding Z-C, Huang L, Blazar BR, et al. Polyfunctional CD4+ T cells are essential for eradicating advanced B-cell lymphoma after chemotherapy. Blood. 2012;120:2229–39. https://doi.org/10.1182/blood-2011-12-398321.
13. Hung K, Hayashi R, Lafond-Walker A, et al. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med. 1998;188:2357–68.
14. Mattes J, Hulett M, Xie W, et al. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. J Exp Med. 2003;197:387–93.
15. Tran E, Turcotte S, Gros A, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. Science. 2014;344:641–5. https://doi.org/10.1126/science.1251102.
16. Corthay A. Does the immune system naturally protect against cancer? Front Immunol. 2014;5:197. https://doi.org/10.3389/fimmu.2014.00197.
17. Chow MT, Möller A, Smyth MJ. Inflammation and immune surveillance in cancer. Semin Cancer Biol. 2012;22:23–32. https://doi.org/10.1016/j.semcancer.2011.12.004.
18. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8. https://doi.org/10.1038/ni1102-991.
19. Burnet M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J. 1957;1:841–7.
20. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science. 1974;183:534–6.
21. Hünig T, Bevan MJ. Specificity of cytotoxic T cells from athymic mice. J Exp Med. 1980;152:688–702.
22. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity. 1994;1:447–56.
23. Kaplan DH, Shankaran V, Dighe AS, et al. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. Proc Natl Acad Sci U S A. 1998;95:7556–61.
24. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410:1107–11. https://doi.org/10.1038/35074122.
25. van den Broek ME, Kägi D, Ossendorp F, et al. Decreased tumor surveillance in perforin-deficient mice. J Exp Med. 1996;184:1781–90.
26. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science. 2011;331:1565–70. https://doi.org/10.1126/science.1203486.
27. Dunn S. Cancer Immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991–8.
28. Grulich AE, van Leeuwen MT, Falster MO, Vajdic CM. Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. Lancet. 2007;370:59–67. https://doi.org/10.1016/S0140-6736(07)61050-2.
29. Tiriveedhi V, Tucker N, Herndon J, et al. Safety and preliminary evidence of biologic efficacy of a mammaglobin-a DNA vaccine in patients with stable metastatic breast cancer. Clin Cancer Res. 2014;20:5964–75. https://doi.org/10.1158/1078-0432.CCR-14-0059.
30. Odunsi K, Matsuzaki J, James SR, et al. Epigenetic potentiation of NY-ESO-1 vaccine therapy in human

ovarian cancer. Cancer Immunol Res. 2014;2:37–49. https://doi.org/10.1158/2326-6066.CIR-13-0126.
31. Schwartzentruber DJ, Lawson DH, Richards JM, et al. gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. N Engl J Med. 2011;364:2119–27. https://doi.org/10.1056/NEJMoa1012863.
32. Odunsi KK, Matsuzaki JJ, Karbach JJ, et al. Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. Proc Natl Acad Sci U S A. 2012;109:5797–802. https://doi.org/10.1073/pnas.1117208109.
33. Hoos A, Eggermont AMM, Janetzki S, et al. Improved endpoints for cancer immunotherapy trials. JNCI. J Natl Cancer Inst. 2010;102:1388–97. https://doi.org/10.1093/jnci/djq310.
34. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus Ipilimumab in advanced melanoma. N Engl J Med. 2013;369:122–33. https://doi.org/10.1056/NEJMoa1302369.
35. Topalian SL, Sznol M, McDermott DF, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. J Clin Oncol. 2014;32:1020–30. https://doi.org/10.1200/JCO.2013.53.0105.
36. Robert C, Schachter J, Long GV, et al. Pembrolizumab versus Ipilimumab in advanced melanoma. N Engl J Med. 2015;372(26):2521–32. https://doi.org/10.1056/NEJMoa1503093.
37. MacKie RM, Reid R, Junor B. Fatal melanoma transferred in a donated kidney 16 years after melanoma surgery. N Engl J Med. 2003;348:567–8. https://doi.org/10.1056/NEJM200302063480620.
38. Campoli M, Ferrone S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. Oncogene. 2008;27:5869–85. https://doi.org/10.1038/onc.2008.273.
39. Garcia-Lora A, Martinez M, Algarra I, et al. MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. Int J Cancer. 2003;106:521–7. https://doi.org/10.1002/ijc.11241.
40. Méndez R, Rodríguez T, Del Campo A, et al. Characterization of HLA class I altered phenotypes in a panel of human melanoma cell lines. Cancer Immunol Immunother. 2008;57:719–29. https://doi.org/10.1007/s00262-007-0411-3.
41. Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. Cancer Immunol Immunother. 2012;61:249–54. https://doi.org/10.1007/s00262-011-1153-9.
42. Lampen MH, van Hall T. Strategies to counteract MHC-I defects in tumors. Curr Opin Immunol. 2011;23:293–8. https://doi.org/10.1016/j.coi.2010.12.005.
43. Aptsiauri N, Carretero R, Garcia-Lora A, et al. Regressing and progressing metastatic lesions: resistance to immunotherapy is predetermined by irreversible HLA class I antigen alterations. Cancer Immunol Immunother. 2008;57:1727–33. https://doi.org/10.1007/s00262-008-0532-3.
44. Rolland P, Deen S, Scott I, et al. Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer. Clin Cancer Res. 2007;13:3591–6. https://doi.org/10.1158/1078-0432.CCR-06-2087.
45. Carretero R, Romero JM, Ruiz-Cabello F, et al. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. Immunogenetics. 2008;60:439–47. https://doi.org/10.1007/s00251-008-0303-5.
46. Hicklin DJ, Wang Z, Arienti F, et al. beta2-Microglobulin mutations, HLA class I antigen loss, and tumor progression in melanoma. J Clin Invest. 1998;101:2720–9. https://doi.org/10.1172/JCI498.
47. Jiménez P, Cantón J, Collado A, et al. Chromosome loss is the most frequent mechanism contributing to HLA haplotype loss in human tumors. Int J Cancer. 1999;83:91–7.
48. Maleno I, López-Nevot MA, Cabrera T, et al. Multiple mechanisms generate HLA class I altered phenotypes in laryngeal carcinomas: high frequency of HLA haplotype loss associated with loss of heterozygosity in chromosome region 6p21. Cancer Immunol Immunother. 2002;51:389–96. https://doi.org/10.1007/s00262-002-0296-0.
49. Klippel ZK, Chou J, Towlerton AM, et al. Immune escape from NY-ESO-1-specific T-cell therapy via loss of heterozygosity in the MHC. Gene Ther. 2014;21:337–42. https://doi.org/10.1038/gt.2013.87.
50. Khan ANH, Gregorie CJ, Tomasi TB. Histone deacetylase inhibitors induce TAP, LMP, Tapasin genes and MHC class I antigen presentation by melanoma cells. Cancer Immunol Immunother. 2008;57:647–54. https://doi.org/10.1007/s00262-007-0402-4.
51. Setiadi AF, Omilusik K, David MD, et al. Epigenetic enhancement of antigen processing and presentation promotes immune recognition of tumors. Cancer Res. 2008;68:9601–7. https://doi.org/10.1158/0008-5472.CAN-07-5270.
52. Martini M, Testi MG, Pasetto M, et al. IFN-gamma-mediated upmodulation of MHC class I expression activates tumor-specific immune response in a mouse model of prostate cancer. Vaccine. 2010;28:3548–57. https://doi.org/10.1016/j.vaccine.2010.03.007.
53. Mantovani A, Savino B, Locati M, et al. The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev. 2010;21:27–39. https://doi.org/10.1016/j.cytogfr.2009.11.007.
54. Mukaida N, Sasaki S-I, Baba T. Chemokines in cancer development and progression and their potential as targeting molecules for cancer treatment. Mediators Inflamm. 2014;2014:170381. https://doi.org/10.1155/2014/170381.
55. Prestwich R, Errington F, Hatfield P, et al. The immune system—is it relevant to cancer development, progression and treatment? Clin Oncol.

2008;20:101–12. https://doi.org/10.1016/j.clon.2007.10.011.
56. Stewart TJ, Smyth MJ. Improving cancer immunotherapy by targeting tumor-induced immune suppression. Cancer Metastasis Rev. 2011;30:125–40. https://doi.org/10.1007/s10555-011-9280-5.
57. Li MO, Wan YY, Sanjabi S, et al. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol. 2006;24:99–146. https://doi.org/10.1146/annurev.immunol.24.021605.090737.
58. Mocellin S, Marincola F, Rossi CR, et al. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev. 2004;15:61–76.
59. Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. Nat Rev Immunol. 2002;2:46–53. https://doi.org/10.1038/nri704.
60. Pockaj BA, Basu GD, Pathangey LB, et al. Reduced T-cell and dendritic cell function is related to cyclo-oxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer. Ann Surg Oncol. 2004;11:328–39.
61. Kalinski P. Regulation of immune responses by prostaglandin E2. J Immunol. 2012;188:21–8. https://doi.org/10.4049/jimmunol.1101029.
62. Gabrilovich DI, Ishida T, Nadaf S, et al. Antibodies to vascular endothelial growth factor enhance the efficacy of cancer immunotherapy by improving endogenous dendritic cell function. Clin Cancer Res. 1999;5:2963–70.
63. Stagg J, Smyth MJ. Extracellular adenosine triphosphate and adenosine in cancer. Oncogene. 2010;29:5346–58. https://doi.org/10.1038/onc.2010.292.
64. Jin D, Fan J, Wang L, et al. CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. Cancer Res. 2010;70(6):2245–55. doi: https://doi.org/10.1158/0008-5472.CAN-09-3109.
65. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med. 2007;204:1257–65. https://doi.org/10.1084/jem.20062512.
66. Lu T, Gabrilovich DI Molecular pathways: tumor-infiltrating myeloid cells and reactive oxygen species in regulation of tumor microenvironment. Clin Cancer Res. 2012;18(18):4877–82. doi: https://doi.org/10.1158/1078-0432.CCR-11-2939.;
67. Bronte V, Kasic T, Gri G, et al. Boosting antitumor responses of T lymphocytes infiltrating human prostate cancers. J Exp Med. 2005;201:1257–68. https://doi.org/10.1084/jem.20042028.
68. Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med. 2007;13:828–35. https://doi.org/10.1038/nm1609.
69. Molon B, Ugel S, Del Pozzo F, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208:1949–62. https://doi.org/10.1084/jem.20101956.
70. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005;5:641–54. https://doi.org/10.1038/nri1668.
71. Kropf P, Baud D, Marshall SE, et al. Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. Eur J Immunol. 2007;37:935–45. https://doi.org/10.1002/eji.200636542.
72. Terness P, Bauer TM, Röse L, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med. 2002;196:447–57.
73. Grimm M, Kim M, Rosenwald A, et al. Tumour-mediated TRAIL-Receptor expression indicates effective apoptotic depletion of infiltrating CD8+ immune cells in clinical colorectal cancer. Eur J Cancer. 2010;46:2314–23. https://doi.org/10.1016/j.ejca.2010.05.025.
74. O'Connell J, Bennett MW, O'Sullivan GC, et al. The Fas counterattack: cancer as a site of immune privilege. Immunol Today. 1999;20:46–52.
75. Motz GT, Santoro SP, Wang L-P, et al. Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. Nat Med. 2014;20:607–15. https://doi.org/10.1038/nm.3541.
76. Martin-Orozco N, Li Y, Wang Y, et al. Melanoma cells express ICOS ligand to promote the activation and expansion of T-regulatory cells. Cancer Res. 2010;70:9581–90. https://doi.org/10.1158/0008-5472.CAN-10-1379.
77. Linsley PS, Bradshaw J, Greene J, et al. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. Immunity. 1996;4:535–43.
78. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol. 2002;2:116–26. https://doi.org/10.1038/nri727.
79. Onodera T, Jang MH, Guo Z, et al. Constitutive expression of IDO by dendritic cells of mesenteric lymph nodes: functional involvement of the CTLA-4/B7 and CCL22/CCR4 interactions. J Immunol. 2009;183:5608–14. https://doi.org/10.4049/jimmunol.0804116.
80. Fallarino F, Grohmann U, Hwang KW, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol. 2003;4:1206–12. https://doi.org/10.1038/ni1003.
81. Jacobs JFM, Nierkens S, Figdor CG, et al. Regulatory T cells in melanoma: the final hurdle towards effective immunotherapy? Lancet Oncol. 2012;13:e32–42. https://doi.org/10.1016/S1470-2045(11)70155-3.
82. Riella LV, Paterson AM, Sharpe AH, Chandraker A. Role of the PD-1 pathway in the immune response. Am J Transplant. 2012;12:2575–87. https://doi.org/10.1111/j.1600-6143.2012.04224.x.

83. Taube JMJ, Anders RAR, Young GDG, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med. 2012;4:127ra37. https://doi.org/10.1126/scitranslmed.3003689.
84. Pilon-Thomas S, Mackay A, Vohra N, Mulé JJ. Blockade of programmed death ligand 1 enhances the therapeutic efficacy of combination immunotherapy against melanoma. J Immunol. 2010;184:3442–9. https://doi.org/10.4049/jimmunol.0904114.
85. Wang B-J, Bao J-J, Wang J-Z, et al. Immunostaining of PD-1/PD-Ls in liver tissues of patients with hepatitis and hepatocellular carcinoma. World J Gastroenterol. 2011;17:3322–9. https://doi.org/10.3748/wjg.v17.i28.3322.
86. Lesterhuis WJ, Punt CJ, Hato SV, et al. Platinum-based drugs disrupt STAT6-mediated suppression of immune responses against cancer in humans and mice. J Clin Invest. 2011;121:3100–8. https://doi.org/10.1172/JCI43656DS1.
87. Ahmadzadeh M, Johnson LA, Heemskerk B, et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood. 2009;114:1537–44. https://doi.org/10.1182/blood-2008-12-195792.
88. Ngiow SF, Teng MWL, Smyth MJ. Prospects for TIM3-targeted antitumor immunotherapy. Cancer Res. 2011;71:6567–71. https://doi.org/10.1158/0008-5472.CAN-11-1487.
89. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol. 2005;6:1245–52. https://doi.org/10.1038/ni1271.
90. Huard B, Prigent P, Tournier M, et al. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. Eur J Immunol. 1995;25:2718–21. https://doi.org/10.1002/eji.1830250949.
91. Goldberg MV, Drake CG. LAG-3 in cancer immunotherapy. Curr Top Microbiol Immunol. 2011;344:269–78. https://doi.org/10.1007/82_2010_114.
92. Hannier S, Tournier M, Bismuth G, Triebel F. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. J Immunol. 1998;161:4058–65.
93. Hemon P, Jean-Louis F, Ramgolam K, et al. MHC class II engagement by its ligand LAG-3 (CD223) contributes to melanoma resistance to apoptosis. J Immunol. 2011;186:5173–83. https://doi.org/10.4049/jimmunol.1002050.
94. Liang B, Workman C, Lee J, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol. 2008;180:5916–26.
95. Sabatos CA, Chakravarti S, Cha E, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat Immunol. 2003;4:1102–10. https://doi.org/10.1038/ni988.
96. Sakuishi K, Apetoh L, Sullivan JM, et al. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J Exp Med. 2010;207:2187–94. https://doi.org/10.1084/jem.20100643.
97. Ngiow SF, Scheidt Von B, Akiba H, et al. Anti-TIM3 antibody promotes T Cell IFN-mediated antitumor immunity and suppresses established tumors. Cancer Res. 2011;71:3540–51. https://doi.org/10.1158/0008-5472.CAN-11-0096.
98. Zhou Q, Munger ME, Veenstra RG, et al. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. Blood. 2011;117:4501–10. https://doi.org/10.1182/blood-2010-10-310425.
99. Woo S-R, Turnis ME, Goldberg MV, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res. 2012;72:917–27. https://doi.org/10.1158/0008-5472.CAN-11-1620.
100. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12:252–64. https://doi.org/10.1038/nrc3239.
101. Taube JM, Young GD, McMiller TL, et al. Differential expression of immune-regulatory genes associated with PD-L1 display in melanoma: implications for PD-1 pathway blockade. Clin Cancer Res. 2015;21(17):3969–76. doi: https://doi.org/10.1158/1078-0432.CCR-15-0244.
102. Kluger HM, Zito CR, Barr ML, et al. Characterization of PD-L1 expression and associated T-cell infiltrates in metastatic melanoma samples from variable anatomic sites. Clin Cancer Res. 2015;21(13):3052–60. doi: https://doi.org/10.1158/1078-0432.CCR-14-3073.
103. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013;73:1733–41. https://doi.org/10.1158/0008-5472.CAN-12-2384.
104. Dolen Y, Esendagli G. Myeloid leukemia cells with a B7-2(+) subpopulation provoke Th-cell responses and become immuno-suppressive through the modulation of B7 ligands. Eur J Immunol. 2013;43:747–57. https://doi.org/10.1002/eji.201242814.
105. Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med. 2013;5:200ra116. https://doi.org/10.1126/scitranslmed.3006504.
106. Hosoi A, Matsushita H, Shimizu K, et al. Adoptive cytotoxic T lymphocyte therapy triggers a counter-regulatory immunosuppressive mechanism via recruitment of myeloid-derived suppressor cells. Int J Cancer. 2014;134:1810–22. https://doi.org/10.1002/ijc.28506.

107. Wong JL, Obermajer N, Odunsi K, et al. Synergistic COX2 induction by IFN and TNF self-limits Type-1 immunity in the human tumor microenvironment. Cancer Immunol Res. 2016;4:303–11. https://doi.org/10.1158/2326-6066.CIR-15-0157.
108. Tumeh PC, Harview CL, Yearley JH, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515:568–71. https://doi.org/10.1038/nature13954.
109. McGray AJR, Bernard D, Hallett R, et al. Combined vaccination and immunostimulatory antibodies provides durable cure of murine melanoma and induces transcriptional changes associated with positive outcome in human melanoma patients. Oncoimmunology. 2012;1:419–31.
110. Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res. 2007;13:5256–61. https://doi.org/10.1158/1078-0432.CCR-07-0892.
111. Gajewski TF, Fuertes M, Spaapen R, et al Molecular profiling to identify relevant immune resistance mechanisms in the tumor microenvironment. Curr Opin Immunol. 2010;23(2):286–92. doi: https://doi.org/10.1016/j.coi.2010.11.013.
112. Gajewski TF. Molecular profiling of melanoma and the evolution of patient-specific therapy. Semin Oncol. 2011;38:236–42. https://doi.org/10.1053/j.seminoncol.2011.01.004.
113. Gajewski TF. Cancer immunotherapy. Mol Oncol. 2012;6:242–50. https://doi.org/10.1016/j.molonc.2012.01.002.
114. Gajewski TF, Woo S-R, Zha Y, et al. Cancer immunotherapy strategies based on overcoming barriers within the tumor microenvironment. Curr Opin Immunol. 2013;25:268–76. https://doi.org/10.1016/j.coi.2013.02.009.
115. Woo S-R, Corrales L, Gajewski TF. The STING pathway and the T cell-inflamed tumor microenvironment. Trends Immunol. 2015;36:250–6. https://doi.org/10.1016/j.it.2015.02.003.
116. Baniyash M (2006) Chronic inflammation, immunosuppression and cancer: new insights and outlook. Seminars in Cancer Biology.
117. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol. 2009;182:4499–506. https://doi.org/10.4049/jimmunol.0802740.
118. Umansky V, Sevko A. Overcoming immunosuppression in the melanoma microenvironment induced by chronic inflammation. Cancer Immunol Immunother. 2012;61:275–82. https://doi.org/10.1007/s00262-011-1164-6.
119. Kanterman J, Sade-Feldman M, Baniyash M. New insights into chronic inflammation-induced immunosuppression. Semin Cancer Biol. 2012;22:307–18. https://doi.org/10.1016/j.semcancer.2012.02.008.
120. Baniyash M, Sade-Feldman M, Kanterman J. Chronic inflammation and cancer: suppressing the suppressors. Cancer Immunol Immunother. 2014;63:11–20. https://doi.org/10.1007/s00262-013-1468-9.
121. Wang J, Yoshida T, Nakaki F, Hiai H. Establishment of NOD-Pdcd1−/−mice as an efficient animal model of type I diabetes. Proc Natl Acad Sci U S A. 2005;102(33):11823–8.
122. Guleria I. A critical role for the programmed death ligand 1 in fetomaternal tolerance. J Exp Med. 2005;202:231–7. https://doi.org/10.1084/jem.20050019.
123. Habicht AA, Dada SS, Jurewicz MM, et al. A link between PDL1 and T regulatory cells in fetomaternal tolerance. J Immunol. 2007;179:5211–9.
124. Zhang Y, Chung Y, Bishop C, et al. Regulation of T cell activation and tolerance by PDL2. Proc Natl Acad Sci U S A. 2006;103:11695–700. https://doi.org/10.1073/pnas.0601347103.
125. Matsumoto KK, Inoue HH, Nakano TT, et al. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. J Immunol. 2004;172:2530–41.
126. Okazaki T, Okazaki I-M, Wang J, et al. PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. J Exp Med. 2011;208:395–407. https://doi.org/10.1084/jem.20100466.
127. Liu Y, Shu Q, Gao L, et al. Increased Tim-3 expression on peripheral lymphocytes from patients with rheumatoid arthritis negatively correlates with disease activity. Clin Immunol. 2010;137:288–95. https://doi.org/10.1016/j.clim.2010.07.012.
128. Keir ME, Freeman GJ, Sharpe AH. PD-1 regulates self-reactive CD8+ T cell responses to antigen in lymph nodes and tissues. J Immunol. 2007;179:5064–70.
129. Dalton DK, Wittmer S. Nitric-oxide-dependent and independent mechanisms of protection from CNS inflammation during Th1-mediated autoimmunity: evidence from EAE in iNOS KO mice. J Neuroimmunol. 2005;160:110–21. https://doi.org/10.1016/j.jneuroim.2004.11.004.
130. Yaswen L, Kulkarni AB, Fredrickson T, et al. Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse. Blood. 1996;87:1439–45.
131. Shull MM, Ormsby I, Kier AB, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359:693–9. https://doi.org/10.1038/359693a0.
132. Pallotta MTM, Orabona CC, Volpi CC, et al. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. Nat Immunol. 2011;12:870–8. https://doi.org/10.1038/ni.2077.
133. Fallarino FF, Grohmann UU, Puccetti PP. Indoleamine 2,3-dioxygenase: From catalyst to signaling function. Eur J Immunol. 2012;42:1932–7. https://doi.org/10.1002/eji.201242572.
134. Vermeire KK, Heremans HH, Vandeputte MM, et al. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. J Immunol. 1997;158:5507–13.

135. Ferber IA, Brocke S, Taylor-Edwards C. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J Immunol. 1996;156(1):5–7.
136. Krakowski MM, Owens TT. Interferon-gamma confers resistance to experimental allergic encephalomyelitis. Eur J Immunol. 1996;26:1641–6. https://doi.org/10.1002/eji.1830260735.
137. Tran EHE, Prince ENE, Owens TT. IFN-gamma shapes immune invasion of the central nervous system via regulation of chemokines. J Immunol. 2000;164:2759–68.
138. Ring GHG, Dai ZZ, Saleem SS, et al. Increased susceptibility to immunologically mediated glomerulonephritis in IFN-gamma-deficient mice. J Immunol. 1999;163:2243–8.
139. Zaidi MR, Merlino G. The two faces of interferon- in cancer. Clin Cancer Res. 2011;17:6118–24. https://doi.org/10.1158/1078-0432.CCR-11-0482.
140. Yang JC, Hughes M, Kammula U, et al. Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. J Immunother. 2007;30:825–30. https://doi.org/10.1097/CJI.0b013e318156e47e.
141. Hodi FSF, O'Day SJS, McDermott DFD, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23. https://doi.org/10.1056/NEJMoa1003466.
142. Robert CC, Thomas LL, Bondarenko II, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med. 2011;364:2517–26. https://doi.org/10.1056/NEJMoa1104621.
143. Lynch TJT, Bondarenko II, Luft AA, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J Clin Oncol. 2012;30:2046–54. https://doi.org/10.1200/JCO.2011.38.4032.
144. Brahmer JRJ, Drake CGC, Wollner II, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol. 2010;28:3167–75. https://doi.org/10.1200/JCO.2009.26.7609.
145. Topalian SLS, Hodi FSF, Brahmer JRJ, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366:2443–54. https://doi.org/10.1056/NEJMoa1200690.
146. Brahmer JRJ, Tykodi SSS, Chow LQML, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366:2455–65. https://doi.org/10.1056/NEJMoa1200694.
147. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med. 2015;372(21):2018–28. doi: https://doi.org/10.1056/NEJMoa1501824.
148. Sharma P, Allison JP. The future of immune checkpoint therapy. Science. 2015;348:56–61. https://doi.org/10.1126/science.aaa8172.
149. Postow MA, Chesney J, Pavlick AC, et al (2015) Nivolumab and Ipilimumab versus Ipilimumab in untreated melanoma. N Engl J Med. 2015;372(21):2006–17. doi: https://doi.org/10.1056/NEJMoa1414428.
150. Curran MA, Montalvo W, Yagita H, Allison JP. PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. Proc Natl Acad Sci U S A. 2010;107:4275–80. https://doi.org/10.1073/pnas.0915174107.
151. Duraiswamy J, Kaluza KM, Freeman GJ, Coukos G. Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res. 2013;73:3591–603. https://doi.org/10.1158/0008-5472.CAN-12-4100.
152. Winograd R, Byrne KT, Evans RA, et al. Induction of T-cell immunity overcomes complete resistance to PD-1 and CTLA-4 blockade and improves survival in pancreatic carcinoma. Cancer Immunol Res. 2015;3:399–411. https://doi.org/10.1158/2326-6066.CIR-14-0215.
153. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined Nivolumab and Ipilimumab or monotherapy in untreated melanoma. N Engl J Med. 2015;373(13):1270–1. doi: https://doi.org/10.1056/NEJMoa1504030.
154. Baitsch L, Legat A, Barba L, et al. Extended co-expression of inhibitory receptors by human CD8 T-cells depending on differentiation, antigen-specificity and anatomical localization. PLoS One. 2012;7:e30852. https://doi.org/10.1371/journal.pone.0030852.
155. Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, et al. Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc Natl Acad Sci U S A. 2010;107:7875–80. https://doi.org/10.1073/pnas.1003345107.
156. Blackburn SD, Shin H, Haining WN, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009;10:29–37. https://doi.org/10.1038/ni.1679.
157. Fourcade J, Sun Z, Pagliano O, et al. PD-1 and Tim-3 regulate the expansion of tumor antigen-specific CD8^{+} T cells induced by melanoma vaccines. Cancer Res. 2014;74:1045–55. https://doi.org/10.1158/0008-5472.CAN-13-2908.
158. Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature. 2015;520:373–7. https://doi.org/10.1038/nature14292.

Imaging the Tumor Microenvironment

15

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15.1 Introduction

The microenvironment of solid tumors is heterogeneous and complex, and consists of cancer, stromal and immune cells [1], as well as areas of necrosis [2] (Fig. 15.1). The process of cancer metastasis is highly dependent on the microenvironment of both the primary tumor, as well as the connective tissue stroma at the site of future metastasis, as tumor cells interact with the endothelium of this organ [3]. Inflammation plays a major role in cancer development, which has been viewed as a dysregulated form of protective tissue repair and growth response [4]. Leukocyte infiltration in tumors, including macrophages and neutrophils, is now well known to be one of the "hallmarks of cancer" [1], and can exert both tumor-suppressive and tumor-promoting effects [4]. There is broad diversity of imaging biomarkers for many aspects of the tumor microenvironment. Specific imaging of the cell types and/or physiological factors in the microenvironment can provide critical information regarding cancer aggressiveness and response to treatment. This chapter will discuss several approaches utilizing a multitude of modalities that have been investigated for imaging the tumor microenvironment in mouse models and in humans.

15.2 Imaging Tumor-Associated Inflammation

It has long been known that some tumors are densely infiltrated by cells of both the innate (e.g. macrophages, neutrophils) and adaptive (e.g. T-cells) arms of the immune system [5]. It is now accepted that virtually every tumor contains immune cells at some level, ranging from barely detectable by immunohistochemistry to gross inflammation that can be imaged by standard histology [1, 6] and in vivo imaging [7]. It has also

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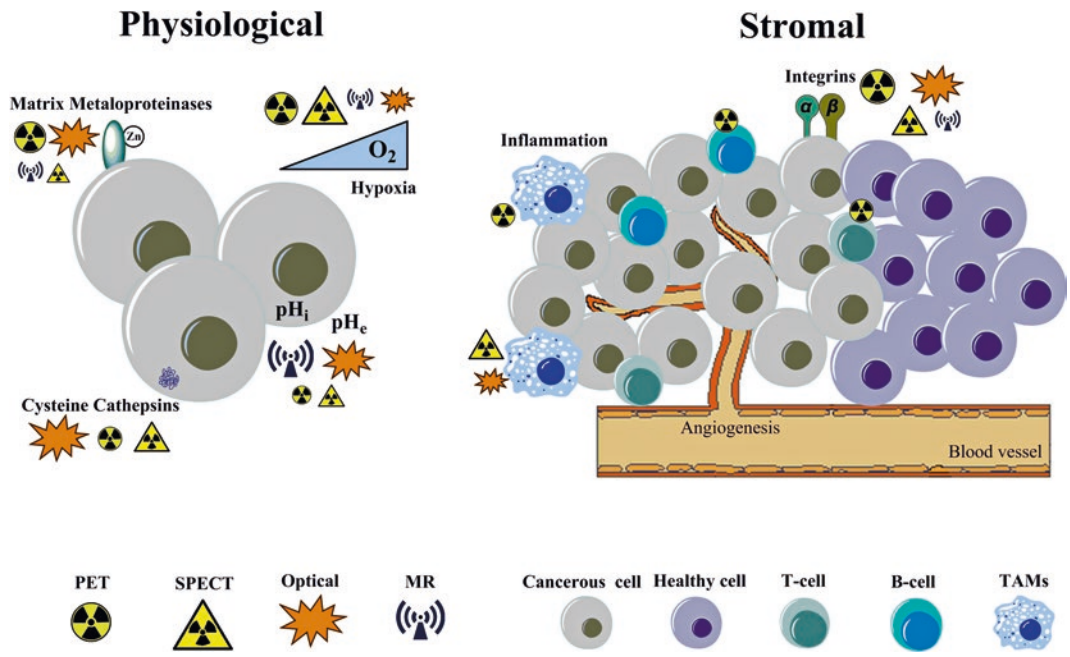

Fig. 15.1 Physiological and stromal factors are targeted to image the tumor microenvironment by various modalities. Unique components of the tumor microenvironment include: hypoxia, internal and external pH ratios, enzymes such as matrix metaloproteinases and cysteine cathepsins, as well as integrins, tumor-associated macrophages which induce angiogenesis and inflammation, respectively

been shown that neutrophils, activated macrophages and dendritic cells (DCs) are present at the tumor margins [8]. Inflammation brings bioactive molecules to the tumor microenvironment, including growth and survival factors that promote tumor cell growth, and matrix-modifying enzymes that contribute to angiogenesis, invasion and metastasis [9–11]. Inflammation may also be causative in promoting the transformation of early neoplasias to fully developed cancer [11]. In vivo imaging of various aspects of inflammation in cancer is vitally important, and can aid in the early diagnosis of tumor growth and progression. In this section we will discuss imaging of tumor-associated macrophages (TAMs) and T-cells that have roles in both cancer progression and regression.

Macrophages in cancer. Monocytes, which originate in bone marrow and spleen, are recruited to tumors by tumor-derived chemokines and growth factors where they differentiate in macrophages [12]. M1 polarized macrophages benefit the host by mediating a classical inflammatory response against such pathogens as bacteria and viruses and can act as antigen presenting cells for antibody production against foreign proteins [13]. They also have a protective role in tumors and suppress the activities of tumor promoting cells [14]. However, recent evidence has emerged that in cancer, M2 polarized macrophages promote tumor growth. For example, TAMs have an M2 phenotype and secret enzymes that remodel the extracellular matrix and promote metastasis. They also suppress the adaptive immune response. Moreover, high numbers of TAMs in tumors have been linked to low survival rates [15]. Therefore, non-invasive diagnostic imaging of TAMs could have prognostic power to stratify patients for therapy. Because a characteristic of macrophages is there ability phagocytose large particles, a particularly attractive approach is to use both non-targeted and targeted nanoparticles [16].

Non-targeted nanoparticles. To take advantage of the phagocytotic activity of M2-polarized macrophages, non-targeted nanoparticles have

been investigated (Fig. 15.2). In one approach, radiolabeled high-density lipoprotein based nanoparticles (rHDL) incorporating the long-lived positron-emitting nuclide ^{89}Zr and a NIR dye (DiO) were developed [17]. These nanoparticles were composed of phospholipids and apolipoprotein. When injected into tumor-bearing mice, histologic analysis of tumor tissues was consistent with particle accumulation in TAM dense tumor regions. By changing the conjugation site of the ^{89}Zr chelator, DFO, to either a phospholipid or to apoA-I, different biodistributions were observed. Moreover, disaggregated tumors showed the greatest uptake of the particles in TAMs with lower accumulation in dendritic cells and in the tumor cells, although macrophage targeting was not exclusive. That lack of exclusivity was attributed to unanticipated particle interaction with the different cell types.

Targeted nanoparticles. To enhance efficiency of nanoparticle uptake in TAMs, they can be conjugated with ligands that are targeted to cell surface receptors on macrophages. In an example of this approach, ^{64}Cu- and DiO-labeled

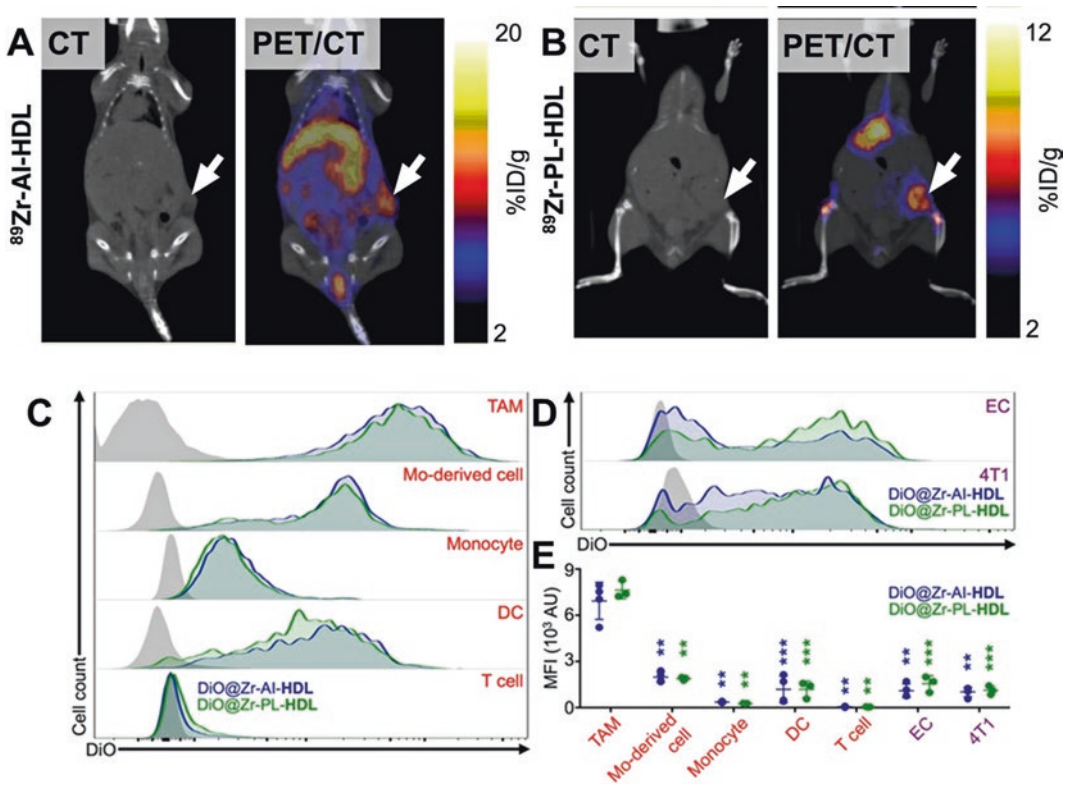

Fig. 15.2 ^{89}Zr-labeled high-density lipoprotein based nanoparticles incorporate in M2-polarized macrophages by phagocytosis in orthotopic 4T1 mouse mammary tumor-bearing mice. *Top* Accumulation of ^{89}Zr-HDL nanotracers in tumor tissues can be visualized by in vivo PET imaging. CT (*left*) and PET/CT fusion (*right*) images of ^{89}Zr-AI-HDL (**a**) and ^{89}Zr-PL-HDL (**b**) obtained at 24 h after injection in mice bearing orthotopic 4T1 tumors (indicated by *arrows*). *Bottom* Both DiO@Zr-PL-HDL and DiO@Zr-AI-HDL preferentially target tumor-associated macrophages. 4T1 cell–induced orthotopic breast tumors were used to isolate single cells. (**c**) Representative DiO levels in five immune cells, namely TAMs, monocyte-derived cells (Mo-derived cells), monocytes, dendritic cells (DCs), and T cells. (**d**) Representative DiO levels in ECs and tumor cells (4T1). Cells from a phosphate-buffered saline-injected mouse served as controls (*gray* histograms to *left*). (**e**) Quantification of DiO levels presented as mean fluorescence intensity (MFI). Importantly, no statistical significance was found when comparing DiO levels of same cell type from two HDL formulations. Statistics were calculated with 2-tailed Student t test with unequal variance by comparing with TAM from same group. **P < 0.01. ***P < 0.001. Adapted from reference [17]

mannose coated liposomes were used to target mannose receptors (CD206) overexpressed on TAMs in lung tumors induced through chemical treatment [18]. Evidence for mannose-mediated uptake was that targeted accumulation in both tumor and spleen exceeded non-target accumulation and that confocal microscopy of lung tissue sections demonstrated co-localization of DiO-labeled liposomes and a pan macrophage marker F4/80.

Molecular imaging agents for macrophages. CD206 has also been targeted with non-nanoparticle molecular imaging agents [19]. A nanobody (Nb cl1) against CD206 radiolabeled with ^{99m}Tc was investigated for visualizing macrophages in tumor bearing mice, both wild type and CD206 knock out mice, and a non-binding control (BCII10) was included. The in vivo results in mice bearing 3LL tumors showed there was no uptake of the CD206 targeted ^{99m}Tc-Nb cl1 in the CD206 knockout mice, demonstrating that the tumor itself does not express significant levels of CD206. However, in WT mice, the targeted antibody localized to the tumor as well as tissue resident macrophages in the liver. The non-binding control did not accumulate significantly in CD206 enriched tissues, which demonstrates CD206 mediated accumulation. Numerous ex vivo experiments supported CD206 mediated accumulation of Nb cl1 including localization of AF647-Nb cl1 in tumor tissue that co-stained with F4/80, a pan macrophage marker.

CD11b is a marker for macrophages as well as dendritic cells but may not exclusively mark M2-polarized macrophages. An ^{18}F-labeled variable heavy chain (VHH) raised against CD11b, readily visualized tumor associated inflammation in a syngeneic mouse model of melanoma (B16F10) by small animal PET, whereas ^{18}F-FDG failed to generate a clear delineation of the tumor [7]. Ex vivo two-photon microscopy demonstrated that the dye-conjugated anti-CD11b VHH had high uptake in CD11b enriched lymphoid organs and virtually no uptake in the corresponding knock-out mice thus supporting the specific high uptake observed in vivo. Flow cytometry of the disaggregated tissues stained with the dye-labeled anti-CD11b VHH were consistent with the findings of the microscopy.

Molecular imaging agents for T-cells. Imaging CD8-expressing cells will be important to track T-cells for non-invasive monitoring of these populations during immunotherapy [20]. Utilizing known sequences for murine CD8 monoclonal antibodies, minibodies (Mbs) were constructed. The Mbs retained high affinity for CD8 and readily stained disaggregated CD8 positive cells from thymus, spleen, and lymph nodes. Unlike the parental antibodies, the Mbs did not deplete T-cells from the host mice. After Cu-64 labeling, uptake in CD8+ tissues was readily visualized by preclinical PET. Uptake was clearly visualized in lymph nodes and spleen (Fig. 15.3) 4 h post injection.

15.3 Imaging Hypoxia in the Tumor Microenvironment

Hypoxia (low oxygenation) occurs in the tumor microenvironment where cells are rapidly growing and taxing the oxygen availability from its surrounding blood supply [21–23]. Chronic and acute/cycling hypoxia are two categories found in the tumor microenvironment [24–26]. Chronic hypoxia arises from diffusion limited oxygen availability, and acute hypoxia occurs from transient perfusion disruptions. In both cases, the oxygen supply is reduced compared to normal supply (normoxia). There are several reviews on different modes of imaging probes targeting hypoxia to study the tumor microenvironment, which include polarographic O_2 microelectrodes, various positron emission tomography (PET) and single photon emission computed tomography (SPECT) tracers, magnetic resonance imaging (MRI) methods and fluorescence probes [27–31]. PET tracers for imaging hypoxia have had much success in the clinic and have been compared amongst each other [32–38]. Recently investigated imaging probes targeting hypoxia in the tumor microenvironment will be discussed.

PET/SPECT tracers for hypoxia imaging: Huang et al. compared ^{18}F-labeled 2-deoxyglucose

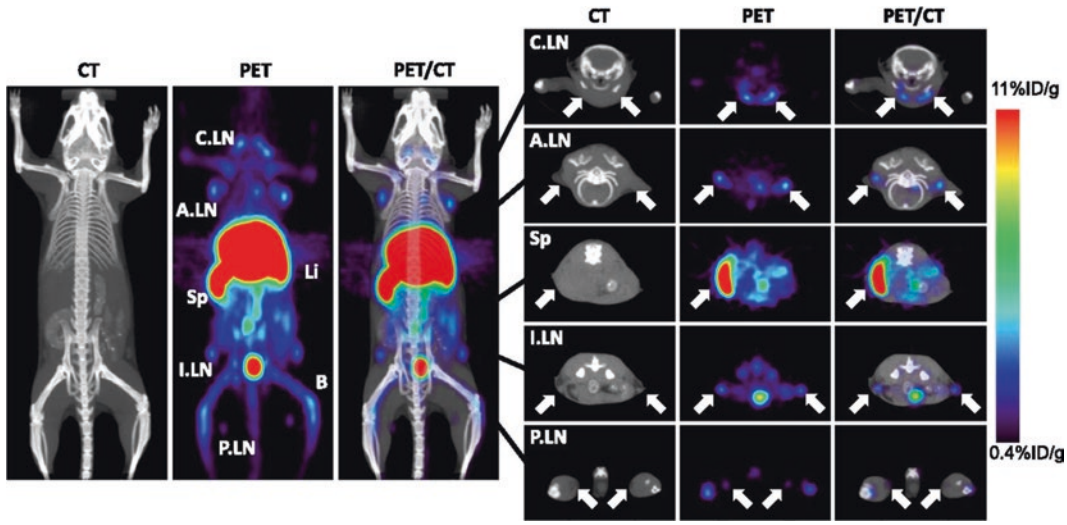

Fig. 15.3 Immuno-PET imaging of CD8 positive T-cells with ^{64}Cu-NOTA-2.43 Mb was observed in thymus, spleen and lymph nodes 4 h after i.v. injection in B/6 mice. The white arrows (2-mm transverse MIPs) are used to highlight uptake in various lymph nodes (*right*) and the spleen seen in the whole-body 20-mm coronal MIPs (*left*). *A.LN* axillary lymph nodes; *B* bone, *C.LN* cervical lymph nodes, *I.LN* inguinal lymph nodes, *Li* liver, *MIPs* maximum intensity projections, *P.LN* popliteal lymph nodes, *Sp* Spleen. Adapted from reference [20]

(FDG), 3′-deoxy-3′-[^{18}F]fluorothymidine (FLT) and 1-(2-nitroimidazolyl)-3-[^{18}F]fluoro-2-propanol (FMISO) distribution in subcutaneously implanted and disseminated peritoneal NSCLC A549 and HTB177 tumors in mice by digital autoradiography and compared this to immunohistochemistry images of tumor hypoxia, proliferation, stroma and necrosis [2]. The accumulation of FDG and FLT were broadly similar for both cell lines and for subcutaneous and disseminated tumors. FDG was found to have higher uptake in regions of high pimonidazole binding (hypoxia), although there were some mismatched regions. Areas of higher FDG activity tended towards low proliferation and perfusion, while stroma and necrotic areas showed lower FDG uptake. FLT accumulation in the tumor correlated with high uptake of bromodeoxyuridine and negative staining for pimonidazole, and showed low uptake in stroma and necrosis. As expected, FMISO activity correlated with pimonidazole binding in A549 s.c. tumors, corresponding to low proliferation and perfusion. Stroma and necrotic areas of the tumor had low FMISO accumulation. Generally speaking, FDG and FMISO are similar in areas of hypoxia, stroma and necrosis, whereas FLT better images non-hypoxic regions of the tumor.

A limitation to be considered when imaging with PET tracers is the uptake time, as it influences the lesion-to-background ratio as well as the signal-to-noise ratio. The most widely used hypoxia imaging PET tracer is FMISO [39, 40]. In humans, FMISO was found to give better quality images of hypoxic tumors at 4 h compared to 2 h [41]. Additional studies have shown an accurate reflection of hypoxia detected by FMISO and expression of hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) in 32 patients with gliomas [42]. FMISO also images hypoxia in micrometastases growing in mice xenograft models of human non-small cell lung cancer (NSCLC) A549 and HTB177 cells [43]. Other F-18 labeled PET tracers like 3-^{18}F-fluoro-2-(4-((2-nitro-1-himidazol-1-yl)methyl)-1H-1,2,3,-triazol-1-yl)-propan-1-ol (^{18}F-HX4) and ^{18}F-fluoroazomycin arabinoside (^{18}F-FAZA) also were found to image hypoxia in NSCLC patients [44, 45] and mice bearing human SiHa cervix tumor xenografts [46]. Comparisons have been done of FMISO, ^{18}F-HX4 and ^{18}F-FAZA in rhabdomyosarcoma

R1-bearing WAG/Rij rats evaluating optimal time to image, tumor-to-blood ratios, spatial reproducibility, and sensitivity to oxygen changes [47]. The comparisons found each F-18 PET tracer had advantages, which could be capitalized upon depending on the imaging requirements. FMISO has also been compared with FDG in patients with pancreatic adenocarcinoma, although there was no significant correlation between SUV_{max} for the two tracers [48]. FMISO was also compared to ^{18}F-HX4 in hypoxia imaging in heat and neck cancer patients, and tumor-to-muscle ratios were similar for both imaging probes [49].

Another F-18 PET hypoxia imaging probe is ^{18}F-fluoroerythronitroimidazole (^{18}F-FETNIM), which has been studied in patients with NSCLC. Tumor-to-mediastinum (T/Me) ratios were quantified on PET/CT and correlated with expression of HIF-1α, glucose transporter 1 (GLUT-1) and VEGF [50]. ^{18}F-FETNIM hypoxia imaging was used to determine a hypoxia threshold, quantifying variability in untreated esophageal squamous cell carcinoma (SCC) in patients, and evaluating clinical response after concurrent chemoradiotherapy [51]. Overall ^{18}F-FETNIM PET/CT showed similar uptake in esophageal SCC and baseline SUV_{max} might have predictive value. Preliminary results for a modified version of this ^{18}F hypoxia imaging probes were investigated; a sulfoxide derivative, (^{18}F-F1) was imaged in vitro (S9 liver homogenate fractions) and in vivo (SK-RC-52 tumor model BALB/c nude mice) and found to have good retention in tumors [52].

^{18}F-labeled PET hypoxia imaging probes have also been examined for detecting changes before and during treatment. ^{18}F-FAZA was used to successfully identify and quantify tumor hypoxia before and during concurrent chemoradiotherapy in patients with advanced head and neck SCC [53]. In a handful of studies, FMISO PET imaging was shown to have prognostic value used to evaluate hypoxia tumors' microenvironment after treatment [54–56].

Other radionuclides have been used to image hypoxia in the tumor microenvironment such as ^{99m}Tc, ^{62}Cu, ^{64}Cu, ^{67}Ga, and ^{68}Ga. Current findings will be briefly presented. A ^{99m}Tc-pyrimidine-4,5-diamine (^{99m}Tc-PyDA) was developed and studied in vitro stability and in vivo hypoxia targeting [57]. In vivo studies with ^{99m}Tc-PyDA in mice with injected with Ehrlich ascites carcinomas showed selective uptake in hypoxia tumor tissues. Derivatives of metronidazole (4-isocyano-N-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl]butanamide (M1) and 1-(4-isocyanobutanoyl)-4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl]piperazine (M2)) have also been investigated as ^{99m}Tc tracers (^{99m}Tc-NS3M1 and ^{99m}Tc-NS3M2) for imaging hypoxia in mice bearing induced 3LL Lewis murine lung carcinoma [58]. Tracer ^{99m}Tc-NS3M1 was found to have favorable tumor-to-muscle ratio in vivo. Another SPECT tracer, ^{99m}Tc-meropenem was demonstrated to have higher selectivity in tumor hypoxia tissue compared to ^{18}F -FDG-PET and ^{99m}Tc-nitroimidazole, and was also shown to differentiate from inflamed and infected tissues in mouse models [59]. A bioreductive ^{99m}Tc hypoxia imaging probe (^{99m}Tc-SD32) was found to be retained in FM3A murine breast tumor cells under hypoxic compared to normoxic conditions [60].

Copper-64, Cu-60 and Cu-62 have been used to make PET hypoxia imaging probes. Patients with glioma were imaged with ^{62}Cu-diacetyl-bis (N4-methylthiosemicarbazone) (^{62}Cu-ATSM) to distinguish tumor grades and tissue hypoxia correlated well to MR imaging and HIF-1α expression [61]. ^{64}Cu-ATSM autoradiography and PET were compared with FDG PET in spontaneous canine sarcomas and carcinomas [62, 63]. Correlations between ^{64}Cu-ATSM and ^{18}F-FDG PET were found at later time points (3 hours post injection), and similar distribution in heterogeneous tumor regions were found for ^{64}Cu-ATSM autoradiography and pimonidazole immunohistochemistry. The well-known ^{64}Cu-ATSM and ^{18}F-FMISO have been compared under several conditions and hypoxia tumor types, findings have noted advantages in both [64]. Derivatives of ^{64}Cu-ATSM have been investigated in EMT6 carcinoma cells; revealing an imagine probe (^{64}Cu-ATSM/en) with higher hypoxia selectivity and lower non-target organ uptake than

^{64}Cu-ATSM in EMT6 tumor-bearing mice [65]. ^{60}Cu-ATSM has also been investigated in women with cervical cancer, and was found to be a predictor of recurrence [66].

Gallium-68 and Ga-67 nitroimidazole and metronidazole derivatives have also been used as PET/SPECT imaging probes for hypoxia. One nitroimidazole derivative, ^{68}Ga-HP-DO3A-nitroimidazole (^{68}Ga-HP-DO3A-NI), was studied in vitro in A549 lung cancer cells and in vivo in SCID mice with A549 tumor xenografts [67]. ^{68}Ga-HP-DO3A-NI showed favorable pharmacokinetics with little accumulation in liver, heart or lung, and selectivity for hypoxic tissue compared to controls. Two metronidazole derivatives (^{67}Ga-DOTA-MN1 and ^{67}Ga-DOTA-MN2) were investigated [68]. ^{67}Ga-DOTA-MN2 showed higher accumulation and selectivity in vivo (NFSa mouse fibrosarcoma or FM3A cells inoculated s. c. in female C3H/He mice) compared to controls and ^{67}Ga-DOTA-MN1); ^{68}Ga-DOTA-MN2 was successfully imaged at 1 h by small-animal PET. In another study, ^{68}Ga-DOTA conjugated to nitroimidazole (amide bond, ^{68}Ga-4; thiourea bond, ^{68}Ga-5) were explored and ^{68}Ga-5 was found to have higher uptake in vitro cancer cell lines (HeLa, CHO, and CT-26) than ^{68}Ga-4 [69]. Interestingly, in mice xenografted with CT-26 mouse colon cancer cells, ^{68}Ga-4 had higher standard uptake values (SUV) in tumors than ^{68}Ga-4.

MR contrast agents for imaging hypoxia. Although not as prevalent as for PET, MRI contrast agents have been developed to image hypoxia in vivo (Fig. 15.4) [70, 71]. MRI for hypoxia imaging has mainly utilized dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). DCE-MRI has been shown to analyze areas of hypoxia in tumors in vitro or in vivo [72–74].

Optical probes for imaging hypoxia. Optical imaging of hypoxia in the tumor microenvironment has been investigated with various probes [75, 76]. Nanoparticles (NPs), including polystyrene NPs and boron NPs have been explored [77, 78]. Fluorescent, phosphorescent, and Förster energy transfer (FRET) off-on probes have also been developed to explore imaging hypoxic microenvironments [79, 80]. Ruthenium (Ru) and iridium (Ir) complexes are intriguing phosphorescent hypoxia imaging probes that offer real-time imaging. Ruthenium complexes containing nitroimidazole (Ru-NI1) can monitor oxygen fluctuations in vitro (A549 human lung adenocarcinoma epithelial cells) and in vivo (A549 cells injected s.c. in female BALB/c nu/nu mice) [81]. Other ruthenium complexes like $[Ru(dpp)_3]^{2+}Cl_2$ NPs have successfully imaged

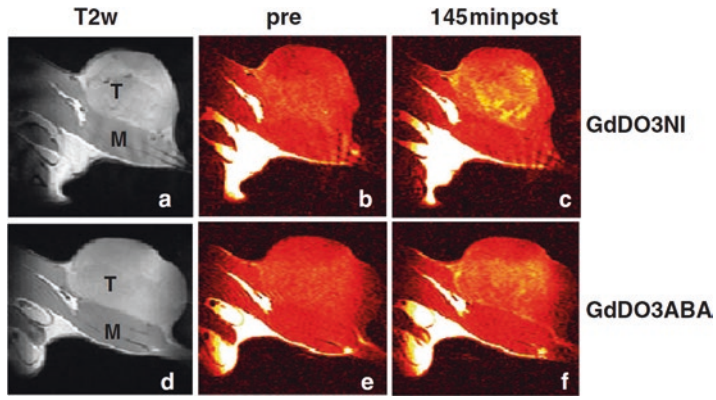

Fig. 15.4 T2-weighted MRI imaging of hypoxia was performed in Dunning prostate R3327-AT1 tumor-bearing rats. Grayscale magnetic resonance T2-weighted (**a**, **d**) and color T1-weighted (**b**, **c**, **e**, **f**) images of Copenhagen rat thighs bearing syngeneic Dunning prostate R3327-AT1 tumors following injection of 0.1 mmol GdDO3NI per kilogram body weight (**a**–**c**) or 0.1 mmol GdDO3ABA per kilogram body weight (**d**–**f**) before injection (**b**, **e**) and 145 min after injection (**c**, **f**). The tumor and thigh muscle are labeled T and M, respectively [70]

hypoxia in U87MG cells and zebrafish embryos [82]. Ruthenium complexes comprised of hydrophobic components like pyrene (Ru-Py) also exhibited hypoxia selective imaging in A549 cells and in female BALB/cSlc nu/nu mice [83]. Although not as common, iridium complexes ($Ir(btp)_2acacBTP$) were found to image hypoxia in vitro (HeLa, CHO, SCC-7, U251) and in vivo tumor-bearing (SCC-7, U87, RAMOS, HT-29, LL-2) female athymic nude mice [84].

15.4 Imaging pH Changes in Tumor Cells and the Microenvironment

The acidic pH found in the tumor microenvironment has been attributed to the fast cell growth and division, which consumes oxygen and nutrients. Tumor cells adapt to the resulting anaerobic conditions producing lactic acid that ultimately affects the intracellular and extracellular pH. The elevated intracellular pH (pH_i) from glycolysis triggers efflux of lactate and protons via monocarboxylate transporters and Na-driven proton extrusion resulting in decreased extracellular pH (pH_e) [85, 86]. Diseased tissue has been shown to have lower local pH_e ranges (5.66–7.78) than normal tissues (7.4) [87, 88]. In normal cells, pH_i is lower than pH_e, but in cancer cells the pH gradient is reversed and stimulates cancer progression and cellular function [89]. Targeting low pH is another characteristic utilized to image the tumor microenvironment.

In the early 1980s, tumor pH measurements were done with pH electrodes, but tissue and capillary damage from probe insertion as well as loss of sensitivity in finer probes led to the development of other non-invasive probes [90–92]. The characteristics of an ideal pH probe are (1) the probe should exist in ionized and unionized forms, where the ratio of the two forms are proportional at the pH of interest; (2) the probe should not change tissue pH; (3) tissue compartmentalization of the probe should be known; (4) the probe should be nontoxic; (5) the measurements of pH should be independent of probe concentration; and (6) pH measurements should be rapid such that temporal changes in pH can be measured [93]. Currently, various pH probes generally use the physical properties of acidic protons for MRS and MRI, acid-cleavability (pH-activatable) for fluorescent probes and pH (low) insertion peptide (pHLIP®) for SPECT/PET tracers.

MRI probes for imaging pH changes. Hyperpolarized nuclei increase sensitivity of NMR and MRI experiments, and hyperpolarized ^{13}C and ^{89}Y have been used for in vitro and in vivo mapping of pH in normal and inflammation mouse models [86, 94, 95]. Chemical exchange saturation transfer (CEST) is a common MRI technique that selectively saturates exchangeable protons that are transferred to bulk water signal [96]. Biosensor imaging of redundant deviations in shifts (BIRDS) which measures exchangeable protons like $-OH$ and $-NH_y$, where $2 \geq y \geq 1$, was compared with CEST for pH imaging [97]. BIRDS showed good sensitivities, eliminating the need to use water resonance as a reference thereby offering a new method to calibrate CEST. AcidoCEST is a known MRI technique to measure acidosis within tumors. Several groups have measured tumor pH_e using acidoCEST MRI in conjunction with the repurposed CT contrast agents, iopromide and/or iopamidol. In vivo studies in xenograft tumor models of Raji lymphoma and MCF-7 breast cancer showed iopromide to measure a greater tumor region compared to iopamidol [98]. Processing methods were evaluated in acidoCEST MRI with iopromide, finding MCF-7 to be more acidic than MDA-MB-231 tumor models in mice [99]. A CEST-fast imaging with steady-state free precession (FISP) method was successfully used to detect iopromide in the MDA-MB-231 mouse model, and tumor pH_e monitored after bicarbonate treatment [100]. Iopamidol has also been used in CEST MRI to generate pH maps in vivo (mouse model of acute kidney injury) over 21 days; the study was able to differentiate among functional regions of damaged kidneys [101]. A ratiometric CEST imaging method using iobitridol, an X-ray contrast agent, has been developed under different radiofrequency (RF) irradiation power levels in vitro and in vivo (xenografted adenocarcinoma TSA tumors s.i. in mouse model) [102]. The RF power-based ratiometric pH MRI method improves in vivo pH sensitivity in pH imaging.

Paramagnetic chemical exchange saturation transfer (PARACEST) is another MRI technique using paramagnetic lanthanides designed with chelator groups that are CEST selective such that the ratio of the two CEST effects can be used to remove the influence of concentration in the pH measurements [103]. PARACEST and CEST-FISP MRI were successfully used in combination to detect pH_e in tumor tissue (MDA-MB-231 mammary carcinoma and MCF-7) in vivo using Yb-DO3A-oAA [104, 105]. A glycol chitosan (GC) with pH-titratable primary amines coupled to Gd-DOTA (GC-NH_2-GdDOTA) has shown in vitro and in vivo (T6–17 cells injected into nu/nu nude mice) provided diagnostic information about the tumor environment while giving high spatiotemporal resolution [106]. A variety of pH probes have been developed using MRI to image the tumor microenvironment. Representative pH_e probes also include mixed micelles that are destabilized at different pH, which can generate pH maps of solid tumors in vivo [107], manganese oxide-based hybrid mesoporous composite nanocapsules for pH-responsive imaging as well as ultrasonography in vitro and in vivo [104], and glycol chitosan coupled to superparamagnetic iron oxide nanoparticles (SPIO) that associates with tumor tissues in vivo [108]. Dual imaging pH probes have also been advanced to image the tumor microenvironment. One example is lanthanide complexes (DO3A) conjugated to rhodamine that gives higher fluorescence at lower pH due to ring opening of the spirolactam while providing MR images [109]. In vitro studies in HEK293 cells and primary mouse islet cells demonstrated the dual MR/fluorescent probe to distinguish between tumor and healthy cells as well as in vivo images from BALB/c nude mice bearing M21 melanoma xenografts.

Fluorescent probes for imaging pH changes. Fluorescence imaging of pH in the tumor microenvironment has seen a boon in the last decade. Several exciting pH probes have been explored. A near-infrared (NIR) fluorescent probe containing a hemicyanine (NIR-Ratio-BTZ) can be protonated/deprotonated on the hydroxyl group to give two photostable, sensitive, ratiometric and reversible states for imaging pH_i in real-time in vitro (HeLa cells) and in vivo (nude mice with LPS-mediated inflammatory response) [110] (Fig. 15.5). Water soluble naphthalene diimides (NDIs), which do not emit as a free base but strongly emit once protonated, were tested in vitro (PC-3 cells) and showed successful imaging of pH_i in tumor microenvironments [111]. A pH probe that has two NIR fluorophores coupled by an acid cleavable linker (DilRB-S) was investigated for visualizing tumors by imaging pH_e [112]. A high target-to-background ratio was obtained for DilRB-S in vivo (HCCLM3-GFP and HepG2 cells inoculated in male nude mice) and gave insight in evaluating metastatic potential of tumors studied. Another NIR ratiometric pH_i probe takes advantage of intramolecular charge transfer (ICT) with a coumarin-indole (π-bonded donor-acceptor) conjugate showing dual-emission changes with pH changes while imaging KB and HeLa cells [113].

Nanoparticles have been explored as a scaffold for pH probes. Gold nanoclusters protected by BSA (reference fluorophore) conjugated to fluorescein-isothiocyanate (response to pH) and folic acid (targeting folate acceptors on folic rich cancer cells) were studied to develop ratiometric pH_i probes for in vitro imaging (HeLa cells and lung cell carcinoma cells A549) [114]. Multifunctional nanoparticles (MNPs) made of silica coated iron oxide with benzo[a]phenoxazine (NIR dye) on the surface that fluorescence at pH lower than 6.0 were developed and tested in vitro (4T1 and 293T tumor cells) and in vivo to visualize the acidic tumor microenvironment with minimal toxicity [115]. An ultra pH-sensitive probe (UPS) was developed by conjugating a near-IR dye (Cy5.5) to a copolymer with cRGD targeting that self assembles into micelles [116]. These fluorescent pH probes have shown exponential nonlinear fluorescence activation at low pH indicative of the tumor microenvironment in vivo (nu/nu mice) in s.c. A549 lung carcinoma, MDA-MB-231 breast cancer, HN5 and HCC4034 head-neck cancer, SF-188 glioma, LN-229 glioma, 3LL lung carcinoma, Mia Paca-2 pancreatic cancer and PC-3 prostate tumors. Another micelle platform made from block copolymers (MPEG-PAE) with encapsulated fluores-

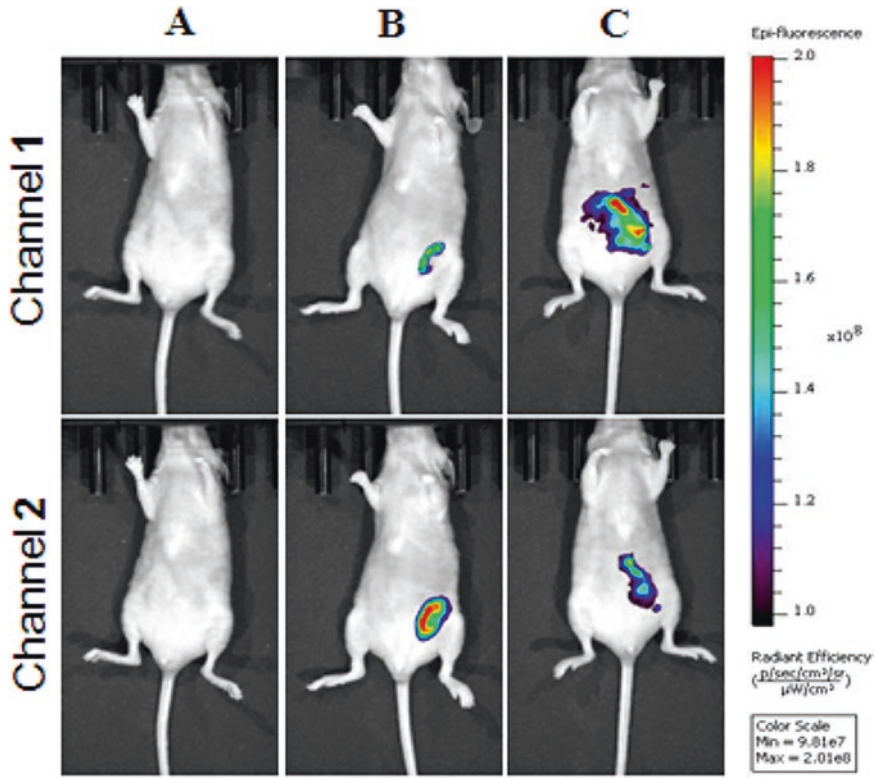

Fig.15.5 A near-infrared fluorescence probe with a hemicyanine dye that can be protonated or deprotonated on the hydroxyl group provides a ratiometric approach for imaging pH. Representative fluorescence images (*pseudocolor*) of mice injected with NIR-Ratio-BTZ during LPS-mediated inflammatory response in vivo. (**a**) Only LPS was injected for control. (**b**) Saline was injected in the peritoneal cavity of mouse, followed by injection of NIR-Ratio-BTZ (50 μM). (**c**) LPS was injected into the peritoneal cavity of the mouse, followed by injection with NIR-Ratio-BTZ (50 μM). The mice were imaged with an excitation filter 580 nm and two emission channels of Channel 1 (650 nm) and Channel 2 (720 nm) [110]

cent dye (TRITC) and black hole quencher (BHQ) have shown to break micelles at acidic pH with high tumor (MDA-MB-231 cells) specificity and sensitivity [117]. Biodegradable NIR fluorescence nanoprobes (InNP1 and Rd.-InNP1) for imaging acidic tumor microenvironments were shown to have high tumor-to-normal (T/N) ratio in vitro (human glioblastoma U87MG) and in vivo (s.c. U87MG tumor xenografts in female SCID mice) [118].

Another interesting platform for NIR fluorescent pH probes are quantum dots (QDs) decorated with fluorescent dyes. A quantum dot ($CuInS_2$/ZnS) surrounded by lauric acid and 2,3-dimethylmaleic anhydride modified ε-polylysine (QD@ε-PL-g-LA/DMA) exhibited a positive charge in acidic conditions and reverses to negatively charged at pH 7.4, showed proof of concept in HeLa cells [119]. Stable luminescent ZnSe/ZnS quantum dots conjugated with SNARF-5F fluorophore and porphyrins were explored with FRET demonstrating proof-of-concept for imaging pH in tumor microenvironment [120]. QD-mOrange-Arg9 and QD-mCherry-Arg9 dyes exhibited improved sensitivity and photostability to image pH_i in vitro (HeLa cells) compared to BCECF (commonly used fluorescent dye for pH imaging) [121]. In another study, nanogels with CdSe QDs in the interior of a polymer (hydroxypropylcellulose-poly(acrylic acid)) underwent pH dependent volume phase transition providing a low pH response for imaging [122]. Cellular imaging on mouse melanoma B16F10 cells with drug (temozolo-

mide) loaded nanogels gave a method to track release of drug with the low pH of the tumor microenvironment.

Ratiometric pH_i imaging was successfully performed by in HeLa Kyoto cells expressing SypHer2 (fluorescent indicator) in vitro and in vivo to compare to pathomorphology and hypoxia staining of tumors [123]. Another genetically encoded pH_i probe was a red fluorescent protein mKeima-A213S mutant (pHRed) notably imaged in vitro in Neuro2A cells expressing pHRed [124]. Photoacoutic nanoprobes have also been used to image the low pH of the tumor microenvironment. Mouse breast tumor model (EMT-6 cells) could be identified from normal tissue using a dextran based pH-sensitive near-IR nanoprobe [125]. In low pH environments, the nanoprobe's hydrazone bonds cleave causing the resonance absorption peaks in the near-IR region to change creating a different photoacoustic output.

PET imaging of acidic pH. A novel approach to imaging acidic pH in the tumor microenvironment was recently reported, where a pro-drug strategy was employed [126]. A caged derivative of FDG was developed, which is selectively degraded to the parent FDG upon exposure to acidic pH, allowing it to be taken up by adjacent cells. The acid labile pro-drug was based on the glycosylamine linkage, where cleavage is tunable based on the pK_a of the parent amine. In comparing PET imaging of a FDG-glyosylamine (FDG-amine 4; Fig. 15.6) with the parent FDG, only the tumor having an acidic microenvironment was imaged with FDG-amine 4, whereas with FDG other tissues that readily take up glucose were imaged (brain, heart, brown fat).

pHLIP probes. One type of acidic pH probe that has gained increased application in imaging the tumor microenvironment are pH (low) insertion peptides (pHLIPs) [127, 128]. Three fluorescently labeled pHLIPs (Alexa546-WT, Alexa546-Var3, Alexa546-Var7) have successfully accumulated in tumors of metastatic 4T1 mammary tumors and spontaneous breast tumors in FBV/N-Tg (MMTV-PyMT) 634Mul transgenic mice [129]. Fluorescently labeled pHLIPs have also been shown to localize and detect pancreatic ductal adenocarcinoma (PDAC), PDAC and PanIN lesions in human xenografts in mouse models [130]. Topical application of Alexa647 labeled pHLIPs in intact fresh human tissue specimens with head and neck squamous cell carcinoma have reported differences in clinically abnormal and normal tissues which concurred with pathologic evaluations [131]. pHLIPs have also been coupled to radiotracers for in vivo pH imaging with single-photon emission computed tomography (SPECT). Lewis lung carcinoma (LLC), lymph node carcinoma of the prostate (LNCaP) and prostate adenocarcinoma (PC-3) tumor xenografts in mice were studied using ^{99m}Tc-pHLIP (^{99m}Tc-AH114567) and showed adequate imageability and correlation with tumor extracellular acidity [132].

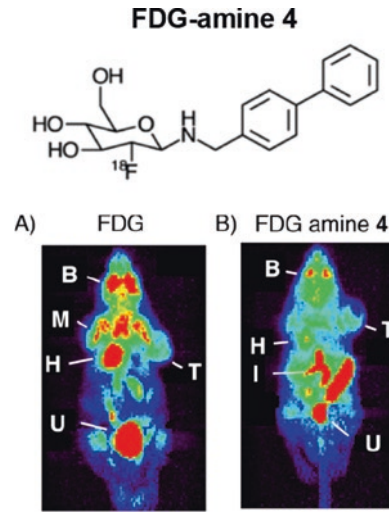

Fig. 15.6 Another approach to imaging pH with PET involves a caged derivative of FDG that selectively degrades to the parent FDG upon exposure to acidic pH. Structure of FDG-amine 4, which at low pH decomposes to FDG (*top*). (**a**) PET imaging with FDG in PC3 prostate tumor-bearing mice (*T* tumor, *B* brain, *M* muscle and brown fat, *H* heart, *U* urinary bladder). The tumor is imaged, as well as typical organs that take up FDG, such as the brain, heart and brown fat. (**b**) PET imaging with the pH sensitive FDG-amine 4 shows primarily tumor uptake, where the acidic microenvironment cleaves at the glycosylamine site, leaving FDG to be taken up by the tumor (from [126])

15.5 Imaging Enzymes in the Tumor Microenvironment

Matrix Metalloproteinases. Matrix metalloproteinases (MMPs) along with other proteases are involved in cellular proteolysis known to be elevated in disease states leading to tumor invasion and metastasis [133, 134]. MMPs are an attractive target for imaging the tumor microenvironment either with activatable probes or MMP inhibitors (MMPIs). There are several good reviews that discuss MMPs as a target for imaging [135, 136]. Another MMP biomarker, membrane type-1 matrix metalloproteinase (MT1-MMP) is a protease that activates MMP-2, which mediates cleavage of extracellular matrix components indicated in tumor progression and metastasis [137–140].

PET/SPECT Agents for imaging MMPs. MMPIs consist of natural and synthetic inhibitors that bind zinc via moieties that include hydroxamate, phosphonate, thiol, carboxylate or barbiturate [135]. A recent paper reported a ^{18}F-labeled hydroxamate-based inhibitor (ML5) for imaging of both MMPs, and disintegrin and metalloproteinase (ADAM) levels in vivo to visualize and quantify overexpression of MMPs and ADAMs [141]. ML5 that was acylated directly with N-succinimidyl-4-[^{18}F]fluorobenzoate showed nanomolar affinity for MMPs and ADAMs, and was brought for PET imaging of HT1080 tumor-bearing mice. The tumor showed modest, but potentially specific, uptake in the tumor. Co-administration of a blocking dose blocked many tissues, including tumor. A cyclic decapeptide (CLP: Cys-Leu-Pro-Gly-His-Trp-Gly-Phe-Pro-Ser-Cys) was studied for its inhibitory selectivity toward MMP-2/9 [142]. The CLP was labeled with ^{99m}Tc with high yield, stable in serum, and accumulated in the uterus, lung, liver and spleen related to MMPs of normal rats, which could contribute to future imaging of metastatic tumors that overexpress MMPs. Another interesting dual imaging probe combined an RGD motif ($\alpha_v\beta_3$ integrin binding), ^{64}Cu-DOTA, PLGVR (MMP-2 cleavage substrate), and ^{123}I-Y for imaging $\alpha_v\beta_3$ integrin positive (M21) and negative (M21L) human melanoma cell localization and pathophysiology [143]. The PET/SPECT imaging probe (c(RGDfE)K(^{64}Cu-DOTA) PLGVR^{123}I-Y) was successfully shown to target $\alpha_v\beta_3$ integrin and detect MMP-2 activity with IC_{50} value in the nanomolar range (83.4 ± 13.2 nM).

There are a few non-inhibitory type imaging probes for MMPs. Specific human MMP-9 (K_d = 20 nM) cleavable RNA aptamers were developed and imaged with ^{99m}Tc for ex vivo imaging of human brain tumors [144]. A truncated aptamer was shown to retain binding affinity and discriminate MMP-9 vs. other human MMPs. MT1-MMP imaging probes consisting of single chain antibody fragments (MT1-scFv) and a dimer (MT1-diabody) were labeled with ^{111}In and imaged in vitro and in vivo [145]. Both probes showed similar tumor accumulation (1–1.5%ID/g) and corresponding MT1-MMP positive areas in ex vivo autoradiography; however, there was extensive uptake in the kidneys (~100%ID/g for the diabody and ~200%ID/g for the scFv), and high liver uptake as well (20–40%ID/g). A study to determine the tumor specificity of ^{177}Lu/^{125}I radiolabeled MMP-2/9 activatable cell-penetrating peptides (ACPPs) was performed on BT-20 (low expression of MMP-9) and s.c. HT1080 (high expression of MMP-9) tumor-bearing mice [146]. The work showed similar uptake in both tumor-bearing mouse model and suggested that probe activation (cleavage) occurs in the vasculature instead of by tumor-specific MMP-9.

Advancement of PET and SPECT imaging probes for the tumor microenvironment are currently being developed, and progress has been made on some innovative probes (discussed above). The results from these studies have provided options and inspiration for future imaging probes and currently utilized radiotracers. Although the nuclear approach in designing imaging probes has advantages of sensitivity and depth, there has been minimal success in the discovery of an MMP-2/9 specific tracer to compete with other imaging modalities (specifically, fluorescence).

Fluorescence Imaging of MMPs. MMP ACPPs are emerging tissue-specific proteases

without nonspecific activation imaging probes for MMP overexpression in tumor microenvironments [147, 148]. Using MMP as a biomarker has been shown to be sensitive enough for optical tomography to determine tumor angiogenesis and invasiveness [149]. Skin squamous cell xenografts were studied and imaged in vivo at different angiogenic and invasive stages using an activatable fluorescence probe [149] (Fig. 15.7). In another study, a nanoprobe system that is activated by MMP-2 was detected by FRET with high efficiency and low toxicity in MMP-2 overexpressed tumors [150].

Protein nanocages have also been used to selectively image MMP-2 activity in metastatic tumors [151] (Fig. 15.8). The nanocages were conjugated to metastatic cancer cell-targeted protein (CTT: CTTHWGFTLC) and imaged in near-IR fluorescence in vitro and in vivo. Gold nanoparticles functionalized with a MMP-2 cleavable peptide (CPLGLAGG) and doxorubicin has been tested in tumor-bearing mice [152]. Tumor growth was inhibited and imaged simultaneous by fluorescence upon MMP cleavage. Imaging of MMP-2/9 was investigated using near-infrared triple-helical peptide conjugated to cypate dyes [153]. In mice bearing human fibrosarcoma xenografted tumors, fluorescence imaging at the tumor site was indicated by cleavage of cypate$_3$-THP. Anti-MT1-MMP monoclonal antibody (MT1-hlC7L) conjugated to micelles with encapsulated NIR dyes were used to image C6 glioma tumor-bearing mice [154]. The probes were specific in detecting MT1-MMP expressing tumors.

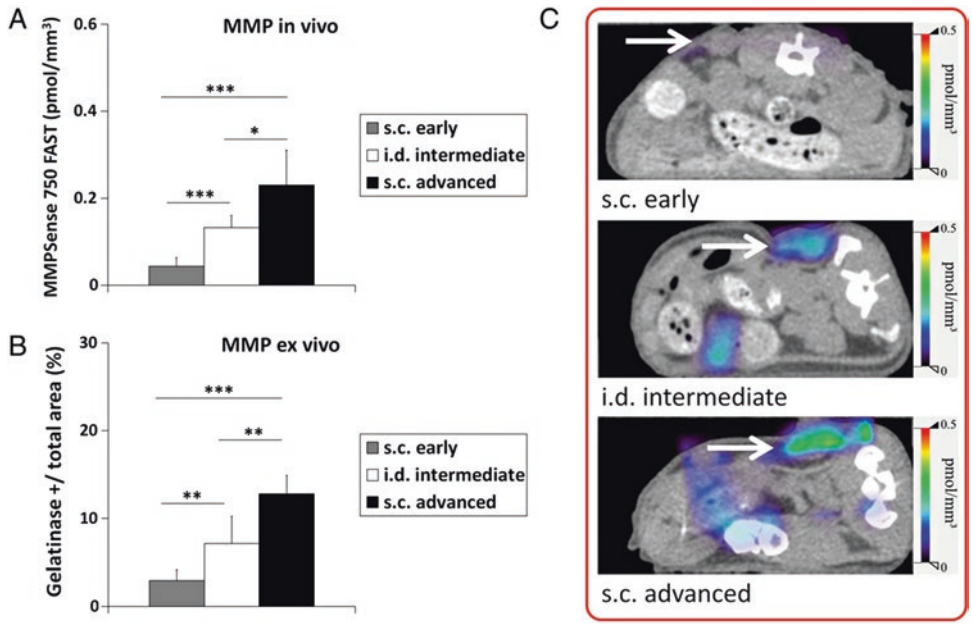

Fig. 15.7 Matrix metalloproteinases can be imaged by activatable fluorescence probes. MMP activity differs significantly between the HaCaT-A-5RT3 tumors at differential angiogenic and invasive stages. (**a**) Assessment of MMP activity in vivo by FMT-μCT imaging reveals significant differences in intratumoral concentrations of activated MMPSense 750 FAST between s.c. advanced (n = 5), i.d. intermediate (n = 5), and s.c. early (n = 7) tumors. Highest concentrations are recorded for s.c. advanced tumors at the highly angiogenic and invasive stage, whereas lowest concentrations are found in s.c. early tumors at the onset of angiogenesis and invasion. (**b**) Quantification of in situ zymography of MMP activity on tumor sections confirms the in vivo data. (**c**) Representative FMT/μCT fusion images of tumor-bearing mice (*transverse plane*) show the fluorescent signals of activated MMPSense 750 FAST in s.c. early, i.d., and s.c. advanced tumors (tumors indicated by a *white arrow*). The additional fluorescent signals found in the intestine region can occur from hepatobiliary excretion of the probe through the intestine [149]

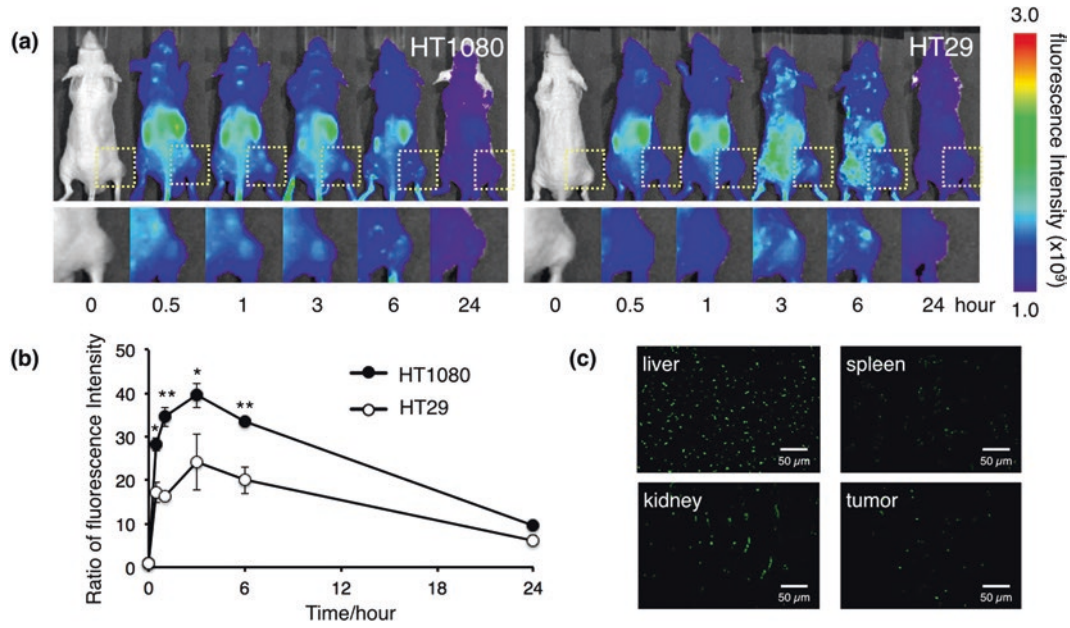

Fig. 15.8 Protein nanocages can selectively image MMP-2 activity in metastatic tumors *in vivo*. (**a**) *In vivo* real-time NIR fluorescence imaging of intravenously injected Alexa Fluor 750-labeled HspG41C-CTT in HT1080 and HT29 tumor-bearing mice. Time-dependent, tumor-targeting specificities of the nanocages were monitored by the IVIS system. Square regions indicate solid tumor growths of subcutaneously injected cancer cells; (**b**) Fluorescence signal intensity ratio of the tumor/background obtained from *in vivo* images. $*p < 0.05$; $**p < 0.01$; (**c**) Organ sections of tumor-bearing mice injected intravenously with Alexa Fluor 488-labeled HspG41C-CTT at 3 h post-injection [151]

Silicon-rhodamine-based near-IR dark quenchers (SiNQs) were shown to quench fluorescence over 780 nm region and detected MMP activity in vitro and in vivo [155]. Improvements on fluorophore pairs used as ACPPs was explored using $Ir(ppy)_3$ and Cy5 for tumor-related MMP-2/9 imaging [156]. In an in vitro evaluation, quenching of the luminophores and shortening of luminescence was reversed upon MMP cleavage. Using a MMP-activatable photoacoustic probe (Alexa750-CXeeeeXPLGLAGrrrrXK-BHQ3) FCT133 thyroid tumors implanted s. c. in nude mice were imaged [157]. Alexa750 fluorescence and photoacoustic imaging showed active forms of MMP-2 and MMP-9 in FTC133 tumor homogenates.

Due to the specificity and efficacy of the fluorescent activatable probes, only a small number of inhibitory type fluorescent probes for MMPs. A Cy5.5-labeled MMP inhibitor (Cy5.5-AF489) was compared to commercially available ACPPs (MMPSenseTM 680 and MMPSenseTM 750 FAST) in xenografted mice [158]. The MMP inhibitor Cy5.5-AF489 showed faster imaging and signal in MMP-active tumors compared to ACPPs.

MRI probes for imaging MMPs. MRI can be used to image MMP biomarkers as well. Iron oxide nanoparticles (IONPs) were designed to undergo cleavage by MMPs resulting in a nanocluster with enhanced T_2 properties [159]. In vitro studies on cells expressing MMP-2/9 and CXCR4 showed T_2 signal enhancements. ACPPs have demonstrated dual targeting for integrin αvβ3 and MMP-2 for imaging and chemotherapy (MMAE: monomethylauristatin) in vivo [160]. Mice treated with cyclic-RGD-PLGC(Me) AG-MMAE-ACPP had improved probe penetration into MDA-MB-231 tumors. Another group has imaged IONPs (ferumoxytol) with a MMP-14 ACPP azademethylcolchicine (ICT) in MMTV-PyMT breast cancer cells in vitro [161].

Theranostic nanoparticles (TNPs) showed proof of concept in tumor specificity, drug delivery and imaging. An interesting activatable ^{19}F-probe was designed to be "off" until cleavage by MMP or nitroreductase (NTR) [162]. Upon cleavage the probe aggregates disassemble turning "on" an imaging signal. Imaging of MMP secreted from tumor cells was shown in vitro, although thus far no in vivo imaging has been reported.

Imaging Cysteine Cathepsins. Cathepsin family of proteases have important roles in normal and diseased cellular physiology [163]. In certain cancers, cysteine cathepsins are upregulated and have been used as cancer biomarkers [164, 165]. The majority of imaging probes using cathepsin activation/cleavage involve near-infrared (NIR) fluorescence agents. There are a handful of PET tracer probes that target cysteine cathepsin as discussed below.

A unique PET tracer probe that inhibits cathepsins was achieved by synthesizing a fluorine-containing azadipeptide nitrile ([^{18}F]**3**) [166]. The azadipeptide nitrile was alkylated with 2-[^{18}F]fluoroethynosylate. In vitro, in vivo, and ex vivo studies were done as well as biodistrubtion and biokinetics in rats, NMRI mice and NMRI nude mice bearing NCI-H292 tumor xenografts. The tracer [^{18}F]**3** showed slow blood clearance, reversible tumor accumulation of the tracer in tumor-bearing mice, confirmed the presence of cathepsins (L, S, K and B) and visualized tumor-associated cathepsins in vivo with tumor:muscle ratios of 10–15, and tumor:blood ratios <1. Another group used a cathepsin inhibitor, acyloxymethyl ketones (AOMKs), with ^{64}Cu-Z-FK(DOTA)-AOMK (^{64}Cu-GB170) as a PET tracer for in vitro and in vivo studies [167]. Small animal PET imaging on MDA-435 and C2C12/Ras tumor-bearing mice with ^{64}Cu-GB170 confirmed in vivo tumor uptake, and selectivity for cysteine cathepsins. SPECT imaging was also employed to optimize cathepsin-targeting polymers to reduce non-target accumulation [168]. N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers were made with cathepsin S linkers and radiolabeled with ^{177}Lu-DOTA. Normal CF-1 and HPAC tumor-bearing SCID mice were imaged to study structure-activity relationship with the length of the linker group, which was found to be tissue specific in vivo. Poly-L-glutamate (PLG) as a tumor protease activated MRI probe visualized cathepsins in tumors in vivo [169]. Using the CEST effect from amine protons of glutamate moieties generated from cathepsin cleavage of PLG, cathepsins were successfully imaged in rat brain gliosarcoma model in a high resolution (9.4T) MRI.

Cathepsin-activatable fluorescence probes can be divided into peptide and nonpeptide probes, although peptide based probes have been more widely studied. For example, hydroxymethylrhodamine (HMRG)-based fluorescence probes (Z-Phe-Arg-HMRG and Z-Arg-Arg-HMRG) are colorless until they are hydrolyzed by cathepsins resulting in strong fluorescence signal [170]. The probes were visualized in human ovarian cancer cells lines (SHIN-3, SKOV-3, and OVCAR-3) in mouse models in vivo. Another group made an activatable peptide probe on glycol chitosan nanoparticles (280 nm in diam.) specific for cathepsin B cleavage [171]. They were able to discriminate in vivo among three metastatic mouse models (4T1-luc2 liver metastases, RFP-B16F10 lung metastases, HT1080 peritoneal metastases). Other peptide probes based on FRET have also shown selectivity upon cleavage with cathepsin B [172, 173]. Cathepsin-activatable fluorescent probe sensitivity has also been studied comparing tumor and normal muscle in mice models for soft tissue sarcoma before and after radiation therapy [174], and in human cathepsin E positive cells (MPanc96-E) implanted in nude mice [175]. Both studies found significant sensitivity in vivo and in vitro. Certain modifications to probes, such as conjugation of palmitoic acid (lipidation), have resulted in favorable properties to increase the retention of a cathepsin S-specific agent in tumor cells in vivo and in vitro [176]. An interesting non-peptidic, cathepsin, S-directed quenched activity based probe (BMV083) was made and imaged in vivo in a breast cancer model [177]. The agent showed high tumor-specific fluorescence and targeting to M2 phenotypic macrophages.

15.6 Imaging Integrins in the Tumor Microenvironment

Integrins have become effective targets for imaging the tumor microenvironment since the discovery of their role in cell adhesion. There are 24 different types of integrins composed of α and β heterodimeric subunits. Integrin receptors can be classified into four categories based on their ligand binding ability; RGD receptors, collagen receptors, laminin receptors, and leukocyte-specific receptors [178, 179]. Certain integrins such as $\alpha_v\beta_6$ are upregulated in tumors yet are almost undetectable in normal tissue. An overwhelming majority of integrins used in targeting the tumor microenvironment focus on the RGD receptors [180], but other integrin receptors are also being investigated. Using integrins to image the tumor microenvironment has been successful in PET, SPECT, fluorescence and MR imaging. A brief compilation of integrin probes related to imaging the tumor microenvironment are discussed.

PET tracers for imaging integrins. PET tracers coupled to integrin ligands have provided a feasible means of imaging integrins that are upregulated in angiogenesis. Progress has been made using ^{18}F, ^{64}Cu and ^{68}Ga radionuclides. For example, a study evaluating the formulation of an integrin $\alpha_v\beta_3$ imaging probes in U87MG tumor-bearing mice found that ^{18}F-AlF-NOTA-E[PEG$_4$-cRGDfk)]$_2$ had highest tumor uptake and lowest liver accumulation compared to ^{18}F-AlF-NOTA-E[c(RGDfK)]$_2$ and ^{18}F-AlF-NOTA-PEG$_4$-E[c(RGDfK)]$_2$ [181]. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were targeted using [^{18}F]fluciclatide, an RGD peptide developed by industry, in 18 patients (melanoma and renal cell carcinoma (RCC)) to compare PET imaging of angiogenesis with integrin expression in tumors [182] (Fig. 15.9). All tumors showed significant [^{18}F]fluciclatide uptake as well as correlation with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins expression in melanoma and RCC tumor tissue (Fig. 15.9). An $\alpha_5\beta_1$ integrin receptor was targeted with [^{18}F]FProp-Cys(*)-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys(*)-OH in human melanoma M21 ($\alpha_v\beta_3$-positive and $\alpha_5\beta_1$-negative), human melanoma M21-L ($\alpha_v\beta_3$-negative and $\alpha_5\beta_1$-negative), and human prostate carcinoma DU145 ($\alpha_v\beta_3$-negative and $\alpha_5\beta_1$-positive) cells to study receptor-specific binding [183]. Blocking studies with mice bearing $\alpha_5\beta_1$-negative M21 tumors gave conflicting results compared to the in vitro studies, which showed high affinity for $\alpha_5\beta_1$ integrin, warranting further investigations. Integrin $\alpha_v\beta_3$ receptor was also targeted using ^{18}F-E[c(RGDfK)$_2$] in healthy KM mice and U87MG tumor-bearing mice to study the biodistribution of the PET tracer [184]. It was found to target tumors with high uptake (5.2 ± 0.56%ID/g) 1 h post injection. Other peptide ligands like cysteine knot peptides, R_o1 and S_o2, which have 3–6 nM affinity for integrin $\alpha_v\beta_6$, have been radiolabeled with ^{18}F-fluorobenzoate for PET imaging of BxPC3 pancreatic adenocarcinoma xenografts on mice [185]. The radiolabeled peptides, ^{18}F-fluorobenzoate-R_o1 and ^{18}F-benzoate-S_o2, showed 2.3 ± 0.6 and 1.3 ± 0.4%ID/g, respectively in BxPC3 xenografted tumors at 0.5 h post injection. Another well-known $\alpha_v\beta_3$ integrin PET probe is [^{18}F]Galacto-RGD, which has been shown to image high-grade human carotid plaques in patients correlating well with ex vivo autoradiography of surgical specimens [186]. An $\alpha_6\beta_1$ integrin PET probe was developed using a peptide-porphyrin azide-alkyne conjugation [187]. The probe was radiolabeled with ^{68}Ga and found have higher activity in HeLa cells with higher $\alpha_6\beta_1$ integrin expression compared to the U87 cells which display minimal integrin expression.

Several synthetic strategies have emerged to incorporate radionuclides more efficiently to integrin ligands for imaging upregulated integrins in the tumor microenvironment. One methods involves coordinating aluminum [^{18}F]fluoride into NOTA chelators [188, 189]. An $\alpha_v\beta_6$ integrin targeted peptide (A20FMDV2) was evaluated as a radiotracer (Al[^{18}F]-NOTA-PEG$_{28}$-A20FMDV2) in vitro and in vivo in $\alpha_v\beta_6$(+) and $\alpha_v\beta_6$(−) cells and xenograft mice, respectively [190]. The Al[^{18}F]-NOTA radiolabeling was found to be efficient, and the tracer showed $\alpha_v\beta_6$ selectivity in vitro and in vivo, although kidney uptake was significant even after 4 h post

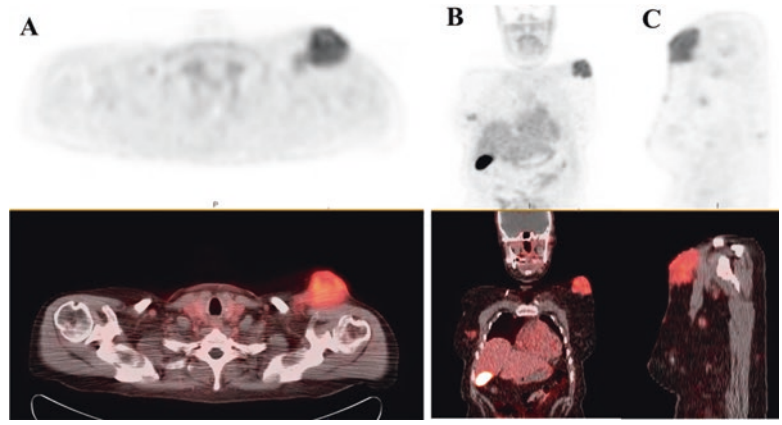

Fig. 15.9 Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were imaged with [^{18}F] fluciclatide, an RGD peptide, in 18 patients (melanoma and renal cell carcinoma (RCC)) to compare PET imaging of angiogenesis with tumor integrin expression. [^{18}F] Fluciclatide PET/CT axial (**a**) sagittal (**b**) and coronal (**c**) images in patient four (malignant melanoma) show focal radiotracer uptake within a left supraclavicular mass, with SUV80% max 6.5, as well as in other soft tissue nodules [182]

injection. Another integrin probe, 2-[^{18}F]fluoroethyl triazolyl conjugated c(RGDyK) peptide, was designed using Cu(I)-catalyzed conjugation showing good stability in vivo (U87MG tumors) and tumor-to-background ratio of 1.6 ± 0.3%ID/g 1.5 h post injection [191].

Copper-64 is an attractive radioisotope for PET integrin probes to image the tumor microenvironment. Integrin $\alpha_v\beta_6$ has been targeted by several groups with various ^{64}Cu PET probes as a biomarker for several cancers such as non-small cell lung cancer (NSCLC). One groups developed a divalent probe, ^{64}CuAcD10, with low kidney accumulation and good tumor uptake in mice with either H2009 or H460 xenografts (Ajay N [192].). Another study evaluated chelators (CB-TE1A1P, DOTA, NOTA and BaBaSar) for ^{64}Cu by radiolabeling an $\alpha_v\beta_6$ integrin targeting peptide, A20FMDV2, respectively [193]. The findings suggest that the best chelator depended on the stability, selectivity or pharmacokinetics desired out of the PET probe (Fig. 15.10). Other ^{64}Cu chelates have been used to image known upregulated integrins in the tumor microenvironment such as $\alpha_4\beta_1$ and $\alpha_v\beta_3$. ^{64}Cu-(CB-TE2A)-LLP2A was investigated for imaging bone marrow derived cells involved in bone metastasis in nude mice injected with MDA-MB-231/firefly luciferase human breast tumor cells (VL4–4-negative) [194]. Higher uptake in bone corresponded with mice that eventually had bone metastases, and this was confirmed by flow cytometry for the presence of hematopoietic progenitor cells. Dually radiolabeled peptides for imaging and targeting $\alpha_v\beta_3$ integrin have been developed containing an RGD sequence for binding $\alpha_v\beta_3$ integrin, DOTA for radiolabeling with ^{64}Cu, PLGVRY for MMP2 cleavage, and terminal tyrosine labeled with ^{125}I (c(RGDfE)K(DOTA)PLGVRY) [143]. The dually radiolabeled probe showed high affinity of $\alpha_v\beta_3$ integrin in substrate competition and cell binding assays.

SPECT agents for imaging integrins. Several SPECT probes have been explored to target integrins for imaging the tumor microenvironment using ^{99m}Tc, ^{125}I, and ^{111}In. An $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin SPECT imaging probe was developed to evaluate its dosimetry in seven healthy and three breast cancer patients and in mice with MCF7 tumors [195]. The RGD peptide was coupled to ethylenediamine-N,N′-diacetic (EDDA) which favors renal excretion with ^{99m}Tc-labeled hydrazinonicotinamide (HYNIC). The probe, ^{99m}Tc-EDDA/HYNIC-E-[c(RGDfK)]$_2$ showed high tumor uptake in patients with malignant lesions and rapid bowel clearance. A ^{99m}Tc-labeled cRGD was used to monitor hepatic stellate cells (HSC) which express $\alpha_v\beta_3$ integrins in fibrotic rat

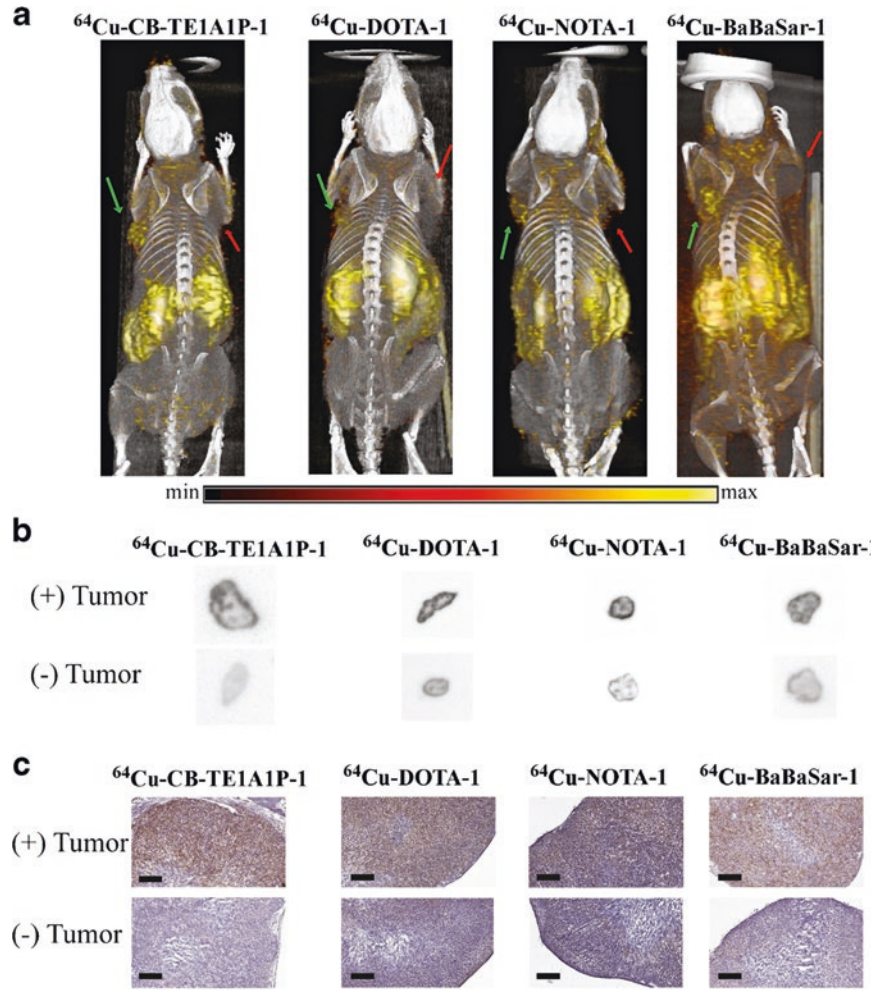

Fig. 15.10 PET imaging of $\alpha_v\beta_6$ integrin with a ^{64}Cu-labeled peptide (A20FMDV2) conjugated with CB-TE1A1P, DOTA, NOTA and BaBaSar chelators was performed to determine the best chelator for optimal tumor uptake and normal tissue biodistribution. (**a**) Reconstructed 3D PET/CT images showing (+) (*green arrow*) and (−) (*red arrow*) tumors. Mice were anesthetized using 2–3 % isoflurane and received 150–250 μCi of formulated radiotracer via tail vein. All images were acquired 4 h p.i. using 20 min static scans. (**b**) Autoradiography slices (20 μm) of (+) and (−) tumors, sectioned at 4 h p.i. and exposed overnight. Each slice read at a 50-μm resolution. (**c**) Histology slices (5 μm) from (+) and (−) tumors after immunohistochemistry staining for αvβ6 viewed at ×4 magnification. Scale bar = 400 μm [193]

livers [196]. The study found the tracer to distinguish the different stages of liver fibrosis in rats. Other SPECT probes for imaging the tumor microenvironment also target $\alpha_v\beta_6$ integrins. One such probe (^{99m}Tc-HHK) was studied in BxPC-3 (integrin $\alpha_v\beta_6$-positive) and HEK293 (integrin $\alpha_v\beta_6$-negative) in vitro and in vivo [197]. The study found ^{99m}Tc-HHK showed high specificity for integrin $\alpha_v\beta_6$, with highest uptake at 0.5 h post injection. An $\alpha_v\beta_6$ integrin targeting cysteine knot peptide, S_o2, coupled to a single amino acid chelate (SAAC) to give ^{99m}Tc-SAAC-S_o2 and evaluated in vitro and in vivo studies [198]. In vitro studies compared HCC4009 and BxPC-3 cell lines (integrin $\alpha_v\beta_6$-positive) and H838 and 293 T cell lines (integrin $\alpha_v\beta_6$-negative) to find significant uptake in integrin $\alpha_v\beta_6$-positive cells. In vivo studies in nude mice bearing HCC4009 and H838

tumor xenografts showed uptake in antigen positive tumors and high tumor-to-background ratios (6.81 ± 2.32%ID/g) at 6 h post injection.

Probes for $\alpha_v\beta_6$ integrin targeting were designed to image pancreatic ductal adenocarcinoma (PDAC) were examined in a biodistribution assay, in vivo blocking study and SPECT imaging of tumor-bearing mice [199]. The study found one of four probes (^{125}I-HFMDV2) showed the highest affinity for $\alpha_v\beta_6$ integrin in AsPC-1 cells and 3–5 times greater uptake in AsPC-1 xenografts compared to MIA PaCA-cells and xenografts. Another study developed an indium-111-labeled tetra[DTPA]-A20FMDV2 to investigate the development of fibrosis in a murine model (bleomycin-induced lung injury) by monitoring lung hydroxyproline, $\alpha_v\beta_6$ integrin, and *itgb6* messenger RNA [200]. The SPECT probe enabled quantifiable detection in lungs 1 h post injection compared to several controls.

Fluorescent probes for imaging integrins. Optical imaging with various near-infrared (NIR) fluorescence probes has been successful in imaging the tumor microenvironment. One study targeted $\alpha_v\beta_6$ integrin binding peptide (HK) with a NIR phthalocyanine dye (Dye-SA-B-HK) to image subcutaneous and orthotopic BxPC-3 cancer xenografts in mice for optical image-guided surgery and phototherapy [201] (Fig. 15.11) and found impressive antitumor effects both in vitro and in vivo. Other targeting agents have included activatable cell-penetrating peptides (ACPP) in combination with integrin targeting coupled to Cy5 dye to achieve improved specificity and sensitivity in imaging MDA-MB-231 tumor-bearing mice [160]. This study also showed the dual targeting probe combined with a chemotherapeutic (monomethylauristatin, MMAE), finding improved efficacy in MDA-MB-231 orthotopic human and syngeneic Py230 murine breast tumors. Gold nanostars (Au NS) were explored as a platform for targeting integrin with cyclic RGD and fluorescent probe (MPA) or anti-cancer drug (DOX) [152]. The study demonstrated the photo-thermal therapy and chemotherapy in MDA-MB-231 cell lines and in S180 tumor-bearing mice. Imaging intracranial medulloblastoma has also been investigated using $\alpha_5\beta_1$ integrin-binding cysteine knot peptide (EETI 2.5F and EETI 2.5F-Fc) conjugated to a fluorescent dye (AF680) in tumor-bearing mice [202]. The optical imaging probes were found to be to cross the blood-brain-barrier to the tumor showing impressive brain tumor imaging compared to other cysteine knot peptides conjugated to c(RGDfK). A fluorescent imaging probe (tetraphenylsilole, TPS) coupled to two integrin-targeting peptide (cRGD) uses aggregation induced emission (AIE) to image the tumor microenvironment in vitro [203]. Quantitative detection was achieved using the imaging probe (TPS-2cRGD) in MCF-7 and HT29 cancer cells, targeting $\alpha_v\beta_3$-integrin. Another study tested a small peptidomimetic $\alpha_v\beta_3$ integrin antagonist coupled to NIR dye (IntegriSense) for binding specificity in vitro and in vivo [204]. The integrin NIR fluorescent agent exhibited selectivity toward $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin in vitro and provided real-time quantification in tumors in vivo. In addition to fluorescence imaging, Cerenkov luminescence imaging (CLI) has also been shown in male athymic mice with DX3puroβ6 ($\alpha_v\beta_6$-positive) and DX3puro ($\alpha_v\beta_6$-negative) tumors [205]. CLI agents, ^{90}Y-DOTA-PEG$_{28}$-A20FMDV2 and ^{90}Y-DOTA-Ahx-A20FMDV2 were investigated showing good correlation between CLI quantification and biodistribution, but also having low uptake in $\alpha_v\beta_6$-positive tumors.

MR agents for imaging integrins. MR imaging has more recently been employed to image integrins in the tumor microenvironment. Superparamagnetic iron oxide (SPIO)-loaded cRGD PEGylated polyion complex vesicles (PICsomes) targeting $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have recently been investigated to image the neovasculature in an orthotopic glioblastoma model [206]. The 40% PEG on distal end of the PICsomes to cRGD moieties was found to accumulate in tumor neovasculature after 24 h, and were able to identify tumor lesions using T_2-weighted MRI. Other forms of nanoparticles like magnetoliposomes have been studied to also target integrins [207]. The study evaluated tumor angiogenesis targeting of anti- $\alpha_v\beta_3$ antibody guided 3-step pretargeting approach using modi-

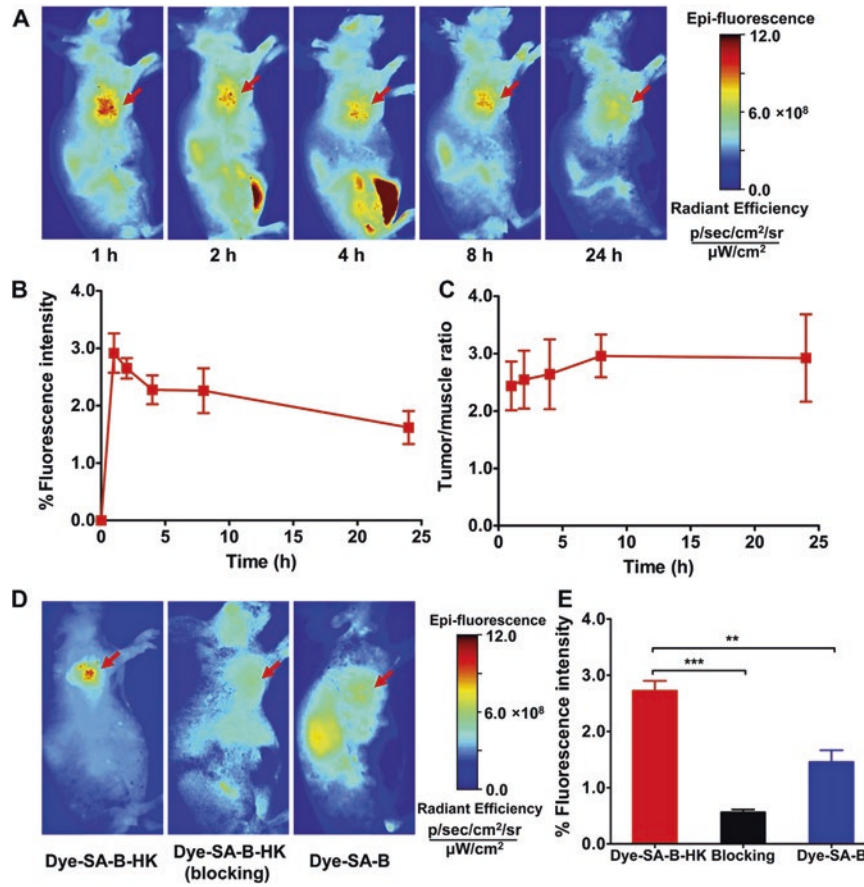

Fig. 15.11 Optical imaging in combination with photodynamic therapy has been performed in a mouse model of pancreatic cancer with near-infrared phthalocyanine dye (Dye-SA-B) conjugated to an $\alpha_v\beta_6$ integrin targeted peptide (HK). (**a**) Representative near-infrared fluorescence sagittal images of Dye-SA-B-HK in BxPC-3 tumor-bearing nude mice at 1, 2, 4, 8, and 24 h postinjection. (BeC) Quantified in vivo tumor uptake (**b**) and tumor-to-muscle ratios (**c**) of Dye-SA-B-HK in BxPC-3 tumor-bearing nude mice. Results are expressed as mean ± SD (n ¼ 5). (DeE) In vivo nearinfrared fluorescence sagittal imaging (**d**) and quantified tumor uptake (E) of BxPC-3 tumor-bearing nude mice at 2 h after intravenous injection of Dye-SA-B-HK (with or without the blocking of an excess dose of the HK peptide) or Dye-SA-B. Results are expressed as mean ± SD (n 1/4 5 per group). **P < 0.01, ***P < 0.001. *Arrows* indicate the tumors in all cases [201]

fied SPIO in liposomes. The magnetoliposomes showed greater signal enhancement in tumor area (7.0%) by T_2-weighted MR images compared to the control (2.0%).

15.7 Conclusions

There are many aspects of the tumor microenvironment and having a way to non-invasively monitor changes of both cell types and physiological parameters of the extracellular milieu is of great importance, both in humans and in small animal models of cancer. The literature reviewed highlights numerous pathways and strategies for imaging not just the growth of tumors, but the changes in the microenvironment over time and after treatment. Some of these are already being investigated in humans. Additionally, there is a wealth of research on investigating novel approaches in small animal models of cancer, both for gathering mechanistic information non-invasively over time, as well as for translation of imaging strategies to humans.

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
2. Huang T, Civelek AC, Li J, Jiang H, Ng CK, Postel GC, Shen B, Li XF. Tumor microenvironment-dependent 18F-FDG, 18F-fluorothymidine, and 18F-misonidazole uptake: a pilot study in mouse models of human non-small cell lung cancer. J Nucl Med. 2012;53(8):1262.
3. Yanar M, Abel F, Haalck T, Klutmann S, Schumacher U. The microenvironment in the human lung partly determines the site of the first metastasis. Anticancer Res. 2014;34:3845–9.
4. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nat Rev Cancer. 2013;13:759–71.
5. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med. 1986;315:1650–9.
6. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene. 2010;29:1093–102.
7. Rashidian M, Keliher EJ, Bilate AM, Duarte JN, Wojtkiewicz GR, Jacobsen JT, Cragnolini J, Swee LK, Victora GD, Weissleder R, Ploegh HL. Noninvasive imaging of immune responses. Proc Natl Acad Sci U S A. 2015;112:6146–51.
8. Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res. 2007;13:1472–9.
9. Denardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev. 2010;29:309–16.
10. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2010;140:883–99.
11. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell. 2010;141:39–51.
12. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest. 2007;117:1155–66.
13. Wang Y-C, He F, Feng F, Liu X-W, Dong G-Y, Qin H-Y, Hu X-B, Zheng M-H, Liang L, Feng L, Liang Y-M, Han H. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. Cancer Res. 2010;70:4840–9.
14. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A. Tumor associated macrophages and neutrophils in tumor progression. J Cell Physiol. 2013;228:1404–12.
15. Chen J, Yao Y, Gong C, Yu F, Su S, Chen J, Liu B, Deng H, Wang F, Lin L. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. Cancer Cell. 2011;19:541–55.
16. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 2012;122:787–95.
17. Pérez-Medina C, Tang J, Abdel-Atti D, Hogstad B, Merad M, Fisher EA, Fayad ZA, Lewis JS, Mulder WJM, Reiner T. PET imaging of tumor-associated macrophages with 89Zr-Labeled high-density lipoprotein nanoparticles. J Nucl Med. 2015;56:1272–7.
18. Locke LW, Mayo MW, Yoo AD, Williams MB, Berr SS. PET imaging of tumor associated macrophages using mannose coated 64Cu liposomes. Biomaterials. 2012;33:7785–93.
19. Movahedi K, Schoonooghe S, Laoui D, Houbracken I, Waelput W, Breckpot K, Bouwens L, Lahoutte T, de Baetselier P, Raes G. Nanobody-based targeting of the macrophage mannose receptor for effective in vivo imaging of tumor-associated macrophages. Cancer Res. 2012;72:4165–77.
20. Tavare R, McCracken MN, Zettlitz KA, Knowles SM, Salazar FB, Olafsen T, Witte ON, Wu AM. Engineered antibody fragments for immuno-PET imaging of endogenous CD8+ T cells in vivo. Proc Natl Acad Sci U S A. 2014;111:1108–13.
21. Harris AL. Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer. 2002;2:38–47.
22. Murdoch C, Muthana M, Lewis CE. Hypoxia regulates macrophage functions in inflammation. J Immunol. 2005;175:6257–63.
23. Semenza GL. Angiogenesis ischemic and neoplastic disorders. Annu Rev Med. 2003;54:17–28.
24. Lee C-T, Boss M-K, Dewhirst MW. Imaging tumor hypoxia to advance radiation oncology. Antioxid Redox Signal. 2014;21:313–37.
25. Vaupel P, Mayer A. Hypoxia in tumors: pathogenesis-related classification, characterization of hypoxia subtypes, and associated biological and clinical implications. Adv Exp Med Biol. 2014;812:19–24.
26. Hsieh CH, Chang HT, Shen WC, Shyu WC, Liu RS. Imaging the impact of Nox4 in cycling hypoxia-mediated U87 glioblastoma invasion and infiltration. Mol Imaging Biol. 2012;14:489–99.
27. Avni R, Cohen B, Neeman M. Hypoxic stress and cancer: imaging the axis of evil in tumor metastasis. NMR Biomed. 2011;24:569–81.
28. Horsman MR, Mortensen LS, Petersen JB, Busk M, Overgaard J. Imaging hypoxia to improve radiotherapy outcome. Nat Rev Clin Oncol. 2012;9:674–87.

29. Mendichovszky I, Jackson A. Imaging hypoxia in gliomas. Br J Radiol. 2014;84(2):S145–58.
30. Sun X, Niu G, Chan N, Shen B, Chen X. Tumor hypoxia imaging. Mol Imaging Biol. 2011;13:399–410.
31. Wang L, Li C. Fluorescence nanoprobe imaging tumor by sensing the acidic microenvironment. Boca Raton: Science Publishers; 2012.
32. Carlin S, Humm JL. PET of hypoxia: current and future perspectives. J Nucl Med. 2012;53:1171–4.
33. Carlin S, Zhang H, Reese M, Ramos NN, Chen Q, Ricketts S-A. A comparison of the imaging characteristics and microregional distribution of 4 hypoxia PET tracers. J Nucl Med. 2014;55:515–21.
34. Hoigebazar L, Jeong JM. Hypoxia imaging agents labeled with positron emitters. Recent Results Cancer Res. 2013;194:285–99.
35. Kelada OJ, Carlson DJ. Molecular imaging of tumor hypoxia with positron emission tomography. Radiat Res. 2014;181:335–49.
36. Kersemans V, Cornelissen B, Hueting R, Tredwell M, Hussien K, Allen PD, Falzone N, Hill SA, Dilworth JR, Gouverneur V. Hypoxia imaging using PET and SPECT: the effects of anesthetic and carrier gas on [64 Cu]-ATSM,[99m Tc]-HL91 and [18 F]-FMISO tumor hypoxia accumulation. PLoS One. 2011;6:e25911.
37. Kurihara H, Honda N, Kono Y, Arai Y. Radiolabelled agents for PET imaging of tumor hypoxia. Curr Med Chem. 2012;19:3282–9.
38. Yip C, Blower PJ, Goh V, Landau DB, Cook GJ. Molecular imaging of hypoxia in non-small-cell lung cancer. Eur J Nucl Med Mol Imaging. 2015;42:956–76.
39. Bell C, Dowson N, Fay M, Thomas P, Puttick S, Gal Y, Rose S. Hypoxia imaging in gliomas with 18F-fluoromisonidazole PET: toward clinical translation. Semin Nucl Med. 2015;45:136–50.
40. Rajendran JG, Krohn KA. F-18 fluoromisonidazole for imaging tumor hypoxia: imaging the microenvironment for personalized cancer therapy. Semin Nucl Med. 2015;45:151–62.
41. Kobayashi K, Hirata K, Yamaguchi S, Kobayashi H, Terasaka S, Manabe O, Shiga T, Magota K, Kuge Y, Tamaki N. FMISO PET at 4 hours showed a better lesion-to-background ratio uptake than 2 hours in brain tumors. J Nucl Med. 2015;56:373.
42. Kawai N, Lin W, Cao W-D, Ogawa D, Miyake K, Haba R, Maeda Y, Yamamoto Y, Nishiyama Y, Tamiya T. Correlation between 18F-fluoromisonidazole PET and expression of HIF-1α and VEGF in newly diagnosed and recurrent malignant gliomas. Eur J Nucl Med Mol Imaging. 2014;41:1870–8.
43. Huang T, Civelek AC, Zheng H, Ng CK, Duan X, Li J, Postel GC, Shen B, Li X-F. 18F-misonidazole PET imaging of hypoxia in micrometastases and macroscopic xenografts of human non-small cell lung cancer: a correlation with autoradiography and histological findings. Am J Nucl Med Mol Imaging. 2013;3:142.
44. Trinkaus ME, Blum R, Rischin D, Callahan J, Bressel M, Segard T, Roselt P, Eu P, Binns D, Macmanus MP. Imaging of hypoxia with 18F-FAZA PET in patients with locally advanced non-small cell lung cancer treated with definitive chemoradiotherapy. J Med Imaging Radiat Oncol. 2013;57:475–81.
45. Zegers CM, van Elmpt W, Wierts R, Reymen B, Sharifi H, Öllers MC, Hoebers F, Troost EG, Wanders R, van Baardwijk A. Hypoxia imaging with [18 F] HX4 PET in NSCLC patients: defining optimal imaging parameters. Radiother Oncol. 2013;109:58–64.
46. Busk M, Mortensen LS, Nordsmark M, Overgaard J, Jakobsen S, Hansen K, Theil J, Kallehauge J, D'Andrea F, Steiniche T. PET hypoxia imaging with FAZA: reproducibility at baseline and during fractionated radiotherapy in tumour-bearing mice. Eur J Nucl Med Mol Imaging. 2013;40:186–97.
47. Peeters SG, Zegers CM, Lieuwes NG, van Elmpt W, Eriksson J, van Dongen GA, Dubois L, Lambin P. A comparative study of the hypoxia PET tracers [18 F] HX4,[18 F] FAZA, and [18 F] FMISO in a preclinical tumor model. Int J Radiat Oncol Biol Phys. 2015;91:351–9.
48. Segard T, Robins PD, Yusoff IF, Ee H, Morandeau L, Campbell EM, Francis RJ. Detection of hypoxia with 18F-fluoromisonidazole (18F-FMISO) PET/CT in suspected or proven pancreatic cancer. Clin Nucl Med. 2013;38:1–6.
49. Chen L, Zhang Z, Kolb HC, Walsh JC, Zhang J, Guan Y. 18F-HX4 hypoxia imaging with PET/CT in head and neck cancer: a comparison with 18F-FMISO. Nucl Med Commun. 2012;33:1096–102.
50. Hu M, Xing L, Mu D, Yang W, Yang G, Kong L, Yu J. Hypoxia imaging with 18F-Fluoroerythronitroimidazole integrated PET/CT and immunohistochemical studies in non–small cell lung cancer. Clin Nucl Med. 2013;38:591–6.
51. Yue J, Yang Y, Cabrera A, Sun X, Zhao S, Xie P, Zheng J, Ma L, Fu Z, Yu J. Measuring tumor hypoxia with 18F-FETNIM PET in esophageal squamous cell carcinoma: a pilot clinical study. Dis Esophagus. 2012;25:54–61.
52. Laurens E, Yeoh SD, Rigopoulos A, Cao D, Cartwright GA, O'Keefe GJ, Tochon-Danguy HJ, White JM, Scott AM, Ackermann U. Radiolabelling and evaluation of novel haloethylsulfoxides as PET imaging agents for tumor hypoxia. Nucl Med Biol. 2012;39:871–82.
53. Servagi-Vernat S, Differding S, Hanin F-X, Labar D, Bol A, Lee JA, Grégoire V. A prospective clinical study of 18 F-FAZA PET-CT hypoxia imaging in head and neck squamous cell carcinoma before and during radiation therapy. Eur J Nucl Med Mol Imaging. 2014;41:1544–52.

54. Murakami M, Zhao S, Zhao Y, Chowdhury NF, Yu W, Nishijima K-I, Takiguchi M, Tamaki N, Kuge Y. Evaluation of changes in the tumor microenvironment after sorafenib therapy by sequential histology and 18F-fluoromisonidazole hypoxia imaging in renal cell carcinoma. Int J Oncol. 2012;41:1593–600.
55. Tachibana I, Nishimura Y, Shibata T, Kanamori S, Nakamatsu K, Koike R, Nishikawa T, Ishikawa K, Tamura M, Hosono M. A prospective clinical trial of tumor hypoxia imaging with 18F-fluoromisonidazole positron emission tomography and computed tomography (F-MISO PET/CT) before and during radiation therapy. J Radiat Res. 2013;54:1078–84.
56. Zips D, Zöphel K, Abolmaali N, Perrin R, Abramyuk A, Haase R, Appold S, Steinbach J, Kotzerke J, Baumann M. Exploratory prospective trial of hypoxia-specific PET imaging during radiochemotherapy in patients with locally advanced head-and-neck cancer. Radiother Oncol. 2012;105:21–8.
57. Sakr T, Essa B, El-Essawy F, El-Mohty A. Synthesis and biodistribution of 99m Tc-PyDA as a potential marker for tumor hypoxia imaging. Radiochemistry. 2014;56:76–80.
58. Giglio J, Fernández S, Pietzsch H-J, Dematteis S, Moreno M, Pacheco JP, Cerecetto H, Rey A. Synthesis, in vitro and in vivo characterization of novel 99m Tc-‘4+ 1’-labeled 5-nitroimidazole derivatives as potential agents for imaging hypoxia. Nucl Med Biol. 2012;39:679–86.
59. Sakr T, Motaleb M, Ibrahim I. 99mTc–meropenem as a potential SPECT imaging probe for tumor hypoxia. J Radioanal Nucl Chem. 2012;292:705–10.
60. Kimura S, Umeda IO, Moriyama N, Fujii H. Synthesis and evaluation of a novel 99m Tc-labeled bioreductive probe for tumor hypoxia imaging. Bioorg Med Chem Lett. 2011;21:7359–62.
61. Tateishi K, Tateishi U, Sato M, Yamanaka S, Kanno H, Murata H, Inoue T, Kawahara N. Application of 62Cu-diacetyl-bis (N4-methylthiosemicarbazone) PET imaging to predict highly malignant tumor grades and hypoxia-inducible factor-1α expression in patients with glioma. Am J Neuroradiol. 2013;34:92–9.
62. Hansen AE, Kristensen AT, Jørgensen JT, McEvoy FJ, Busk M, Van der Kogel AJ, Bussink J, Engelholm SA, Kjær A. 64Cu-ATSM and 18FDG PET uptake and 64Cu-ATSM autoradiography in spontaneous canine tumors: comparison with pimonidazole hypoxia immunohistochemistry. Radiat Oncol. 2012;7:89.
63. Hansen AE, Kristensen AT, Law I, McEvoy FJ, Kjær A, Engelholm SA. Multimodality functional imaging of spontaneous canine tumors using 64 Cu-ATSM and 18 FDG PET/CT and dynamic contrast enhanced perfusion CT. Radiother Oncol. 2012;102:424–8.
64. Bourgeois M, Rajerison H, Guerard F, Mougin-Degraef M, Barbet J, Michel N, Cherel M, Faivre-Chauvet A. Contribution of [64Cu]-ATSM PET in molecular imaging of tumour hypoxia compared to classical [18F]-MISO—a selected review. Nucl Med Rev Cent East Eur. 2011;14:90–5.
65. Bonnitcha PD, Bayly SR, Theobald MB, Betts HM, Lewis JS, Dilworth JR. Nitroimidazole conjugates of bis (thiosemicarbazonato) 64 Cu (II)–potential combination agents for the PET imaging of hypoxia. J Inorg Biochem. 2010;104:126–35.
66. Grigsby PW, Malyapa RS, Higashikubo R, Schwarz JK, Welch MJ, Huettner PC, Dehdashti F. Comparison of molecular markers of hypoxia and imaging with (60)Cu-ATSM in cancer of the uterine cervix. Mol Imaging Biol. 2007;9:278–83.
67. Wu Y, Hao G, Ramezani S, Saha D, Zhao D, Sun X, Sherry AD. [68 Ga]-HP-DO3A-nitroimidazole: a promising agent for PET detection of tumor hypoxia. Contrast Media Mol Imaging. 2015;10(6):465–72.
68. Sano K, Okada M, Hisada H, Shimokawa K, Saji H, Maeda M, Mukai T. In vivo evaluation of a radiogallium-labeled bifunctional radiopharmaceutical, Ga-DOTA-MN2, for hypoxic tumor imaging. Biol Pharm Bull. 2013;36:602–8.
69. Hoigebazar L, Jeong JM, Hong MK, Kim YJ, Lee JY, Shetty D, Lee Y-S, Lee DS, Chung J-K, Lee MC. Synthesis of 68 Ga-labeled DOTA-nitroimidazole derivatives and their feasibilities as hypoxia imaging PET tracers. Bioorg Med Chem. 2011;19:2176–81.
70. Gulaka PK, Rojas-Quijano F, Kovacs Z, Mason RP, Sherry AD, Kodibagkar VD. GdDO3NI, a nitroimidazole-based T 1 MRI contrast agent for imaging tumor hypoxia in vivo. J Biol Inorg Chem. 2014;19:271–9.
71. Pacheco-Torres J, López-Larrubia P, Ballesteros P, Cerdán S. Imaging tumor hypoxia by magnetic resonance methods. NMR Biomed. 2011;24:1–16.
72. Egeland TA, Gulliksrud K, Gaustad JV, Mathiesen B, Rofstad EK. Dynamic contrast-enhanced-MRI of tumor hypoxia. Magn Reson Med. 2012;67:519–30.
73. Gulliksrud K, Øvrebø KM, Mathiesen B, Rofstad EK. Differentiation between hypoxic and non-hypoxic experimental tumors by dynamic contrast-enhanced magnetic resonance imaging. Radiother Oncol. 2011;98:360–4.
74. Stoyanova R, Huang K, Sandler K, Cho H, Carlin S, Zanzonico PB, Koutcher JA, Ackerstaff E. Mapping tumor hypoxia in vivo using pattern recognition of dynamic contrast-enhanced MRI data. Transl Oncol. 2012;5:437–IN2.
75. Wu Y, Zhang W, Li J, Zhang Y. Optical imaging of tumor microenvironment. Am. J. Nucl Med Mol Imaging. 2013;3:1–15.
76. Zheng X, Wang X, Mao H, Wu W, Liu B, Jiang X. Hypoxia-specific ultrasensitive detection of tumours and cancer cells in vivo. Nat Commun. 2015;6:5834.
77. Napp J, Behnke T, Fischer L, WÜRth C, Wottawa M, Katschinski DRM, Alves F, Resch-Genger U, Schäferling M. Targeted luminescent near-infrared

polymer-nanoprobes for in vivo imaging of tumor hypoxia. Anal Chem. 2011;83:9039–46.
78. Palmer GM, Fontanella AN, Zhang G, Hanna G, Fraser CL, Dewhirst MW. Optical imaging of tumor hypoxia dynamics. J Biomed Opt. 2010;15:7–066021.
79. Okuda K, Okabe Y, Kadonosono T, Ueno T, Youssif BG, Kizaka-Kondoh S, Nagasawa H. 2-Nitroimidazole-tricarbocyanine conjugate as a near-infrared fluorescent probe for in vivo imaging of tumor hypoxia. Bioconjug Chem. 2012;23:324–9.
80. Takahashi S, Piao W, Matsumura Y, Komatsu T, Ueno T, Terai T, Kamachi T, Kohno M, Nagano T, Hanaoka K. Reversible off–on fluorescence probe for hypoxia and imaging of hypoxia–normoxia cycles in live cells. J Am Chem Soc. 2012;134:19588–91.
81. Son A, Kawasaki A, Hara D, Ito T, Tanabe K. Phosphorescent ruthenium complexes with a nitroimidazole unit that image oxygen fluctuation in tumor tissue. Chemistry. 2015;21:2527–36.
82. Liu J, Liu Y, Bu W, Bu J, Sun Y, Du J, Shi J. Ultrasensitive nanosensors based on upconversion nanoparticles for selective hypoxia imaging in vivo upon near-infrared excitation. J Am Chem Soc. 2014;136:9701–9.
83. Komatsu H, Yoshihara K, Yamada H, Kimura Y, Son A, Nishimoto SI, Tanabe K. Ruthenium complexes with hydrophobic ligands that are key factors for the optical imaging of physiological hypoxia. Chemistry. 2013;19:1971–7.
84. Zhang S, Hosaka M, Yoshihara T, Negishi K, Iida Y, Tobita S, Takeuchi T. Phosphorescent light–emitting iridium complexes serve as a hypoxia-sensing probe for tumor imaging in living animals. Cancer Res. 2010;70:4490–8.
85. Gillies RJ. In vivo molecular imaging. J Cell Biochem Suppl. 2002;39:231–8.
86. Gallagher FA, Kettunen MI, Day SE, Hu D-E, Ardenkjær-Larsen JH, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM. Magnetic resonance imaging of pH in vivo using hyperpolarized 13C-labelled bicarbonate. Nature. 2008;453:940–3.
87. Engin K, Leeper DB, Cater JR, Thistlethwaite AJ, Tupchong L, McFarlane JD. Extracellular pH distribution in human tumours. Int J Hyperth. 1995;11:211–6.
88. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res. 1989;49:6449–65.
89. Damaghi M, Wojtkowiak JW, Gillies RJ. pH sensing and regulation in cancer. Front Physiol. 2013;4:370.
90. van den Berg AP, Wike-Hooley JL, van den Berg-Blok AE, van der Zee J, Reinhold HS. Tumour pH in human mammary carcinoma. Eur J Cancer Clin Oncol. 1982;18:457–62.
91. Bicher HI, Hetzel FW, Sandhu TS, Frinak S, Vaupel P, O'Hara MD, O'Brien T. Effects of hyperthermia on normal and tumor microenvironment. Radiology. 1980;137:523–30.
92. Calderwood SK, Dickson JA. pH and tumor response to hyperthermia. Adv. Radiat. Biol. 1983;10:135–90.
93. Gallagher FA, Kettunen MI, Brindle KM. Imaging pH with hyperpolarized 13C. NMR Biomed. 2011;24:1006–15.
94. Jindal AK, Merritt ME, Suh EH, Malloy CR, Sherry AD, Kovács Z. Hyperpolarized 89Y complexes as pH sensitive NMR probes. J Am Chem Soc. 2010;132:1784–5.
95. Scholz DJ, Janich MA, Köllisch U, Schulte RF, Ardenkjaer-Larsen JH, Frank A, Haase A, Schwaiger M, Menzel MI. Quantified pH imaging with hyperpolarized 13C-bicarbonate. Magn Reson Med. 2015;73:2274–82.
96. Ward KM, Balaban RS. Determination of pH using water protons and chemical exchange dependent saturation transfer (CEST). Magn Reson Med. 2000;44:799.
97. Huang Y, Coman D, Ali MM, Hyder F. Lanthanide ion (III) complexes of 1,4,7,10-tetraazacyclododecane-1, 4, 7, 10-tetraaminophosphonate for dual biosensing of pH with chemical exchange saturation transfer (CEST) and biosensor imaging of redundant deviation in shifts (BIRDS). Contrast Media Mol Imaging. 2015;10:51–8.
98. Moon BF, Jones KM, Chen LQ, Liu P, Randtke EA, Howison CM, Pagel MD. A comparison of iopromide and iopamidol, two acidoCEST MRI contrast media that measure tumor extracellular pH. Contrast Media Mol Imaging. 2015;10:446–55.
99. Chen LQ, Randtke EA, Jones KM, Moon BF, Howison CM, Pagel MD. Evaluations of tumor acidosis within in vivo tumor models using parametric maps generated with AcidoCEST MRI. Mol Imaging Biol. 2015;17:488–96.
100. Chen LQ, Howison CM, Jeffery JJ, Robey IF, Kuo PH, Pagel MD. Evaluations of extracellular pH within in vivo tumors using acidoCEST MRI. Magn Reson Med. 2014;72:1408–17.
101. Longo DL, Busato A, Lanzardo S, Antico F, Aime S. Imaging the pH evolution of an acute kidney injury model by means of iopamidol, a MRI-CEST pH-responsive contrast agent. Magn Reson Med. 2013;70:859–64.
102. Longo DL, Sun PZ, Consolino L, Michelotti FC, Uggeri F, Aime S. A general MRI-CEST ratiometric approach for pH imaging: demonstration of in vivo pH mapping with iobitridol. J Am Chem Soc. 2014;136:14333–6.
103. Aime S, Delli Castelli D, Terreno E. Novel pH-reporter MRI contrast agents. Angew Chem Int Ed Engl. 2002;114:4510–2.
104. Chen Y, Yin Q, Ji X, Zhang S, Chen H, Zheng Y, Sun Y, Qu H, Wang Z, Li Y, Wang X, Zhang K, Zhang L, Shi J. Manganese oxide-based multifunctionalized mesoporous silica nanoparticles for pH-responsive

MRI, ultrasonography and circumvention of MDR in cancer cells. Biomaterials. 2012;33:7126–37.
105. Sheth VR, Li Y, Chen LQ, Howison CM, Flask CA, Pagel MD. Measuring in vivo tumor pHe with CEST-FISP MRI. Magn Reson Med. 2012;67:760–8.
106. Nwe K, Huang C-H, Tsourkas A. Gd-labeled glycol chitosan as a pH-responsive magnetic resonance imaging agent for detecting acidic tumor microenvironments. J Med Chem. 2013;56:7862–9.
107. Lee YJ, Kang HC, Hu J, Nichols JW, Jeon YS, Bae YH. pH-sensitive polymeric micelle-based pH probe for detecting and imaging acidic biological environments. Biomacromolecules. 2012;13:2945–51.
108. Crayton SH, Tsourkas A. pH-Titratable superparamagnetic iron oxide for improved nanoparticle accumulation in acidic tumor microenvironments. ACS Nano. 2011;5:9592–601.
109. Rivas C, Stasiuk GJ, Gallo J, Minuzzi F, Rutter GA, Long NJ. Lanthanide(III) complexes of Rhodamine-DO3A conjugates as agents for dual-modal imaging. Inorg Chem. 2013;52:14284–93.
110. Li Y, Wang Y, Yang S, Zhao Y, Yuan L, Zheng J, Yang R. Hemicyanine-based high resolution ratiometric near-infrared fluorescent probe for monitoring pH changes in vivo. Anal Chem. 2015;87:2495–503.
111. Doria F, Folini M, Grande V, Cimino-Reale G, Zaffaroni N, Freccero M. Naphthalene diimides as red fluorescent pH sensors for functional cell imaging. Org Biomol Chem. 2015;13:570–6.
112. Wang L, Fan Z, Zhang J, Changyi Y, Huang C, Gu Y, Xu Z, Tang Z, Lu W, Wei X. Evaluating tumor metastatic potential by imaging intratumoral acidosis via pH-activatable near-infrared fluorescent probe. Int J Cancer. 2015;136:E107–16.
113. Liu X-D, Xu Y, Sun R, Xu Y-J, Lu J-M, Ge J-F. A coumarin–indole-based near-infrared ratiometric pH probe for intracellular fluorescence imaging. Analyst. 2013;138:6542–50.
114. Ding C, Tian Y. Gold nanocluster-based fluorescence biosensor for targeted imaging in cancer cells and ratiometric determination of intracellular pH. Biosens Bioelectron. 2015;65:183–90.
115. Liu X, Chen Q, Yang G, Zhang L, Liu Z, Cheng Z, Zhu X. Magnetic nanomaterials with near-infrared pH-activatable fluorescence via iron-catalyzed AGET ATRP for tumor acidic microenvironment imaging. J Mater Chem B. 2015;3:2786–800.
116. Wang Y, Zhou K, Huang G, Hensley C, Huang X, Ma X, Zhao T, Sumer BD, Deberardinis RJ, Gao J. A nanoparticle-based strategy for the imaging of a broad range of tumors by nonlinear amplification of microenvironment signals. Nat Mater. 2014;13:204–12.
117. Ko JY, Park S, Lee H, Koo H, Kim MS, Choi K, Kwon IC, Jeong SY, Kim K, Lee DS. pH-sensitive nanoflash for tumoral acidic pH imaging in live animals. Small. 2010;6:2539–44.
118. Li C, Xia J, Wei X, Yan H, Si Z, Ju S. pH-activated near-infrared fluorescence nanoprobe imaging tumors by sensing the acidic microenvironment. Adv Funct Mater. 2010;20:2222–30.
119. Dong C, Liu Z, Zhang L, Guo W, Li X, Liu J, Wang H, Chang J. pHe-induced charge-reversible NIR fluorescence nanoprobe for tumor-specific imaging. ACS Appl Mater Interfaces. 2015;7:7566–75.
120. Lemon CM, Curtin PN, Somers RC, Greytak AB, Lanning RM, Jain RK, Bawendi MG, Nocera DG. Metabolic tumor profiling with pH, oxygen, and glucose chemosensors on a quantum dot scaffold. Inorg Chem. 2014;53:1900–15.
121. Dennis AM, Rhee WJ, Sotto D, Dublin SN, Bao G. Quantum dot–fluorescent protein FRET probes for sensing intracellular pH. ACS Nano. 2012;6:2917–24.
122. Wu W, Aiello M, Zhou T, Berliner A, Banerjee P, Zhou S. In-situ immobilization of quantum dots in polysaccharide-based nanogels for integration of optical pH-sensing, tumor cell imaging, and drug delivery. Biomaterials. 2010;31:3023–31.
123. Shirmanova MV, Druzhkova IN, Lukina MM, Matlashov ME, Belousov VV, Snopova LB, Prodanetz NN, Dudenkova VV, Lukyanov SA, Zagaynova EV. Intracellular pH imaging in cancer cells in vitro and tumors in vivo using the new genetically encoded sensor SypHer2. Biochim Biophys Acta. 2015;1850:1905–11.
124. Tantama M, Hung YP, Yellen G. Imaging intracellular pH in live cells with a genetically encoded red fluorescent protein sensor. J Am Chem Soc. 2011;133:10034–7.
125. Huang G, Si Z, et al. Dextran based pH-sensitive near-infrared nanoprobe for in vivo differential-absorption dual-wavelength photoacoustic imaging of tumors. J Mater Chem. 2012;22:22575–81.
126. Flavell RR, Truillet C, Regan MK, Ganguly T, Blecha JE, Kurhanewicz J, Vanbrocklin HF, Keshari KR, Chang CJ, Evans MJ, Wilson DM. Caged [(18) F]FDG glycosylamines for imaging acidic tumor microenvironments using positron emission tomography. Bioconjug Chem. 2016;27:170–8.
127. Andreev OA, Engelman DM, Reshetnyak YK. pH-sensitive membrane peptides (pHLIPs) as a novel class of delivery agents. Membr Biol. 2010;27:341–52.
128. Andreev OA, Engelman DM, Reshetnyak YK. Targeting diseased tissues by pHLIP insertion at low cell surface pH. Front Physiol. 2014;5:97.
129. Adochite R-C, Moshnikova A, Carlin SD, Guerrieri RA, Andreev OA, Lewis JS, Reshetnyak YK. Targeting breast tumors with pH (low) insertion peptides. Mol Pharm. 2014;11:2896–905.
130. Cruz-Monserrate Z, Roland CL, Deng D, Arumugam T, Moshnikova A, Andreev OA, Reshetnyak YK, Logsdon CD. Targeting pancreatic ductal adenocarcinoma acidic microenvironment. Sci. Rep. 2014;4:4410/1–8.
131. Loja MN, Luo Z, Greg Farwell D, Luu QC, Donald PJ, Amott D, Truong AQ, Gandour-Edwards RF,

Nitin N. Optical molecular imaging detects changes in extracellular pH with the development of head and neck cancer. Int J Cancer. 2013;132:1613–23.
132. Macholl S, Morrison MS, Iveson P, Arbo BE, Andreev OA, Reshetnyak YK, Engelman DM, Johannesen E. In vivo pH imaging with 99mTc-pHLIP. Mol Imaging Biol. 2012;14:725–34.
133. Rothberg JM, Sameni M, Moin K Live-cell imaging of tumor proteolysis: impact of cellular and noncellular microenvironment. Biochim Biophys Acta. 2012;1824:123–32.
134. Mason SD, Joyce JA. Proteolytic networks in cancer. Trends Cell Biol. 2011;21:228–37.
135. Matusiak N, van Waarde A, Bischoff R, Oltenfreiter R, van de Wiele C, Dierckx RA, Elsinga PH. Probes for non-invasive matrix metalloproteinase-targeted imaging with PET and SPECT. Curr Pharm Des. 2013;19:4647–72.
136. Narunsky L, Oren R, Bochner F, Neeman M. Imaging aspects of the tumor stroma with therapeutic implications. Pharmacol Ther. 2014;141:192–208.
137. Chen H, Zhang X, Dai S, Ma Y, Cui S, Achilefu S, Gu Y. Multifunctional gold nanostar conjugates for tumor imaging and combined photothermal and chemo-therapy. Theranostics. 2013;3:633–49. 17 pp
138. D'Alessio S, Ferrari G, Cinnante K, et al. Tissue inhibitor of metalloproteinases-2 binding to membrane-type 1 matrix metalloproteinase induces MAPK activation and cell growth by a non-proteolytic mechanism. J Biol Chem. 2008;283:87–99.
139. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature. 1994;370:61–5.
140. Uekita T, Itoh Y, Yana I, Ohno H, Seiki M. Cytoplasmic tail-dependent internalization of membrane-type 1 matrix metalloproteinase is important for its invasion-promoting activity. J Cell Biol. 2001;155:1345–56.
141. Matusiak N, Castelli R, Tuin AW, Overkleeft HS, Wisastra R, Dekker FJ, Prély LM, Bischoff RPH, van Waarde A, Dierckx RAJO. A dual inhibitor of matrix metalloproteinases and a disintegrin and metalloproteinases, [18F]FB-ML5, as a molecular probe for non-invasive MMP/ADAM-targeted imaging. . Bioorganic. Med Chem. 2015;23:192–202.
142. Altiparmak BL, Yurt F, Citak A. Design of radiolabeled gelatinase inhibitor peptide (99mTc-CLP) and evaluation in rats. Appl Radiat Isot. 2014;89:130–3.
143. Efrem Mebrahtu AZ, Hur MA, Laforest R, Lapi SE. Initial characterization of a dually radiolabeled peptide for simultaneous monitoring of protein targets and enzymatic activity. Nucl Med Biol. 2013;40:190.
144. Da Rocha Gomes S, Miguel J, Azéma L, Eimer S, Ries C, Dausse E, Loiseau H, Allard M, Toulmé JJ. 99mTc-MAG3-Aptamer for imaging human tumors associated with high level of matrix Metalloprotease-9. Bioconjug Chem. 2012;23:2192–200.
145. Kondo N, Temma T, Shimizu Y, Watanabe H, Higano K, Takagi Y, Ono M, Saji H. Miniaturized antibodies for imaging membrane type-1 matrix metalloproteinase in cancers. Cancer Sci. 2013;104:495–501.
146. van Duijnhoven SMJR, Marc S, Nicolay K, Gruell H. In vivo biodistribution of radiolabeled MMP-2/9 activatable cell-penetrating peptide probes in tumor-bearing mice. Contrast Media Mol Imaging. 2015;10:59–66.
147. van Duijnhoven SMJR, Marc S, Nicolay K, Gruell H. Tumor targeting of MMP-2/9 activatable cell-penetrating imaging probes is caused by tumor-independent activation. J Nucl Med. 2011;52:279–86.
148. Zhao T, Harada H, Teramura Y, Tanaka S, Itasaka S, Morinibu A, Shinomiya K, Zhu Y, Hanaoka H, Iwata H, Saji H, Hiraoka M. A novel strategy to tag matrix metalloproteinases-positive cells for in vivo imaging of invasive and metastatic activity of tumor cells. J Control Release. 2010;144:109–14.
149. Al Rawashdeh W, Arns S, Gremse F, Ehling J, Knüchel-Clarke R, Kray S, Spöler F, Kiessling F, Lederle W. Optical tomography of MMP activity allows a sensitive noninvasive characterization of the invasiveness and angiogenesis of SCC xenografts. Neoplasia. 2015;16:235–46.
150. Wang Y, Lin T, Zhang W, Jiang Y, Jin H, He H, Yang VC, Chen Y, Huang Y. A prodrug-type, MMP-2-targeting nanoprobe for tumor detection and imaging. Theranostics. 2015;5:787–95.
151. Kawano T, Murata M, Piao JS, Narahara S, Hamano N, Kang JH, Hashizume M. Systemic delivery of protein nanocages bearing CTT peptides for enhanced imaging of MMP-2 expression in metastatic tumor models. Int J Mol Sci. 2015;16:148–58.
152. Chen WH, Xu XD, Jia HZ, Lei Q, Luo GF, Cheng SX, Zhuo RX, Zhang XZ. Therapeutic nanomedicine based on dual-intelligent functionalized gold nanoparticles for cancer imaging and therapy in vivo. Biomaterials. 2013;34:8798–807.
153. Zhang X, Bresee J, Fields GB, Barry Edwards W. Near-infrared triple-helical peptide with quenched fluorophores for optical imaging of MMP-2 and MMP-9 proteolytic activity in vivo. Bioorg Med Chem Lett. 2014;24:3786–90.
154. Shimizu Y, Temma T, Hara I, Makino A, Kondo N, Ozeki E, Ono M, Saji H. In vivo imaging of membrane type-1 matrix metalloproteinase with a novel activatable near-infrared fluorescence probe. Cancer Sci. 2014;105:1056–62.
155. Myochin T, Hanaoka K, Iwaki S, Ueno T, Komatsu T, Terai T, Nagano T, Urano Y. Development of a series of near-infrared dark quenchers based on Si-rhodamines and their application to fluorescent probes. J Am Chem Soc. 2015;137:4759–65.
156. Rood MT, Raspe M, ten Hove JB, Jalink K, Velders AH, van Leeuwen FW. MMP-2/9-specific

activatable lifetime imaging agent. Sensors (Basel). 2015;15:11076–91.
157. Levi J, Kothapalli SR, Bohndiek S, Yoon JK, Dragulescu-Andrasi A, Nielsen C, Tisma A, Bodapati S, Gowrishankar G, Yan X, Chan C, Starcevic D, Gambhir SS. Molecular photoacoustic imaging of follicular thyroid carcinoma. Clin Cancer Res. 2013;19:1494–502.
158. Waschkau B, Faust A, Schäfers M, Bremer C. Performance of a new fluorescence-labeled MMP inhibitor to image tumor MMP activity in vivo in comparison to an MMP-activatable probe. Contrast Media Mol Imaging. 2013;8:1–11.
159. Gallo J, Kamaly N, Lavdas I, Stevens E, Nguyen QD, Wylezinska-Arridge M, Aboagye EO, Long NJ. CXCR4-targeted and MMP-responsive iron oxide nanoparticles for enhanced magnetic resonanceimaging. Angew Chem Int Ed Engl. 2014;53:9550–4.
160. Crisp JL, Savariar EN, Glasgow HL, Ellies LG, Whitney MA, Tsien RY. Dual targeting of integrin avb3 and matrix Metalloproteinase-2 for optical imaging of tumors and chemotherapeutic delivery. Mol Cancer Ther. 2014;13:1514–25.
161. Ansari C, Tikhomirov GA, Hong SH, Falconer RA, Loadman PM, Gill JH, Castaneda R, Hazard FK, Tong L, Lenkov OD, Felsher DW, Rao J, Daldrup-Link HE. Development of novel tumor-targeted theranostic nanoparticles activated by membrane-type matrix metalloproteinases for combined cancer magnetic resonance imaging and therapy. Small. 2014;10:566–75.
162. Matsuo K, Kamada R, Mizusawa K, Imai H, Takayama Y, Narazaki M, Matsuda T, Takaoka Y, Hamachi I. Specific detection and imaging of enzyme activity by signal-amplifiable self-assembling 19F MRI probes. Chem Eur J. 2013;19:12875–83.
163. Reiser J, Adair B, Reinheckel T. Specialized roles for cysteine cathepsins in health and disease. J Clin Invest. 2010;120:3421–31.
164. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. Nat Rev Cancer. 2006;6:764–75.
165. Palermo C, Joyce JA. Cysteine cathepsin proteases as pharmacological targets in cancer. Trends Pharmacol Sci. 2008;29:22–8.
166. Loeser R, Bergmann R, Frizler M, Mosch B, Dombrowski L, Kuchar M, Steinbach J, Guetschow M, Pietzsch J. Synthesis and radiopharmacological characterisation of a Fluorine-18-labelled Azadipeptide nitrile as a potential PET tracer for in vivo imaging of cysteine cathepsins. ChemMedChem. 2013;8:1330–44.
167. Ren G, Blum G, Verdoes M, Liu H, Syed S, Edgington LE, Gheysens O, Miao Z, Jiang H, Gambhir SS, Bogyo M, Cheng Z. Non-invasive imaging of cysteine cathepsin activity in solid tumors using a 64Cu-labeled activity-based probe. PLoS One. 2011;6:e28029.
168. Shi W, Ogbomo SM, Wagh NK, Zhou Z, Jia Y, Brusnahan SK, Garrison JC. The influence of linker length on the properties of cathepsin S cleavable 177Lu-labeled HPMA copolymers for pancreatic cancer imaging. Biomaterials. 2014;35:5760–70.
169. Haris M, Singh A, Mohammed I, Ittyerah R, Nath K, Nanga RPR, Debrosse C, Kogan F, Cai K, Poptani H, Reddy D, Hariharan H, Reddy R. In vivo magnetic resonance imaging of tumor protease activity. Sci Rep. 2014;4:6081.
170. Fujii T, Kamiya M, Urano Y. In vivo imaging of Intraperitoneally disseminated tumors in model mice by using activatable fluorescent small-molecular probes for activity of cathepsins. Bioconjug Chem. 2014;25:1838–46.
171. Ryu JH, Na JH, Ko HK, You DG, Park S, Jun E, Yeom HJ, Seo DH, Park JH, Jeong SY, Kim I-S, Kim B-S, Kwon IC, Choi K, Kim K. Non-invasive optical imaging of cathepsin B with activatable fluorogenic nanoprobes in various metastatic models. Biomaterials. 2014;35:2302–11.
172. Kisin-Finfer E, Ferber S, Blau R, Satchi-Fainaro R, Shabat D. Synthesis and evaluation of new NIR-fluorescent probes for cathepsin B: ICT versus FRET as a turn-ON mode-of-action. Bioorg Med Chem Lett. 2014;24:2453–8.
173. Tian R, Li M, Wang J, Yu M, Kong X, Feng Y, Chen Z, Li Y, Huang W, Wu W, Hong Z. An intracellularly activatable, fluorogenic probe for cancer imaging. Org Biomol Chem. 2014;12:5365–74.
174. Cuneo KC, Mito JK, Javid MP, Ferrer JM, Kim Y, Lee WD, Bawendi MG, Brigman BE, Kirsch DG. Imaging primary mouse sarcomas after radiation therapy using cathepsin-activatable fluorescent imaging agents. Int J Radiat Oncol Biol Phys. 2013;86:136–42.
175. ABD-Elgaliel WR, Cruz-Monserrate Z, Logsdon CD, Tung C-H. Molecular imaging of Cathepsin E-positive tumors in mice using a novel protease-activatable fluorescent probe. Mol BioSyst. 2011;7:3207–13.
176. Hu H-Y, Vats D, Vizovisek M, Kramer L, Germanier C, Wendt KU, Rudin M, Turk B, Plettenburg O, Schultz C. In vivo imaging of mouse tumors by a lipidated cathepsin substrate. Angew Chem Int Ed Engl. 2014;53:7669–73.
177. Verdoes M, Edgington LE, Scheeren FA, Leyva M, Blum G, Weiskopf K, Bachmann MH, Ellman JA, Bogyo M. A nonpeptidic cathepsin S activity-based probe for noninvasive optical imaging of tumor-associated macrophages. Chem Biol. 2012;19:619–28.
178. Goodman SL, Picard M. Integrins as therapeutic targets. Trends Pharmacol Sci. 2012;33:405–12.
179. Goswami S. Importance of integrin receptors in the field of pharmaceutical, medical science. Adv Biol Chem. 2013;3:224–52. 29 pp

180. Danhier F, Breton AL, Préat VR. RGD-based strategies to target alpha (v) beta (3) integrin in cancer therapy and diagnosis. Mol Pharm. 2012;9:2961–73.
181. Guo J, Lang L, Hu S, Guo N, Zhu L, Sun Z, Ma Y, Kiesewetter DO, Niu G, Xie Q, Chen X. Comparison of three dimeric 18F-AlF-NOTA-RGD tracers. Mol Imaging Biol. 2014;16:274–83.
182. Mena E, Owenius R, Turkbey B, Sherry R, Bratslavsky G, Macholl S, Miller MP, Somer EJ, Lindenberg L, Adler S, Shih J, Choyke P, Kurdziel K. [18F]Fluciclatide in the in vivo evaluation of human melanoma and renal tumors expressing αvβ3 and αvβ5 integrins. Eur J Nucl Med Mol Imaging. 2014;41:1879–88.
183. Haubner R, Rangger C, Virgolini IJ, Maschauer S, Prante O, Einsiedel J, Gmeiner P, Eder IE. H-CRRETAWAC-OH, a lead structure for the development of radiotracer targeting integrin α5β1? Biomed Res Int. 2014;2014:243185.
184. Bao X, Wang M-W, Xu J-Y, Zheng Y-J, Jiang J-J, Zhang Y-J. Biodistribution in healthy KM mice and micro PET/CT imaging in U87MG tumor-bearing nude mice of a new 18F-labeled cyclic RGD dimer. Zhongguo Aizheng Zazhi. 2013;23:408–12.
185. Hackel BJ, Kimura RH, Miao Z, Liu H, Sathirachinda A, Cheng Z, Chin FT, Gambhir SS. 18F-fluorobenzoate-labeled cystine knot peptides for PET imaging of integrin αvβ6. J Nucl Med. 2013;54:1101–5.
186. Beer AJ, Pelisek J, Heider P, Reeps C, Eckstein HH, Saraste A, Metz S, Seidl S, Kessler H, Wester H-J, Schwaiger M. PET/CT imaging of integrin αvβ3 expression in human carotid atherosclerosis. JACC Cardiovasc Imaging. 2014;7:178–87.
187. Bryden F, Rosca EV, Boyle RW. PET/PDT theranostics: synthesis and biological evaluation of a peptide-targeted Ga-68 porphyrin. Dalton Trans. 2014;44:4925–32.
188. McBride WJ, D'Souza CA, Sharkey RM, Karacay H, Rossi EA, Chang C-H, Goldenberg DM. Improved F-18 labeling of peptides with a fluoride-aluminum-chelate complex. Bioconjug Chem. 2010;21:1331–40.
189. WJ MB, Sharkey RM, Goldenberg DM, McBride WJ. Radiofluorination using aluminum-fluoride (Al18F). EJNMMI Res. 2013;3:36.
190. Hausner SH, Bauer N, Sutcliffe JL. In vitro and in vivo evaluation of the effects of aluminum [18F] fluoride radiolabeling on an integrin αvβ6-specific peptide. Nucl Med Biol. 2014;41:43–50.
191. Bejot R, Goggi J, Moonshi SS, Robins EG. A practical synthesis of [18F]FtRGD: an angiogenesis biomarker for PET. J Label Compd Radiopharm. 2013;56:42–9.
192. Singh AN, McGuire MJ, Li S, Hao G, Kumar A, Sun X, Brown KC. Dimerization of a phage-display selected peptide for imaging of αvβ6- integrin: two approaches to the multivalent effect. Theranostics. 2014;4:745–60.
193. Hu LY, Bauer N, Knight LM, Li Z, Liu S, Anderson CJ, Conti PS, Sutcliffe JL. Characterization and evaluation of (64)Cu-labeled A20FMDV2 conjugates for imaging the integrin αvβ 6. Mol Imaging Biol. 2014;16:567–77.
194. Shokeen M, Zheleznyak A, Wilson JM, Jiang M, Liu R, Ferdani R, Lam KS, Schwarz JK, Anderson CJ. Molecular imaging of very late antigen-4 (α4β1 integrin) in the premetastatic niche. J Nucl Med. 2012;53:779–86.
195. Ortiz-Arzate Z, Santos-Cuevas CL, Ocampo-Garcia BE, Ferro-Flores G, Garcia-Becerra R, Estrada G, Gomez-Argumosa E, Izquierdo-Sanchez V. Kit preparation and biokinetics in women of 99mTc-EDDA/HYNIC-E-[c(RGDfK)]2 for breast cancer imaging. Nucl Med Commun. 2014;35:423–32.
196. Li F, Song Z, Li Q, Wu J, Wang J, Xie C, Tu C, Wang J, Huang X, Lu W. Molecular imaging of hepatic stellate cell activity by visualization of hepatic integrin αvβ3 expression with SPECT in rat. Hepatology. 2011;54:1020–30.
197. Liu Z, Liu H, Ma T, Sun X, Shi J, Jia B, Sun Y, Zhan J, Zhang H, Zhu Z, Wang F. Integrin αvβ6-targeted SPECT imaging for pancreatic cancer detection. J Nucl Med. 2014;55:989–94.
198. Zhu X, Li J, Hong Y, Kimura RH, Ma X, Liu H, Qin C, Hu X, Hayes TR, Benny P, Gambhir SS, Cheng Z. 99mTc-Labeled cystine knot peptide targeting integrin αvβ6 for tumor SPECT imaging. Mol Pharm. 2014;11:1208–17.
199. Ueda M, Fukushima T, Ogawa K, Kimura H, Ono M, Yamaguchi T, Ikehara Y, Saji H. Synthesis and evaluation of a radioiodinated peptide probe targeting αvβ6 integrin for the detection of pancreatic ductal adenocarcinoma. Biochem Biophys Res Commun. 2014;445:661–6.
200. John AE, Luckett JC, Tatler AL, Awais RO, Desai A, Habgood A, Ludbrook S, Blanchard AD, Perkins AC, Jenkins RG, Marshall JF. Preclinical SPECT/CT imaging of αvβ6 integrins for molecular stratification of idiopathic pulmonary fibrosis. J Nucl Med. 2013;54:2146–52.
201. Gao D, Gao L, Zhang C, Liu H, Jia B, Zhu Z, Wang F, Liu Z. A near-infrared phthalocyanine dye-labeled agent for integrin αvβ6-targeted theranostics of pancreatic cancer. Biomaterials. 2015;53:229–38.
202. Moore SJ, Hayden GMG, Bergen JM, Su YS, Rayburn H, Scott MP, Cochran JR. Engineered knottin peptide enables noninvasive optical imaging of intracranial medulloblastoma. Proc Natl Acad Sci U S A. 2013;110:14598–603.
203. Shi H, Liu J, Geng J, Tang BZ, Liu B. Specific detection of integrin αvβ3 by light-up bioprobe with aggregation-induced emission characteristics. J Am Chem Soc. 2012;134:9569–72.
204. Kossodo S, Pickarski M, Lin S-A, Gleason A, Gaspar R, Buono C, Ho G, Blusztajn A, Cuneo G, Zhang J, Jensen J, Hargreaves R, Coleman P, Hartman G, Rajopadhye M, Duong LT, Sur C, Yared W, Peterson

J, Bednar B. Dual in vivo quantification of integrin-targeted and protease-activated agents in cancer using fluorescence molecular tomography (FMT). Mol Imaging Biol. 2010;12:488–99.
205. Satpati D, Hausner SH, Bauer N, Sutcliffe JL. Cerenkov luminescence imaging of αvβ6 integrin expressing tumors using 90Y-labeled peptides. J Label Compd Radiopharm. 2014;57:558–65.
206. Kawamura W, Miura Y, Kokuryo D, Toh K, Yamada N, Nomoto T, Matsumoto Y, Sueyoshi D, Liu X, Aoki I. Density-tunable conjugation of cyclic RGD ligands with polyion complex vesicles for the neovascular imaging of orthotopic glioblastomas. Sci Technol Adv Mater. 2015;16:1–13.
207. Yan C, Wu Y, Feng J, Chen W, Liu X, Hao P, Yang R, Zhang J, Lin B, Xu Y, Liu R. Anti-αvβ3 antibody guided three-step pretargeting approach using magnetoliposomes for molecular magnetic resonance imaging of breast cancer angiogenesis. Int J Nanomedicine. 2013;8:245–55.

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https://doi.org/10.1007/978-3-319-67577-0

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