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Tumor-Induced Immune Suppression

Mechanisms and Therapeutic Reversal

Second Edition

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Preface

Since publication of the first edition of this monograph the field of tumor immunology and immunotherapy made tremendous progress. The second edition reflects those changes. The chapters were revised to reflect new information and several new chapters were added. The development of any field of science follows spiral motion from basic observations to greater understanding of more and more complex mechanisms. Along this road, many basic facts are being rediscovered over time, at new, more sophisticated levels. However, for people outside the field, this spiral motion is usually lost and the movement is often reminiscent of a pendulum. The period of enthusiasm is followed by widespread disappointment to be replaced by the renewed enthusiasm.

Tumor immunology and cancer immune therapy are classic examples of this paradigm. Initial realization that some immune mechanisms could be involved in control of tumor growth and hopes that the treatment of cancer with bacterial pathogens or simple vaccines could cure cancer made tumor immunology an exciting area of research in the first 30 years of last century. However, the period of high expectations was followed by long hiatus of skepticism or even oblivion when clinical results did not meet expectation. Moreover, some experimental results suggested that the immune system was not involved in regulation of tumor progression.

In late 1980s, when the nature of some tumor-associated antigens was identified and researchers discovered limitations of original experimental systems used to determine the role of the immune system in cancer, interest in the field returned. With the identification of many regulatory activities in T cell activation, more molecularly-targeted approaches were described. Many clinical trials were initiated and hopes for quick progress were again high. However, at the beginning of this century, lack of sufficient success in clinical trials turned the pendulum back to skepticism.

Fortunately, this skepticism was placed in very a different environment than in previous years. Much more was learned about the mechanisms by which the immune system responds to tumors and how it is regulated. One of the areas that developed fast during the last 20 years was immune suppression in cancer. Research in this field did not slow down and in recent years, has produced real pre-clinical successes. Now, the field is gaining momentum again. Interest in tumor immunology and immunotherapy is high, and numerous clinical trials are being conducted, with

encouraging results. This includes FDA approval of both a prostate cancer vaccine and a monoclonal antibody which blocks CTLA-4-dependent inhibition. However, despite many positive signs, it is clear that the level of responses is still rather limited and only a fraction of the patients truly benefit from these therapies. One of the major factors that limits the effect of cancer immune therapy is the persistence of suppressive mechanisms that arise in the tumor microenvironment, which limit the durability of anti-tumor immune responses.

This monograph will present readers with a broad and comprehensive overview of these mechanisms. They range from immune suppressive cytokines and molecules expressed by tumor cells to immune suppressive T cells and myeloid cells. Each factor has its own history, elaborate pathway and functional consequences. The litany of mechanisms present in tumor-bearing hosts is so powerful and redundant, that it raises a question how a host can actually survive such an onslaught, given the need for maintaining immunity to pathogens. Importantly, it is well known that neither tumor-bearing mice nor cancer patients are profoundly immune suppressed until very late in tumor progression. Even in that situation, it is not clear whether these consequences are due to specific immune suppressive mechanisms or metabolic changes associated with tumor-induced cachexia. Patients don't suffer from opportunistic infections and could be immunized, albeit with some difficulties, against viral pathogens.

It seems that there are two possible explanation for this paradox. One is that there is a strong compartmentalization of immune suppression associated with cancer. The tumor site provides a profound immune suppressive microenvironment, whereas in peripheral lymphoid organs, non-specific suppression is rather limited and the main operational mechanism is tumor-specific immune tolerance. Several chapters in this book will discuss these issues.

However, there could be another explanation. It is possible that various immune suppressive factors are not that redundant after all and instead, are essentially tumor-specific. In this scenario, a tumor has a "driver" immune suppressive mechanism that determines the outcome of the response and "passenger" mechanisms, which may be present but not critical. One example is the role of myeloid-derived suppressor cells (MDSC) and regulatory T (Treg) cells in melanoma. In the B16F10 melanoma model, Treg cells play a prominent role whereas MDSCs appear to be a "passenger" factor. The situation is reversed in the Ret transgene-induced melanoma model, where MDSC are the critical "driver" factor determining the suppressive mechanism. This paradigm can be observed in other tumor models where different immune suppressive factors may exert different roles.

Immune suppressive factors are attractive therapeutic targets with a goal of boosting immune responses and enhancing antitumor activity. However, universal approaches to therapeutic correction of the situation may be prone to failure. There is also a risk of targeting redundant or inconsequential suppressive mechanisms which might also have adverse effects to immunotherapy. We need to approach this therapeutic intervention with open eyes to avoid mistakes made in previous years. Therefore future studies should address several major questions.

- There is a need to determine “driver” immune suppression factors for each type of tumor and specific factors that could cause this. This may be used for more precise targeting;
- It may worth considering the creation of a standard diagnostic panel, where major factors of immune suppression are tested in each particular tumor;
- Compensatory changes need to be monitored, with consideration of targeting multiple mechanisms as necessary;
- Monitoring different suppressive mechanisms during relapse.

In recent years, a new paradigm of cancer treatment was developed. It suggests that conventional cancer therapy (radiation, chemotherapy) can synergize with immune-based therapy of cancer. The role of immune suppressive networks in this combinatorial therapy is only beginning to emerge. It is tempting to speculate that elimination of immune suppression could play an important role in this process. However, the results are mainly obtained in tumor-bearing mice and more work needs to be done in the clinical setting, which will give a more realistic validation to the hypothesis. The field of tumor immunology is now engaged in a renaissance, with very high hopes for successful immune therapeutics. However, in order to be successful, we need to revisit our understanding of the regulation of the tumor microenvironment. We believe that this monograph will help readers to do this.

Contents

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Chapter 1
Regulatory T Cells and Cancer

Mary Jo Turk

Abstract Regulatory T cells (T_{reg}) are key mediators of tumor immune suppression, and elevated T_{reg} proportions have now been identified in association with all major types of human cancer. Suppression of antitumor immunity is mediated by both natural (nT_{reg}) and induced T_{reg} (iT_{reg}) subsets, which express Foxp3, and they have been shown to engage a wide range of tumor-associated antigens. Preexisiting T_{reg} are actively recruited to tumors through chemokine and cytokine signals and become activated by dendritic cells (DCs) and myeloid-derived suppressor cells (MDSCs) within tumors. Th0 cells are also efficiently converted to Foxp3-expressing iT_{reg} in response to TGF-β produced by tumor cells and antigen-presenting cells (APCs) in the tumor microenvironment. T_{reg} exert suppression of tumor-specific T-cell responses through a variety of mechanisms including cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), interleukin 35 (IL-35), interleukin 10 (IL-10), and transforming growth factor beta (TGF-β). Therapies that inhibit these pathways, or directly deplete T_{reg} populations, are an effective means for enhancing antitumor immunity. Clinical trials are now beginning to reveal that blocking T_{reg} responses is a necessary component of successful cancer immunotherapy.

Keywords Regulatory T cell · Cancer · T_{reg} · iT_{reg} · Foxp3 · CD25 · CTLA-4 · VEGF · Neuropilin · CCL22 · CCL2 · IDO · PD-1 · IL-35 · GITR

1 Introduction

1.1 History

Regulatory T cells (T_{reg}) are major mediators of tumor-induced immune suppression. Some of the earliest clues indicating that T_{reg} could suppress antitumor immunity were found in the early 1980s in conjunction with the phenomenon of concomitant

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tumor immunity. North and colleagues reported that mice bearing progressive Meth A fibrosarcomas would spontaneously reject an inoculum of the same tumor at a distal site [1]. However, after several days of primary tumor growth, concomitant immunity was spontaneously abolished by a population of Ly-1^+2^- "suppressor T cells" [1]. These suppressor cells were undoubtedly T_{reg}, as contemporary studies now show [2]. However, T cell-mediated suppression of antitumor immunity was largely ignored throughout the following decades due to skepticism about other fundamental experiments in the field [3].

T_{reg} experienced a rebirth in 1999 when Sakaguchi and colleagues identified them by cell-surface markers CD4 and CD25 [4]. This work established that T_{reg} are a thymically derived T-cell subset that prevents profound autoimmune diseases [4]. Anti-CD25-depleting antibodies became a powerful new tool for addressing the role of T_{reg} in cancer. In 1999, Shimizu and Sakaguchi reported that treatment of tumor-bearing mice with an anti-CD25 monoclonal antibody (mAb) promoted immune-mediated tumor regression [4], with similar findings reported by Gallimore in 2002 [5]. Anti-CD25 was soon administered in conjunction with cytotoxic T-lymphocyte antigen 4 (CTLA-4) blockade, demonstrating its ability to synergistically promote CD8 T-cell responses against melanoma [6]. This fundamental work initiated a slow but steady resurgence in the study of T_{reg} responses to cancer.

In 2004, the first natural major histocompatibility complex-II (MHC-II)-restricted epitope for T_{reg} was reported [7]. Elegant cloning work by Wang and colleagues demonstrated that $CD4^+CD25^+$ T_{reg} from human melanoma tumors recognized the unmutated self-antigen LAGE-1 [7]. Returning to the model of concomitant tumor immunity, our work that year further established that T_{reg} prevent the generation of natural CD8 T cell-mediated immunity against the poorly immunogenic B16 melanoma [8]. Depletion of T_{reg} with an antibody to CD4 initiated the priming of CD8 T-cell responses to shared melanoma/melanocyte differentiation antigens in response to tumor growth [8]. Adoptive transfer experiments in tumor-bearing hosts confirmed that $CD4^+CD25^+$ T cells from naïve hosts give rise to T_{reg} that exert dominant suppression over CD8 T cell-mediated immunity [8]. The following year, Antony and Restifo demonstrated that $CD4^+CD25^+T_{reg}$ suppress gp100-specific CD8 T cells in the adoptive T-cell therapy setting [9]. Collectively, these studies solidified the theory that T_{reg} exert dominant suppression over antitumor immunity.

Almost a decade later, our knowledge of T_{reg} has grown exponentially. Fueled by extensive work in cancer, autoimmune disease, transplantation tolerance, and infectious diseases, we now understand many of the mechanisms governing T_{reg} function. This chapter synthesizes current knowledge of T_{reg} behavior in mouse tumor models and human cancer patients, with a goal of providing a broad and detailed understanding of how T_{reg} function in hosts with cancer.

1.2 T_{reg} Definitions

1.2.1 $CD4^+$ T_{reg}

The present chapter focuses on subsets of $CD4^+$ T_{reg} that express the transcription factor Foxp3. Foxp3 has been shown to be necessary for T_{reg} cell lineage development in the thymus and for T_{reg} suppressive function [10], [11]. There are two major subsets of $Foxp3^+CD4$ T cells: natural (thymic) and induced (adaptive) T_{reg} (nT_{reg} and iT_{reg}, respectively). Thus, in addition to its thymic expression, Foxp3 also becomes expressed on a subset of conventional CD4 T cells (Th0 cells) upon encounter with factors and cells present in tumor-bearing hosts [12]. This process of tumor-driven T_{reg} conversion will be discussed in Sect. 2.4.

Suppressive, $Foxp3^{neg}$ subsets of $CD4^+$ T cells, such as Tr1 and Th3 cells— which are thought to suppress through IL-10 and TGF-β, respectively—have also been identified in conjunction with cancer [13]. Additionally, hepatic tumor-associated $CD4^+Foxp3^{neg}$ T cells have been shown to suppress through membrane-bound TGF-β [14]. However, as compared to classical, $Foxp3^+$ T_{reg}, there is less convincing *in vivo* evidence that $Foxp3^{neg}$ subsets can suppress antitumor immunity.

1.2.2 $CD8^+$ T_{reg}

Studies have also shown that $CD8^+$ T_{reg} can function in cancer. $CD8^+CD28^-$ T cells with *in vitro* suppressive function have been identified in multiple types of human tumors [15]. In human ovarian cancer, $CD8^+$ T cells have been shown to suppress in an IL-10-dependent manner [16], and in human prostate tumors, suppressive $CD8^+$ T cells also express Foxp3 [17]. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) cancer model, Hurwitz and colleagues reported that $CD8^+$TcR-I cells regulate antitumor immunity in a TGF-β-dependent manner, although these cells were predominantly $Foxp3^{neg}$ [18]. Thus, there is a small but growing literature that $CD8^+$ T_{reg} play a role in suppressing antitumor immunity.

1.3 Evidence for the Suppressive Role of T_{reg} in Cancer

1.3.1 T_{reg} in Human Cancers: Prognostic Significance

Elevated proportions of T_{reg} have been identified in association with all major types of human cancer. In humans, T_{reg} are generally defined based on their expression of Foxp3, high levels of CD25, and the ability to exert *in vitro* suppressive function. Despite this, Foxp3 has also been found in *in vitro* activated human effector T cells [19]. Therefore, there remained some doubt regarding Foxp3 as a specific marker of T_{reg} in humans. To address this, recent studies showed that primary $CD4^+CD25^+Foxp3^+$ cells from tumors of patients are equally as suppressive as *bona fide* $Foxp3^+$ T_{reg}

taken from peripheral blood [20]. Thus, Foxp3 can be used to define a population of suppressive T_{reg} in association with human cancers [20].

Among the earliest studies to identify T_{reg} in human tumors, Curiel and colleagues reported that $CD4^+CD25^+Foxp3^+$ T_{reg} in human ovarian carcinoma were associated with poor prognosis [21]. Since then, T_{reg} have been linked to poor outcomes for many types of cancer. In pancreatic ductal carcinomas, and in hepatocarcinomas, high proportions of $CD4^+CD25^+Foxp3^+$ T_{reg} mark patients with poor prognosis [22], [23]. Higher proportions of $Foxp3^+T_{reg}$ infiltrating non-small cell lung cancer tumors are associated with a worse recurrence-free survival after surgery [24]. In melanoma patients, the proportion of $CD25^+Foxp3^+$ cells among tumor-infiltrating lymphocytes is significantly elevated in patients with later disease recurrence [25], and Foxp3 expression correlates with worse progression-free survival in patients with stage III disease [26]. Similarly, in patients with breast cancer, high T_{reg} proportions correlate with the most aggressive forms of the disease [27].

On the other hand, T_{reg} proportions can serve as a positive prognostic factor in some cases. This has been shown for hematological malignancies including follicular and Hodgkin's lymphomas [28], [29], and also for solid tumors including head and neck cancer [30] and colorectal cancer [31]. It has been speculated that this dichotomy may be due to the ability of T_{reg} to suppress the production of innate inflammatory and pro-angiogenic factors that contribute to tumor progression in certain cancers [32]. T_{reg} have also been shown to restrict low-avidity T-cell responses, and thus promote high-avidity CD8 T-cell responses to infectious pathogens [33], which could potentially explain their beneficial role in cancer. While further studies are needed to address a potentially complex role for $Foxp3^+$ cells in human diseases, mouse models have provided definitive evidence that T_{reg} function in a suppressive manner in tumor-bearing hosts.

1.3.2 Unequivocal Evidence from Studies in Foxp3-Diphtheria Toxin Receptor Mice

Many therapies currently exist for depleting T_{reg} and/or blocking their suppressive function in mouse models. Anti-CD25 and anti-CD4 mAbs were mentioned briefly above, and various other methods are discussed in Sect. 3.1. Each of these therapies has pronounced effects on stimulating antitumor immunity; however, none of them are absolutely specific for T_{reg}. Currently, the only means to specifically deplete $Foxp3^+$ T_{reg} *in vivo* is through the use of Foxp3-diphtheria toxin receptor (DTR) mice. Created independently by two groups, Foxp3-DTR mice express a DTR–green fluorescent protein (GFP) fusion protein under control of the Foxp3 promoter, which renders $Foxp3^+$ T_{reg} sensitive to depletion by *in vivo* administration of diphtheria toxin (DT) [34], [35]. Because effector CD8 and CD4 T cells remain virtually unaffected by DT treatment, studies in Foxp3-DTR mice have provided the most compelling and definitive evidence that T_{reg} play an immunosuppressive role in cancer.

The earliest studies involving Foxp3-DTR mice showed that T_{reg} depletion leads to rapid and aggressive autoimmune scurfy-like disease [34]–[36]. Therefore, studies in tumor-bearing animals have only involved short-term, temporary DT treatment. Regardless, the effects of T_{reg} depletion on antitumor immunity are unequivocal. DT treatment of B16-ovalbumin (OVA) tumor-bearing mice beginning as late as day 7, when tumors were 2–4 mm in diameter, substantially reduced tumor growth by a mechanism requiring CD8 T cells [37]. Further combination of DT with CpG oligodeoxynucleotides and OVA vaccination led to complete tumor regression [37]. Similar studies in Foxp3-DTR mice with autochthonous methylcholanthrene (MCA)-induced cancers showed that a single depleting dose of DT, administered at the time of carcinogen exposure, protected mice from tumorigenesis in a natural killer (NK) cell-dependent fashion [38]. Repeated DT dosing also cured a proportion of mice with established MCA fibrosarcomas by a mechanism requiring host CD8 T cells and interferon gamma (IFN-γ) [38]. Thus, studies provide definitive evidence that Foxp3-expressing T_{reg} exert dominant suppression over innate and adaptive immunity during tumor initiation, establishment, and progression.

2 T_{reg} Characteristics and Behavior

2.1 Natural Versus Induced T_{reg} (nT_{reg} vs. iT_{reg})

As mentioned above, Foxp3 drives the development of T_{reg} in the thymus, and can also become expressed by conventional CD4 T cells in the periphery. The phenomenon of acquired Foxp3 expression by conventional T cells is referred to as T_{reg} conversion, with converted T_{reg} referred to as iT_{reg}. The relative contribution of nT_{reg} and iT_{reg} to tumor-induced immune suppression remains an open question. Based on *in vitro* studies, it has been postulated that T_{reg} in human cancer patients are comprised overwhelmingly of iT_{reg}-producing and IL-10-producing Tr1 cells, rather than nT_{reg} [13]. However, due to experimental limitations in determining the origins of T_{reg} from human cancer patients, mouse models have also been useful for exploring this question.

Studies in CT26 tumor-bearing mice showed that $Foxp3^+$ T_{reg} accumulate in spleen and draining lymph nodes even after treatment with depleting anti-CD25 mAb and thymectomy [39]. Because these mice lacked detectable thymic T_{reg}, this finding implicated conversion as the major process driving T_{reg} accumulation in tumor-bearing hosts [39]. On the other hand, in mice bearing hemagglutinin (HA)-expressing A20 lymphoma, that were adoptively transferred with HA-specific CD4 T cells, T_{reg} accumulation in tumors was due mainly to nT_{reg} expansion, with a smaller contribution from iT_{reg} conversion [40]. More recently, the T-cell receptor (TCR) repertoires of $Foxp3^+$ and $Foxp3^{neg}$ cells were analyzed by TCR clonotyping in mice with MethA-induced carcinomas. In both tumors and tumor-draining lymph nodes, TCR repertoires of these subsets were found to be distinctly nonoverlapping [41]. As iT_{reg} generated from Th0 cells are expected to have the same range of specificities

as the CD4 peripheral repertoire, this suggests that tumor-associated T_{reg} may not derive from the conversion of conventional CD4 T cells [41]. Collectively, these studies show that both nT_{reg} and iT_{reg} can participate in tumor immune suppression.

Phenotypically, it remains unclear how tumor-associated nT_{reg} and iT_{reg} can be differentiated. Helios was originally implicated as a specific marker of nT_{reg} [42]. However, more recent studies show that helios can be expressed by iT_{reg} under *in vitro* activation conditions [43], on activated conventional CD4 and CD8 T cells [44], and by iT_{reg} *in vivo* [45]. More recently, the vascular endothelial growth factor (VEGF) receptor Neuropilin-1 (Nrp-1) was found at high levels on nT_{reg} but at low levels on iT_{reg} [46]. As blocking Nrp clearly influences T_{reg} responses to tumors ([47]; see Sect. 2.3.2), further analysis of Nrp-1 on T_{reg} from mouse and human tumors may provide a much-needed insight into this question.

2.2 *Antigen Specificity*

In theory, circulating $Foxp3^+$ T_{reg} are thought to recognize both self- and non-self-antigens. iT_{reg} or "adaptive" T_{reg} are generated from conventional CD4 T cells (or Th0 cells), and thus can have the same range of specificities as the CD4 T-cell repertoire. On the other hand, nT_{reg} or "thymic" T_{reg} are generated through high-affinity interactions with self-antigen in the thymus. Indeed, recent studies using TCR retrogenic technology show that the generation of nT_{reg} is directly proportional to TCR avidity, with higher avidity TCRs giving rise to a larger proportion of T_{reg} [48]. However, even thymocytes with low-avidity TCRs could develop into T_{reg}, demonstrating a broader avidity range for nT_{reg} differentiation than originally appreciated [48]. Furthermore, even in the absence of thymically expressed OVA, it was shown that OVA-specific nT_{reg} can be generated [48]. Thus, presumably through cross-reactivity with self-antigen in the thymus, foreign antigen-specific nT_{reg} can also be positively selected [48].

In patients with cancer, only a few notable studies report the antigen specificity of T_{reg}. This is likely due to the low frequency of T_{reg} with any given specificity and the difficulty in assessing suppressive function using low cell numbers [49]. However, these studies collectively show that T_{reg} are capable of responding to all major classes of tumor antigens. As mentioned earlier, the first of these studies showed that suppressive $CD4^+CD25^+$Foxp3-expressing T cells from human melanoma tumors recognize an epitope from the unaltered cancer testes antigen LAGE [7]. Subsequent studies from the same group showed that T_{reg} taken from solid tumors could also recognize the ARTC1 peptide, a mutated tumor-specific antigen [50]. Circulating $Foxp3^+$CD4 T cells from patients with melanoma have also been shown to be specific for the self-antigens gp100, TRP-1, NY-ESO-1, and survivin, whereas these specificities were not found in T_{reg} from healthy individuals [51]. Cervical cancer patient lymph node biopsy samples were found to contain human papillomavirus

(HPV)-specific $CD4^+Foxp3^+$ T cells with *in vitro* suppressive function [52] illustrating that T_{reg} also respond to tumor-expressed viral antigens. Thus, T_{reg} target antigens appear to be similar to those of effector T cells.

Studies in patients with colorectal carcinoma further demonstrate that T_{reg} suppress effector T-cell responses in an antigen-specific manner. T_{reg} from colon cancer patients were found to be specific for certain tumor antigens (including carcinoembryonic (CEA), telomerase, human epidermal growth factor receptor 2 (Her2/neu), and mucin 1 (MUC-1)), but not other antigens (including survivin and p53) [53]. Interestingly, *in vitro* depletion of T_{reg} preferentially led to effector/memory T-cell responses against the antigens recognized by T_{reg} [53]. In the mouse CT26 model, depletion of T_{reg} with anti-CD25 led to recognition of a cryptic cytotoxic T lymphocyte (CTL) epitope from an endogenous retrovirus, again suggesting that only certain antigens are under the control of T_{reg} suppression [54].

2.3 *Mechanisms of T_{reg} Recruitment to Tumors*

T_{reg} clearly recognize a broad range of tumor-expressed antigens, and they accordingly accumulate where antigen is most available—within the tumor microenvironment. Most tumor-bearing hosts are not broadly immunosuppressed; therefore, it is generally believed that T_{reg} exert their most potent suppressive functions within the local tumor microenvironment and draining lymph nodes. Indeed, a low ratio of T_{reg} to effector T cells within the melanoma tumor microenvironment has been shown to be an important determinant of effective antitumor immunity [55]. Thus, T_{reg} must be actively recruited to the tumor microenvironment before they can function optimally to suppress antitumor immunity. This section describes the molecular interactions that are known to promote the accumulation of T_{reg} in tumors (Fig. 1.1).

2.3.1 VEGF and Neuropilin-1

VEGF was originally identified in the mid-1980s as a tumor-secreted factor that increased vascular permeability and promoted angiogenesis [56]–[58]. More recently, a novel role for VEGF in promoting T_{reg} responses has been discovered. $Foxp3^+$ T_{reg} have been shown to express receptors for VEGF including VEGFR2 and Nrp-1 [59], [60]. As mentioned in Sect. 1.2.1, Nrp-1 is expressed at high levels on nT_{reg} but low levels on iT_{reg}, and can thus serve as a marker for differentiating these subsets [46].

Studies in mouse models demonstrate a key role for VEGF in promoting the infiltration of $Foxp3^+$ T_{reg} into tumors. In B16 melanoma, VEGF blockade using an adenovirus-expressed soluble VEGF-R was shown to substantially reduce the proportion of T_{reg} in tumors, and to improve the efficacy of a tumor vaccine [61]. B16 tumors overproducing VEGF also had a markedly enhanced accumulation of T_{reg} [61]. Both anti-VEGF and sunitinib, which target multiple receptor tyrosine

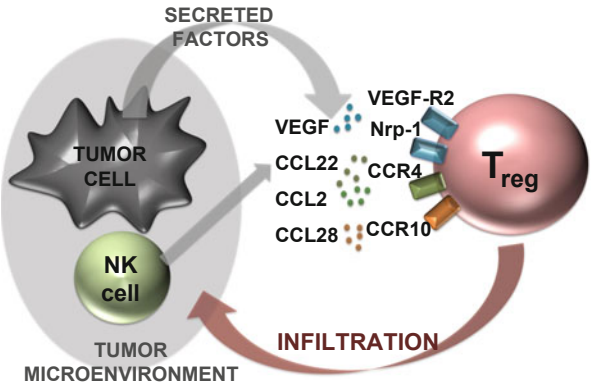

Fig. 1.1 Mechanisms of T_{reg} recruitment to the tumor microenvironment. Tumor cells secrete VEGF, which binds to T_{reg}-expressed VEGF-R2 and Nrp-1 and mediates recruitment into tumors. Tumor cells also secrete chemokines CCL22, CCL2, and CCL28, which recruit T_{reg} through the cognate receptors CCR4 and CCR10. NK cells within tumors also express CCL22, which can lead to T_{reg} recruitment

kinases including VEGFR, were also shown to reduce T_{reg} proportions in the CT26 colon tumor model [59]. Expression of the VEGF receptor Nrp-1 is clearly an important factor in T_{reg} responsiveness to VEGF, as it was recently shown that Nrp-1 expression on T_{reg} is required for T_{reg}-mediated suppression of antitumor immunity in the MT/ret spontaneous melanoma model [47]. Consistent with its role as a receptor for VEGF, Nrp-1 was crucial for T_{reg} accumulation into melanomas in response to tumor-derived VEGF, but was not required for T_{reg} development or suppressive function [47]. By selectively eliminating Nrp-1 on T_{reg}, these studies differentiated the direct effects of VEGF on T_{reg} from vascular effects that may indirectly influence T_{reg} behavior.

Recent clinical trials have now begun to confirm a role for VEGF in promoting T_{reg} responses in patients. At present, three studies report that sunitinib treatment decreases T_{reg} proportions in patients with metastatic renal cell carcinoma [62]–[64]. Very recent clinical studies involving bevacizumab, a humanized mAb to VEGF-A, have also demonstrated inhibition of T_{reg} increases in the blood of metastatic colorectal cancer patients [59]. Thus, sunitinib and bevacizumab may serve as important components in future cancer immunotherapy protocols.

VEGF and Nrp-1 also appear to have roles in T_{reg} function beyond driving recruitment. VEGF has also been shown to directly trigger T_{reg} cell proliferation [59], and Nrp-1 can mediate interactions between T_{reg} and DCs [65]. These studies collectively illustrate the overlap between factors that drive tumor angiogenesis and various aspects of T_{reg}-mediated immune suppression.

2.3.2 CCL22, CCL2, and CCL28

Chemokines and their receptors also play an important role in recruiting T_{reg} to tumors. Analyses of human ovarian carcinoma samples have revealed that ovarian cancer cells and associated macrophages produce chemokine (C-C motif) ligand 22 (CCL22), whereas $Foxp3^+$ T_{reg} express the associated receptor C-C chemokine receptor type 4 (CCR4) [21]. CCL22 was shown to mediate trafficking of T_{reg} both

in vitro and into tumors of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice reconstituted with human ovarian tumor cells [21]. It was subsequently shown that CCL22 drives the recruitment of T_{reg} to lungs of mice bearing Lewis lung carcinomas (LLC) [66]. LLC cells themselves did not secrete CCL22, but NK cell-infiltrating tumors were major producers of the chemokine [66]. Also, in the MT/ret melanoma model, tumors were shown to produce high levels of CCL2 (an agonist for CCR4), and tumor-infiltrating T_{reg} were found to be overwhelmingly CCR4-positive [67]. Thus, CCL22 and CCL2 produced by tumor cells, or innate immune cells in the tumor microenvironment, can attract CCR4-expressing T_{reg}.

Hypoxia-induced production of CCL28 has also been shown to mediate T_{reg} recruitment into tumors. In the ID8 ovarian cancer model, it was shown that hypoxia induces tumor cell production of CCL28, which recruits CCR10-expressing T_{reg} to the intraperitoneal tumor microenvironment [68]. Recruited T_{reg} then specifically produced VEGF-A within the tumor [68]. Taken together with studies described above, this suggests that T_{reg} recruitment into tumors can be a self-sustaining event, with T_{reg}-produced VEGF recruiting additional T_{reg}. VEGF also drives tumor angiogenesis, which may more comprehensively explain why T_{reg} are associated with poor outcomes in cancer patients.

2.4 *Mechanisms of T_{reg} Activation and Conversion in Tumors and Draining Lymph Nodes*

2.4.1 Activation of nT_{reg}

Early studies by Fission and colleagues showed that a subset of T_{reg} repeatedly encounter self-antigens in the periphery, which induce their continuous proliferation [69]. More recent studies suggest that these $CD44^{hi}$ T_{reg} are the earliest responders to tumors, thereby functioning as "memory T_{reg}" [70]. In tumor-draining lymph nodes of mice bearing either the 4T1 transplantable breast tumor or an autochthonous mammary carcinoma, it was shown that $Foxp3^+$ T cells proliferate earlier and more rapidly as compared with effector T cells [70]. These memory T_{reg} prevented the priming of de novo effector T-cell responses to tumors [70]. Accordingly, T_{reg} taken from B16-granulocyte–macrophage colony-stimulating factor (GMCSF) melanoma tumor-draining lymph nodes (but not contralateral lymph nodes) have been shown to be immediately suppressive ex vivo without a need for *in vitro* stimulation [71]. Thus, $Foxp3^+$ T_{reg} appear to be activated in a rapid and sustained fashion by antigens in tumor-draining lymph nodes, while maintaining suppressive function and avoiding exhaustion (Fig. 1.2).

In addition to the direct recognition of antigen, T_{reg} are also activated by factors produced by tumor-associated antigen-presenting cells (APCs). pDCs expressing the tryptophan-catabolizing enzyme indolamine 2,3-dioxygenase (IDO) directly activate resting T_{reg} in tumor-draining lymph nodes, thereby inducing suppressive function [71]. It was shown that tryptophan catabolism by IDO activates T_{reg} through

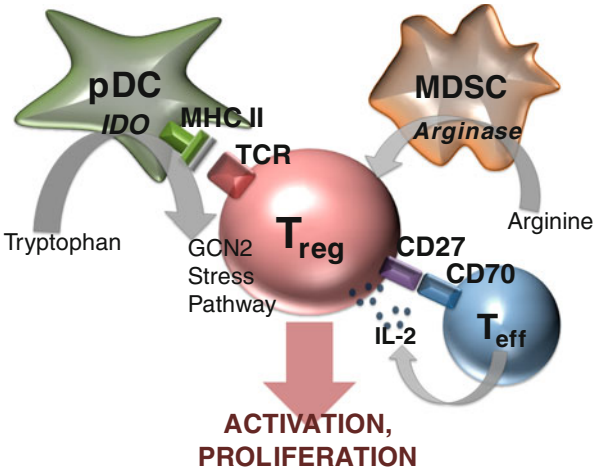

Fig. 1.2 Mechanisms of T_{reg} activation within tumors and draining lymph nodes. Foxp3$^+$ T_{reg} are activated by plasmacytoid dendritic cells (pDCs) expressing IDO in tumor-draining lymph nodes. This activation requires MHC expression by antigen presenting cells, and activation of the GCN2 stress pathway in T_{reg}. Myeloid-derived suppressor cells (MDSCs) also activate T_{reg} in tumors by a mechanism requiring arginase. T_{reg} activation can also depend on CD70 expression and IL-2 production by tumor-infiltrating effector T cells

the general control nonderepressible-2 (GCN2) stress pathway, which is associated with amino acid starvation [71]. Importantly, the competitive inhibitor 1-methyl-tryptophan could reverse T_{reg} activation by pDCs [71]. While IDO function clearly promoted T_{reg} activation, DC expression of MHC-II was also needed, confirming a requirement for antigen recognition by T_{reg} [71], [72].

Myeloid-derived suppressor cells (MDSCs) are another type of APC that have been found to activate Foxp3$^+$ T_{reg} in tumor-bearing hosts. MDSCs associated with a murine B cell lymphoma model were shown to expand natural Foxp3$^+$ T_{reg} [73]. Expansion of T_{reg} populations was dependent on arginase production by MDSCs, and could, thus, be inhibited with sildenafil or N-hydroxy-L-arginine (NOHA) [73]. While lymphoma-associated MDSCs could activate preexisting Foxp3$^+$ T_{reg}, TGF-β did not play a role in this process, and Th0 cells were not converted to iT_{reg} [73].

Finally, there are reports that other factors in the tumor microenvironment can contribute to T_{reg} activation. Tumor cell-derived high-mobility group box 1 (HMGB1), a protein associated with tumor cell invasion and metastasis, was shown to be important for the induction of Foxp3$^+$ cells in the 4T1.2 Neu mouse breast tumor model [74]. In the MC57 tumor model, signaling through CD27 directly on T_{reg}, likely by CD70 expressed on other tumor-infiltrating CD4 T cells, was shown to be important for T_{reg} accumulation in tumors and the suppression of antitumor immunity [75]. CD27 engagement on effector T cells also induced IL-2 production, which prevented T_{reg} cell apoptosis [75]. Thus, both tumor cells and tumor-infiltrating leukocytes can cooperate to promote the survival and activation of Foxp3$^+$ T_{reg} in the tumor microenvironment.

2.4.2 Generation of iT_{reg}

TGF-β plays a fundamental role in the conversion of Th0 cells to iT_{reg}. First identified by Sporn as a secreted product of murine sarcoma cells in 1982, TGF-β was shown to promote neoplastic cell transformation and anchorage-independent growth [76], [77]. Shortly thereafter, its role in the suppression of T-cell responses was recognized *in vitro* [78]. In 2001, mice harboring a TGF-β dominant negative receptor were shown to mount robust T-cell responses to B16 melanoma and EL4 thymoma tumors, establishing TGF-β responsiveness as a key determinant in the suppression of antitumor immunity [79]. However, a direct link between TGF-β and T_{reg} induction was not demonstrated until 2003, when TGF-β was shown to induce Foxp3 expression in conventional $CD4^+CD25^{neg}$ T cells [12]. In separate studies, TGF-β was found to be dispensable for nT_{reg} development in the thymus, although important for T_{reg} maintenance in the periphery [80] (Fig. 1.3).

Several studies in mouse models have since implicated TGF-β in the conversion of Th0 cells to iT_{reg} within the tumor microenvironment. In a rat carcinoma model, as well as the B16 melanoma model, immature DCs were shown to be key producers of TGF-β. TGF-β from these tumor-licensed DCs induced the proliferation of preexisting T_{reg} and the generation of iT_{reg} [81]. TGF-β produced by TRAMP prostate cancer cells was also shown to convert $CD4^+CD25^{neg}$ cells into iT_{reg} *in vitro* [82]. *In vivo*, complete neutralization of TGF-β with the mAb clone 1D11 prevented the accumulation of T_{reg} in renal cell carcinoma (RENCA) tumors growing in lungs [82] and in transplantable PanO2 pancreatic tumors, which are strong producers of TGF-β [83]. Furthermore, T cells expressing a TGF-β-dominant negative receptor were used to show that TGF-β responsiveness in CD4 T cells is required for the generation of iT_{reg} in response to B16 melanoma tumor growth [84] and PanO2 tumors [83]. Thus, TGF-β from either tumor cells or immune cells in the tumor microenvironment acts locally on CD4 T cells to induce their conversion to iT_{reg}.

MDSCs are another important mediator of iT_{reg} conversion in tumor microenvironments. In mice bearing MCA26 tumors expressing the neoantigen HA, naïve HA-specific transgenic T cells were efficiently converted to $Foxp3^+$ iT_{reg} by a process requiring $Gr1^+CD115^+$MDSCs [85]. These tumor-associated MDSCs induced Foxp3 expression through a mechanism involving IL-10 and IFN-γ, but not inducible nitric oxide synthase (iNOS) [85]. Further work showed that MCA26 colon tumor-associated MDSCs require CD40 to drive T_{reg} proliferation, which can explain why blockade of CD40 in mice with large tumors actually impaired the efficacy of immunotherapy [86]. Accordingly, blockade of the SCF/cKit pathway resulted in decreased MDSC and Treg accumulation in MC26 tumors [87].

Despite convincing evidence that MDSCs promote T_{reg} responses to cancer, one recent study suggests that tumor-associated MDSCs can also impair iT_{reg} generation. Suppressive $CD11b^+$Ly-$6G^+$MDSCs taken from mice bearing LLC or 4T1 breast carcinoma were found to impair iT_{reg} conversion by TGF-β *in vitro* [88]. This impairment relied on a mechanism involving reactive oxygen species and, surprisingly,

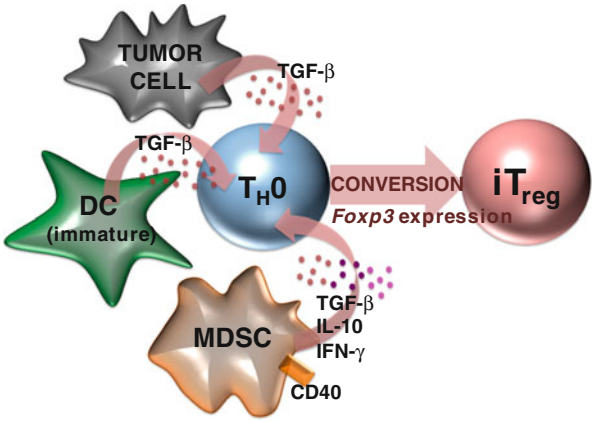

Fig. 1.3 Mechanisms of iT_{reg} conversion by tumor cells and associated antigen presenting cells. Tumor cells and immature DCs produce TGF-β, which results in the conversion of CD4 T cells to Foxp3-expressing iT_{reg} within tumors. MDSCs can also produce TGF-β, and have been shown to drive iT_{reg} conversion through a mechanism involving IL-10, IFN-γ, and CD40

IDO [88]. Whether MDSCs serve such diametric roles in regulating T_{reg} responses to tumors *in vivo* remains to be seen.

2.5 *Mechanisms of T_{reg}-Mediated Suppression of Antitumor Immunity*

After nT_{reg} and iT_{reg} have been recruited and activated within the tumor microenvironment, they begin to suppress T-cell responses locally. T_{reg} can suppress CD4 and CD8 T cells at both the priming and effector phases of the response. They do so through a variety of mechanisms involving secreted factors and interactions with APCs. While a myriad of suppressive mechanisms have been attributed to T_{reg} in general, the present section deals predominantly with those mechanisms that are operational in models of cancer (Fig. 1.4).

2.5.1 CTLA-4

Studies by Allison and colleagues in the 1990s showed that CTLA-4 blockade could induce potent antitumor immunity either as a monotherapy or in combination with vaccines [89], [90]. Accordingly, humanized anti-CTLA-4 (ipilimumab or YERVOY™) is now a Food and Drug Administration (FDA)-approved drug for the treatment of metastatic melanoma. However, because both T_{reg} and activated effector T cells express CTLA-4, the relative importance of blocking CTLA-4 on these two subsets was not fully known until recently. *In vitro* studies showed that CTLA-4 blockade expands human T_{reg} and effector T cells, but enables T_{reg} to maintain their suppressive function, suggesting that CTLA-4 blockade preferentially drives effector T-cell function [91]. However, in 2008, Sakaguchi showed that selective CTLA-4 deficiency in T_{reg} induces potent immunity against radiation leukemia (RL)

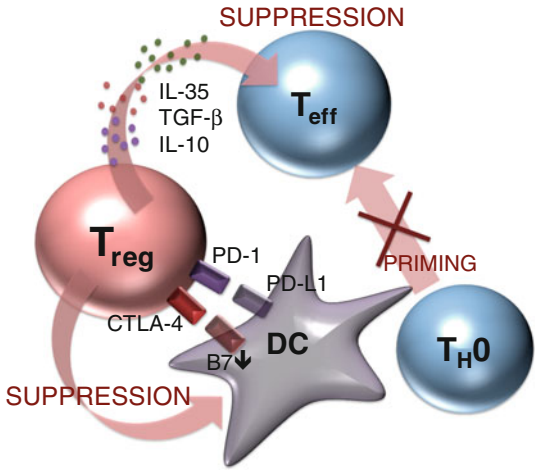

Fig. 1.4 Mechanisms of T_{reg} suppression of antitumor effector T cells. T_{reg} production of IL-35, TGF-β, IL-10, has been implicated in the direct suppression of effector T-cell responses. T_{reg} also impair DC function through CTLA-4 and PD-1. CTLA-4 expressed by T_{reg} has been shown to downregulate DC expression of the costimulatory molecule B7, potentially leading to impaired effector T-cell priming

male leukemia tumors, demonstrating that CTLA-4 also exerts direct control over T_{reg} function in cancer [92].

Further evidence supporting a role for CTLA-4 on T_{reg} comes from elegant studies in *CTLA-4*$^{-/-}$ mice bearing a functional replacement with human/mouse chimeric CTLA-4 that interacts with mouse B7 [93]. In contrast to *CTLA-4*$^{-/-}$ mice that suffer from an early fatal lymphoproliferative syndrome, chimeric CTLA-4 mice survive to adulthood, and could, thus, serve as a source of functional T cells [93]. Using combinations of T_{reg} and effector T cells expressing either chimeric or wild-type CTLA-4 to reconstitute B16 tumor-bearing mice, and then treating mice with corresponding human or mouse CTLA-4 blocking antibodies, CTLA-4 blockade was restricted to either the regulatory or conventional T-cell compartment [93]. Results of these experiments showed that the full antitumor effect of CTLA-4-blocking antibodies required direct engagement of both effector and T_{reg} [93]. Thus, CTLA-4 expression on T_{reg} is important for their immunosuppressive role *in vivo*.

The mechanism whereby CTLA-4 mediates T_{reg} suppression likely involves direct interaction with APCs. In support of this, T_{reg}-surface CTLA-4 was required to engage B7 for dendritic cells (DCs) to fully induce IDO expression [71]. CTLA-4 deficiency also impaired the ability of T_{reg} to downregulate the expression of costimulatory molecules CD80 and CD86 on DCs [92]. Thus, suppression through CTLA-4 likely involves a three-cell model whereby T_{reg} act on DCs to induce an immunosuppressive phenotype, thereby, preventing the priming of tumor-specific effector T cells.

Very recent studies also elucidate a role for anti-CTLA-4 in directly depleting T_{reg} within the tumor microenvironment [94]. It was found that T_{reg} in B16 melanoma tumors express elevated levels of CTLA-4 and are depleted by anti-CTLA-4 in an FcγR-dependent fashion [94]. Accordingly, anti-CTLA-4 therapy was ineffective against B16 tumors in *FcγRIV*$^{-/-}$ mice [94]. Future studies are warranted to determine if ipilimumab functions through similar mechanisms in patients with melanoma.

2.5.2 IL-35

Vignali and colleagues have shown that T_{reg} produce high levels of IL-35, which directly suppresses effector T-cell proliferation [95], [96]. In hosts bearing either B16 or MC38 tumors, infiltrating Foxp3$^+$ T_{reg} were shown to produce IL-35, which further promoted the production of IL-35 by Foxp3^{neg} CD4 T cells, a population termed iTr35 cells [96]. By reconstituting tumor-bearing *RAG*$^{-/-}$ mice with nT_{reg} and IL-35-responsive or nonresponsive CD4 T cells, it was shown that IL-35 responsiveness in CD4 T cells is required for optimal suppression of CD8 T-cell responses to melanoma [96]. Thus, IL-35 may participate in the decades-old theory of infectious tolerance whereby tumor-associated T_{reg} confer suppressive function to other T-cell subsets [97]. T_{reg} specific for human prostate cancer antigens were recently shown to suppress through IL-35 *in vitro* [98], although IL-35 expression by human T_{reg} remains controversial. Based on these findings, IL-35 may prove to be a major mechanism of T_{reg}-mediated suppression in cancer.

2.5.3 IL-10 and TGF-β

T_{reg}-produced IL-10 and TGF-β can directly suppress effector T-cell responses, and both of these cytokines have been implicated as mediators of infectious tolerance [97]. With regard to cancer, the most compelling evidence that IL-10 and TGF-β mediate T_{reg} suppression come from *in vitro* studies involving human T cells. Foxp3$^+$ cells isolated from patients with head and neck squamous cell carcinoma have been shown to secrete both IL-10 and TGF-β, which mediated suppression of effector T-cell responses [99]. IL-10-containing and TGF-β-containing exosomes, derived from human tumor cells, have also been shown to induce T_{reg} that can in turn suppress through IL-10 and TGF-β [100]. Recently, T_{reg} isolated from human hepatocellular carcinoma were shown to suppress the function of γδT cells through IL-10 and TGF-β [101].

While TGF-β has been shown to mediate T_{reg} suppression *in vitro*, it is unclear that similar mechanisms govern T_{reg} suppression *in vivo* [102]. TGF-β was found to be the major mechanism of suppression of TRAMP prostate tumor-infiltrating CD8$^+$ TcR-I cells [18]. These cells express some T_{reg} markers such as CD25 and GITR, but were predominantly Foxp3^{neg} [18]. Thus, while TGF-β is considered important for generating iT_{reg} and maintaining T_{reg} in the periphery (see Sect. 2.4.2.), *in vivo* data do not yet support TGF-β as a major mediator of T_{reg} suppressive function in tumor models.

Similarly, IL-10 has been shown to be a mediator of T_{reg} suppression at mucosal surfaces, but not in somatic tissues [103]. Accordingly, there exists controversy regarding IL-10 as mediator of T_{reg} suppression in tumor models. One study showed that *IL-10*$^{-/-}$ T_{reg} from 4T1 tumor-bearing mice were less suppressive as compared to wild-type T_{reg} [74]. However, other studies with *IL-10*$^{-/-}$ cells demonstrate that APC-derived (but not T_{reg}-derived) IL-10 is important for suppression [104]. Interestingly, recent studies show that IL-10 can actually support immune responses against carcinogen-induced tumors [105]. In this setting, host IL-10 deficiency

resulted in increased numbers of MDSC and T_{reg} in tumors [105]. Thus, despite its role in T_{reg}-mediated suppression at mucosal surfaces, IL-10 could actually support immune surveillance of some cancers.

2.5.4 PD-1

There is growing evidence that the programmed death-1 (PD-1) pathway plays a role in T_{reg}-mediated suppression of antitumor immunity. PD-1, expressed predominantly on exhausted CD8 T cells, is a negative regulator of T-cell function [106]. The ligand for PD-1, PD-L1 (B7-H1), is expressed on a variety of cells in tumor microenvironments including tumor cells themselves, T_{reg}, and MDSCs [107], [108]. Clinical trials of a monoclonal anti-PD-1 blocking antibody have already demonstrated encouraging responses in patients with various solid cancers [109]. Tumor cell-expressed PD-L1 clearly mediates immune suppression, and expression of PD-L1 on cancer cells is associated with responsiveness to therapy [110]. However, studies are now beginning to shed light on a role for PD-1 on T_{reg} as well.

In samples of T cells taken from melanoma patients, PD-1 blockade was found to enhance effector T-cell proliferation and inhibit the suppressive function of PD-L1 expressing T_{reg} [111]. Furthermore, in the B16 model, T_{reg} from tumor-draining lymph nodes could suppress via the PD-1/PD-L1 pathway [71]. This mechanism may be particular to T_{reg} induced by IDO-expressing pDC, because the function of conventional T_{reg} (induced by anti-CD3 and IL-2) could not be abrogated by PD-1 blockade [71]. However, in a mouse model of acute myelogenous leukemia, studies with $PD1^{-/-}$ T_{reg} demonstrated that PD-1 expression on T_{reg} and PD-L1 expression on APCs were both required for CD8 T-cell suppression *in vitro* [112]. Additional *in vivo* mechanistic studies (such as those described for CTLA-4-blocking antibodies in Sect. 2.5.1) will be required to dissect the relative importance of inhibiting PD-L1 on specific cell subsets. However, these initial studies suggest an immunosuppressive role for PD-L1 on T_{reg}.

2.5.5 Other Potential Mechanisms of Suppression

Numerous other suppressive mechanisms have been attributed to T_{reg}, although formal evidence of their role in the suppression of antitumor immunity remains lacking. Regardless, the potential involvement of two additional mechanisms bears mention. The first of these is the generation of adenosine. T_{reg} have been shown to express CD39 and CD73 ectoenzymes that can generate extracellular adenosine from adenosine triphosphate (ATP), and extracellular adenosine has been implicated as a mechanism of T_{reg} suppression both *in vitro* and *in vivo* [112]. Human Tr1 generated *in vitro* were also shown to produce high levels of adenosine [113]. Extracellular adenosine is known to accumulate in the tumor microenvironment as a result of hypoxia [114]. Adenosine responsiveness through the A2A adenosine receptor was also shown to directly promote proliferation and suppressive function of T_{reg}, which could provide a possible mechanism to amplify suppression in the tumor microenvironment [13], [115].

A second likely mechanism is the production of granzymes [116]. Gondek and Noelle showed that activated T_{reg} upregulate expression of granzyme B (GzB), which suppressed T-cell responses *in vitro*, in part through target cell apoptosis [116]. Accordingly, GzB expression specifically in T_{reg} was shown to be crucial for the establishment of long-term allograft survival *in vivo* [117]. Because GzB is also a mediator of CD8 T-cell responses against tumors, experiments involving T_{reg}-specific deletion of GzB will be important to elucidate a specific role for T_{reg}-produced GzB in cancer models.

2.5.6 T_{reg} Promotion of Tumor Angiogenesis and Metastasis

In addition to their primary role as suppressors of antitumor immunity, recent studies have implicated T_{reg} in the promotion of tumor invasiveness. As mentioned above, studies demonstrating that tumor hypoxia recruits T_{reg} through the CCL28/CCR10 axis also showed that recruited T_{reg} produce high levels of VEGF-A within the tumor microenvironment [68]. This study provided the first evidence that T_{reg} could directly promote tumor angiogenesis [68]. Recent work in the mouse mammary tumor virus (MMTV)-Erbb2 transgenic mouse model also demonstrated that T_{reg} can directly promote metastasis [118]. Breast tumor metastasis to the lungs involved Receptor activator of nuclear factor kappa-B ligand expression on T_{reg}, which stimulated $RANK^+$ breast cancer cells to metastasize [118]. These direct tumor-promoting functions of T_{reg} are only beginning to be explored, and the extent to which they contribute to poor outcomes in patients with cancer remains to be seen.

3 Targeting T_{reg} as Cancer Immunotherapy

Strategies to block the negative checkpoint inhibitors CTLA-4 and PD-1 were already entering cancer clinical trials before their inhibitory effects on T_{reg} were fully appreciated. However, it is now clear that the most effective immunotherapies for cancer must involve disabling regulatory T cells. Methods for impairing T_{reg} function can be divided into three main categories: depletion, costimulation, and retroconversion. This section provides a discussion of these strategies with a focus on the most promising and well-described approaches.

3.1 Depletion of T_{reg}

3.1.1 Cyclophosphamide

Cyclophosphamide has a notable history as one of the earliest methods for depleting T_{reg}. First shown to have antitumor properties in the late 1950s [119], cyclophosphamide was rapidly translated as a therapy for children with acute leukemia [120].

In 1979, studies by Glaser were the first to demonstrate that antitumor effects of cyclophosphamide could be due to the depletion of suppressive T cells [121]. Studies by North in the 1980s confirmed this by showing that cyclophosphamide could induce regression of a syngeneic lymphoma in mice, if given around the time of tumor implantation [122]. This therapeutic effect could be inhibited by transfer of "suppressive L3T4$^+$ T cells from normal donor mice," indicating that cyclophosphamide was preferentially destroying a suppressor cell population [122]. In 2004, our work showed that cyclophosphamide elicited concomitant immunity against the poorly immunogenic B16 melanoma, further suggesting its role in the depletion of T_{reg} [8].

Now it is widely accepted that cyclophosphamide can deplete T_{reg} associated with cancer; however, its effects have been found to be highly dose dependent. Low doses preferentially but partially deplete CD4$^+$CD25$^+$Foxp3$^+$ T_{reg} and also decrease their homeostatic proliferation and suppressive capability [123]. Appropriately dosed and timed cyclophosphamide was also shown to deplete rapidly proliferating T_{reg}, thereby, driving high-avidity T-cell responses in the neu-NT breast tumor model [124]. At higher doses, T_{reg} are more completely depleted, but toxicity is observed against CD8 and CD4 effector T-cell subsets [123].

Multiple clinical studies in humans have recapitulated these findings in mouse models [125], [126]. In end-stage cancer patients, metronomic (low dose, daily) dosing of cyclophosphamide was shown to decrease T_{reg} numbers and suppressive function [127]. Similar effects of metronomic cyclophosphamide have been observed in the blood of patients with hepatocellular carcinoma, wherein alpha fetoprotein (AFP)-specific CD4 T-cell responses also increased [128]. In patients with solid tumors, cyclophosphamide decreased T_{reg} populations and did not impair CD8 T-cell responses to an oncolytic adenovirus [129]. However, in one study involving patients with metastatic melanoma, no decrease in T_{reg} populations was observed as a result of metronomic cyclophosphamide [130]. Thus, the effectiveness of T_{reg} depletion may vary depending on the type and/or stage of cancer.

In addition to T_{reg} depletion, recent studies have demonstrated additional immunomodulatory effects of cyclophosphamide [126]. It was shown that cyclophosphamide can promote the generation of Th17 responses in mice [131], [132], and in patients with solid tumors [132]. Cyclophosphamide has also been shown to promote immunogenic cancer cell death [133] and drive immunogenic tumor antigen release in the B16 melanoma model [134]. Thus, cyclophosphamide has complex immune-modulating properties beyond the depletion of T_{reg}.

3.1.2 CD25 Depletion

The discovery that T_{reg} constitutively express high levels of the IL-2R-α chain CD25 prompted an ongoing series of experiments to deplete T_{reg} with anti-CD25 antibodies in tumor-bearing hosts. However, because CD25 is also expressed by activated T cells, anti-CD25 treatment is typically given early to avoid the depletion of effectors. Studies have shown that anti-CD25 promotes T-cell responses in a variety of mouse

tumor models [135]–[138]. Our own studies with B16 melanoma showed that anti-CD25 did not affect primary tumor growth, but could elicit concomitant immunity and postsurgical immunity against melanoma tumor rechallenge [8], [139].

In cancer patients, clinical trials have been conducted using the humanized anti-CD25 mAb daclizumab, a therapy that was originally developed in the 1990s for the prevention of acute kidney graft rejection [140]. In one such study, 30 melanoma patients received daclizumab prior to DC vaccination [141]. Patients achieved efficient depletion of $CD25^{hi}$ cells including $Foxp3^+$ T_{reg} within 4 days of treatment. Vaccine antigen-specific T-cell responses were also generated; however, daclizumab may have prevented T cells from acquiring effector functions. Daclizumab also had no effect on progression-free survival in these patients. More recently, daclizumab was given as an infusion to breast cancer patients 1 week prior to peptide vaccination [142]. In this study, rapid and significant reduction in numbers of $Foxp3^+$ T_{reg} (as high as 77 % reduction) was again observed, and nine out of ten patients generated vaccine antigen-specific T-cell responses. However, in contrast to the melanoma study, these T cells were functional with regard to CD107A mobilization and IFN-γ secretion. Promising results of this second study merit further investigation, although the effectiveness of daclizumab may vary depending on the type of vaccine with which it is administered.

Another strategy to deplete CD25-expressing T_{reg} is through the use of the IL-2-DT fusion protein known as denileukin diftitox, or Ontak. Ontak is cytotoxic to IL-2R-α-expressing cells and was originally FDA approved for the treatment of cutaneous T-cell lymphoma. In patients with metastatic disease, a single dose of Ontak was shown to reduce T_{reg} proportions within 4 days, and also enhance vaccine antigen-specific CD8 T-cell responses by ELISPOT [143]. In another study of patients with CEA-expressing malignancies, four doses of Ontak were shown to eventually reduce blood $CD4^+CD25^+Foxp3^+$ cell counts [144]. In a phase I trial in metastatic melanoma, more frequent and prolonged Ontak dosing was shown to have effects on non-T_{reg} T cells in blood, but also induced partial clinical responses in 5 out of 16 patients [145]. A subsequent phase II trial in stage IV melanoma demonstrated a partial response in 16 % of patients [146]. Thus, levels of antitumor immunity achieved by Ontak treatment alone are suboptimal.

3.1.3 CD4 Depletion

Another means for depleting T_{reg} is by targeting CD4. In mice bearing progressive B16 melanoma, we showed that treatment with anti-CD4 (mAb clone GK1.5) efficiently removed T_{reg} and drove the systemic priming of melanoma antigen-specific CD8 T-cell responses that were protective against tumor rechallenge [8]. Similarly, Fu and Shreiber showed that injection of anti-CD4 directly into established murine fibrosarcomas could lead to the regression of large tumors [147]. Treatment with anti-CD4 in conjunction with PD-L1 blockade and tumor cell vaccination also drove the rejection of established RENCA tumors [148].

The obvious concern with this approach is the co-depletion of CD4-expressing helper T cells. T-cell help has been thought to be crucial for the optimal priming of CD8 T-cell responses and also for the generation of functional CD8 T-cell memory. However, in studies with B16 melanoma, we observed that CD4 depletion in tumor-bearing hosts actually promoted the development of CD8 memory T-cell responses to melanoma [139]. Importantly, T_{reg} depletion with anti-CD4, followed by surgical melanoma excision, also drove the development of melanocyte-specific autoimmune disease (i.e., vitiligo), which was required to maintain memory CD8 T cells [149]. Even complete and prolonged CD4 T-cell depletion did not significantly impair the priming, maintenance, function, or protective capabilities of CD8 T-cell responses to melanoma in hosts with vitiligo [150]. Thus, factors in the autoimmune host may supersede the requirement for CD4 T-cell help in the generation of CD8 T-cell memory to tumor/self-antigens. Moreover, as CD4 T-cell responses in late-stage melanoma patients have been found to be overwhelmingly Th2 polarized [151], complete loss of the CD4 T-cell compartment may be particularly beneficial to the host. These studies suggest that CD4-depleting mAbs such as zanolimumab, which is currently undergoing clinical trials as a tumor cell-depleting therapy in cutaneous T-cell lymphoma (CTCL) patients [152], may be promising T_{reg}-depleting therapies in the future.

3.1.4 FR4 Depletion

In 2007, Sakaguchi and colleagues reported that T_{reg} preferentially express high levels of the protein folate receptor 4 (FR4 or FRδ) [153]. Whereas an important role for this receptor in T_{reg} function or homeostasis has yet to be shown, FR4 is expressed constitutively by nT_{reg} and also on iT_{reg} upon conversion *in vitro* [153]. Using a mouse mAb to FR4 (clone TH6), *in vivo* depletion of T_{reg} beginning when tumors were well established (day 8), resulted in impressive total rejection of a majority of MethA and CT26 tumors. Anti-FR4 treatment did not deplete a population of peripheral effector/memory-like T cells that expressed lower levels of FR4, demonstrating its selective ability to deplete $FR4^{hi}$ T_{reg} [153]. Accordingly, *in vitro* pretreatment of tumor antigen-stimulated T cells with anti-FR4 effectively depleted regulatory T cells, but not activated/effector T cells, providing a superior T-cell population for adoptive therapy against advanced MethA tumors. Thus, anti-FR4 appears to be a potent means for selectively depleting T_{reg} without apparent harm to effector T cells.

More recently, a direct comparison between anti-FR4, anti-CD25, and anti-CD4 T_{reg} depletion strategies was conducted in the RENCA and MethA tumor models [154]. While each therapy was effective, there existed subtle differences in the effector mechanisms that they initiated. All three treatments induced antitumor immunity that was mediated by CD8 T cells. However, tumor immunity induced by anti-FR4 also required NK cells. Requirements for effector cytokines also differed between the groups. Anti-CD25 treatment required IFN-γ, IL-4, and IL-13; anti-FR4 treatment did not require IFN-γ but was partially dependent on Th2 cytokines; and anti-CD4

treatment did not appear to require any one specific cytokine [154]. These important studies demonstrate the variable downstream mechanisms underlying different T_{reg} depletion strategies and suggest that a single strategy may not be ideal in all circumstances.

3.2 T_{reg} Costimulation

As described in Sect. 2.5, blockade of CTLA-4 and PD-1 are two therapeutic approaches that can overcome T_{reg} suppressive function *in vivo*. Numerous cancer patients are already benefiting from these therapies in the clinic. However, in addition to blocking negative checkpoint regulators, it has been shown that engaging surface costimulatory molecules on T_{reg} can also attenuate their suppressive function. Evidence for the importance of two such molecules—the glucocorticoid-induced tumor necrosis factor (TNF) family-related receptor (GITR), and CD134 (OX40)—in T_{reg} responses to tumors is presented below.

3.2.1 GITR Stimulation

Similar to CD25, the TNF family-related receptor GITR is expressed constitutively by T_{reg}, but only upon activation by CD8 and CD4 effector T cells [155], [156]. In 2004, we showed that GITR stimulation (using mAb clone DTA-1) in mice bearing progressive B16 melanoma induced concomitant immunity against secondary tumors [8]. Concomitant immunity induced by anti-GITR was similar to that observed upon treatment with anti-CD25, anti-CD4, or cyclophosphamide, suggesting a role for anti-GITR in directly inhibiting T_{reg} function [8]. The following year, it was shown that treatment with agonistic anti-GITR induced total rejection of more established highly immunogenic tumors, indicating the potency of anti-GITR as a monotherapy [157]. Early, frequent, and high-dose administration of anti-GITR has also been shown to induce rejection of more poorly immunogenic B16 tumors [8], [158], [159].

Because GITR is expressed by both T_{reg} and activated effector T cells, the relative importance of GITR stimulation on these different subsets remained an open question during these initial studies. GITR costimulation can clearly influence effector T cells and has been shown to enhance their survival, proliferation, cytokine production, and resistance to T_{reg}-mediated suppression [160]–[162]. Recently, Cohen and Wolchok examined the cellular effects of GITR stimulation and found that T_{reg} accumulation and stability within B16 tumors was reduced upon treatment with agonistic anti-GITR [163]. Studies in $RAG^{-/-}$ mice reconstituted with $GITR^{-/-}$ T_{reg} or effector T cells further showed that GITR stimulation directly on both populations was required for optimal impairment of B16 tumor growth [163]. Interestingly, in similar $RAG^{-/-}$ reconstitution experiments, we found that systemic concomitant immunity against B16 melanoma did not require GITR expression on T_{reg}, but rather that agonistic

anti-GITR acted solely by stimulating effector CD8 T cells [164]. Thus, while GITR is clearly an important costimulatory molecule for effector T cells, the importance of GITR stimulation directly on T_{reg} remains in question.

In our studies comparing agonistic anti-GITR to T_{reg} depletion with anti-CD4, we observed that GITR stimulation was not sufficient to break tolerance to self-antigens, but rather that anti-GITR preferentially drove the recognition of tumor-specific antigens [164]. We also found that GITR stimulation had the unique ability to induce antitumor immunity without concurrent autoimmunity [164]. This may set GITR agonists apart from checkpoint inhibitors, such as anti-CTLA-4, which have pronounced autoimmune side effects. A humanized, agonistic anti-GITR antibody is currently being tested in patients with unresectable melanoma and other solid tumors [165], which should provide important insights into this therapeutic approach.

3.2.2 OX40 Stimulation

OX40 (CD134) is another member of the TNFR superfamily that is expressed on T_{reg} and activated T cells. Most $Foxp3^+$ T_{reg} infiltrating CT26 tumors were shown to express high levels of OX40 [166]. It was also shown that systemic pretreatment of mice with agonistic anti-OX40 antibody could protect against CT26 tumor challenge, and that direct injection of anti-OX40 into established tumors could induce complete tumor rejection [166]. Moreover, agonistic anti-OX40 was more effective than anti-CD25 or anti-CD4 T_{reg} depletion in this setting. The mechanism of tumor rejection in OX40-stimulated mice was shown to be CD8 T-cell mediated, with anti-OX40 potentially having direct effects on T_{reg}, effector T cells, and also enhancing DC migration to tumor-draining lymph nodes [166].

Subsequent studies by Hirshchorn and Houghton showed that combination therapy with OX40 and cyclophosphamide was potent enough to regress established B16 tumors [134]. Data showed that cyclophosphamide upregulated expression of OX40 on T_{reg}, enabling anti-OX40 to subsequently induce T_{reg} apoptosis within tumors. Thus, the combination of cyclophosphamide and anti-OX40 resulted in a more favorable CD8 to T_{reg} ratio within tumors [134]. This study illustrated the unexpected synergistic effects between two T_{reg}-modulating therapies. Agonistic OX40 antibodies are currently being used in multiple cancer clinical trials, with one study employing anti-OX40, cyclophosphamide, and radiation therapy in metastatic prostate cancer patients [167].

3.3 iT_{reg} Plasticity and Retroconversion in Tumors

3.3.1 The Principle of Retroconversion

iT_{reg} and inflammatory Th17 cell subsets are closely related by their requirement for TGF-β during priming. Because IL-6 is also required for the generation of Th17

cells, it has been recognized that the introduction of inflammatory stimuli can result in the conversion of T_{reg} to Th17-like cells. Indeed, using reporter mice in which $Foxp3^+$ cells and their progeny are permanently marked, it was shown that 10–20 % of peripheral CD4 T cells had actually lost Foxp3 expression and became capable of producing IFN-γ and IL-17 [168]. This generation of "ex-Foxp3 cells" was increased by pancreatic destruction and associated antigen recognition [168]. Vaccination with CpG (a TLR9 agonist) was similarly shown to result in T_{reg} expression of IL-2, IL-17, TNF-α, and CD40 L [169]. In contrast to "ex-Foxp3 cells," CpG-stimulated T_{reg} maintained Foxp3 expression, although microscopy revealed that Foxp3 was sequestered in an unusual pattern in the nucleus [169]. These studies establish the principle of T_{reg} plasticity and lead to the question of whether T_{reg} retroconversion can take place efficiently within tumor microenvironments.

3.3.2 T_{reg} Retroconversion in Cancer

T_{reg} retroconversion has been examined in mice bearing B16 tumors expressing OVA. In tumor-draining lymph nodes, T_{reg} were shown to be converted to Th17-like cells by IL-6-producing pDC upon treatment with the IDO inhibitor 1-methyl-D-tryptophan [170]. This conversion also took place upon adoptive transfer of vaccine-activated OVA-specific CD8 T cells (OT-1 cells), which blocked IDO production by DC through B7/CD28 interactions. While Th17-like cells lost suppressive function and gained helper function, these cells still maintained Foxp3 expression [170]. Thus, T_{reg} retroconversion by blockade of IDO appears to be either transient or incomplete.

In $RAG^{-/-}$ mice bearing MB49 tumors, H-Y antigen-specific T_{reg} were shown to lose Foxp3 expression and become "ex-T_{reg}" [171]. Significant retroconversion was observed in both nT_{reg} and iT_{reg} populations [171]. It is likely that the lymphopenic environment in these mice contributed to Foxp3 loss, as studies investigating Foxp3 expression by T_{reg} in lymphoreplete mice argue against substantial plasticity of nT_{reg} [103]. Retroconversion of tumor-associated T_{reg} can also be achieved *in vitro*. Human T_{reg} ($CD4^+CD25^+CD127^{low}$) sorted from epithelial ovarian cancer and cultured with tumor-associated CD3-negative cells and IL-2, demonstrated Foxp3 downregulation, loss of suppressive function, and the ability to produce IL-17 [172]. Whether driving T_{reg} retroconversion will be a durable approach to tumor immunotherapy remains to be seen.

3.3.3 Controversy Regarding T_{reg} Plasticity

The idea that T_{reg} can acquire helper functions in the face of inflammation has been considered inconsistent with their role of suppressing autoimmunity, and it has been argued that "ex-Foxp3 cells" were, in fact, never actually true T_{reg} [173]. In defining regulatory T cells, it has become important to consider epigenetic factors that regulate Foxp3 expression. The Foxp3 locus contains a TGF-β sensor/enhancer region whereby TGF-β binding and TCR stimulation promote Foxp3 expression

that is unstable, for example, in iT_{reg} [174]. The Foxp3 locus also contains an enhancer/stabilizer region that must be demethylated to cause stable Foxp3 expression as is found in natural T_{reg} [174], [175]. It has recently been shown that the apparent plasticity of $Foxp3^+$ cells may be explained by this heterogeneity, with only a minor population of peripheral $Foxp3^+$ cells giving rise to "ex-Foxp3" Th17 cells in response to lymphopenia or inflammatory cytokine signals [176]. These cells are presumably iT_{reg}, because naturally occurring $Foxp3^+$ cells, whose enhancer regions are demethylated, were resistant to producing IL-17 and losing Foxp3 [176].

In the future, it will become important to profile T_{reg}-specific demethylated region (TSDR) methylation status in tumor-infiltrating T_{reg}, particularly during vaccination and immunotherapy. If TSDR demethylation can be perturbed by therapy, this would indicate that T_{reg} can be durably reprogrammed. While naturally occurring thymic T_{reg} may be resistant to retroconversion, tumor-induced iT_{reg} may be a more realistic target for this type of therapy.

4 Future Classifications of T_{reg} in Cancer

During the past few years, more detailed classifications for T_{reg} have been developed based on the types of effector T-cell responses that they regulate. These definitions serve to explain how T_{reg} can home to an array of different inflamed tissues, and suppress a wide range of effector T-cell responses [177], [178]. Thus, T_{reg} subsets have now been described in association with each major type of helper T cells.

Th1-like-T_{reg} were first discovered by Koch and Campbell in 2009 [179]. These T_{reg} express the Th1-specific transcription factor T-bet in response to IFN-γ, and were shown to be induced in models of infection with *Leishmania* or *mycobacterium tuberculosis* [179]. T-bet was shown to induce expression of CCR3, which enabled T_{reg} to traffic to sites of inflammation and also promoted homeostasis during persistent infection. T-bet expression in T_{reg} was also shown to be required for the control of autoimmunity in scurfy mice, indicating the importance of Th1-like-T_{reg} for the maintenance of peripheral tolerance [179]. T-bet-expressing T_{reg} have also recently been identified in humans [180]. It remains to be seen whether T-bet is expressed by T_{reg} within tumor microenvironments, although one could speculate that Th1-like-T_{reg} play a role in suppressing Th1 and CD8 T-cell responses to cancer.

Th2 and Th17-type T_{reg} have also been identified in mice and humans [180]–[182]. Th2-like-T_{reg} express the Th2-associated transcription factor IRF4 as well as CCR8, which enables their migration to sites of Th2 inflammation [181]. Similarly, Th17-like-T_{reg} require STAT-3 to produce IL-10 and suppress Th17-mediated autoimmune colitis [182], [183]. Alternatively, T_{reg} expressing the Th17-specific transcription factor related orphan receptor (ROR-γt) have been shown to produce IL-17 [184], [185]. Thus, expression of Th17-associated factors such as STAT-3, instead of ROR-γt, may enable T_{reg} to suppress Th17 responses without undergoing retroconversion to Th17 cells as discussed above. Future studies will be needed to determine whether these specific types of T_{reg} are generated in response to tumor initiation or progression.

5 Conclusions

Our knowledge of T_{reg} has grown exponentially over the last decade, and it is now difficult to envision the tumor microenvironment without considering T_{reg} as a dominant suppressive force. Both nT_{reg} and iT_{reg} comprise the Foxp3-expressing T-cell populations within tumors, where they respond to a multitude of tumor-associated antigens and immunosuppressive factors produced by tumor cells and associated APCs. It is now possible to deplete T_{reg} in cancer patients; however, clinical studies suggest that more durable, potent, and specific methods are needed. Other types of cancer therapy will likely be required in addition to T_{reg} depletion to achieve durable antitumor effects. Interestingly, cancer drugs that were originally developed for other indications, such as bevacizumab and sunitinib, have recently been implicated in blocking T_{reg} responses to cancer. Thus, possible T_{reg}-modulating function should be considered when evaluating new cancer drugs. Future studies are expected to illuminate how epigenetic factors govern the stability of tumor-associated T_{reg}, and how helper T cell-associated transcription factors may govern the behavior of T_{reg} in cancer. Armed with a wealth of knowledge about T_{reg}, we are now entering a new era in the understanding of tumor-induced immune suppression.

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Chapter 2
Th17 Cells in Cancer

Chrystal M. Paulos, Michelle H. Nelson and Xue-Zhong Yu

Abstract T helper (Th)17 cells regulate host defense and exacerbate autoimmune diseases, yet their role in tumor immunity remains controversial with reports that Th17 cells could either promote or suppress tumor growth, depending on the type of malignancy or means of therapeutic intervention. This review discusses how inflammatory signals (such as cytokines and co-stimulatory/co-inhibitory molecules) induced in the tumor milieu regulate the functional fate of Th17 cells, which ultimately affect the cells' capacity to mount immune responses against cancer. We review recent findings regarding the factors that influence the generation, plasticity, and memory phenotype of Th17 cells and their relevance to cancer immunotherapy. Further, we discuss recent reports concerning the interaction of Th17 cells with regulatory T lymphocytes and cytotoxic cluster of differentiation 8 ($CD8^+$) T cells present in tumor tissue. Unraveling the mysteries surrounding basic and translational aspects of Th17 cell biology promises to have important implications for patients with advanced malignancies.

Keywords Th17 · IL-17A · RORγt · Plasticity · Immunology · Cancer · Immunotherapy · Tumor microenvironment · Transplantation

1 Introduction

Cancer persists as a clinical problem not only because of the disease, in its myriad forms, but also because it remains stubbornly resistant to even some of the most advanced therapeutic regimens. Often, by the time the patient requires treatment, the

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disease has already stymied the first line of defense: the immune system. Despite being exquisitely effective against disease brought about by foreign bodies, the immune system can seem impotent against cancerous self-tissue. Certainly, the immune system plays a vital role in recognizing and killing cancer cells; but, more often, the body finds itself overwhelmed by the malignancy. Under certain treasonous circumstances, the immune system can even promote the growth and spread of tumor tissue in the patient.

These facts may paint the immune system as at best neutral, at worst a foe in the treatment of cancer. However, the burgeoning field of immunotherapy still sees a promise in harnessing the immune system to arrest the advance of tumor malignancies in patients. Understanding the conditions necessary to coax the patient's own immune elements into halting or even turning back the spread of cancer provides the primary impetus for a flurry of current research into the role of T cells in tumor immunity. This review focuses on the exciting discoveries concerning the recently discovered (2005) cluster of differentiation 4 $(CD4)^+$ T-cell subset called Th17, and its potential role in tumor immunity.

$CD4^+$ T cells are among the chief regulators of the immune system. These cells can differentiate into different lineages of T helper (Th) cells with distinct biological functions [1]. In 1986, Th cells were divided into two distinct subsets: Th1 cells that produce interferon-γ (IFN-γ) promote cell-mediated immunity and Th2 cells that produce interleukin-4 (IL-4) support humoral immune responses. Both subsets were found to enhance antitumor immunity by inducing the expansion and cytotoxic function of $CD8^+$ T cell (cytotoxic T lymphocytes, CTLs) responses to the tumor. In contrast, regulatory $FoxP3^+$ $CD4^+$ T cells (regulatory T (Treg) cells) were found to suppress antitumor immunity by inhibiting CTLs. In 2005, IL-17-expressing T cells (Th17 cells) were discovered as an independent subset. Studies on the newly described Th17 cells quickly established their contributions to inflammation, autoimmunity, and host defense, but the role of these cells in tumor immunity remains both unclear and hotly debated [2], [3]. Th17 cells have been found to eradicate tumors when adoptively transferred into their autologous host. However, naturally arising endogenous Th17 cells have also been found to promote tumor progression. In this chapter, we discuss the basic and translational properties of Th17 cells in the context of cancer. We discuss Th17 cell plasticity, enabling them to convert into other $CD4^+$ T-cell subsets (such as Th1 or Treg-like cells) profoundly altering the cells' functional and phenotypic fate (specifically, their capacity to kill tumors). This chapter brings together the latest research on the disparate means by which cytokines, co-stimulation/co-inhibition, T-cell receptor (TCR) signal strength, transcriptional factors, and epigenetic mechanisms regulate Th17 cells in tumor tissue. We also highlight the critical importance of interplay between Th17 cells and other immune cells in regulating cancer growth. Finally, we discuss recent proto-clinical findings involving human Th17 cells in cancer and speculate how these results may lead to new treatments for cancer patients.

2 Cd4 T Cell Subsets in Tumor Immunity

2.1 Th17 Cells: The Th1/Th2 Paradigm Demystified

Twenty-eight years ago, Mosmann and Coffman introduced the Th1/Th2 paradigm of T helper cell differentiation to explain the adaptive immune system's mode of clearing intracellular and extracellular pathogens [4]. Subsequent investigation revealed that the Th1/Th2 paradigm could not fully account for the development of inflammatory responses to self-tissue or tumor tissue [5], [6]. The hunt was on for the T-cell subset(s) responsible for driving inflammation to fill this knowledge gap [7]. Eight years ago, a new effector $CD4^+$ T helper cell subset that produces IL-17A was discovered: Th17 cells [8]–[10]. The cytokines and transcription factors that promote Th17 cell generation were soon identified, and it became clear that Th17 cells represent an independent subset of T helper cells with distinct functions in regulating inflammation—functionally divergent from that possessed by Th1 or Th2 cells. The discovery of Th17 cells thus expanded the Th1/Th2 paradigm and provided a clearer picture of the immune system's agent responsible for tissue inflammation, autoimmunity, and tumor immunity.

2.2 T Helper Subsets in Tumor Immunity

$CD4^+$ T cells localized in tumor tissue take immunological cues from the tumor milieu (Fig. 2.1), leading to differentiation into one of several T helper (Th) subsets (Th1, Th2, Th17) [6], [11] or into a suppressive subset (Tregs) [12]. The cellular effect on cancer development will depend on the phenotypic outcome of $CD4^+$ T-cell differentiation. Th1 and Th2 cells are the effector cells that express T-bet and GATA-3 [13], [14], respectively. Both subsets elicit antitumor effects, with Th1-polarized cells traditionally regarded as the more effective tumor killers. In contrast, Treg cells are believed to impair antitumor immunity by suppressing cytotoxic $CD8^+$ T-cell responses [15], [16]. (For further information on Treg cells in cancer, we refer the reader to Chap. 1 of this book.) The effect of Th17 cells on cancer is more ambiguous: Some investigators have reported Th17 cell acceleration of tumor growth, while others report that Th17 prompted eradication of established tumors [2], [3]. A satisfying, all-encompassing explanation for these conflicting results has not been forthcoming, but recent results have provided a clue: Th17 cell form and function are uniquely sensitive to a host of factors in the context of tumor. The type of cancer tissue (e.g., prostate versus pancreatic), the therapeutic approach (e.g., vaccine versus adoptive cell transfer therapy; *vide infra*), and the stimuli to which the cells are exposed during activation (e.g., cytokines, co-stimulatory molecules, TCR signal strength) all significantly impact the development of Th17 cells, providing the cells with an apparently broad range of phenotypic and functional prospects. Thus, understanding the nature of Th17 cell responses in the tumor microenvironment will be essential for advancing efficacious cancer therapies. Herein, we review recent findings concerning the means by which cytokines and other signals modulate Th17 cell development, and their consequences for regulation of tumor immunity.

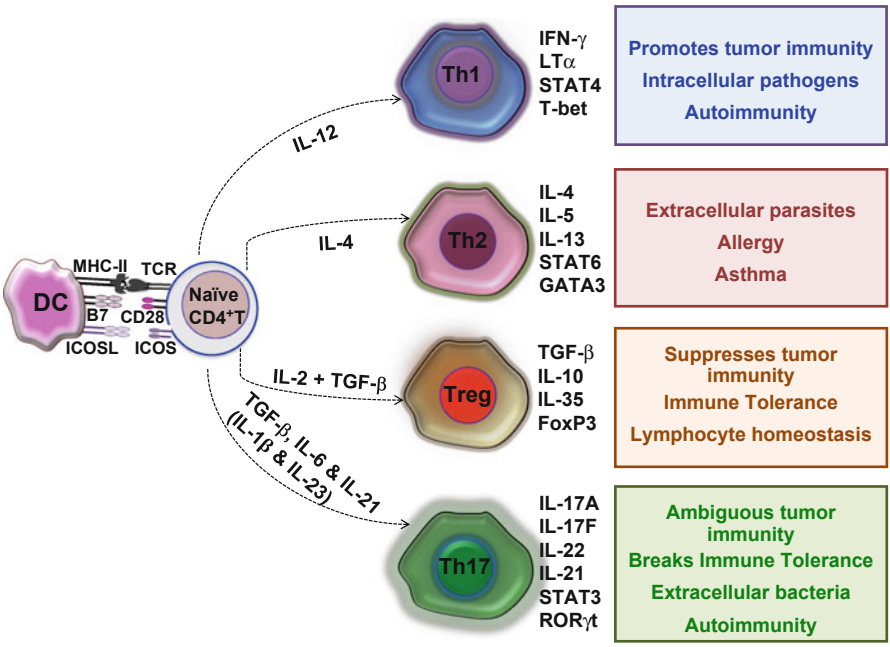

Fig. 2.1 Differentiation of helper T cell subsets. Post activation of $CD4^+$ T cells with antigen presenting cells, e.g. with dendritic cells that present peptide via MHCII and costimulation with CD28 and ICOS, these cells can differentiate into various effector (Th1, Th2 or Th17 cells) or regulatory subsets (Treg cells). Their differentiation depends on the local cytokines environment they encounter during activation. The differentiation of each of these effector T cell subsets is controlled by distinct sets of transcription factors. In the presence on interleukin-6 (IL-6) and transforming growth factor-beta (TGF-b), naïve $CD4^+$ T cells differentiate into a Th17 cell phenotype, which are characterized by expression of transcription factors retinoic acid receptor-related orphan receptor-gt (RORgt) and signal transducer and activator of transcription 3 (STAT3). IL-21 and IL-23 cytokines can promote and stabilize this phenotype during their expansion. Once programmed, these cells secrete IL-17A, IL-17F, IL-21 and IL-22. While the role of Th17 cells is controversial in tumor immunity, they play a key role in enhancing autoimmunity and host defense. The cytokines and transcription factors that control the development of Th1, Th2 and Treg cells are also shown herein and they also distinctly regulate immune response to foreign, self and tumor antigens

3 TH17 Cells in Cancer: Basic Th17 Biology and Interaction with Other Immune Cells in the Body

3.1 Cytokines, Transcription Factors, and Extracellular Markers

Th17 cell development is distinct from the development of Th1, Th2, and Treg cells and is characterized by unique transcription factors and cytokine requirements [8]. As shown in Fig. 2.1, naïve $CD4^+$ T cells undergo differentiation into Th17 cells when exposed to transforming growth factor beta (TGF-β), IL-6, IL-1-β, and IL-21

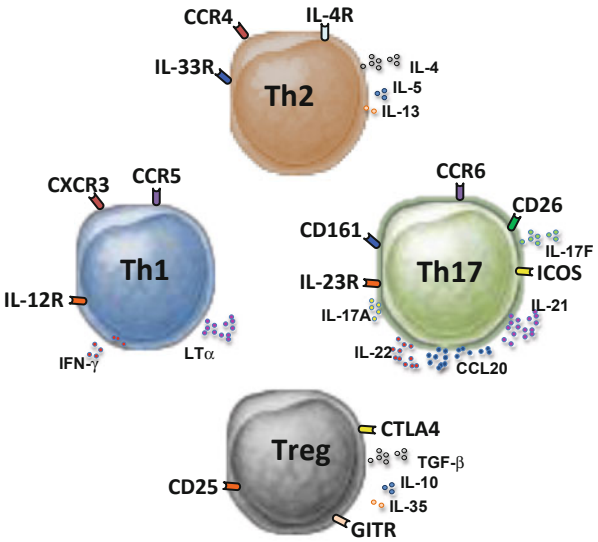

Fig. 2.2 Extracellular markers on Th1, Th2, Th17, and Treg cell subsets. Th17 cells can be distinguished from Th1, Th2 and Treg cells in the peripheral blood $CD4^+$ T cells of healthy and diseased human donors by their expression of chemokine receptors and other cell surface molecules. As depicted, Th1 express CXCR3, CCR5, IL-12R but not CCR4 or CCR6 on their cell surface; and Th2 express CCR4, IL-33R and IL-4R but not CXCR3 or CCR6 on their surface; and Th17 express a number of different markers on their cell surface, including CCR6, CD161, IL-23R, ICOS and CD26. Finally, Treg cells have been found to express a high level on CD25, CTLA4 and express a low level of CD127 on their cell surface

via signal transducer and activator of transcription 3 (STAT-3)-dependent signaling [17], [18]. Th17 cells are maintained long term in the presence of IL-23 [19]. Conversely, IL-12 supports Th1 development and suppresses Th17 generation or converts them to a Th1 phenotype. Likewise, IL-4 promotes Th2 and suppresses Th17 development. TGF-β and IL-2 support Treg cell generation [2], [6] but differentially regulate whether a Th17 cells will possess inflammatory or regulatory functions (*vide infra*). As depicted in Fig. 2.1, Th17 cells are characterized by their capacity to secrete IL-17A, IL-17F, IL-21, IL-22, and chemokine (C–C motif) ligand 20 (CCL20) [20]–[22]. Moreover, the Th17 phenotype is controlled by the master transcription factors retinoic acid-related orphan receptors (RORs)γt, RORα, aryl hydrocarbon receptor (AHR), and IFN-regulatory factor 4 (IRF4) [18], [23]–[26]. In addition to these cytokines and transcription factors, Th17 cells can be identified by their surface expression of the chemokine receptor (CCR6), the IL-23 receptor (IL-23R), the inducible costimulator (ICOS), and the lectin-like receptor (CD161) (Fig. 2.2) [27]–[30]. Quite recently, human Th17 cells were found to express high levels of the dipeptidyl peptidase IV called CD26 on the cellular surface; CD26 is a multifunctional ectoenzyme involved in T cell activation and has been implicated in autoimmune pathophysiology [31]. In contrast, Treg cells express low levels of

CD26 and high levels of ectonucleotidases CD39 and CD73 [32]. CD26 expression has previously been reported to crop up in multiple inflammatory conditions. Originally linked to Th1 cells prior to the discovery of Th17 [33], in fact $CD26^{bright}$ Th17 cells are enriched in the inflamed tissue of patients with inflammatory bowel disease [31]. Further, CD26 upregulation correlates with disease activity in human autoimmune manifestations linked to the presence of pathogenic Th17 cells, such as rheumatoid arthritis (RA). Elevated CD26 expression as well as the high expression of ICOS, IL-23R, CCR6, and CD161 allow Th17 cells to be distinguished from other T cell counterparts (Th1, Th2, and Treg) in patients with cancer as well. Please refer to additional references [27], [34] and Fig. 2.2 for a detailed description of the markers on Th1, Th2, Th17, and Treg cells. Importantly, these phenotypic markers permit the determination of Th17 distribution and functionality in tumor tissue—a prerequisite for elucidating the still murky role of Th17 cells in either promotion or suppression of tumor growth.

3.2 Th17 Cell Distribution and Impact on Tumor Immunity

While Th17 cells are abundant in the mucosal tissues and support gut-related homeostasis, few Th17 cells (~ 0.1 %) reside in the peripheral blood of healthy individuals or cancer patients [3], [35], [36]. However, significantly greater numbers of Th17 cells infiltrate tumors, especially as compared to Th17 density in the adjacent, non-tumor tissues of patients. This heightened presence of Th17 cells in tumor tissue holds true for a vast range of malignancies, including melanoma, ovarian, pancreatic, colon, and prostate cancer (Table 2.1) [37]–[55]. These observations imply that tumors produce factors that promote Th17 cell trafficking to the diseased site; the responsible parties include factors such as monocyte chemotactic protein 1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), and tumor-secreted prostaglandin E2 [40], [56]. Tumor-associated fibroblasts, monocytes, and macrophages also promote Th17 cell infiltration and expansion in hepatocellular carcinomas [54]. Nitric oxide (NO)-producing myeloid-derived suppressor cells from patients with ovarian cancer were also recently found to support the Th17 cell development [57]. Collectively, these data suggest that new targets can be manipulated to modulate Th17 responses in cancer.

Murine tumors similarly house large Th17 cell populations [58], permitting researchers to examine Th17 capacity to either promote tumor growth or enhance antitumor immunity. The results have added to the confusion concerning Th17 cells and cancer. Proinflammatory cytokines secreted by Th17 cells in vivo, such as IL-17A, have been reported to impair immune surveillance and promote tumor growth [59], [60]. In contrast, other studies reported that Th17 cells mediate the regression of large, established, and poorly immunogenic melanoma tumors in mice to a greater extent than Th1 cells [61], [62]. Those studies followed an adoptive T cell transfer (ACT) therapy approach, which takes advantage of $CD4^+$ T lymphocytes that express a TCR recognizing tyrosinase tumor antigen. Exploitation of the TCR

leads to rapid expansion of Th17 populations to large numbers ex vivo for reinfusion into the autologous tumor-bearing host mouse [62], [63]. This approach effectively parallels ACT trials in human patients, and has allowed investigators to examine how infused TCR-specific $CD4^+$ (or $CD8^+$) T cells interact with other immune cells in the body—interactions that may either enhance or impair treatment outcome, and may hold the key to understanding the Janus-faced effects of either protumor or antitumor Th17 cells.

3.3 Interplay Between Th17 Cells and Other Immune Cells in the Tumor

Until recently, $CD4^+$ T cells had been regarded as mere suppliers of growth factors in support of $CD8^+$ effectors T cells. $CD8^+$ T cells, believed to be the more important antitumor immune actors, kill tumors by direct cytotoxicity. However, mounting evidence has revealed that $CD4^+$ Th cells—particularly Th17 cells—are capable of mediating tumor regression, not in an ancillary role but as the primary cytotoxic agents [62], [64]. In fact, in some cases the ability of $CD4^+$ T cells to reject tumor significantly improves on the antitumor capacity of $CD8^+$ T cells. This result has sparked heated debate, fueled by reports suggesting an indirect regulatory—as opposed to direct cytotoxic—role for Th17 cells in tumor destruction, via interaction with Treg and $CD8^+$ T cells [61], [65]. The following sections address this debate.

Th17–$CD8^+$ T cell dynamics. Th17 cells mount antitumor immune responses not merely in experimental models of cancer that involve ACT therapy; in some studies, vaccines have successfully induced endogenous antitumor effects. A positive example involved forming a vaccine from pancreatic cancer cells (Pan02). Pan02 cells, which normally secrete TGF-β but not IL-6, were transduced to secrete IL-6 and transplanted into syngeneic mice [66]. As expected, an increase in host tumor-infiltrating Th17 cells was observed in mice with IL-6-transduced tumors. This is not surprising given that TGF-β and IL-6 are critical cytokines for promoting Th17 cell generation. In this model, vaccinated mice displayed a significant delay in tumor growth and enhanced survival prospects compared to mice treated with wild-type, untransduced Pan02 tumor cells. Additional investigation revealed results with interesting implications for the species responsible for the antitumor response: Murine Pan02 tumors transduced to secrete IL-6 not only induced host Th17 cells but also drove concomitant recruitment of IFN-γ-producing $CD8^+$ T cells to the tumor, which enhance antitumor activity of Th17 cells.

This discovery—bolstering of $CD8^+$ T cell activation by endogenous Th17 cells in mice with IL-6-transduced pancreatic cancer— complements recent findings in the context of adoptively transferred Th17 cells (Fig. 2.3a). Restifo, Dong, and colleagues found that adoptively transferred tyrosinase-related protein-1 (TRP-1) Th17 cells elicited activation of endogenous $CD8^+$ T cells in mice with melanoma. $CD8^+$ activation was crucial for the observed antitumor effect [61]. Subsequent studies revealed that Th17 cells promoted $CD8\alpha^+$ dendritic cell recruitment into the tumor

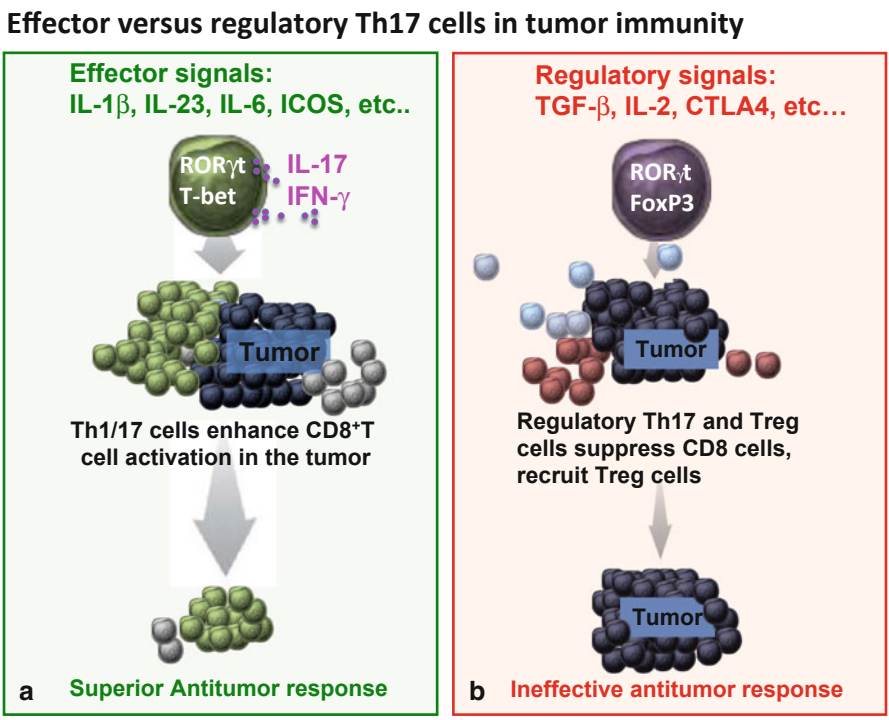

Fig. 2.3 Effector (**a**) versus regulatory (**b**) Th17 cells in tumor immunity. Depending on the cytokine or other signals that Th17 cells encounter during tumor progression, they can transform into either effector or regulatory Th17 lymphocytes that either enhance or suppress tumor regression. **a**) Th17 cells (effectors) activated with IL-1b, IL-23, IL-6 and/or ICOS agonist are poly-functional and capable of mediating potent tumor regression in solid tumors. **b**) Th17 cells (regulatory) programmed with cytokines such as TGF-b, IL-2 and or CTLA4 can dampen their function and persistence, thereby potentially reducing their capability to kill tumors. Regulatory Th17 cells likely do not foster the induction or cooperation of CTLs to the malignant site

tissues as well as the draining lymph nodes, likely inducing the activation and expansion of cytotoxic $CD8^+$ T cells. Th17 cells promoted CCL20 chemokine production by tumor tissues, thereby recruiting $CD8^+$ T cells to the malignant site. Additionally, tumor-bearing CCR6-deficient mice did not respond to Th17 cell therapy. Th17 cells, thus, elicited a protective inflammatory response that promoted the activation of tumor-specific $CD8^+$ T cells perhaps via CCL20/CCR6 homing mechanisms.

A picture of synergistic interaction between Th17 and $CD8^+$ T cells emerges from these antitumor studies; yet recent work from the Antony laboratory suggests caution against overemphasizing this interplay of immune cells. Antony and coworkers reported that tumor-specific TRP-1 $CD4^+$ T cells can eradicate large tumors directly and without the need for endogenous $CD8^+$ T cells or natural killer (NK) cells [67]. These contrasting results highlight the need for follow-up investigations of the role of antitumor $CD4^+$ T cells (as well as Th1 and Th17 cells) on host or infused $CD8^+$

T cells. $CD4^+$ T cell cooperation with cytotoxic $CD8^+$ T cells in tumor immunity is currently under investigation in the laboratories of the authors as well as others.

Th17–Treg dynamics. What remains clear, however, is that Th17 cells—under the right conditions—have positive antitumor effects. For example, adoptively transferred Th17 cells can mediate potent tumor regression in irradiated mice bearing the established B16F10 tumor. Interactions between the Th17 and $CD8^+$ T cells may have certain consequences for the treatment outcome; however, another important question, with ramifications for the efficacy and persistence of these treatments, concerns the proportion and effects of Th17 and Treg cells on each other and on tumor regression. The influence of host Treg cells on either endogenous or infused Th17 cells is unclear, though the potential role of Tregs in dampening antitumor responses (*vide supra*) has been mentioned herein (Fig. 2.3b).

Discussion of the effect of IL-2—often administered to mice in ACT experiments to support the expansion of transferred Th17 cells—on either Th17 or Treg or both cell subsets suffices to exemplify the current lack of conceptual clarity concerning Th17–Treg interactions and their role in antitumor immunity. The Zou group first reported that IL-2 signaling exerts significant but divergent regulatory effects on Th17 and Treg cells in the tumor microenvironment [68]. In other words, while IL-2 bolsters Th17 cell populations, which subsequently—by an unknown mechanism—appear to dampen host Treg populations in the tumor tissue. These findings would suggest that infused Th17 cells may reduce the number and the suppressive function of host Treg cells; abrogation of Treg suppressor function offers one explanation for why the therapeutic outcome in these ACT-treated mice is curative (Fig. 2.3a). However, once again, alternate explanations for conflicting data in the literature may be preferred. For example, Treg cells require IL-2 for their in vivo maintenance and outcompete other cell subsets (including Th17) for the molecule via a high-affinity IL-2 receptor (67). Thus, it is conceivable that Treg cells impair the engraftment of infused Th17 cells by depriving the infused cells of the beneficial cytokine, a situation that would certainly hamper treatment efficacy, as seen in some literature reports (Fig. 2.3b). On the other hand, given that high concentrations of IL-2 impair the expansion and function of Th17 cells, Treg cells may actually support the engraftment and function of adoptively transferred Th17 cells (at least, in the B16F10 model; see below for the discussion of different tumor tissues) by functioning as an IL-2 cytokine sink. If so, then depletion of host Treg cells would impair the persistence and antitumor activity of donor Th17 cells. These conjectural scenarios suggest that experiments with Treg-depleted FoxP3-DTR mice would go a long way toward elucidating the Th17–Treg dynamics in melanoma-bearing mice that are infused with TRP-1 Th17 cells. Such studies are currently underway in several laboratories. Questions concerning the interplay of host Th17 and Treg cells with endogenous $CD8^+$ T cells also need to be addressed. However, as will become evident in the following sections of this chapter, Th17 interactions with other T cell subsets do not present the only riddles surrounding T cell tumor immunity; the incompletely characterized roles of subcellular molecular species (e.g., cytokines and co-stimulatory molecules) in activating or pacifying Th17 cells—whether in isolation or in interaction with other cell subsets—requires comment and further study.

4 Multiple Facets of Th17 Cells in Cancer Development

4.1 Regulatory and Inflammatory Th17 Cells in Cancer

Th17 cells are not uniformly beneficial in mediating antitumor responses; one possible explanation is their inflammatory function, which has been linked to tumor growth. The Th17 cell function may depend on the type of cancer encountered by the cells; and, if so, a number of factors could alter the effect of Th17 cells on a malignancy's pathology: the source of the Th17 cells (arising naturally via tumor growth, or adoptively transferred following ex vivo manipulation); the regulatory or inflammatory functional phenotype of the cells (and what gives rise to the functionality); and/or exposure to therapeutic interventions such as chemotherapy, vaccination, cytokines, or co-inhibitory/co-stimulatory molecules. Understanding how Th17 cells cause inflammation in the context of these factors, as well as how these elements impact patient survival, is of considerable interest in the cancer immunotherapy field.

One thing remains clear: The influence of Th17 cell accumulation in murine and human tumors on cancer progression remains controversial due to the disparity of experimental results of Th17 cell interactions with cancers [2], [3]. Some small measure of consensus is arising from the controversy, however: Th17 cell subsets can possess either regulatory or inflammatory properties depending on the stimuli they encounter, which may explain why Th17 cells have potent antitumor properties in some experimental regimens but actually fosters tumor growth in others. Th17 cell responses to foreign pathogens provides some illumination of this concept, for example, the Sallusto laboratory found that different pathogens favor the generation of either regulatory or effector Th17 cells [69]. Specifically, *Candida albicans*-specific Th17 cells secreted IL-17 and IFN-γ, but no IL-10, whereas *Staphylococcus aureus*-specific Th17 cells secreted IL-17 and IL-10 (upon restimulation). At the molecular level, *C. albicans* IL-1β was essential for differentiation of *C. albicans*-induced, IL-17/IFN-γ double-producing Th17 cells. IL-1β inhibited IL-10 secretion; blockade of IL-1β in vivo rescued cell capacity for IL-10 secretion. The different cytokines presented by *C. albicans* and *S. aureus* prime Th17 cells to produce either effector IFN-γ or regulatory IL-10, respectively (and further identify IL-1β as a regulator, along with IL-2, of Th17 cell function).

An implication for Th17 functionality in the context of cancer follows from these observations with pathogen-primed cells: Different types of tumor tissue may foster the generation of Th17 cells with different phenotypes—either suppressive or inflammatory, with divergent consequences for tumor growth progression. Indeed, high-frequency Th17 cell infiltration into the tumor bed of patients with colon or pancreatic cancer strongly correlates with poor prognosis [70]. Conversely, increased Th17 cell numbers in ovarian tumors have been associated with improved patient survival rates [71]–[75]. The intrinsic properties of tumors that might regulate the anticancer activity of Th17 cells have not been fully identified, but the inconsistent success of Th17 cell-based cancer immunotherapy may arise when varying cytokines produced in tumor microenvironments or co-stimulatory and/or co-inhibitory

molecules expressed on tumor-associated macrophages and dendritic cells steer Th17 cells toward functional phenotypes with vastly different pathological and therapeutic properties. How the tumor microenvironment regulates downstream protein kinase B (Akt) and mammalian target of rapamycin (mTOR) pathways in Th17 cells might also impact their fate, as Kim and coworkers very recently discovered that natural versus induced Th17 cells are regulated differently by Akt and mTOR pathways [76]. Identification of the tumor-localized triggers that shape distinct Th17 cell responses (either pro- or antitumorigenic) will be invaluable for progress in developing effective antitumor therapies.

4.2 Th17 Cells and Tumor-Associated Angiogenesis

A suspected link between inflammation and cancer emerged over a hundred years ago, but today's scientists are still just beginning to unravel the role of inflammation in cancer development at the cellular and molecular levels [77]–[80]. What has the intervening century revealed? Inflammation regulates the tumor microenvironment, driving the proliferation, survival, and migration of cancer cells with the potential results of tumor invasion, migration, and metastasis. Th17 cells are characterized by a potent proinflammatory activity mediated predominantly by their hallmark cytokines: IL-17A, IL-17F, IL-21, and IL-22. Other factors come into play as well, such as granulocyte–macrophage colony-stimulating factor (GM-CSF)—an IL-23-induced RORγt- driven factor that is vital for Th17 cell-mediated inflammation [8]. Thus, Th17 cells, with cues from Th17-specific cytokines, take the helm in regulating inflammation. Consequently, Th17 cells become prime actors in tumor growth, which inflammation promotes in two distinct ways—by driving angiogenesis and suppressing immunity [80]. The following paragraphs discuss angiogenesis; Sect. 2.3.3 addresses immunosuppression by Th17.

Among the cytokines secreted by Th17 cells, IL-17A is best known to perpetuate angiogenesis; when occurring in tumor tissue, angiogenesis facilitates tumor growth, providing the cancer cells their causeway to migrate to healthy tissues in patients. IL-17A contributes to angiogenesis via indirect mechanisms [81], [82] promoting increased production of vascular endothelial growth factor (VEGF), IL-8/CXCL8, and fibroblast growth factor 1 (FGF-1) by tumor cells and tumor-associated fibroblasts [82]–[84]. Tumors transfected with IL-17A grow rapidly and are more vascularized than wild-type tumors in mice. Moreover, genetic removal of IL-17A in mice impairs tumor growth [82]. For example, the growth and metastasis of B16 melanoma and MB49 bladder cancer is reduced in IL-17A-deficient mice relative to wild-type hosts, suggesting that IL-17A-induced angiogenesis plays a therapeutically detrimental role in nourishing the lifecycle of the tumor [60], [85]. Additionally, IL-17A promotes the development of lymphatic vessels, by inducing expression of VEGF and proliferation of lymphatic epithelial cells. This phenomenon favors cancer metastasis to draining lymph nodes. Positive correlations between the density of tumor-infiltrating Th17 cells and the concentration of micro-vessels have been reported in many types

of human cancers [3], [43], [86]. These results suggest that IL-17A-producing Th17 cells (and potentially other immune cells that secrete IL-17A) promote tumor progression by fostering angiogenesis. In another context, for patients with RA, uveitis, and psoriasis, a Novartis blocking monoclonal antibody (AIN457) to IL-17A recently went through clinical trials and provided promising results in the participating patients [87]. Blocking IL-17A may reduce angiogenesis, thereby improving disease prognosis in patients. Combining AIN457 with other effective therapies, such as chemotherapy, ACT, or vaccines, could potentially increase the survival of patients with malignancies that thrive on IL-17A.

Yet, other cytokines secreted by Th17 cells (IL-17F, IL-21, and IL-22) exhibit antiangiogenic properties, convoluting the overall correlation between Th17 cell activity and tumor growth in the context of angiogenesis [88]–[90]. The conditions that prompt Th17 cells to secrete one or more of these cytokines may regulate angiogenesis, and the critical context of the type of tumor Th17 cells encounter may have some bearing on the outcome Th17's regulatory role. In light of the findings by Sallusto's group that different pathogens promote the generation of either effector or regulatory Th17 cells [69], it is possible that different types of cancers will induce Th17 cells that can either facilitate or suppress angiogenesis by differentially regulating IL-17A, IL-17F, IL-22, and IL-21 secretion in patients. Unraveling the anti- and pro-angiogenesis regulatory patterns of Th17 cells requires a complete study of the cytokines, co-stimulatory and co-inhibitor molecules within the tumor milieu of different cancers at various stages of their growth.

4.3 Immunosuppressive Properties of Th17 Cells

Although Th17 cells can eradicate melanomas when adoptively transferred into preconditioned animals, Th17 cells can also function as regulatory cells with the capacity to suppress immune responses to tumors [2]. At least two distinct mechanisms sustaining these immunosuppressive effects have been identified. One, Th17 cells are capable of converting into Treg cells (i.e., Th17–Treg plasticity) [91]; two, Th17 cells can release immunosuppressive adenosine upon TGF-β-dependent ectonucleotidase expression [92] as illustrated in Fig. 2.4 and described in detail below.

4.3.1 Th17–Treg Plasticity Promotes Tumor Suppression

In contrast to Th1 cells, Treg and Th17 cells can transform into other lineage phenotypes [91]. Thus, Th17 and Treg cells are capable of trans-differentiation, a phenomenon referred to as "plasticity." Th17 cells may originate from Treg cells, differentiation mediated by IL-1β interaction with Treg cells expressing the IL-1 receptor (IL-1R) [93], [94]. Treg cells can also undergo complete lineage conversion into Th17, indicating that plasticity is a two-way street, but with the astounding feature that interconversion does not have rigidly binary outcomes. Intermediate

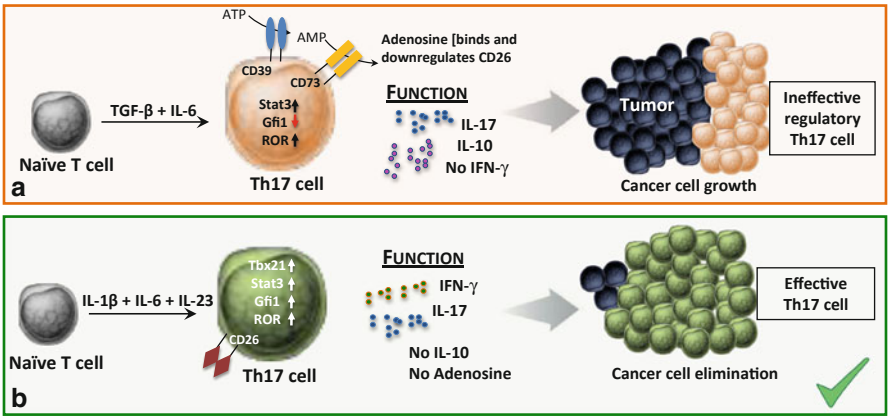

Fig. 2.4 TGF-β induces Th17 cells to express ectonucleotidases and suppress tumor immunity. Th17 cells generated in the presence of TGF-b plus IL-6 fail to secrete IFN-g but do secrete IL-17A and IL-10. **a**) Th17 cells programmed with TGF-b plus IL-6 also express less Gfi1 (growth factor independent protein 1 – repressor of econucleotidase, resulting in CD39 and CD73 ectonucleotidase expression on their cell surface. CD39 and CD73 function to convert ATP to adenosine. Adenosine binds CD26 on Th17 cells and subsequently down-regulates CD26 on their surface. These series of events – induced by TGF-b – contribute to the inhibition of antitumor immunity. **b**) Conversely, programming $CD4^+$ T cells in the absence of TGF-b and in the presence of IL-1b, IL-6 and IL-23 cytokines supports the generation of Th17 cells that secrete IL-17A and express transcription factors RORgt and STAT3. Moreover, these cells also express transcription factor Tbx21 and co-secrete IL-17 and IFN-g but not the immunosuppressive cytokine IL-10. These inflammatory Th17 cells also express the transcription factor growth factor independent protein (Gfi1) and CD26 on their cell surface. Upon their infusion, these cells promote the activation of $CD8^+$ effector T cells and cooperate to mediate tumor regression in an IFN-g and IL-17A-dependent manner

phenotypes that co-express FoxP3 and RORα may also arise. Zeigler and workers reported that FoxP3 and RORα not only homodimerize but also heterodimerize, a consequence that generates Th17/Treg-like cells that secrete IL-17A [95]. These intermediates display immunosuppressive functions against cytotoxic $CD8^+$ T cells [96]. Hence, distinguishing this discrete cell population from bona fide Treg versus Th17 cells will be critical for a clear elucidation of Th17 cells' regulation of tumor immunity in mouse and man.

The ability of Th17 cells to convert into Treg cells was first identified in Th17 clones of $CD4^+$ T cells obtained from a human cancer [97]. These clones initially expressed IL-17A and RORγt. Interestingly, when these clones underwent repetitive stimulations and expansions with anti-CD3 and allogeneic peripheral blood mononuclear cells (PBMCs—i.e., feeders), the percentage of IL-17A-producing cells diminished while the percentage of FoxP3-expressing cells increased. Moreover, an increase in the percentage of cells coproducing IL-17A and FoxP3 was also observed, as these cells were subjected to multiple expansion cycles. Th17 cell conversion to Treg cells may be linked to TCR stimulation or cluster of differentiation 28 (CD28) co-stimulation; CD28 is a signal that supports Treg cell expansion [98].

Epigenetic modification and stabilization of FoxP3 also occurred in Th17 cells after multiple cycles of propagation.

Additional results would indicate that Th17 plasticity is wide-ranging. Under the influence of repeated TCR stimulations, the Th17 cells changed stripes again, differentiating into Th1-like cells that co-express T-bet and IFN-γ [99], [100]. After three rounds of rapid expansion, additional investigation is required to determine if these clones also exhibited suppression of effector $CD8^+$ T cells. Taken together, these findings suggest that excessive TCR stimulation modifies gene expression and the epigenetic status of Th17 clones, notably resulting in the conversion of Th17 cells into immunosuppressive Treg cells and possibly dysfunctional Th1 cells. It is also possible that multiple rounds of TCR stimulation expanded the clones to a short-lived, terminally differentiated T cell population, a consequence that could impair cell functional fate and antitumor performance in vivo. These studies were conducted with anti-CD3 and feeder cells as opposed to antigen-specific activation of the cells with a genuine TCR. The population of cells generated from cloned Th17 cells may differ in phenotype from cells expanded via anti-CD3 versus TCR stimulation [101]. It will be important to understand how specific TCR stimulation with tumor antigen impacts conversion of Th17 to Treg in future experiments.

4.3.2 TGF-β Induces Th17 Cells to Express Ectonucleotidases

TGF-β and IL-6 program naïve $CD4^+$ T cells toward a Th17 cell phenotype. These cytokines have long been thought to be responsible for generating lymphocytes that enhance autoimmune manifestations, particularly autoimmune encephalomyelitis (experimental autoimmune encephalomyelitis, EAE). This hypothesis was experimentally tested using myelin-reactive T cells from immunized mice; Th17 cells were generated either by culturing in the presence of TGF-β plus IL-6 or with IL-23 [102]. As expected, cell population subsets from both cultures secreted IL-17A. Yet, upon transfer into recipient mice, only IL-23-cultured Th17 cells induced the pathologic lesions typically observed in mice with EAE, whereas cells exposed to TGF-β plus IL-6 produced fewer lesions. The authors postulated that TGF-β imprint Th17 cells with an immunosuppressive phenotype. The finding that TGF-β/IL-6-cultured cells produced IL-10 upon recognition of myelin antigen supported this hypothesis; IL-10 is a cytokine that dampens the destructive capacity of myelin antigen. Experimentally, cytokines TGF-β, IL-6, IL-21, IL-23, and IL-1-β have been used in different combinations by a number of groups to generate Th17 cells. The ramification of the results described in this paragraph is that these cytokines cannot be used interchangeably to generate Th17 cells.

Guided by the autoimmune results, Ghiringhelli and colleagues postulated that Th17 cells programmed with IL-23 (plus IL-1β and/or IL-6) will eradicate tumors when transferred into irradiated recipient mice, while those programmed with TGF-β and IL-6 will be less effective [92], [103]. Mechanistic studies recently revealed that TGF-β/IL-6 cultured Th17 cells co-express CD39 and CD73 ectonucleotidases

on their surface; concomitant expression of these two enzymes transforms adenosine triphosphate (ATP) or adenosine diphosphate (ADP) into immunosuppressive adenosine [92]. These signaling events impair the antitumor activity of Th17 cells programmed with TGF-β and IL-6. As depicted in Fig. 2.4a, TGF-β and IL-6 induce CD39 and CD73 expression on Th17 cells by activating transcription factors Gfi1 (growth factor independent protein 1) and STAT-3, resulting in secretion of IL-17 and IL-10 [92]. In contrast, Th17 cells programmed with IL-1β, IL-6, and IL-23 cytokines did not express CD39 or CD73 (Fig. 2.4b). Additional investigation revealed that these cells co-expressed T-bet and RORγt, resulting in secretion of IFN-γ and IL-17 but not IL-10. Thus, Th17 cells programmed with IL-1β, IL-6, and IL-23 cytokines will mediate robust eradication of tumor, as recently reported by the Ghiringhelli and Restifo laboratories [92], [104]. These results suggest that minor changes to the cytokines used in generating Th17 cells can drastically impact the cell-mediated responses to self/tumor tissue.

Beyond the classic cytokines (e.g., TGF-β, IL-6, IL-1β, and IL-23) used to program $CD4^+$ T cells toward a Th17 phenotype, other proinflammatory cytokines' effects on Th17 immune responses to tumors have not been completely elucidated. Given that lymphodepletion with chemotherapy or irradiation can increase inflammatory cytokines in the γ-chain family (e.g., IL-7 and IL-15) and the IL-12 family cytokines (such as IL-12 and IL-23), it is likely such therapies will have a unique, yet still undetermined impact on the long-term function and antitumor activity of Th17 cells in the context of cancer [105], [106]. Moreover, lymphodepletion augments the antitumor activity of adoptively transferred Th17 cells by aggressive depletion of Th17-suppressing immune elements: host Treg cells, myeloid-derived suppressor cells, and certain activating antigen-presenting cells (APCs) [107]. How the removal of these cells impacts Th17 cell biology requires elucidation to fully understand the antitumor activity of T cells in lymphodepleted patients.

5 Antitumor Activity of Th17 Cells in ACT Therapy

5.1 Plasticity of Th17 Cells in Antitumor Immunity

Th17 cell plasticity has attracted the attention of ACT researchers interested in how these cells behave in the context of antitumor therapy. Th17 cells can convert into IFN-γ $CD4^+$ T cells with a Th1-like phenotype: the Restifo laboratory first reported that this phenomenon transforms TRP-1 tumor-specific Th17 cells into tumoricidal Th1-like lymphocytes once infused into lymphodepleted animals [62]. The plasticity can be flexible, however, leading to intermediate cells that co-secrete IL-17A and IFN-γ and co-express RORγt and T-bet; this process affords cells denoted "Th1/Th17." However, depending on the in vivo cues, these cells can completely transform to apparent Th1 cells (denoted "Th1-like"), devoid of all former Th17 markers [108]. Whereas conventional Th17 cells are poorly pathogenic in the context of autoimmunity, the Th1/Th17 cell intermediates resulting from Th17 cell plasticity can be highly

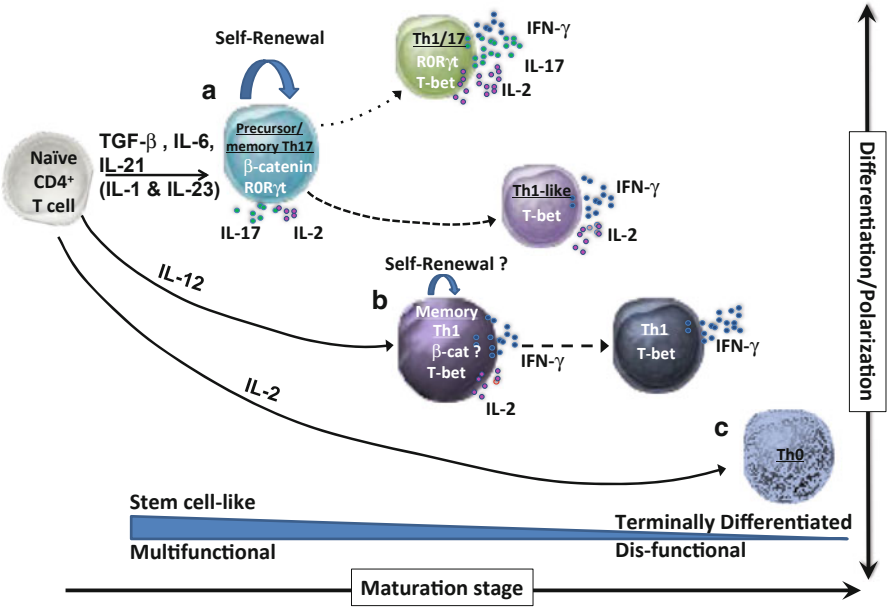

Fig. 2.5 Divergent potential for self-renewal and plasticity within T cell subsets. Upon antigen recognition, naive $CD4^+$ T cells differentiate into distinct effector pathways based on the existing cytokine milieu and the key transcription factors they induce, such as RORgt and/or T-bet, shape their long-term functional fate. The bulk of activated $CD4^+$ T cells will be expanded into poorly functional, terminally differentiated effectors that direct immediate immune responses to antigen but are short-lived. However, a small proportion of these cells will enter into a self-renewing pool and are long-lived. Their durability is dependent on the homeostatic cytokines and innate signals they encounter overtime. Unlike Th1 or Th0 cells (**b and c**, respectively), Th17 cells appear to exist in a progenitor-like stage (**a**, Th17 precursor), with a number of uniquely traits: enhanced self-renewal characteristics, multi-functionality and rapid recall responses to tumor antigen. The potential for divergent late developmental fates of Th17 cells induced by key transcription factors and signals in the Wnt/b-catenin pathway are associated with hemotepoetic stem cells or stem cell-like $CD8^+$ T cells induced upon re-encounter tumor antigen, and their impact in Th1 versus Th17 memory remains elusive and hotly debated

pathogenic [109], [110]. Th17 cell plasticity is perpetuated by lymphodepletion in ACT models, by unknown mechanisms [111], [112]. Due to their capacity to secrete IFN-γ, these protean cells can also exert robust antitumor activity, mediating long-term curative response in mice with melanoma—in contrast to untransformed Th17 cells that enhance tumor growth through pro-angiogeneic functions and suppression of anticancer immune responses through ectonucleotidase-induced adenosine release (see Sect. 2.3.3) [92]. Thus, Th17 cell plasticity toward Th1 or Th1/Th17 cells appears to be a prerequisite for potent antitumor activity and may also, at least in part, explain the disparity in antitumor activity observed by certain Th17 studies.

It is important to note that potent antitumor immune responses do not merely arise from Th17 IFN-γ production, as underscored by the finding that Th1 cells—which secrete significantly more IFN-γ than Th17 cells (Fig. 2.5)—do not elicit tumor

destruction to a comparable extent [62], [104]. Other intrinsic Th17 cell properties, besides IFN-γ production, likely endow the cells with the capacity to kill tumor in vivo. In the following section, we discuss the most salient Th17 property to arise from the most recent antitumor research: durable memory responses to tumors.

5.2 Long-Lived Memory Th17 Cells with Stemness

Recent advances in gene therapy enable researchers to endow T cells with tumor specificity via TCR or chimeric antigen receptors (CARs) that recognize tumor antigen, which has potentially unlocked new ACT treatments of unprecedented efficacy [113]–[115]. Yet, most ACT-based clinical trials in patients have not risen to their extraordinary expectations, marred by the use of short-lived or terminally differentiated T cells [116]. Thus, a need arises for the generation of long-lived memory T cells for immunotherapy. Th17 cells have recently been discovered to display durable persistence, enhanced self-renewal capacities, increased polyfunctionality, and the ability to mount rapid recall responses to tumors [104]. However, effective means of generating antitumor Th17 cells with memory are still in their infancy.

Memory definitions. Whereas much of our understanding of T cell memory has been attained through studies of $CD8^+$ T cells, recent advancements in defining the features of memory $CD4^+$ T cells have trickled into the literature. First, a primer on T cell memory formation: Upon antigen recognition, $CD8^+$ T cells experience clonal expansion followed by a contraction phase and the formation of memory [117]. $CD8^+$ T cells can acquire central memory qualities upon in vitro culture with IL-15; these cells (T_{CM}) possess heightened antitumor potency in vivo compared to effector memory cells (T_{EM}) [118], [119]. Reported memory $CD8^+$ T cells with "stem cell-like" properties (T_{SCM}), generated in vitro, destroy tumor even more effectively than T_{CM} cells [120], [121]. The salient feature of these $CD8^+$ T cells seems to be that the "younger" their phenotype, the more persistent and potent their potential antitumor response [116], [122], [123]. Yet, these younger cells must possess memory responses to tumor antigen to debulk large malignancies.

Naive $CD4^+$ T cells can differentiate into a plethora of distinct functional effectors upon antigenic encounter (e.g., Th1, Th2, Treg, and Th17 cells), adding complexity to the issue of $CD4^+$ T cell memory [124]. Recent findings discussed above further complicate matters. Th17 cell plasticity in their late-developmental programming that allows them to acquire at least some features of Th1 or Treg cells. This plasticity is likely contingent upon the prevailing cytokines, co-stimulation/co-inhibition, and TCR stimulatory strength they receive during recall responses [110]. Th17 diversity poses unique challenges to definitively defining Th17 memory phenotype, whether the molecular mechanisms that maintain hematopoietic stem cells (HSC) self-renewal are functional in Th17 cells becomes a difficult issue to decipher. Reported attempts to do so are described in the following paragraphs.

Murine tumor-specific Th17 cells were recently discovered to be long-lived, to possess a high proliferative potential upon antigenic reencounter, and to self-renew

with enhanced polyfunctionality in vivo compared to tumor-specific Th0 or Th1 cells (Fig. 2.5) [104]. These data were unexpected given that Th17-polarized cells express extracellular markers of terminally differentiated effector memory Th17 cells in vitro (e.g., low CD62L and CCR7 levels; high CD44 levels). Additional investigation revealed that these cells were masquerading as terminally differentiated lymphocytes in vitro; once infused into mice, the cells resumed expression of CD62L and CCR7, indicative of a less-differentiated ("younger") phenotype. Several genes/pathways—the expression of which carries significance for T cell memory responses—were identified as operating in these cells. For example, young phenotype Th17 cells expressed *Lef1* and *Tcf7* (genes linked to the Wnt/β-catenin pathway) to a greater extent than Th1 cells. This pathway is expressed at high levels in HSCs and has more recently been found in $CD8^+$ T_{SCM} cells [125]. In vivo, Th17 cells not only gave rise to Th1-like effector cell progeny but also possessed self-renewal capacity and persisted as IL-17A-secreting cells. Multipotency was required for Th17 cell-mediated tumor eradication because cells deficient in IFN-γ or IL-17A had impaired activity. Thus, the short lifespan of in vitro Th17 cells proves deceptive; in vivo Th17 cells may be a less-differentiated subset possessing superior functionality, persistence, and ability to mount recall responses to tumor challenge—and, fundamentally, all of these properties may be associated with the Wnt-β-catenin pathway.

Mirroring results with murine Th17 cells, Zou and colleagues discovered that human Th17 cells display durable memory responses to self/tumor antigen in a variety of human diseases [126]. Specifically, human Th17 cells were studied in the pathological microenvironments of graft-versus-host disease (GVHD), ulcerative colitis, and advanced cancers in human patients. Th17 cell numbers were increased in the chronic phase of these diseases; the cells phenotypically durable memory T cells. Th17 cells mediated antitumor immunity in xenograft-humanized mouse models of cancer. Furthermore, Th17 cells had a high capacity for proliferative self-renewal, potent persistence, and apoptotic resistance in vivo, as well as plasticity—converting into other subsets. These cells expressed a relatively specific gene signature that incorporated abundant antiapoptotic genes. Yu and coworkers showed that Th17 cells are relatively resistant to activation-induced cell death due to high expression of cellular FLICE inhibitory protein (c-FLIP) [127]. Similarly, Zou and coworkers also found that hypoxia-inducible factor–1α and Notch collaboratively controlled key antiapoptosis Bcl-2 family gene expression and function in Th17 cells [128]. Together, these data indicate that human Th17 cells exhibit the hallmark properties of a long-lived proliferating effector memory T cell population, displaying unique genetic and functional characteristics similar to those found in HSCs. Yet the fact remains that memory T cells (whether they are naturally arising or transferred) become compromised in the tumor microenvironment of patients [129]. The Th17 pathways associated with memory response thus present themselves as attractive targets for manipulation; controlled activation of these pathways in tumor-infiltrating T cells may likely lead to significant therapeutic advances. While the papers discussed in this paragraph together present great promise, the results also emphasize the difficulty in predicting how T cells might perform in vivo based on their—at times deceptive—in vitro phenotype.

5.3 Th1 Versus Th17 Memory Responses to Tumor

Advances in memory Th17 cell biology—especially the apparent similarities between HSC and Th17 cells—raise questions concerning the other T cell subsets: What of "stemness" in Th1 cells? The literature suggests uniformly display antitumor effects (Fig. 2.5) [104]. The poor capacity of Th1 cells derived ex vivo to survive long-term and contribute to the memory pool in ACT cancer models is inconsistent with reports showing the potential of Th1 effector cells to contribute to long-lived memory in infectious disease models [130]. In ACT models, Th17 cells possess a higher self-renewal capacity than Th1 cells [104]. Additionally, Th1 cells show restricted developmental flexibility compared to Th17 cells; Th1 cells only produce IFN-γ, while Th17 cells co-secrete IFN-γ and IL-17A. It is probable that Th17 cells are not unique among effector $CD4^+$ T cell subsets in their potential for durable memory, but merely survive better long-term due to existing in a less differentiated state. Then again, it is possible that ex vivo conditions used to program Th17 versus Th1 in these studies do not faithfully mimic physiological conditions. Resolving the question of Th1 memory durability will require probing the naturally arising Th1 and Th17 tumor-infiltrating lymphocytes (TIL), sorted via transcription factors (such as T-bet and RORγt) or extracellular markers (such as CXCR3 and CCR6).

The potentially confounding factors associated with ex vivo expansion (part of all ACT studies) can be further exemplified. Tumor-specific Th17 cells used in the majority of these experiments are derived from TRP-1-specific $CD4^+$ TCR transgenic mice, the cells of which carry a TCR receptor isolated from a TRP-1-deficient animal [62]. The cells thus possess an unnaturally high affinity for TRP-1. Although high affinity TCRs and CARs are used in some ACT trials, this TCR likely does not model endogenous tumor-specific Th17 cells; the findings then, to some extent, defy extrapolation to patient TIL or vaccination-induced endogenous Th17 cell responses. Interestingly, Purvis and colleagues found that lowering the signal strength of human Th17 cells by decreasing the number of artificial APCs (bearing TCR stimulation and CD28) per Th17 cell increased their functionality, as indicated by the cells' ability to secrete IL-17 [131]. These data imply that lowering TCR affinity might enhance Th17 persistence and multipotency in vivo. The use of TCRs with a range of affinities to the same antigen (experiments performed by the Riley laboratory with TCRs of enhanced affinity to gag-envelope) would address the impact of TCR signal strength on Th17 cell function, memory phenotype, and antitumor activity [132].

5.4 The Role of Lymphodepletion on Th17 Memory Formation

Although infusion of Th17 cells into animals preconditioned with lymphodepletion (using total body irradiation (TBI) or chemotherapeutic reagents) models therapeutic protocols for ACT immunotherapy in cancer patients [133], it is unclear whether the signaling pathways induced by irradiation are similar to those activated during

memory Th17 generation in non-irradiated animals. For example, T cells transferred into irradiated hosts undergo extensive homeostatic proliferation due to an increase in non-physiologic levels of IL-7 and IL-15, two key cytokines that support the expansion and self-renewal of memory T cells [107], [134], [135]. Moreover, lymphodepletion transiently depletes Treg cells, while microbes released from the radiation-compromised gut activate the innate immune system [136]. These immunological changes in the irradiated host may impact Th1 versus Th17 cell memory responses to tumor. Studies that perform transcriptional profiling of effector Th1 and Th17 cell populations, derived in the course of natural immune responses versus those induced by lymphodepletion or infection, may begin to address these important questions. Further, while it has been established that expression parallels exist between CD8 and $CD4^+$ T cell memory subsets that include downstream targets of the Wnt-β-catenin signaling pathway, its (Wnt pathway) contribution to memory cell development remains unclear and controversial [137], [138]. Although tempting to ascribe the longevity of Th17 cell memory responses to β-catenin expression, β-catenin bears indefinite responsibility for cell persistence and antitumor activity; other pathways induced in Th17 cells may govern in vivo durability. Possible agents for imparting durability include enhanced metabolism or increased IL-21 production [139], [140]. Metabolic control of the Treg/Th17 axis can be reviewed extensively elsewhere [141], and understanding the role of metabolism on memory Th17 cells (as well as other T cell subsets) is of interest in the cancer therapy field. Collectively, however, these results represent an important advance in the field of $CD4^+$ T cell memory, and provide clues regarding the potential signaling mechanisms, shared with HSCs, that promote memory [142], [143]. Although the overall differentiation potential of memory lymphocytes is more constrained than that of HSCs, the molecular challenges faced by memory T cells are similar to those of HSCs, as are at least some of the downstream effects on phenotype. For example, both T cells and HSCs must rely on external signals to maintain homeostatic proliferation without terminal differentiation. Both Th17 cells and HSC must maintain malleable epigenetic states at multiple loci that convert to more fixed states upon induction by external activating cues [143]. These observations suggest an overarching hypothesis that memory lymphocytes rely on a subset of stem cell-like self-renewal programs that are reactivated during their generation. The significance of this hypothesis for developing novel cancer therapies remains to be explored.

6 TH17 Cells in Hematologic Malignances and Allogeneic Hematopoietic Stem Cell Transplantation (HSCT)

6.1 Th17 Cells and Related Cytokines in Hematologic Malignancies

While the work discussed thus far focuses on the role of autologous Th17 cells and adoptive immunotherapy for solid tumors, the contribution of Th17 cells and

related cytokines in hematologic malignances is gaining significant attention. In fact, targeting Th17 cells has been considered as a potential therapeutic strategy for patients with acute myeloid leukemia (AML), the most common hematological malignancy, with allogeneic HSCT [144]. Primary human AML cells constitutively produce Th17-polarizing cytokines including IL-1β and IL-6 [145], which can induce fibroblasts to release IL-23 [146]. These observations support the idea that Th17 cells can be differentiated in the leukemic bone marrow milieu. In fact, patients with AML have normal or even elevated levels of circulating Th17 cells as compared with healthy individuals, although these patients are transiently deficient in the number of circulating $CD4^+$ T cells due to intensive chemotherapy [147], [148]. Likewise, elevated frequencies of Th17 cells were also observed in the peripheral blood of patients with chronic lymphocytic leukemia (CLL). Increased circulating Th17 cells correlated greater survival in CLL patients; implicating that Th17 cells represent a prognostic marker for the disease severity. Indeed, Lenalidomide, an effective therapy for CLL patients, was found to reduce Treg cells but increase Th17 cells in their peripheral blood. Collectively, these data underscore that Th17 cells may play a role in eliciting tumor regression in patients with hematologic malignancies [55], [149].

6.2 Th1/Th17 Cells and Related Cytokines in Allogeneic HSCT

Allogeneic HSCT is a promising therapy for treating patients with hematologic malignancies. A major complication in allogeneic HSCT, however, is GVHD, which causes morbidity and mortality in patients after transplantation. During GVHD, alloreactive donor T cells target the intestines, skin, and liver tissue, thus mounting an attack against the host. Importantly, these donor cells can also contribute to graft-versus-tumor (GVT) activity, which prevents tumor relapse and extends the survival of the patient [150].

GVHD can be considered an exaggerated, undesirable manifestation of a normal inflammatory mechanism, in which donor lymphocytes encounter self-antigens in a milieu that fosters tissue destruction. Recent advances in the study of cytokine networks, chemokine gradients, and the direct mediators of cellular cytotoxicity have led to improved understanding of this complex syndrome. How various subsets of cytokine-producing T cells contribute to disease development is an active area in GVHD research. GVHD was originally believed to be a Th1-mediated disease, since Th1 cytokines (IFN-γ and TNF-α) correlate with the disease and in vitro polarized Th1 cells are much more pathogenic than Th2 cells. This concept, however, was challenged by recent observations where T cells deficient in IFN-γ exacerbated GVHD [151]. Furthermore, evidence has emerged that Th17 cells may also play an important role in GVHD. In fact, an elegant study by Yi et al. demonstrated that $CD4^+$ T cells could differentiate into Th1, Th2, and Th17 cells and that each subset may individually contribute in some way to tissue-specific GVHD [152].

The role of Th17 cells in the pathogenesis of GVHD has not been carefully evaluated until very recently. Two recent reports have provided conflicting data on

the role of IL-17A in GVHD [153], [154]. By using in vitro polarized Th17 cells, strong evidence suggests that Th17 cells are highly pathogenic in GVHD induction [155], [156]. The high pathogenicity of Th17 cells is likely associated with their superior ability to expand and that survival is at least partially due to high levels of c-FLIP expression [127]. Although polarized Th17 cells can induce severe GVHD, T cells deficient for RORγt- or IL-17 were still capable of causing severe GVHD [154], [156], [157]. Taken together, these data demonstrate that Th17 cells are sufficient, but not necessary, in the induction of GVHD.

To further evaluate the role of T cell differentiation in GVHD, Yu and colleagues blocked Th1/Th17 lineage-specific transcription factors (T-bet and RORγt) [157]. By comparing the ability of wild-type, T-bet$^{-/-}$, RORγt$^{-/-}$, and T-bet$^{-/-}$/RORγt$^{-/-}$ T cells in the induction of GVHD, they found that RORγt$^{-/-}$ T cells had a comparable ability to cause GVHD as wild-type T cells, whereas T-bet$^{-/-}$ T cells were less pathogenic. The T-bet$^{-/-}$/RORγt$^{-/-}$ T cells failed to induce acute GVHD, but caused minor to modest chronic GVHD in some of recipients at the doses tested. Furthermore, the GVT effect primarily induced by granzyme B^{+} CD8^{+} T cells was largely preserved despite T-bet and RORγt deficiency. Given RORγt$^{-/-}$ T cells had an intact GVT activity whereas T-bet$^{-/-}$ T cells had a compromised GVT activity (Yu et al., unpublished observation), it is possible that Th17-associated cytokines might promote tumor growth in this system (Fig. 2.6c). As expected, T-bet$^{-/-}$/RORγt$^{-/-}$ T cells produced significantly less IFN-γ (Th1 cytokine) and IL-17 (Th17-cytokine), but significantly more IL-4 and IL-5 (Th2-cytokines) as compared to wild-type T cells. In addition, T-bet$^{-/-}$/RORγt$^{-/-}$ donor T cells express significantly less CXCR3 and CCR6, chemokine receptors required for infiltration of alloreactive T cells into GVHD-targeted organs, which could be the reason that significantly fewer T-bet$^{-/-}$/RORγt$^{-/-}$ T cells had accumulated in recipient liver and lung than wild-type T cells. Taken together, these findings provide strong evidence to support the hypothesis that GVHD can be prevented when both Th1 and Th17 lineages are simultaneously blocked. Further efforts must be made to understand the pathogenesis of T cell subsets in GVHD in humans so that effective methods can be developed for the prevention and treatment of GVHD in patients.

7 Type 17 CD8^{+} T Cells in Cancer

7.1 Development, Characteristics, and Plasticity of Tc17 Cells

We have addressed the possible importance of Th17 cell interaction with CD8^{+} T cells, without to this point acknowledging the existence of its CD8^{+} counterpart—Tc17 cells—the significance of which, in terms of cancer treatment, is just beginning to be explored [158]. While Th17 cells have been studied extensively in conjunction with murine and human cancers, fewer investigations have evaluated the antitumor activity of IL-17 producing CD8^{+} T cells. The Restifo laboratory first showed that naive Pmel-1 TCR CD8^{+} T cells can be programmed toward a Tc17 phenotype with

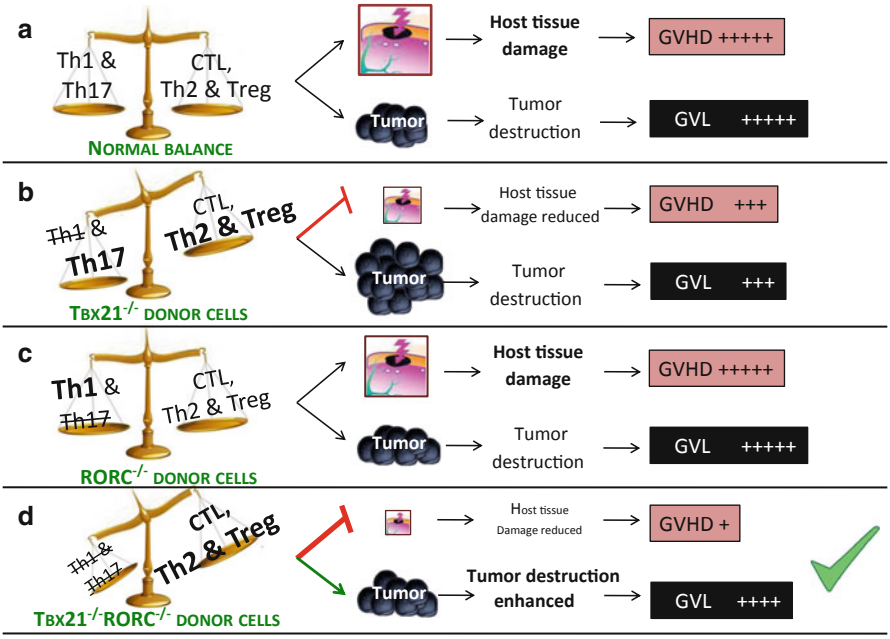

Fig. 2.6 Allo-reactive Th17 and Th1 cells in graft-versus-host diseases and graft-versus leukemia activity. Allogeneic hematopoietic cell transplantation (HCT) is an effective means for treating hematologic malignancies through harnessing the T cell-mediated graft-versus-leukemia (GVL) effect. Unfortunately, HCT benefits are frequently offset by graft-versus-host disease, which destroys health tissue via allogeneic and autoreactive donor T cells. **a**) After transfer into an allogeneic host, naive Th can differentiate into Th1 and Th17 subsets and both can mediate GVHD On the hand, Treg and Th2 cells pay inhibitory roles in GVHD. Typically, Th1 and Th17 cells predominate over Th2 and Treg cells after allogeneic HCT, where tumor (e.g. leukemia) cells are rejected but severe GVHD also occur frequently. The genetic absence of Th1 (**b, Tbx21**$^{-/-}$) but not Th17 (**c, RORC**$^{-/-}$) transcription factors alone reduced GVHD without fully compromising GVL activity. Importantly, concomitant depletion of both transcription factors (**d, Tbx21**$^{-/-}$**RORC**$^{-/-}$) manifested the most optimal outcome. These cells were skewed toward Th2 and regulatory phenotypes, and nearly ameliorated GVHD in a major MHC-mismatched model of HCT. Moreover, the GVL effects mediated by granzyme-positive CD8^{+} T cells were largely preserved despite T-bet and RORgt deficiency

Th17-programming cytokines IL-6 and TGF-β. Likewise, Tc17 cells elicited potent regression of melanoma in irradiated mice and expressed higher levels of *Tcf7* and β-catenin than Tc0 cells [104], [158]. As with Th17 cells, Tc17 convert to IFN-γ/IL-17A- producing cells once infused into irradiated mice. Subsequent studies revealed that Tc17 programmed cells mediated tumor regression and persist to a significantly greater extent than unprogrammed CD8^{+} T cell counterparts in vivo [158].

However, parallels between Th17 and Tc17 cells begin to diverge from there. While Th17 cells engraft and kill tumor with greater efficacy than IL-12 programmed CD8^{+} T cells (i.e., Th1 cells) in lymphodepleted mice (TRP-1 CD4^{+} ACT model), the situation is reversed in the Pmel-1 CD8^{+} ACT model. The Dutton laboratory

first reported that Tc1 cells mediate tumor regression to a significantly greater extent than Tc17 cells [65], [159]. However, both Tc1 and Tc17 cells are superior at mediating tumor regression when compared to Tc0 cells. The mechanism underlying the effectiveness of Tc1 versus Tc17 cells in tumor immunity has not been fully defined.

7.2 Therapeutic Potential of Tc1 Versus Tc17 Cells in Antitumor Immunity

Newly published results from the Yu laboratory revealed that Tc1 and Tc17 cells elicit antitumor immunity against established melanoma through a distinct mechanism [160]. Tc17 cells suppress the growth of melanoma transiently, while Tc1 cells induce long-term tumor regression. After infusion, Tc1 cells maintain their capacity to produce IFN-γ, but did not produce IL-17A. Although Tc17 cells preserved their ability to secrete IL-17A, a population of cells was found to co-secrete both IL-17 and IFN-γ, revealing that Tc17 but not Tc1 cells exhibit plasticity in vivo. Mechanistically, Tc1 cells mediated potent antitumor immunity via the direct IFN-γ signaling of tumor cells. In contrast, Tc17 cells mediated tumor regression independently of IFN-γ signaling. However, IFN-γ still played a critical role in tumor immunity, by creating a microenvironment that supported Tc17 cell-mediated antitumor activity. Taken together, these studies demonstrate that both Tc1 and Tc17 cells can mediate effective antitumor immunity through distinct effector mechanisms; ultimately, however, results demonstrated that Tc1 cells mediate tumor regression to a greater extent than Tc17 cells in mice with lung-metastasis.

Emerging evidence indicates that the Wnt–β-catenin signaling pathway is important for the long-term maintenance of memory $CD8^+$ T cells, as it is with $CD4^+$ T cells. In both murine and human experiments, GSK3β inhibitors (such as TWS119), known to increase β-catenin in the cell, block naïve T cell differentiation into T_{EM} cells while promoting the generation of self-renewing T_{SCM} and T_{CM} cells [104], [125], [142]. Consistent with these findings, chemically induced (by TWS119) expression of a stabilized form of β-catenin inhibited T cell proliferation and prevented the cells from acquiring a fully effector phenotype, thereby enhancing the antitumor activity of T cells in vivo.

8 Clinical Use of Human Th17 Cells for Adoptive Immunotherapy

The promising findings in mice suggest that human Th17 cells may provide useful therapies of cancer patients. To date, clinical trials with ACT have been limited to the use unprogrammed CD4 and/or CD8 lymphocytes (either TIL or gene engineered). In these trials, tumor-reactive lymphocytes are expanded with high dose IL-2 (6,000 IU/ml) and soluble OKT3 (anti-CD3), or are expanded with magnetic beads decorated with CD3 and CD28 agonists [161], [162]. Th17 cells have yet to be exploited

in the clinic; however, one promising case study revealed that a patient infused with ex vivo-propagated $CD4^+$ T cell clones (that recognized tumor-associated antigen NY-ESO1) experienced antitumor responses to bulky metastatic melanoma [163]. Thus, it seems possible that human Th17 cells could be used in next-generation clinical trials to treat cancer patients. Given that human Th17 cells can be engineered for tumor specificity (via TCR or CAR that recognize tumor antigen), the gene therapy approach permits opportunities with either engineered or sorted $CCR6^+$ Th17 cells for the treatment of a broader range of malignancies and a wider patient population [113], [115], [164]–[171]. The approach would circumvent the use of inefficacious short-lived or terminally differentiated T cells obtained from TIL [116], [123]. Such therapies would likely exploit the known cytokines and co-stimulatory molecules that potentiate the antitumor qualities of Th17 cells, as discussed in the following sections.

Although the cytokines that support the generation of human Th17 cells is known, the co-stimulatory molecules important for Th17 cell function and long-term persistence remain poorly understood. The June laboratory recently discovered that the inducible co-stimulator ICOS, but not CD28, is necessary for optimal expansion and function of human Th17 cells. ICOS stimulation-induced c-MAF, RORγt, and T-bet expression in these cells, leading to increased secretion of IL-17A, IL-21, IL-22, CCL20, and IFN-γ compared with cells stimulated with CD28 (Fig. 2.7a) [28], [172]. Conversely, CD28 ligation abrogated the effects of ICOS co-stimulation, dampening RORγt expression and increasing FoxP3 expression, which led to the reduced secretion of IL-17A and IFN-γ compared with cells stimulated with ICOS alone (Fig. 2.7b). Moreover, ICOS promoted the robust expansion of IL-17^+ IFN-γ^+ human Th17 *and* Tc17 cells; the antitumor activity of these cells following adoptive transfer into mice bearing large human tumors ($> 250\,mm^2$) was superior to that of cells expanded solely with CD28. The therapeutic effectiveness of ICOS-expanded cells was associated with enhanced functionality and long-term engraftment in vivo, reminiscent of "stemness" qualities (*vide supra*). These findings reveal a vital role for ICOS signaling in the generation and maintenance of human Th17 cells and suggest that components of the ICOS-induced pathway could be therapeutically targeted to treat cancer or chronic infection; conversely, interruption of this pathway may have utility in treating multiple sclerosis and other autoimmune syndromes.

In another xenograft study, human Th17 cells were also found to elicit the regression of human ovarian tumors in mice when combined with tumor-associated $CD8^+$ T cells [126]. Interestingly, effective therapy required both Th17 cells and $CD8^+$ T cells, as treatment of mice with Th17 cells alone or $CD8^+$ T cells alone was completely ineffective. These findings suggest that it will be critical to combine human Th17 cells with $CD8^+$ T cells to mediate therapeutically meaningful antitumor responses in patients with cancer— though the exact phenotype of the $CD8^+$ cells (Tc1 or Th17) most conducive to suppressing tumor has yet to be identified. Taken together, these data also reveal that the choice of co-stimulation for the expansion of ACT Th17 cells and $CD8^+$ T cells is critical to treatment outcome. It was surprising that CD28 co-stimulation impaired the function and antitumor activity of human Th17 cells, given that CD28 is the fallback method of choice for expanding human

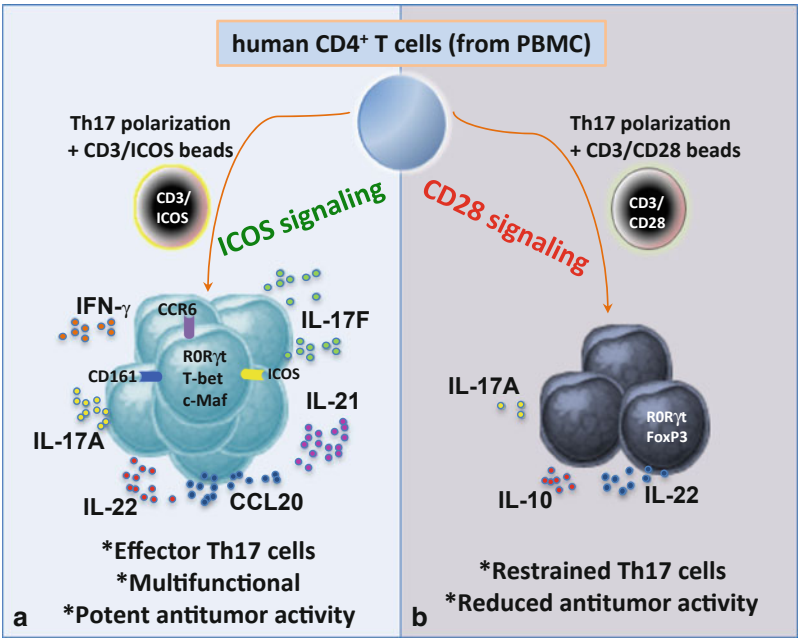

Fig. 2.7 The impact of CD28 and ICOS costimulation on human Th17 cells. CD28 and ICOS costimulation mediate distinct functional fates in human tumor-specific Th17 cells. Bulk human $CD4^+$ T cells were obtained from normal donor healthy individuals and programmed towards a Th17 cell phenotype with IL-1b, IL-6, IL-23, anti-IFN-g and anti-IL-4. TGF-b that naturally exists in the fetal calf serum was also available to the cytokine-programmed cells. These cells were either activated with artificial antigen presenting cells as magnetic beads coated with CD3, ICOS and/or CD28 agonist. As displayed herein, the nature of costimulatory molecules robustly determines the functional fate and antitumor activity of these genetically redirected lymphocytes (endow with tumor specificity with a chimeric antigen receptor (CAR) that recognizes mesothelin, an antigen overexpressed on lung, ovarian and pancreatic cancers. **a**) ICOS costimulation induced RORgt, T-bet and c-Maf transcription factors and strongly potentiated the proliferation and expansion of inflammatory Th17 cells that secrete IFN-g, IL-22 and IL-10 and secrete high levels of IL-17A, IL-17F and CCL20. A high proportion of these programmed cells expressed CCR6, ICOS and CD161 on their cell surface post expansion. **b**) Conversely, CD28 costimulation yields a smaller number of Th17 cells with are functionally restrained in terms of their secretion of these cytokines. CD28 induces FoxP3 and RORgt expression in these cells. Thus, CD28-stimulated Th17 cells are capable of secreting ample amounts of IL-22 and IL-10 but only nominal amounts of IL-17A, IL-17F and IFN-g. In xenografts bearing large human tumors, Th17 cells that eradicate human mesothelioma to a significantly greater extent if they are costimulated with ICOS compared to those co-stimulated with the CD28 signal

Th17 cells in studies of both basic and translational T cell biology. The more recent work from the June laboratory raises the possibility that the full inflammatory potential of human Th17 and Tc17 cells in vivo has not been fully reflected in previous in vitro studies.

While it is clear that ICOS co-stimulation is critical for the generation of potent human Th17 cells, whether ICOS co-stimulation of human $CD8^+$ T cells will enhance their antitumor activity is unknown. In a murine GVHD model, however, ICOS was found to have opposing effects on $CD4^+$ and $CD8^+$ T cells [173]. In the absence of ICOS signaling (using ICOS or ICOSL-deficient recipient mice), the function and expansion of $CD8^+$ T cell was increased while $CD4^+$ Th1 cell function and expansion decreased. These data suggest that ICOS augments the function of $CD4^+$ T cells while diminishing $CD8^+$ T cell function. Possibly CD28 (or other positive molecules) but not ICOS co-stimulation is optimal for the generation of human $CD8^+$ Tc1 or Tc17 cells with enhanced antitumor activity. Collectively, these data reveal that co-stimulatory (and likely co-inhibitory) molecules play distinct roles in regulating memory type 17 $CD4^+$ and $CD8^+$ T cells in tumor immunity.

Exciting work by Nishimura et al. and Vizcardo et al. have demonstrated that exhausted human T cell with short lived memory can be reincarnated to long-lived memory T cells via exploiting induced pluripotent stem cells (iPSCs) technology [174]. Both groups reprogrammed human antigen-specific terminally differentiated $CD8^+$ T cells into iPSCs [175], [176]. Then, they went on to show that differentiation of these iPSCs generated $CD8^+$ T cells that recognize their original cognate antigen with renewed features of stem cell-like memory T cells. These striking findings have significant implications for improving the efficacy of vaccine and T cell-based immunotherapies. Future work to understand how iPSC technology is shaping the generation of T cells with stem cell like qualities, such as Th17, Tc17 and stem cell-like $CD8^+$ T cells, will be insightful for advancing the cancer vaccine and cellular field. Future work that investigates if iPSC reprogrammed human Th17 cells, perhaps stimulated with proper co-stimulation, can eradiate established tumors may likely be fruitful for advancing the ACT field.

9 Conclusions

A vast number of exciting discoveries involving both basic and translational aspects of Th17 cell biology has flooded the cancer research literature since the initial discovery of these cells 8 years ago. It has become clear that these cells represent an independent subset of $CD4^+$ T cells with distinct functions in regulating host defense and promoting autoimmune manifestations, neither of which could be sufficiently accounted for by Th1/Th2 paradigm described 30 years ago. Although the role of Th17 cells in tumor immunity remains ambiguous, their hallmark-associated cytokines and co-stimulatory molecules clearly exert crucial control of cell plasticity and antitumor activity. Recent research has also illuminated the large role that tumor microenvironment plays in determining whether Th17 cells will possess regulatory or pathogenic properties. While tumor-infiltrating Th17 cells from human or murine tumors appear to favor the growth of a variety of malignancies by promoting angiogenesis or suppressing tumor immunity, convincing evidence demonstrates that

adoptively transferred Th17 cells can mediate potent and long-lasting antitumor responses in mice with large tumors. However, the exact nature of how Th17 cells affect the course of tumor development remains poorly understood in some models. Whether Th17 cells adopt either a pro- or antitumorogenic role is largely dependent on the stimulation encountered by the cells. A better understanding of these signals that so divergently impact cell function and immunological fate is of significant interest to the field of cancer immunotherapy. Results could lead to the development of enhanced vaccine and T cell-based therapies for patients with cancer.

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Chapter 3
Mast Cells and Immune Response in Cancer

Mario P. Colombo and Paola Pittoni

Abstract Mast cells are the main promoters of allergic reactions, but they intervene in many other physiological and pathological conditions. For many decades, their involvement in tumor biology has been recognized, but a series of recent studies has greatly contributed to clarifying some important aspects regarding the specific involvement of mast cells in the tumor microenvironment. Data from human samples indicate mast cells as associated to either better or worst prognosis depending on tumor types and stages. Results obtained in mouse models have demonstrated that mast cells influence tumor progression, thanks to their ability to promote angiogenesis, modulate antitumor immune responses, and regulate tumor growth. All these properties are mediated by a huge variety of receptors and effector molecules, which make mast cells an extremely plastic and eclectic immune cell. In light of their pro-tumor role, mast cells may be targeted thanks to old drugs used to treat allergic disease, or to potent tyrosine kinase inhibitors acting on the c-Kit receptor.

Keywords Mast cell · Tumor · Immune response · Immunosuppression · c-Kit · SCF · Angiogenesis · Mouse model

1 Introduction

Mast cells were first described by Ehrlich in his doctoral thesis in the late 1800s as cells of the connective tissues containing granules that reacted metachromatically with aniline dyes [1]. Ehrlich thought that these granules had a nutritional function and termed the cells accordingly ("mast" meaning "well-fed" in German) [2]. The subsequent observation by Ehrlich's student Westphal that mast cells swarmed around tumors was thus attributed to the high nutritional requirements of tumor cells. Only in the following years, it became evident that mast cells were the main players in anaphylaxis and that their granules instead contained histamine, heparin

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(responsible for metachromasy), and proteases implicated in inflammation and allergic responses. Nowadays, more than 130 years after their first description, mast cells have been involved in a number of functions beyond allergy, including host defense against pathogens, immune regulation, and tissue remodeling. A great bulk of experimental and clinical investigations has contributed to our understanding of the complex interaction between mast cells and tumors. In this chapter, we summarize mast cell biology, functions, and products that permit mast cells to promote or suppress tumor growth. Evidences from the clinics linking mast cells to tumor progression are discussed. Also described are the mouse models used for analyzing mast-cell function in vivo and the various mechanisms employed by mast cells in promoting angiogenesis, modulating antitumor immune response, and controlling tumor cell growth. Finally, we discuss current therapeutic options that can be envisaged to target mast cells in neoplastic diseases.

2 Mast Cell Biology

2.1 Origin and Distribution

Mast cells are myeloid cells generated from hematopoietic stem cells. They leave the bone marrow as committed mast cell precursors, circulate throughout the body, and complete their maturation in peripheral tissues, under the influence of local cytokines and growth factors, such as stem cell factor (SCF), interleukin-3 (IL)-3, IL-4, and IL-9 [3], [4]. Mature mast cells preferentially inhabit vascularized districts exposed to the external environment, such as the skin, the respiratory tract, the gastrointestinal tract, and the genitourinary system, as well as the serosal cavities, but they can be found in almost all tissues, mainly associated with blood vessels and nerve terminations [5].

Tissue-resident mast cells are a long-lived population, which, in response to appropriate stimulation, can reenter the cell cycle and proliferate locally; the pool of mast cells in a tissue can also be expanded by recruitment of new bone marrow-derived progenitors [3].

In both, mice and humans, mast cells can be divided into two main subsets, mucosal and connective tissue mast cells, according to their location and granule content. In general, mast cells are characterized by an elevated plasticity, allowing them to easily adapt to different tissue microenvironments by finely tuning their receptors, mediators, and activation threshold, in response to local stimuli [6].

2.2 Activation and Functions

Mast cell progenitors and mature mast cells are characterized by high expression of the c-Kit receptor [7], [8]. The signaling pathway activated through the engagement

of c-Kit by its ligand SCF is crucial for mast cells throughout their whole life, since it governs their development and maturation [9], as well as migration [10] and survival [11]. Moreover, c-Kit engagement can also modulate activation [5], [12], [13].

Mast cell activation is stereotypically associated to the engagement of the high-affinity receptor for immunoglobulin E (IgE), FcεRI, expressed at high levels on mast cell surface. IgE antibodies are important physiological components of the adaptive immune response to helminthic parasites, but are better known for their infamous role of instigators of allergic reactions. When parasites or allergen-bound IgE induce FcεRI cross-linking, a signaling cascade is triggered, which culminates with the release of granule content by mast cells (degranulation) [14].

Mast cell activation can also be achieved through the engagement of other receptors expressed on their surface, such as complement receptors (C3aR, C5aR), Toll-like receptors (TLRs 1–10), and other microbial pattern recognition receptors, such as nucleotide oligomerization domain (NOD) receptors and Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing Protein (NLRP) proteins, that facilitate the detection of bacterial, fungal, and viral pathogens and their products.

Mast cells are also characterized by the expression of an extensive variety of costimulatory molecules, such as cluster of differentiation 80 (CD80), CD86, programmed cell death 1 ligand 1 (PD-L1), PD-L2, OX40, CD28, CD40L, and glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), which can modulate mast cell activation and also mediate their immunoregulatory function [15]. Finally, receptors for several cytokines, such as IL-3, IL-9, IL-18, IL-33, and thymic stromal lymphopoietin (TSLP), confer further levels of mast cell modulation, also considering that the crosstalk between different receptors may result either in co-activation (synergism or additive effects) or in inhibition of response [16].

Under the appropriate stimulation, mast cells can produce a large variety of biologically active molecules (summarized in Fig. 3.1), a feature that characterizes them as one of the most versatile population in the immune system. Mast cell mediators are mostly preformed, stored in granules, and ready to be secreted by degranulation few minutes after FcεRI cross-linking. Granules include histamine, heparin, serotonin, proteases such chymase, tryptase, and carboxypeptidase A, and tumor necrosis factor alpha (TNF-α). In addition, lipid-derived mediators such as leukotrienes and prostaglandins are synthesized *de novo* starting from stored arachidonic acid and released shortly after activation. These early inflammatory mediators confer the ability of mast cells to kill pathogens promptly and neutralize toxins and venoms. They are also responsible for deleterious mast cell-derived effects, like allergic responses, asthma, and life-threatening systemic anaphylaxis.

In addition, mast cells can produce many other mediators by *de novo* protein synthesis, such as cytokines (including IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17; transforming growth factor beta (TGF-β); chemokines (including IL-8, monocyte chemotactic protein 1 (MCP-1); regulated on activation, normal T cell expressed and secreted (RANTES)), growth factors (including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), nerve growth factor (NGF), platelet-derived growth factor (PDGF)), and other molecules, in response to not only IgE

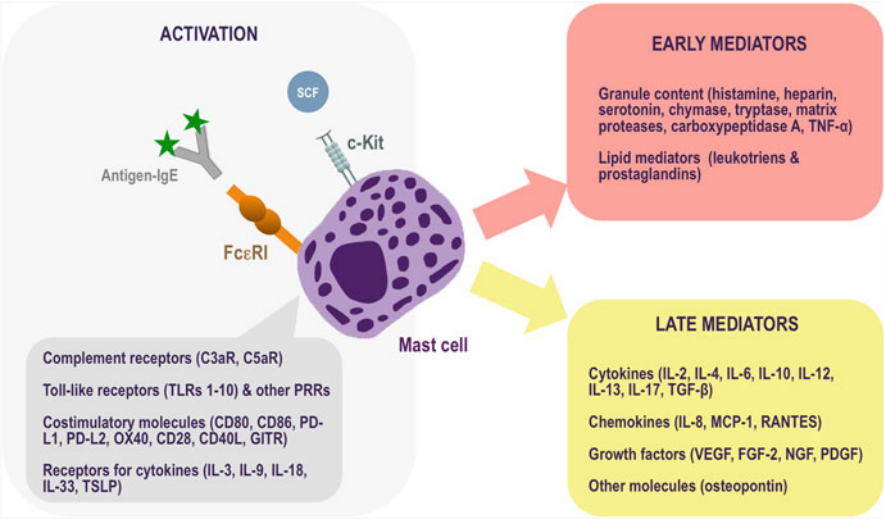

Fig. 3.1 Mast cell activation and mediators. Schematic representation summarizing signals mediating mast cell activation and release of mediators. The two main receptors expressed by mast cells are FcεRI and c-Kit, engaged by antigen-bound IgE and SCF, respectively. The activity of these two receptors is integrated and modulated by a wide variety of other receptors expressed by mast cells. Following activation, mast cells produce a panoply of mediators, which can be classified as early and late, according to the kinetics of their release

binding, but also a variety of other stimuli engaging their heterogeneous receptor repertoire. Mast cells are also able to undergo "piecemeal degranulation," a controlled and selective release of small aliquots of granule-associated material, without overt degranulation, frequently observed in tumors. Therefore, mast cells are not dull executors of stereotyped reactions, but rather smart orchestrators of the behavior of neighboring interacting cells, like stromal, tumor, and immune cells.

3 Mast Cells and Immune Modulation

Several studies in vitro and in vivo, summarized by [17], have shown that mast cells can influence many aspects of immunity. Through the release of their inflammatory mediators, mast cells can stimulate the initiation and regulate the magnitude of innate immune responses. In addition, mast cells can promote the development of acquired immune responses behaving as antigen-presenting cells, as well as by modulating the biology of dendritic cells (DCs), T cells, and B cells.

In general, the products released by mast cell degranulation enhance the maturation and migration of DCs and skew the immune balance towards T helper 2 (Th2) responses. Mast cell-derived TNF-α stimulates DC and Langerhans cells migration; histamine, prostaglandin E2 (PGE2), and prostaglandin D2 (PGD2) inhibit IL-12 production by DCs and promote DC maturation towards an effector DC2 phenotype.

In the absence of degranulation, antigen-presenting mast cells can engage cognate interactions with T cells at the immunological synapse and enhance T-cell proliferation and cytokine production through the expression of costimulatory molecules. Among them, OX40L engages OX40 receptor on T lymphocytes and boosts T-cell activation.

Through the production of disparate cytokines, mast cells can influence the polarization of naïve T cells towards the Th1, Th2, Th17, and regulatory T cells (Tregs) phenotype. Moreover, several mast cell molecules, such as IL-4, IL-6, IL-13, and CD40L, can influence B cell development and function, including IgE production [18].

In recent years, mast cells have been recognized to play immunoregulatory role [19]. A growing body of evidence indicates that mast cells can reduce inflammation and limit the immune response, mainly through the secretion of the suppressive cytokines IL-10 and TGF-β. Mast cell-derived IL-10 has been shown to limit the magnitude, and to promote the resolution, of innate responses to low-dose chronic ultraviolet B (UVB) irradiation, as well as of contact hypersensitivity reactions induced by the dinitrofluorobenzene (DNFB) hapten [20]. Mast cell-derived IL-10 also dampens immune responses after mosquito bites. Mast cell-produced TGF-β has been shown to contribute to the maintenance of homeostasis in the oral cavity and esophagus [21].

Mast cell expression of PD-L1 and PD-L2, the ligands for the inhibitory receptor PD1 expressed by T cells, can mediate T-cell apoptosis, maintenance of anergy and exhaustion, as well as IL-10 production. Mast cells can exert an immunomodulatory role also through the interaction with other regulatory immune cell populations, namely FoxP3+ Tregs and myeloid-derived suppressor cells (MDSCs).

Our group was among the first demonstrating mast cell interaction with Tregs. We uncovered the existence of a bidirectional crosstalk between mast cells and Tregs through the OX40L/OX40 axis. The interaction with Tregs suppresses FcεRI-mediated mast cell degranulation in vitro and dampens the allergic response in vivo [22]. On the Treg side, mast cells counteract Tregs inhibition over effector T cells in vitro and skew the T-cell phenotype towards IL-17-producing cells [23].

The group of Noelle showed that the interaction between mast cells and Tregs is essential for the maintenance of allograft tolerance [24]. In tolerant grafts, Tregs produce IL-9, a cytokine with well-known mast cell-attractant capabilities. Mast cells, recruited and activated in tolerant grafts by IL-9, mediate local immunosuppression by depleting the environment of IL-6 through mast cell protease-6 (Mcp-6) [25] and by conditioning DCs towards tolerance through the production of copious amounts of granulocyte–macrophage colony-stimulating factor (GM-CSF) [26]. In contrast, intragraft or systemic mast cell degranulation induces the loss of Treg suppressive function and allografts are acutely rejected [27].

A very recent report has contributed to disclose the interactions of mast cells and MDSCs [28]. MDSCs are a heterogeneous population of cells, able to mediate opposing immune effects according to the phenotypic subset. Mast cells can enhance both immunosuppressive and immunosupportive functions of MDSCs. In particular, both the clearance of the helmintic parasite *Nippostrongylus brasiliensis* by the

granulocytic MDSC subset and the suppression of the immune response to B16 melanoma by monocytic MDSC subset were dampened in mast cell-deficient mice.

As we will see in the following sections, the above-described properties of mast cells in immunomodulation can become crucial for the outcome of the antitumor immune response.

4 Mast Cells and Human Tumors

The infiltration of the tumor microenvironment by mast cells is a common finding in many types of human cancer. In most cases, mast cells are localized at the edges of the growing tumor, rather than inside. According to the tumor type, grade, and stage, the presence of mast cells can have an either negative or positive connotation for the host. The association of mast cells with lymphoma tumors represents a paradigmatic example. Hodgkin's lymphoma is densely infiltrated with mast cells, and patients bearing tumors with higher mast cell numbers have a worse relapse-free survival [29]. In line with this, patients with primary cutaneous lymphoma with a progressive course show higher mast cell counts than stable patients, and mast cell numbers directly correlate with disease progression [30]. In contrast, high mast cell counts are related to a favorable outcome in diffuse large B-cell lymphoma [31].

Multiple myeloma is frequently associated with mast cell infiltration and neovascularization, which correlate directly with disease severity.

Increased mast cell numbers have been correlated with poor prognosis also in several solid tumors. Among these, high mast cell density was found in invasive melanoma compared to benign nevi and in situ melanoma and represents a factor of negative prognostic significance [32]. Similarly, mast cells infiltrate thyroid carcinomas, but are virtually absent from normal thyroid tissue; furthermore, mast cell presence and intensity of tryptase staining positively correlate with invasive tumor behavior [33].

In contrast, increased mast cell number has been correlated with a good prognosis in other human tumors. In a large cohort of invasive breast cancer patients, stromal mast cell infiltration was indicated to be an independent good prognostic marker [34]. Likewise, in patients with surgically resected non-small cell lung carcinoma, mast cell infiltration of the tumor islets was associated with increase in survival, independently of other favorable prognostic factors including stage [35]. Analogous findings were obtained, among others, for ovarian cancer, endometrial carcinoma, and pleural mesothelioma.

Prostate cancer represents a tumor, in which association of tumor outcome with mast cell density has provided opposing results in different studies. Nonomura et al. evaluated mast cell infiltration in 104 patients and found that mast cell count was higher around cancer foci in patients with higher Gleason scores than in those with low Gleason scores. Prostate-specific antigen-free survival of patients with lower mast cell counts was thus better than that in patients with higher mast cell counts [36]. Oppositely, Fleishmann and colleagues determined mast cell densities in prostate

cancer samples of more than 2,300 hormone-naïve patients. They found that high mast cell densities were significantly associated with less aggressive tumors with good prognosis, in contrast to a poor outcome for patients without intratumoral mast cells [37]. Finally, Johansson and colleagues demonstrated in untreated prostate cancer patients with a long follow-up that the accumulation of intratumoral mast cells has a protective role and relates to a favorable prognosis, whereas mast cells in peritumoral tissue are indicative of poor prognosis [38].

In this complex scenario, studies of experimental carcinogenesis carried out in mast cell-deficient mice have represented invaluable tools to finely dissect the antitumor and protumor role exerted by mast cells. Of note, although there are many similarities between mast cell populations in human and mouse, differences also exist in their anatomical distribution, phenotype, and function; therefore, care must be taken in the evaluation of experimental results obtained in mouse models.

5 Mast Cell-Deficient Mice

5.1 c-Kit Mutant Mice

Up to now, the majority of the in vivo studies on mast cells and cancer have been performed in mouse strains harboring spontaneous inactivating mutations in the *W* locus, which encodes for the c-Kit receptor. C-Kit represents the main growth factor for mast cells; therefore, alterations in its expression result in severe mast cell deficiency. Nonetheless, c-Kit has a pleiotropic role in the development and function of many other stem and mature cells in the organism, so c-Kit mutants display additional abnormalities that impinge on their global phenotype and may lead to confounding results unrelated to mast cell deficiency.

The first c-Kit mutant employed has been the $Kit^{W/W\text{-}v}$ model [39]. This strain harbors two mutated alleles at the *W* locus: the *W* mutation, a point mutation ablating a transmembrane c-Kit portion and impeding protein expression on the cell surface [40], and the *W-v* mutation, a point mutation in the kinase domain, resulting in decreased enzymatic activity [40]. $Kit^{W/W\text{-}v}$ mice exhibit not only profound mast cell deficiency in many organs, but also other c-Kit-related abnormalities, such as defective melanogenesis, sterility, anemia, and neutropenia [reviewed by 41].

A neater model of mast cell deficiency was found in mice bearing the $Kit^{W\text{-}sh}$ mutation, which consists of a genetic inversion in the c-Kit promoter [42]. The 3′ end of this inversion is located upstream the coding region of c-Kit and breaks a positive element that controls c-Kit expression specifically in mast cells [43]. $Kit^{W\text{-}sh/W\text{-}sh}$ mice display profound mast cell deficiency in many organs and impaired melanogenesis but are neither anemic nor sterile [41], and have therefore become a widespread model to investigate mast cell involvement in several disease settings, including cancer [44]–[46]. In these mice, it is possible to adoptively transfer in vitro-cultured, bone marrow-derived, wild-type (wt) or mutant mast cells by intravenous, intraperitoneal, or intradermal route, in order to generate “mast cell knock-in mice.” Mast cell

reconstitution permits to ascertain whether biological differences observed between wt and Kit-mutant mice are effectively due to the lack of mast cells.

Unfortunately, further analysis on *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice disclosed that these mice are characterized by megakaryocyte and myeloid cell expansion associated to splenomegaly, beyond other cardiac aberrancies [47]. These additional defects may impact the outcome of experiments aimed at assessing the role of mast cells in conditions involving immune responses, including cancer development. The reversion of the defective phenotype through mast cell reconstitution, associated to an independent pharmacological approach aimed at inhibiting mast cell function in wt mice (i.e., by means of the mast cell stabilizer cromolyn or tyrosine kinase inhibitors), has been considered the gold standard controls in studies with *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. However, the generation of c-Kit-independent mast cell-deficient mouse models has modernized the scenario of mast cell research.

5.2 Mast Cell-targeted Mutant Mice

The need for c-Kit independent mast cell-deficient mouse models stimulated the generation of several new mutants, in which mast cell ablation has been obtained by expressing ectopic toxic gene products specifically in mast cells. In particular, Dudeck et al. generated mice co-expressing either a Cre-inducible diphtheria toxin receptor (iDTR) or an active diphtheria toxin subunit (R-DTA) and Cre-recombinase under the control of mast cell protease-5 (Mcpt-5) [48], which ensure depletion of connective tissue mast cells. In the toxin receptor-mediated conditional cell knockout (TRECK) model [49], iDTR expression is driven by an intronic enhancer element that normally drives IL-4 expression in mast cells. In models bearing iDTR, mast cell ablation is inducible, and repetitive diphtheria toxin injections are required to reach durable mast cell depletion. In the Cre-Master mouse, the genotoxic effect of Cre-recombinase expressed at high levels under the control of carboxypeptidase-3 (Cpa-3) promoter was exploited to obtain constitutive mast cell eradication [50]. Finally, Galli and collaborators engineered the "Hello *Kit*ty" mouse (so called to stress its independence from *Kit* mutations), in which a Cpa-3-induced Cre drives deletion of the anti-apoptotic factor Mcl1 specifically in mast cells, leading to their selective death [51].

Although these newer models show a more selective mast cell depletion than Kit mutants, they are not devoid of limitations: In these mice, the effect of Cre-mediated expression of diphtheria toxin can vary in mucosal versus connective tissue mast cells, as well as it can induce a partial but significant reduction in other cell types, especially basophils (Fig. 3.2).

As reviewed by [52] and [53], experiments in Kit-independent models have confirmed the role of mast cells in mediating allergic disease and anaphylaxis, as well as their involvement in the response to specific bacteria, parasites, and toxins. However, data obtained in the past, indicating mast cell contribution to the severity of antibody-dependent arthritis, have not been reproduced in these new models. These

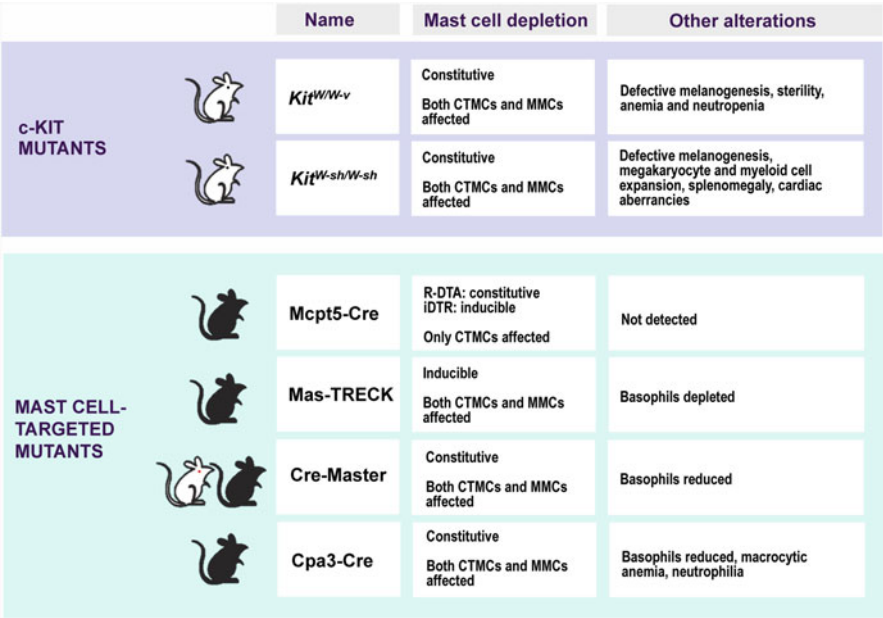

Fig. 3.2 Mast cell-deficient mouse models. Schematic representation summarizing the properties of c-Kit-dependent and mast cell-targeted mouse models of mast cell deficiency. Cartoons represent mouse appearance: white coat with black eyes (due to impaired melanogenesis) for c-Kit mutants and black coat or white coat with red eyes for mast cell-targeted mutants available on the C57Bl/6 or Balb/c background, respectively

discrepancies call for the use of different model systems to confirm the true role of mast cells in particular disease settings. Nevertheless, concordant results underscore the fact that, in certain tissue compartment and associated pathology, no differences exist between total mast cell impairment and more selective mucosal or connective tissue mast cell deletion. Indeed, the first assessment of tumor transplanted into both Kit-dependent ($Kit^{W\text{-}sh/W\text{-}sh}$) and -independent (Mcpt5-Cre/iDTR) mast cell-deficient mice showed similar delayed tumor growth in comparison to mast cell-competent mice [30].

6 Role of Mast Cells in Cancer Promotion

Mast cell recruitment to tumor sites occurs through migration of resident mast cells from neighboring healthy tissue, or through *de novo* recall of bone marrow-derived mast cell progenitors. The main chemoattractant signal for mast cells is SCF: Many mouse and human tumors directly produce SCF, which contributes to mast cell migration and activation in the tumor microenvironment through the feline sarcoma oncogene (FES) protein-tyrosine kinase [53], [54].

Besides SCF, mast cells can be selectively recruited to neoplastic sites by means of CC chemokine ligand 2 (CCL2) and CCL5 chemokines.

The vast majority of reports show tumor-infiltrating mast cells involved in the promotion of cancer cell growth and tumor invasion. Basically, mast cells can support tumor growth in two main ways: (1) as components of the tumor microenvironment impacting on tumor angiogenesis and tissue remodeling [55], [56] and (2) as immune regulators, dampening the antitumor immune response [57]. Considering the signals promoting angiogenesis also capable to inhibit intratumoral accumulation of effector T cells, the two functions above can be considered as interconnected. As recently reviewed by [58], the adhesion and migration of leukocytes are in fact reduced in an angiogenic endothelium. Moreover, tumor endothelium can express mediators that suppress the actions of effector lymphocytes, such as PDL1 and PDL2, FAS ligand, TNF-related apoptosis-inducing ligand (TRAIL), immunosuppressive soluble mediators, and also indoleamine 2,3-dioxygenase (IDO), a well-known enzyme that suppresses T-cell activation through the depletion of tryptophan.

It should be mentioned that mast cells also synthesize compounds that are directly mitogenic for tumor cells, like histamine via the cognate H1 receptors, expressed on tumor cells of various origin.

6.1 Mast Cells, Angiogenesis, and Tissue Remodeling

Angiogenesis is a critical step in tumor progression: The formation of new vessels is essential to provide nourishment to a growing tumor and is also required for the formation of long-distance metastasis. Mast cells have long been known to produce potent pro-angiogenic mediators, such as IL-8, VEGF, FGF-2, PDGF, heparin, and angiopoietins [55]. Studies in several human tumors have revealed a correlation between mast cell numbers and microvascular density [59].

In addition, mast cells are rich in metalloproteases, especially matrix metalloprotease-2 (MMP-2) and MMP-9, which are instrumental for extracellular matrix degradation and tumor invasion. Matrix metalloproteases are also crucial for the cleavage from the extracellular matrix of bioactive angiogenic factors [60], [61].

Coussens and coworkers first demonstrated that early infiltration of mast cells is necessary for the angiogenic switch beginning in hyperplastic lesions in a mouse model of skin squamous carcinogenesis [62], [63]. Through the mast cell-specific proteases Mcp-4 and Mcp-6, stromal fibroblasts receive proliferation and activation signals while MMP-9 is released in the active form. Such premalignant angiogenesis was ablated in the $Kit^{W/W\text{-}v}$ background.

In the bone marrow, regulation of the c-Kit/SCF axis requires MMP-9 to render soluble SCF available to recruit stem cells from the bone marrow niche [64]. This function is exploited in the tumor microenvironment, where mast cell-derived MMP-9 cleaves membrane-bound SCF from the tumor cell surface, which in turn amplifies mast cell attraction and activates an immunosuppressive program [54].

Ang-1 produced by mast cells is essential to stimulate angiogenesis in plasmacytoma [65]. Adrenomedullin (AM), a peptide amide released in hypoxic milieus and abundant in many malignancies, mediates a bidirectional crosstalk between tumor cells and mast cells. Tumor-derived AM stimulates mast cell chemotaxis, histamine degranulation, and release of pro-angiogenic factors whereas mast cell-derived AM directly promotes neo-angiogenesis [66].

In an inducible pancreatic β-cell tumor model, mast cells were shown to be the first cells recruited into transforming foci upon activation of the *Myc* oncogene [45]. In this model, pharmacological (via the mast cell stabilizer cromolyn) or genetic (by using $Kit^{W\text{-}sh/W\text{-}sh}$ mice) mast cell blockade severely dampened the extensive vascular assembly required for macroscopic tumor expansion, a finding clearly demonstrating the causal relationship between tumor-associated mast cells and angiogenesis.

Our group recently contributed to elucidate the involvement of mast cells in prostate carcinogenesis [46]. Previous studies in human prostate tumors were discordant about the association of mast cell infiltration and patient prognosis. Such discrepancies may come from the multifocal origin of prostate cancer, usually characterized by multiple neoplastic foci with heterogeneous characteristics. To dissect the relationships between masts and prostate cancer cells with different degrees of malignancy, we took advantage of the transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, which develop spontaneous prostate cancer quite rich in mast cell infiltration. In particular, mast cells are enriched in areas of well-differentiated (WD) adenocarcinoma, but are lacking in poorly differentiated (PD) areas. This trend was confirmed in human prostate biopsies, suggesting that mast cells may relate differently with tumor cells according to their stage. To better understand these interactions, new tumor cell lines with different degrees of malignancy were derived from TRAMP tumors, encompassing WD or PD characteristics. Cell lines of both types grew efficiently in vivo, but only nodules derived from WD tumors were infiltrated with mast cells; accordingly, WD but not PD tumors secreted large amounts of SCF.

The different mast cell influence on take and growth of transplanted WD and PD tumor cell lines was demonstrated injecting them into $Kit^{W\text{-}sh/W\text{-}sh}$ mice or wt mice that concomitantly received cromolyn. Both approaches resulted in a growth failure of WD but not PD tumor cell lines. Reconstitution of mast cell-deficient mice with wt bone marrow-derived mast cells (BMMCs) rescued WD tumor growth, demonstrating that mast cells are both necessary and sufficient for the development of WD prostate tumors. On the other side, PD nodules seem not to require mast cells for continuous growth. The search of the key factor mediating mast cell protumoral activity identified MMP-9 that was highly expressed by PD lines, but absent in WD lines. Reconstitution of $Kit^{W\text{-}sh/W\text{-}sh}$ mice with MMP-9 ko BMMCs showed that, although recruited at tumor site, these mast cells were unable to support tumor take. This meant that early-stage prostate tumors depend on and exploit mast cells for MMP-9 provision in order to invade the nearby tissue and recruit new blood vessels. Along with neoplastic progression, tumors gain the capacity of producing MMP-9 autocrinously and become mast cell-independent. The same correlation existing between mast cell infiltration, MMP-9 provision, and tumor stage was found in both TRAMP and human prostate cancers.

The correlation between mast cell infiltration and disease progression may exist in tumors other than prostate carcinoma, such as breast cancer. Work on mice bearing 4T1 breast tumors showed that treatment with cromolyn revealed enhanced peritumoral blood clotting and intratumoral hypoxia in treated mice [67].

6.2 Mast Cells and Inflammation-Mediated Tumorigenesis

It is becoming increasingly clear that a chronically inflamed microenvironment fosters tumor initiation and progression [68]. In this context, mast cells may directly contribute to the establishment and maintenance of an inflammatory milieu through their ability to secrete cytokines like TNF-α, IL-1β, IL-6, and IL-17. The colon is the prototypical site where the link between chronic inflammation and increased risk of developing cancer has long been recognized. In a mouse model of colitis-associated carcinogenesis, TNF-α blockade decreased the recruitment of inflammatory cells and the incidence of cancer [69]. Although increased numbers of mast cells [19], as well as pronounced degranulation and secretion of pro-inflammatory cytokines [70], have been observed in patients with inflammatory bowel disease compared to controls, no conclusive studies exist on the association between mast cell prevalence and colon cancer incidence in humans. A trial that treated colon cancer patients with the histamine antagonist cimetidine perioperatively reported a strong survival benefit from the cimetidine treatment [71], but following studies did not confirm such striking results.

The group of Khazaie has greatly contributed to the dissection of the role played by mast cells in colon carcinogenesis. Adenomatous polyps are preneoplastic lesions, occurring frequently in individuals bearing specific genetic alterations affecting the *adenomatous polyposis coli (APC)* gene. Mounting evidence suggests that the progression of polyposis to malignant tumors depends on a receptive stromal microenvironment for tumor expansion. In this context, mast cells were shown as a necessary stromal component for the switch to malignancy. A state of mastocytosis is consistently found in human adenomas. By taking advantage of polyp-prone transgenic mice, Gounaris et al. showed that adenoma formation is mast cell-dependent, as assessed by decreased polyp numbers in mice transplanted with $Kit^{W\text{-}sh/W\text{-}sh}$ bone marrow. Moreover, pharmacological TNF-α neutralization significantly reduced polyp outgrowth [72].

Mast cell promotion of cancer was linked to 5-lipoxygenase activity (5-LO): 5-LO by-products directly potentiate the growth of the intestinal epithelium and intervene also in the recruitment and activation of MDSCs at the polyp site [73].

The same group contributed to disclose another complex innate-adaptive network in the gut, involving mast cells and Tregs: A bidirectional crosstalk is established between these cells in mouse colon polyps and human colorectal cancers, with Tregs fostering mastocytosis and mast cells promoting Tregs skewing into IL-17-producing cells, favoring inflammation and tumor growth [74], [75].

The activation of an inflammatory transcriptional program involving mast cells was described also in other tumor settings. In a mouse hepatocarcinoma model, mast cell activation through c-Kit was shown to exacerbate inflammation and in the tumor microenvironment, via the expression of IL-6, TNF-α, VEGF, cyclooxygenase-2 (Cox-2), inducible nitric oxide synthase (iNOS), CCL2, and intra-tumor accumulation of IL-17 [54]. Here, a loop takes place between local inflammation and immunosuppression, involving mast cells, MDSCs, and Tregs. Mast cells induce MDSC recruitment and activation through CCL2, MDSCs produce IL-17 that indirectly support Treg accumulation and suppressive function, while, in turn, Tregs attract mast cells through IL-9 secretion [76].

IL-6-secreting mast cells, Th17 cells, and granulocytes populate the microenvironment of angioimmunoblastic T-cell lymphoma and cooperate to promote vessel sprouting, local inflammation, and possibly systemic autoimmune manifestations [77].

Mast cells are also essential players in neurofibromatosis. Neurofibromatosis is a complex disorder arising in individuals bearing mutations in the neurofibromin 1 (*Nf1*) gene. The product of *Nf1*, neurofibromin, is a negative regulator of Ras; therefore, its loss results in the aberrant activation of multiple cellular pathways, including c-Kit signaling. The hallmark of neurofibromatosis patients is the formation of neurofibromas, tumors originating from Schwann cells, but also composed of blood vessels, fibroblasts, and mast cells. In a mouse model of neurofibromatosis, it has been demonstrated that Nf1 mutations restricted to Schwann cells were not sufficient to induce tumor formation; additional Nf1 haploinsufficiency in cells of bone marrow origin were required for tumor progression [78]. Mast cells are the main hematopoietic cells infiltrating neurofibromas, and their presence/activation was essential for tumor formation.

Mast cells also bridge humoral immunity and promotion of tumor progression. Indeed, deposition of immune complexes in early neoplastic stroma first activates FcγRs on mast cells that subsequently recruit other pro-inflammatory leukocytes and initiate the angiogenic switch [79].

In this context, it is worth mentioning that mast cells can also be activated by free immunoglobulin light chains (IgLCs), through an unknown receptor [80]. IgLCs are found in the serum and augment under pathological conditions such as autoimmune diseases and tumors. In plasma cell and B-cell malignancies, such as multiple myeloma, chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphomas, tumor cells may display a significant imbalance in heavy and light chain synthesis leading to the secretion of a large excess of IgLCs [81].

6.3 Mast Cells and Modulation of Anticancer Immunity

Immunosuppression is an important aspect of tumor-induced escape from immune surveillance. By means of their above-described immunoregulatory functions, mast cells can contribute to the creation of an immunosuppressive microenvironment favorable for tumor growth.

However, the first clear demonstration of a direct effect of mast cells in the suppression of a protective T-cell response against tumors came from recent data generated by Noelle and collaborators. As a model tumor, they utilized MB49, a male bladder carcinoma line that recruits angiogenesis-promoting mast cells, and expresses Y-linked minor histocompatibility antigens (H-Y antigens). H-Y antigens trigger antitumor T-cell responses in females, but not in males because they are centrally tolerant to H-Y self-antigens. Comparing tumor growth in males and females, they could get insight into the effect of rapid T-cell responses on tumor growth. They showed that $Kit^{W\text{-}sh/W\text{-}sh}$ female mice were capable of controlling tumor growth and surviving systemic tumors significantly better than wt female mice. Enhanced resistance to tumors in $Kit^{W\text{-}sh/W\text{-}sh}$ female mice was T cell-mediated, as demonstrated by adoptive transfer of tumor immunity by T-cell subsets, as well as loss of tumor resistance upon in vivo T-cell depletion. $Kit^{W\text{-}sh/W\text{-}sh}$ female mice had increased frequencies of IFN-γ-producing $CD8^+$ T effector cells in tumor-draining lymph nodes; additionally, $Kit^{W\text{-}sh/W\text{-}sh}$ female mice had significantly increased ratios of intratumoral $CD4^+$ $CD44^{hi}$ and $CD8^+$ $CD44^{hi}$ T cells relative to tumors from wt females [82].

The same group recently disclosed a novel mechanism for mast cell-mediated immune suppression in tumors, based on the deprivation of nutrients necessary for immune cell function. Tryptophan deprivation, mediated by IDO, is a long-known mechanism involved in local immune suppression. Mast cells are the major cellular source of tryptophan hydroxylase-1 (Tph-1), a newly described synthase that utilizes tryptophan as a substrate to produce serotonin and melatonin. In the MB49 model, female Tph-1ko mice have reduced tumor growth kinetics, and half of them completely reject the tumor. Through reconstitution of $Kit^{W\text{-}sh/W\text{-}sh}$ with BMMCs from either wt or Tph-1ko mice, they demonstrated that Tph-1ko but not wt mast cells conferred protection from tumor, confirming that Tph-1 is indeed a major mediator in the maintenance of a suppressive antitumor microenvironment by mast cells [83].

The complex interactions between mast cells and other components of the tumor microenvironment are summarized in Fig. 3.3.

7 Role of Mast Cells in Cancer Inhibition

7.1 Mast Cell Activation Against Tumors

The existence of a potential inverse correlation between allergic diseases and tumor occurrence was postulated already in the mid-1900s, taking cue from clinical observations. The concept that activated mast cells can exert a natural antitumor surveillance was contemplated in several epidemiological studies leading to controversial results. To date, a clear demonstration that individuals with any type of allergy have a decreased risk of cancer development has come only from few cancer types, such as glioma and pancreatic cancer [84].

Still, indications exist that mast cells can exert a direct antitumor activity: Some mast cell mediators, like IL-1, IL-4, IL-6, TNF-α, TGF-β, leukotriene B4 (LTB4),

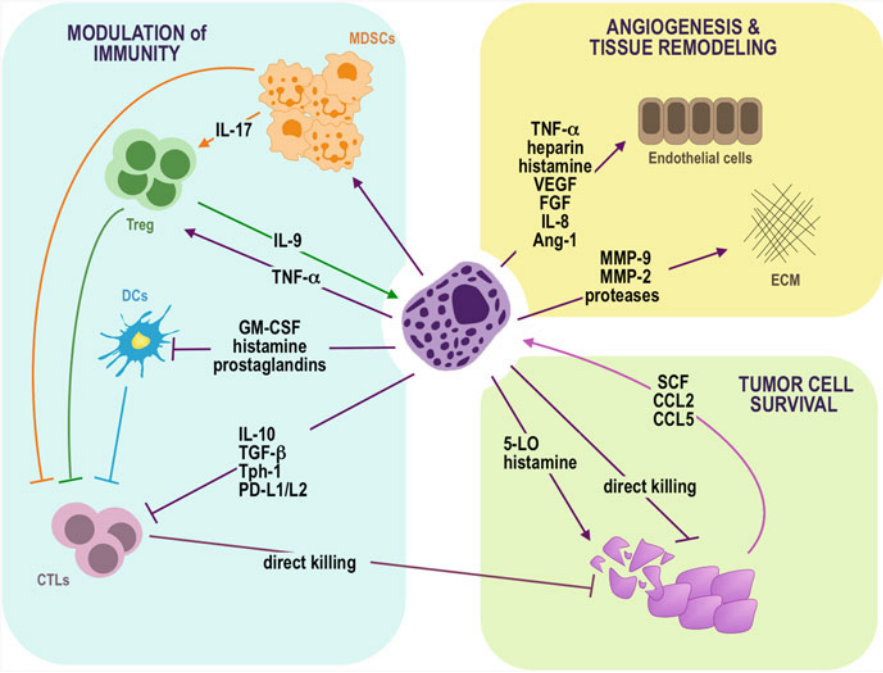

Fig. 3.3 Mast cells in the tumor microenvironment. Schematic representation of interactions between mast cells and other components of the tumor microenvironment. Developing tumor cells secrete molecules promoting mast cell recruitment. In turn, mast cells release a wide range of mediators affecting tumor cell survival, angiogenesis and extracellular matrix (ECM) remodeling, and modulation of immune cells involved in the antitumor immune response. This figure highlights the cell types and molecules mentioned in this chapter

and chymase, as well as chondroitinase and glycosaminoglycan lyases, have been shown to induce inhibition of growth, apoptosis, and disruption of tumor cells in vitro.

Our own data in the setting of experimental prostate cancer showed that in TRAMP mice chronically treated with cromolyn to inhibit mast cell promotion of adenocarcinoma development, half of treated mice showed the desired protection, while the other half of mice developed very aggressive and early developing anaplastic tumors, characterized by neuroendocrine differentiation and c-Kit expression. The same outcome was seen when TRAMP mice were crossed with $Kit^{W\text{-}sh/W\text{-}sh}$ mice [46]. Our findings indicate that functional mast cells somehow inhibit the occurrence of the neuroendocrine tumor variant, through the exertion of an antitumor effect, which could be either direct or mediated by other stromal cells. Interestingly, since in this model mast cell targeting altered the tumor phenotype, a potential influence of mast cells on the homeostasis of prostate stem cells could be envisaged. This hypothesis is corroborated by mast cell preferential location in close vicinity to stem cell compartments, and by mast cell and stem cells expression of c-Kit receptor possibly competing for SCF [85].

7.2 Allergo-Oncology

The idea to exploit the cytotoxic reactions elicited by IgE and subsequent mast cell and basophil activation for tumor control has led to the development of the so-called allergo-oncology [86]. Several approaches have been envisaged and tested in mouse models, all based on the binding of IgE on its receptors and involving an active or a passive activation of the host immune system.

For passive immunotherapy, engineered tumor-associated antigens (TAAs) monoclonal IgE antibodies were developed and proved effective in mouse models. Alternatively, IgE antibodies were used as adjuvant in antitumor vaccination, exploiting the IgE–FcεRI-driven machinery to activate the host immune system. Several reports, reviewed by [87], have shown the initial degranulation of mast cells and basophils induced by tumor cell-bound IgE as effective in promoting recruitment of effector cells, tumor cell killing, and tumor antigen cross-priming, resulting in tumor eradication. As an example, Siccardi and coworkers showed that mice vaccinated twice with IgE-loaded irradiated tumor cells showed antitumor protection with significant delay in tumor growth after a challenge with parental tumor cells [88].

8 Mast Cell Targeted Therapy

Mast cells and their products have been successfully targeted in antiallergic therapies with histamine or leukotriene receptors antagonists, corticosteroids, and mast cell stabilizer drugs. Mast cell stabilizers, such as cromolyn, are chromone agents, which prevent mast cell degranulation by stabilizing the cell membrane, through a still unclear mechanism. Although their use in clinical practice has been abandoned due to their instability and low absorption, mast cell stabilizers have been widely used to block mast cell activity in experimental studies aimed at assessing mast cell involvement in various biological processes, including cancer. As previously mentioned, several studies in mice have demonstrated the essential role of mast cell in tumor promotion by showing tumor impairment upon cromolyn administration. However, a recent study has questioned cromolyn's efficacy and selectivity on murine mast cells [89].

Novel strategies to target mast cells imply the use of imatinib and tyrosine kinase inhibitors (TKI), small molecules able to inhibit c-Kit and other tyrosine kinase receptors, which have been used in clinics for the past 10 years for the therapy of chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GIST) [90]. The use of imatinib to block mast cells has been demonstrated in a rat transplantable model of prostate cancer driven by mast peritumoral accumulation [38].

In addition, imatinib may act as a double-edged sword in tumors associated to both mast infiltration and tumor cell-intrinsic activation of tyrosine kinase receptors. For instance, in mice spontaneously developing neurofibromas, imatinib administration reduces tumor growth, promoting apoptosis and decreasing proliferation of tumor cells on the one side, and leading to mast cell ablation on the other side. Similarly,

in a pediatric patient with a plexiform neurofibroma, imatinib produced the fast contraction of the tumor mass [78]. It is conceivable that both tumor cells and mast cells represent therapeutically relevant cell targets of imatinib in this context.

In light of these results, mast cell targeting would represent a novel and effective approach in cancer therapy. However, due to the opposing role of mast cells in promoting or suppressing cancer, which has been demonstrated even in different stages of the same tumors, mast cell targeting has to be carefully evaluated before being introduced into the clinic.

9 Conclusions

Mast cells are multifaceted cells implicated in a broad spectrum of physiopathological conditions, ranging from inflammation to autoimmunity. Several studies have indicated mast cells as critical components of tumor stroma. As the interest in these cells is growing year after year, new tools are being developed to gain more inside knowledge of these elusive cells. Only when the contributions of mast cells and their myriad effectors on tumor progression and metastasis are finely understood, a fully aware mast cell-targeted therapy might enter clinical oncology.

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Chapter 4
Myeloid-Derived Suppressor Cells in Tumor-Induced T Cell Suppression and Tolerance

Paolo Serafini and Vincenzo Bronte

Abstract Tumor development is often associated with a deep alteration of normal myelopoiesis, leading to a progressive accumulation of various cellular elements, belonging to myelomonocytic lineage, in the tumor bed, in the blood, and in both primary and secondary lymphoid organs. This heterogeneous pool of cells expresses, in the mouse, the common markers CD11b and Gr-1 (Ly6C/G) and is endowed with the ability to suppress antigen and/or polyclonal-driven T cell immune response. These cells, named myeloid-derived suppressor cells (MDSCs), are mobilized from hematopoietic organs by cytokines and other factors produced by the tumors, as well as by strong activation of the immune system, and have a profound influence on the outcome of the T cell-dependent immune responses. MDSCs can restrain T cell function directly in an antigen-independent manner; however, *in vivo*, MDSCs can also process and present tumor-associated antigen and can lead to T cell tolerance in an antigen-specific manner. Furthermore, MDSCs seem to be key players in tumor-induced suppressive network that includes T regulatory (Treg) cells, inhibitory natural killer T (NKT) cells, mast cells, Th17, as well as effector T cells. The importance of MDSCs in human malignancies has been demonstrated in recent years and new approaches targeting their suppressive/tolerogenic action are currently being tested in both preclinical model and clinical trials.

Keywords Human myeloid derived suppressor cells · Mouse myeloid derived suppressor cells · Tumor derived factors · Clinical trials · Mechanisms of action · Tumor progression · MDSC phenotype

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1 History and Nomenclature of Tumor-Conditioned Myeloid Cells with Suppressive Activity on the Immune Response

In the late 1970s, many researchers described the presence of a cellular population that could inhibit different activities of the immune system, both *in vivo* and *in vitro*. These cells, named natural suppressor (NS) cells, inhibited the proliferative responses of T helper lymphocytes to mitogens or alloantigens, antibody production by B lymphocytes, the generation of cytotoxic T lymphocytes (CTLs) independently of antigen and major histocompatibility complex (MHC) restriction [1], and were also suspected to be involved in pathways of tolerance induction. NS cells were shown to appear only briefly in fetal newborn tissues and the placenta during pregnancy as well as during the neonatal maturation of the lymphoid tissues; however, they could be induced in adults by manipulation of the lymphoid tissues with certain treatments such as total lymphoid irradiation, cyclophosphamide administration, and during graft-versus-host-disease [1]. The presence of these cells in several body environments, all characterized by either enhanced hematopoiesis or an intense immune response, suggested their possible involvement in regulating myeloid cell differentiation and controlling lymphocyte and myeloid expansion.

Assigning a characteristic phenotype to NS cells was an unresolved problem for many years, and several discrepancies were found in the marker distribution in cells with suppressive activity on T lymphocytes activated *in vitro*, even though some evidence pointed to the monocytic/macrophage lineage [2]–[5]. The phenotype of NS cells was originally defined "null" because they appeared to lack the usual markers of mature macrophages, T, B, and natural killer (NK) cells. Purified NS cells did not lose their inhibitory activity during *in vitro* culture, nor kill classic NK targets, nor differentiate into macrophages or mature lymphocytes. Unfortunately, despite the importance of these early findings, many experimental limitations (such as a restricted antibody panel to identify their phenotype, the widespread use of culture supernatants with unknown cytokines and growth factors composition, and the absence of high-purity techniques to isolate cell subsets) postponed for many years the progress in understanding their biology. These technical restrictions, combined with experimental difficulties in validating some results and the absence of a clear phenotype, made the very existence of NS doubtful. For these reasons, until the 1990s, the immunosuppressive role of NS/suppressive myeloid cells in tumor-bearing host was still poorly known.

Already in 1989, Subiza et al. showed the expansion of NS in Ehrlich tumor-bearing mice [6], but the first clear involvement of myeloid cells in lowering immune surveillance and in promoting tumor growth was provided in 1995. The administration of an antibody directed against the antigen Gr-1 (recognizing the cross-reacting molecules of lymphocyte antigen 6 complex locus C and G) to immunocompetent mice reduced the growth of an ultraviolet light-induced tumor [7]. The effect of the *in vivo* anti-Gr-1 administration was originally attributed to the elimination of granulocytes, but successive reports from our and other groups suggested that the Gr-1$^+$ cells were mostly CD11b$^+$ and comprised both polymorphonuclear and mononuclear cells, including elements at different maturation stages along the myelomonocytic differentiation lineage [8], [9].

The clear heterogeneity of the $CD11b^+/Gr\text{-}1^+$ cells has generated some confusion, somehow amplified by the use of different terms to define the same cells (i.e., natural suppressor cells, immature myeloid cells, or myeloid suppressor cells). In 2007, a panel of investigators agreed to use the common term of *myeloid-derived suppressor cells* (MDSCs) [10]. The use of the myeloid term highlights the common finding of the enhanced myelopoiesis in tumor-bearing hosts and our incomplete understanding of the relationship between the two main components generated by this altered myeloid differentiation (i.e., granulocytic and monocytic cells). The past 5 years have seen an increase in the functional and correlation studies, and MDSCs have become, together with the regulatory T cells (Treg cells), a worthy biomarker for the immune monitoring of patients with cancer.

2 Mouse MDSCs: Biology and Function

MDSCs represent a heterogeneous population of myeloid cells comprising immature macrophages, granulocytes, dendritic cells (DCs), and other myeloid cells at earlier stages of differentiation that can be identified in mice by expression of CD11b and Gr-1. Co-expression of these markers, together with the immature marker CD31, and the ability to form colonies in agar are consistent with the phenotype of myeloid progenitors [11]–[13]. In healthy mice, $CD11b^+/Gr\text{-}1^+$ cells can be detected in sizable numbers only in the bone marrow (BM, about 30–40 %); however, small numbers of these cells (< 4 %) can also be found in the blood and spleen. $CD11b^+/Gr\text{-}1^+$ cells in tumor-bearing hosts comprise myeloid precursors that can generate mature granulocytes, macrophages, and DCs when cultured *in vitro* with the appropriate cytokines cocktail [13]–[15]. Disturbances in cytokine and chemokine balance, induced by tumor growth, infection, immune stress, and even vaccination, can alter the homeostasis of this population leading to its accumulation in the secondary lymphoid organs and, ultimately, influencing their maturation toward a suppressive phenotype. It must be pointed out that $CD11b^+/Gr\text{-}1^+$ cells in the BM of naïve mice do not show a relevant suppressive activity *ex vivo*, and suppression of T cell function can be observed only when supra-physiologic number of cells are used in *in vitro* assays [16]; however, they can acquire full suppressive function when cultured for few days in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) [17] or with activated $CD4^+$ T cells (Serafini, unpublished data). Also, in mice bearing solid or hematologic tumors, *ex vivo* BM-derived $CD11b^+$ cells show little suppressive activity and low expression of suppressive markers ([18] and Serafini unpublished data). Taken together, these findings seem to indicate that the majority of BM $CD11b^+/Gr\text{-}1^+$ are still pluripotent cells that can differentiate, depending on the kind and/or duration of cytokine/chemokine stimulation, into cells able to either enhance (e.g., myeloid DCs) or restrain (MDSCs) the immune response [17], [19]. Differently from BM, MDSCs in peripheral organs are fully suppressive. In various mouse models, indeed, the dysfunctional immune responses of T lymphocytes in tumor-bearing mice depended almost entirely on the accumulation of

MDSCs in the blood and secondary lymphoid organs. Primary tumor resection, Gr-1$^+$ depletion, pharmacological inhibition, or genetic MDSC inactivation often results, in fact, in a complete correction of T cell dysfunctions [7], [20]–[29].

These studies have also unveiled a functional plasticity of CD11b$^+$/Gr-1$^+$cells in tumor-bearing hosts that was confirmed by *in vitro* experiments in which MDSCs extracted from tumor-bearing mice were cultured with either Th1- or Th2-derived cytokines. The MDSCs' suppressive phenotype was enhanced, in fact, by the addition of Th2 cytokines (i.e., interleukin (IL)-4 or IL-10) to these cultures. Conversely, MDSCs co-cultured with Th1 cytokines *enhanced* antigen-specific T cell cytotoxicity, thereby underscoring the ability of MDSCs to differentiate into functional antigen-presenting cells (APCs) when placed in the appropriate cytokine environment [13]. Moreover, MDSC subsets can appear transiently in cultures of BM cells stimulated with GM-CSF to generate myeloid DCs [17]: These cells were CD11c$^-$ myeloid precursor cells with ring-shaped nuclei and were Gr-1^{low}, CD11b$^+$, CD31$^+$, ER-MP58$^+$, asialoGM1$^+$, and F4/80$^+$ [17], a phenotype very similar to MDSCs described in tumor-bearing mice [30]. Despite these *in vitro* observations and the fact that strong signal such as Flt3L, a combination of GM-CSF and IL-4 [13] or all-*trans*-retinoic acid (ATRA) [31], can force MDSCs to differentiate in fully mature DCs, other *in vivo* data suggest that MDSCs do not simply represent a transitional population along DC maturation but, instead, are cells endowed with a suppressive phenotype [14]. In fact, while CD11b$^+$/Gr-1$^+$ from naïve mice adoptively transferred into naïve congenic mice differentiated into mature CD11c$^+$ MHC class II$^+$ DCs and Gr-1$^-$F4/80$^+$ macrophages within 5 days, MDSCs from tumor-bearing mice retained the phenotype of immature cells for longer time (Gr-1$^+$CD11b$^+$) and the differentiation in macrophages was significantly impaired [31].

2.1 Monocytic and Granulocytic MDSC

In the past years, MDSCs have been classified into two main subsets with different phenotypic and biological properties: the monocytic- (m-MDSC) and polymorphonuclear/granulocytic-like (g-MDSC) [16], [32]–[34]. In tumor-bearing mice, CD11b$^+$Ly6C^{hi}Ly6G$^-$ m-MDSCs are highly immunosuppressive and exert their effect largely in an antigen-nonspecific manner, whereas murine CD11b$^+$Ly6C^{lo}Ly6G$^+$ g-MDSCs are moderately immunosuppressive and promote T cell tolerance via antigen-specific mechanisms. The same phenotypes in tumor-free, naïve mice define, respectively, inflammatory monocytes and polymorphonuclear neutrophils, both lacking the immunosuppressive activity [35]. In the vast majority of tumor models, as well as in cancer patients, granulocytic MDSCs are the predominant subset [32], [36]–[40], representing 70–80 % of the tumor-induced MDSCs compared to 20–30 % of the cells reflecting the monocytic lineage [32], [37], [41]. However, recent evidence [42] indicates that these subsets are not two completely distinct, fully differentiated myeloid populations but rather they may represent two different differentiation states of the same population (see below).

Nevertheless, g-MDSCs and m-MDSCs have been shown to employ different mechanisms of immunosuppression. While g-MDSCs express arginase (Arg) and seem to suppress antigen-specific $CD8^+$ T cells mainly through a reactive oxygen species (ROS)-mediated mechanism [32], m-MDSCs suppress $CD8^+$ T cells, predominantly, via expression of nitric oxide synthase 2 (NOS2) and ARG1 enzymes and through the production of reactive nitrogen species [16], [33], [42]. Nevertheless, both subsets of MDSCs were shown to express PD-L1 (B7-H1) [32], PD-L2 [32], and CD80, and thus multiple mechanisms of immune suppression can be concurrently active. In agreement with this possibility, Movahedi et al. [33] found that g-MDSC suppression was not recovered by ROS, ARG, or NOS2 inhibitors, but required instead interferon-gamma (IFN-γ) acting through a not yet identified signal transducer and activator of transcription factor-1 (STAT1)-independent pathway [33]. It is important to remember that both MDSC subsets often coexist in the same microenvironment, thus allowing the instauration of additional suppressive mechanisms that integrate factors secreted by each subset. Indeed, since g-MDSCs secrete elevated quantities of ROS while m-MDSCs release nitric oxide (NO) via NOS2 activation [16], [32], it is highly probable that reactive nitrogen intermediates (RNIs) are formed that are spontaneously generated when O_2^- and NO react [43]. The hypothesis of an interaction between g-MDSCs and m-MDSCs is also sustained by the analysis of the cytokine that each subset secretes: g-MDSCs secrete high levels of IFN-γ and discrete levels of IL-13, whereas m-MDSCs secrete low levels of both cytokines. We previously showed that MDSCs produce both IL-13 and IFN-γ, which are utilized in an autocrine manner to enhance the production and activity of both ARG1 and NOS2 enzymes [29]. In particular, IFN-γ is required for the upregulation of IL4Rα that mediates IL-13 signaling [29] and promotes the survival of m-MDSC and tumor-associated macrophages (TAMs) [44]. Thus, the elevated production of IFN-γ by g-MDSCs may serve to both maintain m-MDSCs' suppressive activity and prevent their apoptotic death [44].

The assumption that m-MDSCs and g-MDSCs develop along different pathways involving, respectively, monocyte and granulocyte progenitors has recently been challenged by Youn et al. [42]. The authors clearly demonstrated that a large proportion of m-MDSCs in tumor-bearing mice acquired phenotypic, morphological, and functional features of g-MDSCs by a mechanism that involves the epigenetic downregulation of the retinoblastoma protein (Rb1). These data suggest that the two subsets represent different stages of differentiation of the same population [42] and, thus, m-MDSCs not only have the capacity to strongly down-modulate antitumor immunity but also serve as "stem cell-like precursors" that maintain the g-MDSC pool; actually, m-MDSCs proliferate much faster than the g-MDSCs or the normal monocytic counterpart, can form colonies in agar, and generate a wide range of myeloid cells when either adoptively transferred to tumor-bearing hosts or exposed to GM-CSF and IL-6 cytokines *in vitro* [42], [45].

3 MDSCs and Tumor Progression

MDSC appearance has been prevalently reported in transplantable tumor mouse models [46]. These models have been often generated following multiple *in vivo* passages of the transplantable cells, which ultimately can select for clones able to avoid immune recognition. For these aspects, tumors induced by chemical carcinogen or by the activation of tissue-restricted, transgenic oncogenes are often considered more reliable as models for tumor initiation and progression. Indeed, MDSC accumulation has been reported not only in methylcholanthrene (MCA)- or 1,2-dimethylhydrazine-diHCl-induced tumors [47], [48] but also in mice in which the expression of the transforming rat oncogene c-erbB-2, under the control of the mouse mammary tumor virus promoter, drives the spontaneous development of mammary carcinomas with a progression resembling that of human breast cancers [12]. Interestingly, in these latter mice, tumor multiplicity directly correlated with the accumulation of MDSCs in the peripheral blood and in the spleens. Analogously, in the BW-Sp3 lymphoma model, most of the BW-Sp3-bearing mice mount a $CD8^+$ T cell-mediated response resulting in tumor regression. Nonetheless, tumor progression occurs in some of the recipients and is associated with MDSC accumulation. Again, *in vivo* MDSC depletion is sufficient to restore CTL activity [49]. Further examples of MDSC recruitment and activation will be discussed below in relationship with the cytokine driving their differentiation.

Interestingly, in some mouse models, MDSCs' pro-tumoral activity does not require their expansion in the secondary lymphoid organs. In a transformed fibrosarcoma model, in which tumors grow, spontaneously regress, and then recur, Terabe et al. found that IL-13-producing $CD4^+$ NKT cells suppressed $CD8^+$ CTLs to prevent complete tumor elimination [23]. The suppressive mechanism of NKT cells, however, was not direct but involved MDSCs. In fact, IL-13 secreted by NKT cells was sufficient to activate MDSCs to secrete transforming growth factor (TGF)-β that acted as final suppressive molecule. Blocking TGF-β or depleting $Gr\text{-}1^+$ cells *in vivo* prevented tumor recurrence. This negative regulatory circuit was also found to be active in a lung metastasis model of the mouse colon carcinoma CT26 [50].

As discussed later, recent evidences indicate that MDSCs can promote tumor progression not only by suppressing the antitumor immune response but also by promoting tumor angiogenesis through their incorporation in tumor vessel and regulation of vascular endothelial growth factor (VEGF) bioavailability [51] and metastases. These findings were confirmed by Young and Cigal [52] who demonstrated how $CD34^+$ cells cultured in the presence of Lewis lung carcinoma (LLC)-conditioned medium were skewed in their differentiation toward endothelial cells expressing CD31 and CD144. Moreover, a small subset of tumor-infiltrating $CD11b^+$ myeloid cells characterized by the expression of the Tie2, a receptor tyrosine kinase known to be restricted to endothelial cells, was characterized [53]. This population, called Tie2-expressing monocytes (TEMs), was advanced to represent a new hematopoietic lineage of pro-angiogenic cells, selectively recruited to spontaneous and orthotopic tumor sites and required for their neovascularization [53].

4 Tumor-Derived Factors Regulate the Expansion, Recruitment, and Activation of MDSCs

Numerous findings indicate that tumor-derived factors (TDFs) promote not only MDSC recruitment but also maturation toward an immunosuppressive phenotype. Indeed, conditioned media from tumor cell lines can inhibit the *in vitro* differentiation of DCs from their precursors [54], and normal BM cells could give rise to immunosuppressive elements simply by culturing them for a few days with supernatants from a highly metastatic LLC variant [55]. For more than 10 years, efforts have been made to identify these TDFs [26], [56]–[63]. Tumors secrete a large panel of cytokines, chemokines, or other diffusible molecules that, alone or in combination, can induce MDSC recruitment and increase their maturation into fully suppressive cells. To date, a number of candidate proteins have been identified and are discussed below.

4.1 Colony-Stimulating Factor-1 (CSF-1)

CSF-1 signaling through its receptor CSF1R (CD115, c-fms) is a critical regulator of the survival, differentiation, and proliferation of the myeloid lineage including monocytes, macrophages, and MDSCs [64]. Early studies in CSF-1 op/op mice provided the first evidence for the critical role of CSF-1 in TAM infiltration of spontaneous MMTV-PyMT breast tumors [65]. These TAM-depleted tumors exhibited reduced angiogenesis and delayed tumor progression to metastasis, and similar antitumor effects were observed in human breast cancer xenografts by administration of neutralizing antibodies against CSF-1 [66]. CSF-1 has also been shown to stimulate VEGF-A production in monocytes, demonstrating its direct role in myeloid cell-mediated angiogenesis [67]. The secretion of CSF-1, also called macrophage-colony stimulating factor (M-CSF), has been described in various cancers including acute myeloblastic leukemia [68], [69], renal cell carcinoma (RCC) [70], bladder carcinoma [71], and about 70 % of human breast cancers [72]. Its expression in breast cancer is associated with a poor prognosis and is likely involved in tumor progression [72]. CSF-1 can recruit immunosuppressive macrophages and can alter the normal DC maturation [73]. Conditioned media from renal carcinoma cell lines could alter the differentiation of DCs into mature APCs and this effect could be abrogated by the use of neutralizing antibody against IL-6 and CSF-1 [74]. Interestingly, both IL-4 and IL-13 reversed the inhibitory effects exerted by either RCC-conditioned medium or IL-6 and CSF-1 combination on the phenotypic and functional differentiation of $CD34^+$ cells into DCs. IL-4 downregulated M-CSF and IL6R-transducing chain expression, decreased the secondary production of CSF-1, and prevented the loss of GM-CSF receptor α-chain expression, which normally occurs during the differentiation of $CD34^+$ cells [73]. CSF1R expression was observed on MDSCs [33], [75]. The use of GW2580, a selective pharmacologic inhibitor of CSF1R signaling, demonstrated that CSF-1 regulated the tumor recruitment of $CD11b^+Gr\text{-}1^{lo}Ly6C^{hi}$ m-MDSCs in 3LL, B16, and orthotopic RM-1 prostate tumors, drastically inhibiting tumor angiogenesis [76].

4.2 IL-6

High levels of IL-6 have been detected in leukemia, lymphoma, multiple myeloma, melanoma, as well as in breast, lung, ovarian, renal cell, and pancreatic cancers and are associated with a poor prognosis [77]. IL-6 is produced by various cell types including monocytes, macrophages, fibroblasts, keratinocytes, endothelial cells, B cells, T cells, and several transformed cells [78]–[80]. Nevertheless, monocytes, macrophages, and MDSCs seem to be the predominant producers of IL-6 during acute inflammation, with T cells contributing under chronic inflammatory conditions [36], [81]. In these cells, IL-6 expression is regulated through the activation of several transcription factors (TFs) such as nuclear factor-kappa B (NF-κB), CAAT/enhancer-binding protein beta, or activator protein 1 (AP-1). The regulation of IL-6 expression through these TFs enables a rather unspecific upregulation of this cytokine during nearly every type of inflammation including the tumor-associated inflammation.

IL-6 can bind to the membrane-bound IL-6 receptor alpha (mIL-6R, CD126) subunit of the IL-6 receptor on target cells that associates with a homodimer of the second receptor subunit, glycoprotein 130 (gp130, CD130). Additionally, IL-6 can bind to a soluble form of IL-6R (sIL-6R) enabling IL-6 trans-signaling in cells that do not express mIL-6R but only gp130 [82], [83].

The physiological activity of IL-6 is complex, producing both pro-inflammatory and anti-inflammatory effects. In addition, IL-6 affects the differentiation of myeloid lineages, including macrophages and DCs, both *in vitro* and *in vivo* [84] through the activation of the TF STAT3, which exerts a negative regulatory function on the adaptive and innate immune system during tumor development, as described below. The important role of IL-6 in inhibition of DC differentiation has been shown in multiple myeloma [85]. Moreover, soluble factors derived from the BM of patients with multiple myeloma inhibited the generation of DCs, and VEGF- and/or IL-6-specific antibodies neutralized this inhibitory effect [86]. The same neutralizing effect can be accomplished by inhibiting the mitogen-activated protein kinase (MAPK) p38, which is activated in the cultured BM cells by co-culture with myeloma cells or exposure to tumor culture conditioning medium. Inhibiting p38 MAPK activity in BM cells cultured in the presence of tumor culture conditioning medium restored the generation of functional, BM-derived DCs [87].

IL-6 is particularly important in MDSC biology: In fact, not only it is secreted by these cells but it also regulates their differentiation and function by activating STAT3 [18]. Together with GM-CSF, IL-6 can drive both human and murine hematopoietic precursors toward MDSC differentiation [18], and antibody-mediated blockade of IL6R is sufficient to drastically reduce the number of MDSCs in the CMC-1 model of skin squamous cell carcinoma [88], [89]. Additionally, elevated IL-6 level has been reported to correlate with MDSC frequency and function in different mouse tumor models [90]. Finally, IL-6 is one of the key TDFs by which cervical, ovarian, colorectal, renal cell, and head and neck carcinoma human cell lines promote the differentiation of $CD33^+$ and $CD11b^+$ MDSCs [91]. These data, together with the elevated levels of this cytokine in patients affected by different malignancies, suggest that targeting IL-6 signaling might be an option to inhibit MDSC activity in cancer.

4.3 Vascular Endothelial Growth Factor (VEGF)

VEGF plays an important role in the formation of blood vessels during embryogenesis, hematopoiesis, and tumor neovascularization [92]. It is secreted by most tumors in high levels and its expression correlates with a poor prognosis [93]. Neutralizing antibodies against VEGF restored DCs' differentiation from hemopoietic precursors which was blocked by tumor-conditioned media [94]. VEGF has been directly linked with the systemic MDSC expansion. The administration of recombinant VEGF to tumor-free mice, in fact, resulted in inhibition of DC development and was associated with an increase in the number of MDSCs in the spleen [94]. Moreover, tumor progression and multiplicity in transgenic female BALB-neuT mice, which spontaneously develop mammary carcinomas as mentioned earlier, correlated with the increased serum levels of VEGF and the progressive accumulation of MDSCs in the blood and spleen [12]. Furthermore, additional findings showed that the link between MDSCs, VEGF, and tumor progression could be complex and suggest that VEGF can be one of the molecules regulating the crosstalk between tumor and tumor-associated MDSCs [95]. Yang et al. [51], using the MC26 colorectal carcinoma (CRC) and the LLC models, showed that MDSCs could stimulate tumor progression by promoting tumor angiogenesis. Tumor-associated MDSCs, in fact, express high levels of the matrix metalloprotease-9 (MMP-9). Deletion of MMP-9 in these cells completely abolished their tumor-promoting ability. MDSCs were also found to be incorporated directly into tumor endothelium and regulate the bioavailability of VEGF by releasing it from the extracellular matrix [51]. Besides the expression of MMP9, it was recently proposed that MDSCs can regulate VEGF biosynthesis by the expression of NOS2. Nitric oxide, in fact, is a factor upstream the VEGF signaling pathway [96] operating by a mechanism that involves the hypoxia-inducible factor-1 alpha (HIF-1α)-binding site and HIF-1 ancillary sequence site within the hypoxia response element [97]. In transplantable and spontaneous models of MT-RET syngeneic melanoma, production of NO by both cancer cells and MDSCs promoted VEGF production, which, in turn, drove MDSC recruitment, trafficking, and acquisition of immunosuppressive function at the tumor site [98]. These data suggest the existence of a positive loop by which NO-producing intratumoral MDSCs promote the synthesis of VEGF by the tumor cells and stroma, which, in turn, allow the recruitment and differentiation of new MDSCs. Thus, the interruption of this circuit by the use of NOS2 inhibitors [98] or other drugs that influence NO production (i.e., PDE5 inhibitors [28], nitroaspirin [27], and AT38 [99]) could partially explain the antitumor and anti-immunosuppressive effects observed with these drugs.

4.4 GM-CSF

Although GM-CSF has long been considered an immune adjuvant, numerous evidences uncovered its dual role in stimulating as well as suppressing the immune system. Almost 31 % of tested human tumor cell lines (including breast, cervical,

ovarian, prostate, colon, renal cancer, as well as melanoma) secreted this cytokine [25]. GM-CSF is also secreted by many mouse cell lines such as squamous cell carcinoma [100], colon and mammary adenocarcinoma [25], and plasmacytoma [101]. Moreover, Takeda et al. [102] showed that the GM-CSF secretion correlated with the capacity to metastasize when various transplantable mouse tumors were injected subcutaneously. We showed that either tumor-transduced GM-CSF or the administration of recombinant GM-CSF protein in mice were sufficient to recruit MDSCs into the secondary lymphoid organs and suppress antigen-specific $CD8^+$ T cells [25]. MDSCs induced *in vitro* by culturing BM cells with an LLC variant (LN7) supernatants could facilitate tumor engraftment once adoptively transferred into an immunocompetent mouse. The LLC-LN7 supernatants that contained the factor inducing the tumor-promoting cells also had CSF activity. The ability of LLC cells to mediate both effects was completely abrogated by a combination of neutralizing antibodies against GM-CSF and IL-3 [103]. Moreover, mouse GM-CSF and IL-3 can synergize *in vitro* to induce an immunosuppressive phenotype of cultured BM cells [104].

On the other hand, GM-CSF has been shown to elicit powerful immune responses when combined with γ-irradiated tumor cell vaccines in various mouse models and in the clinical setting [105], [106], which supported its widespread use as an immune adjuvant to augment antitumor immunity. Utilizing a bystander vaccine strategy in which the antigen dose and steric hindrance could be maintained constant while altering the GM-CSF dose, we assessed the impact of high versus low concentrations of GM-CSF administered in a vaccine formulation on priming of antitumor immunity. We confirmed the efficacy of low doses of GM-CSF-secreting vaccine and defined a threshold above which the vaccine not only lost its efficacy but also resulted in significant *in vivo* immunosuppression mediated by MDSC recruitment [107]. A systematic analysis of different clinical trials performed with this cytokine suggests that the same phenomenon can take place in humans. Although in some of these studies GM-CSF appeared to help the generation of an immune response, in others no effect or even a suppressive effect was reported. GM-CSF may increase the vaccine-induced immune response when administered repeatedly at relatively low doses (range 40–80 μg for 1–5 days) whereas an opposite effect was often reported at dosages between 100 and 500 μg [108]. These findings support the dual role of GM-CSF on the immune response and highlight several critical parameters such as dose, systemic concentration, and duration of exposure as key factors for GM-CSF effect on the immune system, which need to be considered when utilizing GM-CSF as a vaccine adjuvant or to recover neutropenia in patients with cancer.

Recent data indicate that GM-CSF, with the collaboration of IL-6, can intervene in regulating MDSC function during very early stages of tumor progression. Experimental models of autochthonous pancreatic ductal adenocarcinoma (PDAC), in fact, have shown progressive waves of myelomonocytic cell recruitment after initiation of the transforming program controlled by the active K*ras* oncogene, with $CD11b^+Gr\text{-}1^+$ cells being among the first to be recruited within the developing cancer lesions [109]. K*ras* oncogene-dependent accrual of myelomonocytic cells is mandatory for pancreatic intraepithelial neoplasia (PanIN) initiation and progression,

a process involving GM-CSF and IL-6. In fact, K*ras* oncogene-driven inflammation at the PanIN stage critically relied on GM-CSF for both progression to PDAC and $CD11b^{+}Gr\text{-}1^{+}$ cell recruitment within the pancreatic stroma. Moreover, this circuit was essential to alter tumor-specific $CD8^{+}$ cytotoxic T cells and only the blockade of either GM-CSF production or $CD11b^{+}Gr\text{-}1^{+}$ cell activity allowed to restore antitumor immunity [110], [111]. Interestingly, recruited $CD11b^{+}Gr\text{-}1^{+}$ cells contribute with transformed epithelial cells to the local production of the cytokines IL-6 and IL-11 that activate the STAT3. As discussed further below, STAT3, in turn, induces antiapoptotic and pro-proliferative genes, fueling tumor initiation, promotion, and progression [112], [113].

More recently, an additional role on MDSC biology has emerged for GM-CSF: The presence of this cytokine is necessary to license immature myeloid cells to become fully suppressive MDSCs *in vivo*, in models in which tumorigenesis is driven by the ectopic expression of the SV40 T antigen [114]. In these models, differently from *Kras*, SV40 T oncogene does not cause systemic release of GM-CSF and thus drive the accumulation of non-suppressive immature myeloid cells, which can differentiate into fully suppressive MDSCs *in vivo* only when GM-CSF is exogenously introduced; interestingly, however, MDSCs are competent suppressors *in vitro* when co-cultured with effector T cells (likely because activated T cells can release GM-CSF) [114].

4.5 IL-10

Elevated IL-10 concentrations have been found in patients with solid tumors and hematological malignancies [115] and are used as a marker of tumor progression [116], [117]. IL-10 production has often been correlated with the induction of T cell anergy and, together with TGF-β, is considered one of the key immunosuppressive factors released by tumors [118]. DCs cultured with IL-10 induce T cell anergy and differentiation of suppressive T cells [119]. Myeloid DCs propagated from BALB/c ($H2^{d}$) mouse BM progenitors in IL-10 and TGF-β expressed lower toll-like receptor (TLR)-4 transcripts than lipopolysaccharide (LPS)-stimulated control DCs and were resistant to further maturation [120]. These DCs also expressed comparatively low levels of surface MHC class II, CD40, CD80, CD86, and programmed death-ligand 2 (B7-DC; CD273), and secreted high levels of IL-10, but low levels of IL-12p70 compared with activated control DCs [120]. These "alternatively activated DC" induced alloantigen-specific hyporesponsive T cell proliferation, enhanced IL-10 production by alloactivated T cells, expanded $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells *in vitro*, and prolonged heart allograft survival when administered *in vivo* [120]. MDSCs have been proposed to be the main contributor for IL-10 production in the tumor microenvironment [121], [122]. Indeed, their elimination within the ovarian tumor ascites significantly decreased local IL-10 levels and inhibited tumor progression [123]–[125]. IL-10 production by MDSCs seems to directly inhibit IL-12 production from M1 macrophages [121] and, together with TGF-β, promote Treg cell generation [75].

The production of IL-10 by MDSCs has also been implicated in the autocrine and paracrine activation of IL-10 suggesting the existence of a positive loop by which MDSCs promote their differentiation and function through the secretion of IL-10 [122]. These observations are in line with some of our unpublished observation in which the pretreatment with IL-10 of the immortalized cell line MSC-2 was sufficient to activate ARG1 and NOS2 (P. Serafini, Bronte unpublished data), enzymes crucial for MDSC suppressive pathways [126], and suppress the proliferation of lymphocytes stimulated with allogeneic APCs. Nevertheless, the pro-tumoral and MDSC-favoring action of IL-10 has been recently challenged using transplantable fibrosarcoma models and chemically induced tumors [127]. In these models, genetic depletion of IL-10 was shown to promote tumor development, growth, and metastasis. In particular, a strong accumulation of suppressive MDSCs and Treg cells was found in the tumor and in the secondary lymphoid organs [127]. Furthermore, IL-10-deficient MDSCs were shown to be extraordinary inducers of Treg cells through a mechanism that requires the secretion of IL-1 by MDSCs [127]. The discrepancies in the above-described study might be related to the strain of mice used (BALB/c vs. C57/Bl.6 mice) or to a yet unknown recovery mechanism that modifies MDSC biology in the $IL10^{-/-}$ mice. These findings also highlight the plasticity of MDSCs and the complexity of the system that link IL-1, inflammation, and cancer.

4.6 IL-4 and IL-13

The promiscuous receptor for IL-4 and IL-13 (alias IL4R type II) is composed of the IL4Rα chain and IL13Rα1 chain [128], while IL4Rα and the gamma chain (γc), common to the receptors for different members of the cytokine family comprising IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, associate to compose the IL-4 receptor (alias IL4R type I). Since the IL4Rα chain is the only component that possesses kinase-sensitive tyrosine residues in the cytoplasmic domain, signals from both type I and type II IL4R are transduced by the IL4Rα chain [129]. Upon engagement and dimerization, phosphorylated IL4Rα recruits and phosphorylates STAT6, which dimerizes and migrates to the nucleus to activate the transcription of several proteins including ARG1 [130]. The first evidence of IL-13 involvement in MDSC suppressive function derived from the work of Terabe et al. [23] (2003), who showed that tumor recurrence in a fibrosarcoma murine model was dependent on MDSC activation by IL-13-secreting NKT cell. The authors demonstrated that IL-13 activated $CD11b^{+}Gr\text{-}1^{+}$ cells, which, in turn, directly suppressed $CD8^{+}$ CTLs [23]. Tumor recurrence could be prevented either by MDSC depletion [23] or by IL-13 neutralization [131].

IL4Rα expression on MDSCs and monocytes is required for their suppressive phenotype [29] and survival [44], and genetic ablation of this receptor in monocytes and granulocytes is sufficient to revert MDSC-mediated immune suppression *in vivo* whereas aptamer-mediated blockade is sufficient to promote MDSC and TAM apoptosis. MDSC subsets produce IL-13 and IFN-γ and integrate the downstream

signals of these cytokines to trigger the molecular pathways suppressing antigen-activated $CD8^+$ T lymphocytes [29]. In particular, IFN-γ released by both MDSCs and bystander-activated T cells sustains IL4Rα expression on MDSCs that, once engaged by IL-13, activates survival and suppressive pathways in m-MDSCs [29].

4.7 IFN-γ

IFN-γ plays a central role in coordinating tumor immunity, being the most relevant cytokine for immunosurveillance and immunoediting [132]. IFN-γ exerts its biological effects through interaction with an IFN-γ receptor that is ubiquitously expressed in nearly all cells [133]. IFN-γ upregulates MHC class I expression as well as the expression of genes needed for antigen processing, including the transporters associated with antigen processing (TAP)-1 and TAP-2, and the proteasomal components low molecular weight proteins (LMP)-2 and LMP-7 [134]. For these reasons, IFN-γ is thought to augment the immunogenicity of many tumors. Moreover, IFN-γ can inhibit tumor angiogenesis through either direct or indirect mechanisms. In combination with TNF-α, in fact, IFN-γ directly reduces endothelial cell adhesion and survival by down-modulating the activation of αvβ3 integrin, an adhesion receptor critical for tumor angiogenesis [135]. On the other hand, IFN-γ can indirectly repress angiogenesis by inducing the production of antiangiogenic secondary molecules such as IFN-gamma-inducible protein-10 (IP-10) and monokine induced by IFN-γ (MIG) [136], [137]. Although IFN-γ signaling in the tumor cell has been predominantly viewed as an important molecular pathway for effective antitumor immunity, significant evidences now indicate that, in some cases, it may negatively impact the effectiveness of an antitumor immune response. IFN-γ signaling can, in fact, down-modulate the expression of tumor antigens [138] as well as induce the loss of efficient processing of some tumor antigens by DCs [139]. Morel et al. reported that IFN-γ induction by LMP-2 and LMP-7 immunoproteasome results in less efficient processing of melanoma tumor antigens (e.g., MART-1/Melan-A) allowing for evasion of recognition by CTLs and decreased tumor immunogenicity [139]. Moreover, gene expression profiling of TAMs as well as MDSCs indicates the presence of a distinct IFN-γ signature coupled with an M2 phenotype [140]. The importance of IFN-γ in activating MDSC suppressive activities has been reported by us and other groups. Synthesis of NO in macrophages is catalyzed by NOS2, whose expression is upregulated by a number of cytokines, including IFN-γ, TNF-α, and IL-2 [141]. We showed that IFN-γ together with a cell-mediated signal from activated splenocytes is necessary for generating both the full suppressive activity and high levels of NO secretion on both fresh MDSCs and immortalized cell lines [29], [142]. These signals are produced by activated T cells, and in the absence of an activation signal, T cells do not stimulate NO production in MDSCs [29], [142]. Furthermore, Huang et al. [75] showed that the secretion of IL-10 and TGF-β by $Gr\text{-}1^+CD115^+$ MDSCs was induced and enhanced upon IFN-γ stimulation. These IFN-γ-activated MDSCs, in addition to being able to suppress T cell proliferation *in*

vitro, were able to induce the development/expansion of forkhead box P3 (Foxp3)$^+$ Treg cells *in vivo*, when transferred in tumor-bearing mice [75]. However, since the adoptive MDSC transfer experiments were conducted in irradiated tumor-bearing recipient, these findings do not exclude that additional TDFs or cytokines released by the irradiated host might be necessary for Treg cell expansion [75]. Using the 4T1 murine mammary carcinoma in recipient mice in which T cell response is skewed toward a Th1 response and Th2 response as well as IL-4/IL-13 pathways are impaired by genetic ablation of STAT6 [143], Shina et al. suggested that IFN-γ is not sufficient per se to activate MDSC immunosuppression [144]. In this model, more than 60 % of STAT6$^{-/-}$ mice immunologically rejected spontaneous metastatic mammary carcinoma and survived indefinitely when their primary tumors were removed, whereas 95 % of STAT6-competent BALB/c mice succumbed to metastatic disease. Immunity in post-surgery STAT6-deficient mice was associated with a rapid decrease in the MDSC population and with the IFN-γ-dependent activation of type 1 tumoricidal macrophages. Under peculiar experimental conditions, such as the deletion of STAT6 in all the cells of the host, IFN-γ might thus favor an antitumor response even in the presence of MDSCs. Functional genomic analysis and experiments in cell type selective gene knockout mice have unveiled a complex interaction between Th1 and Th2 cytokines to activate MDSC suppressive program. To effectively exert their suppressive function on antigen-activated CD8$^+$ T cells, MDSCs must: (a) be activated by IFN-γ production from antigen-stimulated T cells, (b) release their own IFN-γ, and (c) be responsive to IL-13 [29]. Cooperation between these two cytokines leads to the activation of ARG1 and NOS2 that mediate MDSC suppressive activity [126].

4.8 Prostaglandins (PGEs)

Prostaglandins (PGEs) are strong immune modulators that are normally secreted in the immune responses by many cells, including macrophages and DCs. Cyclooxygenase-2 (COX2) overexpression is a widely recognized feature of human lung, colon, breast, and prostate cancers [145]. The products of COX2 enzyme activity, prostaglandins and mainly PGE_2, have been implicated in tumor-associated subversion of immune functions, since inhibitors of prostaglandin synthesis typically enhanced antitumor immunity. Freshly excised solid human tumor cells produce substantially more PGE than established tumor cell lines [146]: Interestingly, while primary tumor cell-conditioned media profoundly hampered the *in vitro* DC differentiation from CD14$^+$ monocytes or CD34$^+$ myeloid precursors, the effects of supernatants derived from established tumor cell lines were minor [146]. In these experiments, COX1- and COX2-regulated prostanoids were found to be exclusively responsible for the reduced differentiation of monocyte to DCs. In contrast, both PGE and IL-6 contributed to the tumor-induced inhibition of DC differentiation from CD34$^+$ myeloid precursor cells. DC abnormalities seem to be, at least in part, mediated by the prostaglandin EP2 receptor [147]. For example, in the 3LL lung

carcinoma model, MDSC expression of Arg1 seems to be independent on T cell-produced cytokines but rather is dependent on PGE_2. These tumor cells, in fact, constitutively express COX1 and COX2 and produced high levels of PGE_2 [26]. Genetic or pharmacological inhibition of COX2, but not COX1, blocked ARG1 induction in MDSCs both *in vitro* and *in vivo*. Inhibition of this pathway was sufficient to block ARG1 expression, reverse MDSC-mediated immunosuppression, and elicit a T cell-mediated antitumor response [26]. Furthermore, Celecoxib, a specific inhibitor of COX2, was found to normalize the number of MDSCs in Swiss mice in which intestinal tumor was chemically induced by 1,2-dimethylhydrazine diHCl [48]. Moreover, COX2 inhibition decreased ARG1 and NOS2 expression in the secondary lymphoid organs, promoted T cell infiltration in the tumor, and, overall, reduced tumor multiplicity [48]. Both tumors and MDSCs can actively produce and secrete PGE_2. This production and secretion correlates with arginase overexpression, STAT3 and STAT1 phosphorylation, and IL-10 and macrophage inflammatory protein-2 (MIP-2) production, a phenotype typically associated with MDSC suppressive activity [148].

5 Transcription Factors Regulating MDSC Function

In addition to the TFs that regulate lineage commitment in normal myelopoiesis, different factors for example, the CAAAT enhancer-binding (C/EBP) family, PU.1, and NF-κB, have been clearly associated with the differentiation, trafficking, and function of MDSCs. In particular, members of the STAT family and C/EBP-β were shown to play a central role in the polarization of myeloid cell functions as well as in tumor progression and alteration of immune response to cancer. STAT1, 3, 5, and 6 have been shown to play a major role in transmitting polarizing signals to the nucleus [149] and each of them can play distinct roles in macrophage polarization and MDSC functions. A fundamental component of several signal-transduction pathways associated with STAT is the Janus-activated kinase (JAK) family. These molecules are actively involved in cellular survival, proliferation, differentiation, and apoptosis. In mammals, four members of the JAK family are known: JAK1, JAK2, JAK3, and TYK2 [150]. Receptor oligomerization induced by cytokine binding triggers JAK activation by either auto- or trans-phosphorylation. Subsequent to ligand binding, activated JAKs phosphorylate receptors on target tyrosine residues, generating docking sites for STATs through the STAT Src homology 2 (SH2) domain. Activated JAKs recruit and phosphorylate STATs, which leads to their dimerization and nuclear translocation, where they modulate the expression of target genes. As discussed below, different members of the STAT family as well as C/EBP-β can integrate their individual signaling pathways or even rescue the pharmacological inactivation of one of the members, conferring to MDSCs the suppressive function, resistance to pharmacologic depletion, and a unique cell plasticity.

5.1 STAT1

It is known that STAT1 negatively regulates angiogenesis, tumorigenicity, and metastasis of tumor cells [151]. Since STAT1 mediates IFN-dependent signaling, this TF is an important mediator of antitumor immunity [152]. On the other hand, using analysis of STAT activity in combination with STAT knockout mice, STAT1 emerged as an important player in tumor-associated MDSC suppressive activity [30]. Tumor microenvironment and the inflammation, caused by both tumor growth and tissue invasion, in fact, promoted the differentiation and the activation of Gr-1^+ precursor recruited at the tumor site into ARG1 and NOS2-expressing MDSCs through a STAT1-dependent mechanism [30]. MDSCs, isolated from the tumor of STAT1-deficient mice, in fact, failed to upregulate ARG1 and NOS2 and were unable to suppress the proliferation of anti-CD3/anti-CD28-stimulated splenocytes [30]. Moreover, in a mouse squamous cell carcinoma, STAT1 deficiency enhanced IL-12-mediated tumor regression by a T cell-dependent mechanism [153]. In agreement with the role of STAT1 as central mediator of IFN-γ biological activities, administration of neutralizing antibodies against IFN-γ inhibited tumor growth in IL-12-treated $STAT1^{+/+}$mice [153]. These data might have an experimental confirmation by the fact that IFN-γ produced by activated T cells and by MDSCs themselves is required to trigger NOS2 activation and synergize with IL4Rα-ARG1 pathways in MDSCs isolated from tumor mass [29]. In line with this picture, mice deficient for the suppressor of cytokine signaling-1 factor (SOCS-1), which are characterized by hyperactivation of STAT1, displayed spontaneous development of CRCs [154]. The negative role of STAT1 activation in cancer seems to be confirmed in some human cancer since, by analyzing TAMs derived from 211 patients affected with follicular lymphoma, the presence of STAT1 in TAMs was an important independent prognostic factor that correlated with an adverse outcome [155]. The importance of STAT1 in the m-MDSC biology was highlighted in B6 mice injected subcutaneously with the lymphoma cell lines BW-Sp3 and EG7 [33]. IFN-γ blockade or disruption of STAT1 signaling significantly impaired suppression by m-MDSCs; although IFN-γ was still required for g-MDSC activity, it did not rely on STAT1 signaling or on NO production [33].

5.2 STAT3

STAT3 is activated in many human cancers, including 82 % of prostate cancers [156], 70 % of breast cancers [157], more than 82 % of squamous cell carcinoma of the head and neck [158], and 71 % of nasopharyngeal carcinoma [159]. STAT3 participates in oncogenesis through the upregulation of genes encoding apoptosis inhibitors (Bcl-x_L, Mcl-1, and survivin), cell-cycle regulators (cyclin D_1 and c-Myc), and inducers of angiogenesis such as VEGF [160]. Different studies demonstrate that STAT3 activation in tumors might play an important role not only in maintaining the transformed phenotype in tumor cells but also in inhibiting immune surveillance [161]. The STAT3 signaling pathway in tumor cells can, in fact, inhibit the production of

pro-inflammatory danger signals and induce expression of factors that inhibit DC functional maturation [161] while promoting MDSC differentiation and functional activity. STAT3 expression in macrophages has been associated with their ability to induce T cell tolerance, whereas targeted disruption of *Stat3* gene in these cells stimulated the production of pro-inflammatory cytokines and abrogated their tolerogenic features [162]. Noteworthy, ablating STAT3 in hematopoietic cells triggers an intrinsic immune-surveillance system that inhibits tumor growth and metastasis [163]. Incubation of hematopoietic progenitor cells with tumor cell-conditioned medium resulted in activation of JAK2 and STAT3 and was associated with an accumulation of MDSCs. Importantly, MDSCs derived from tumor-bearing mice demonstrated constitutive activation of JAK2/STAT3 pathway [164]. Inhibition of STAT3 activation in hematopoietic progenitor cells via dominant-negative STAT3D retroviral vector or with the use of JAK2/STAT3-specific small molecule inhibitors abrogated the effect of TDFs on the generation/activation of MDSCs [164], [165]. The importance of STAT3 expression in the myeloid lineage during tumor progression is highlighted by the use of STAT3-specific siRNA-stable conjugated with TLR9-targeting CpG oligonucleotides [166]. When systemically injected in tumor-bearing mice, these chimeric molecules specifically transfected the $TLR9^{+}$ myeloid cells in the tumor and in the secondary lymphoid organs, leading to a spontaneous antitumor immune response that resulted in a drastic inhibition of B16 melanoma growth [166]. Indeed, STAT3 phosphorylation activates different key pathways regulating MDSC survival, differentiation, and immunosuppressive and pro-angiogenic function. STAT3 signaling drives the expression of the antiapoptotic protein Bcl-xL and pro-proliferative factors c-myc, cyclin D1, and survivin (reviewed in [36], [167], [168]). Besides promoting MDSC survival, STAT3 seems to be implicated in their differentiation. For example, G-CSF-induced STAT3 phosphorylation promotes, in myeloid progenitor cells, C/EPBβ expression [169] that, as discussed later, is required for MDSC differentiation [18]. Additionally, STAT3 can activate in hematopoietic precursors, the calcium-binding pro-inflammatory proteins S100A8 and 9, which results in inhibition of differentiation to DC, accumulation of MDSCs, and their migration at the tumor site [170]. STAT3-dependent upregulation of S100A8 and 9 seems to be implicated in the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) expression [171], which leads to the production of superoxide, one of the mechanisms by which MDSCs promote T cell anergy and tolerance. STAT3 activation can directly promote NOX2 activation by upregulating the transcription of the NOX2 subunits $p47^{phox}$ and $gp91^{phox}$ [41].

STAT3 is also essential for signaling through the IL-10 receptor since mice lacking STAT3 in macrophages and neutrophils have a strikingly similar phenotype as IL-10-deficient mice [172]. It is interesting to note that these mice develop chronic enterocolitis with age, likely through a complex contributory network including polarized immune response toward the Th1-phenotype, overexpression of pro-inflammatory cytokines, and deficiency of the immunosuppressive action of macrophages and neutrophils [172]. Activation of STAT3, via IL-10, upregulates the α-chain of the IL-4 receptor that leads to an increased IL-4-dependent expression of ARG1 [173]. Furthermore, IL-10 synergizes with LPS in inducing NOS2. However,

NOS2 regulation by activation of STAT3 needs further study since STAT3 has been reported either to activate [174], [175] or repress [176] NOS2 expression. Interestingly, the RB6-8C5 anti-Gr-1 antibody recognizing the Ly6G and Ly6C antigens, normally used for temporarily depleting MDSCs *in vivo*, is able to trigger STAT3, STAT1, and STAT5 activation on hematopoietic precursors [177] and thus, possibly, promotes rather than inhibits MDSC differentiation. This possibility could explain the MDSC rebound effect observed upon chronic administration of this antibody (Serafini and Bronte unpublished data) and suggest caution in interpreting the use of this reagent for mechanistic analyses in MDSC biology.

In summary, STAT3 activation seems to play a key role in MDSC biology affecting their function by different means.

5.3 STAT5

STAT5 is one of the main factors activated by GM-CSF in the myeloid lineage [178], [179]. Considering the important role of GM-CSF in MDSC differentiation and recruitment, STAT5 involvement in regulating MDSC activity is largely expected but it has been addressed only in few studies. Indeed, STAT5 has been implied in MDSC survival. The multi-target tyrosine kinase inhibitor sunitinib, which blocked MDSC expansion in tumor-bearing mice via inhibition of STAT3 signaling in myeloid cells, prevented MDSC accumulation and restored normal T cell function in the spleens of tumor-bearing mice [180]. On the contrary, at the tumor site, MDSCs were protected in both mice and humans [180]. STAT5 activation by GM-CSF was found to be the key signaling that promoted MDSC survival [180], and this pathway was able to redirect the sunitinib-sensitive g-MDSCs toward sunitinib-resistant, monocytic-like MDSCs [181].

5.4 STAT6

STAT6 is another member of the STAT family which has attracted attention since mice deficient for the STAT6 gene have enhanced immunosurveillance against primary and metastatic tumors. STAT6 is a downstream TF for IL4R and IL13R whose role in the activation of MDSCs has been established by different studies. More than 60 % of STAT6$^{-/-}$ mice immunologically reject spontaneous metastatic mammary carcinoma and survive indefinitely if their primary tumors are removed, whereas 95 % of STAT6-competent BALB/c mice succumb to metastatic disease [144], [182]. The authors suggested that STAT6 deficiency prevents signaling through the type 2 IL4R, thereby blocking the production of ARG1 and promoting the synthesis of NO by myeloid cells. The importance of this pathway in MDSC-mediated suppression has been further demonstrated in the fibrosarcoma model described earlier. In this model, in fact, tumor recurrence was completely prevented in STAT6$^{-/-}$ mice [131]. Besides

regulating the activation and function of MDSCs upon IL4Rα engagement, STAT6 is particularly important for MDSC survival and expansion [44]. Indeed, blockade of STAT6 signaling through an anti-IL4Rα aptamer is sufficient to induce apoptosis in MDSCs and TAMs [44]. The role of STAT6 in promoting MDSC expansion is also highlighted by their reduced accumulation after trauma in STAT6$^{-/-}$ mice. Moreover, the few MDSCs that accumulate in STAT6$^{-/-}$ mice after physical injury showed impaired suppressive activity due to the reduction in ARG1 expression [183]. These results might be extended to humans since interruption of STAT6 signaling in the myeloid lineage has been proposed as a mechanism by which platinum-based chemotherapy prime and enhanced an antitumor immune response [184].

*5.5 C/EBP-*β

The C/EBP-β is a basic leucine zipper (b-ZIP) TF extremely important for the differentiation of the myeloid lineage. Three different C/EBP-β isoforms are translated (starting from 3′ in frame AUG) from the same mRNA: a 38 kd liver-activating protein 1 (LAP_1 or LAP*), a 36 kd liver-activating protein (LAP_2 or LAP), and a 28 kd liver-inhibiting protein (LIP). The relative expression of each of these isoforms has a dramatic effect on myeloid cell differentiation, inflammation, and insulin resistance in tissues. In particular, LAP_1 upregulation is associated with MDSC [18] and M2 macrophage [130], [185]–[188] differentiation, while LAP_1 downregulation seems to promote the differentiation of M1 macrophage [185], [187] and immunogenic DC [188], [189]. Although LAP_1 action in granulopoiesis is still controversial, LAP_2 seems to be required for granulocyte differentiation [190]. The importance of C/EBP-β in MDSCs differentiation is proven by the lack of their accumulation in the spleen of tumor-bearing C/EBP-β$^{-/-}$ mice and by the failure of C/EBP-β-deleted BM cells to differentiate *in vitro* into functional MDSCs [18]. Interestingly, a direct link between STAT3 and C/EBP-β exists [169]: STAT3 transduce the G-CSF signal and induce C/EPB-β expression in myeloid progenitor cells [169]. Once induced, C/EBP-β can activate, by binding to their promoters, different genes including c-myc [36], IL-6 [191], and the gene encoding for the common signaling β-chain receptor that regulates the signal transduction for GM-CSF, IL-3, and IL-5 [192]. Thus, C/EBP-β could be a master regulator that promotes MDSC proliferation, reactivity to the environment through induction of the common β-chain receptor, and the differentiation of other MDSCs by IL-6 induction.

6 Mechanisms OF MDSC-Dependent Immune Suppression

Although it is clear that MDSCs can inhibit the immune response against cancer, it must be pointed out that MDSCs can be present in various functional differentiation grades that might explain the prevalence of the different immunosuppressive

mechanisms described in different tumor models [63]. These functional and phenotypic differences can be related to the status of the disease, MDSC localization in different anatomical compartments, or the different microenvironments that each tumor can establish. MDSC suppressive/tolerizing activity *in vivo* can be dependent on the expression of MHC class I on their surface as elegantly shown by Kusmartsev et al. (2005). Using an experimental system based on the adoptive transfer of transgenic T cells into naïve recipients, the authors showed that Gr-1$^+$ MDSCs as well as DCs from tumor-bearing mice were able to uptake and process tumor-associated antigens. However, while DCs did not reduce the generation of tumor-specific T cells CD8$^+$-producing IFN-γ, MDSCs were able to induce anergy of CD8$^+$ T cell that no longer responded to peptide stimulation [193]. This tolerogenic state could be rescued *in vivo* through immunization with mature DCs. Taken together, these data suggest that the tolerogenic state is reversible and that the balance between mature DCs and MDSCs in secondary lymphoid organs can determine the final outcome of the immune response. Although these cells can produce high levels of ARG1 and ROS [193], their tolerogenic activity seemed to be dependent mostly on NO production since NG-monomethyl-L-arginine (L-NMMA), an inhibitor of various NOS, completely reverted the ability of these MDSCs to tolerize CD8$^+$ T cells [194].

In other situations, however, MDSCs were shown to be powerful and unselective inhibitors since they inhibited not only peptide, mitogen, or anti-CD3/CD28-activated CD4$^+$ and CD8$^+$ T cells [46] but also activated NK and NK T cells [195], [196]. All together, these data indicate that MDSCs can also suppress T and NK cells in an antigen- and MHC-independent fashion. The concept of antigen independence might be misleading, since MDSCs inhibit only activated T lymphocytes, either naïve or memory, whereas resting lymphocytes are spared. The necessity of activation of effector T cells, combined with the fact that MDSCs need to be in strict contact with T cells to deliver the inhibitory signals [8], suggests that MDSC suppressive activities are endowed with some degree of selectivity, even in the absence of an MHC-restricted suppression. MDSCs can restrain the immune response through different mechanisms that operate singularly or in combination. Such mechanisms can be direct or indirect, in this latter case involving the generation or the expansion of other regulatory population such as CD4$^+$ CD25$^+$ Treg cells.

6.1 Direct Mechanisms of Immune Suppression

6.1.1 TGF-β

The transforming growth factor-β (TGF-β) is a critical regulator of thymic T cell development and a crucial player in peripheral T cell homeostasis, tolerance to self-antigens, survival, and differentiation during the immune response [197], [198].

The link between TGF-β and MDSCs was shown first by Young et al. who demonstrated that myeloid progenitor cells derived from tumor-bearing mice produced increased amounts of TGF-β, NO, IL-10, and PGE_2. NO and TGF-β, however, were

the mediators by which MDSCs inhibited *in vitro* the anti-CD3 antibody-induced T cell proliferation [199]. Gr-1$^+$ cells were proposed to bind the immunoglobulin-G (IgG)–TGF-β complex on their Fc receptors and the binding could trigger these immature myeloid cells to suppress CTL response [200]. Moreover, as described above, MDSCs can be activated by IL-13 to secrete TGF-β [23]. In addition to secreting TGF-β, MDSCs can also express the membrane-bound form and thus deliver this cytokine by direct contact with the target cells [201]. This possibility has been shown on orthotopic mouse models of melanoma and liver and lung cancers. In these settings, MDSCs were shown to inhibit NK activation and production of IFN-γ through the membrane expression of TGF-β. The capacity of MDSCs to secrete TGF-β was recently confirmed in human samples. For example, healthy donor monocytes exposed to a glioma cell line acquired an MDSC-like phenotype, the capacity to produce TGF-β, and to suppress CD3/CD28-stimulated T cells [202]. Similarly, results were found in CD14$^+$ HLA-DR$^{-/low}$ MDSCs isolated from patients with head and neck squamous cell carcinoma (HNSCC). These cells showed a marked expression of PD-L1 and production of TGF-β [203]. TGF-β, per se, exerts an antiproliferative effect on T cells [204], arresting their cell cycle typically in the G1 phase [205], [206] by inducing the expression of the cell-cycle inhibitors, p27KIP1 and p21CIP1 [207], or by inhibiting IL-2 secretion [208]. Importantly TGF-β has been shown to inhibit the differentiation of CD4$^+$ T cells into Th1 or Th2 cells by suppressing the expression of T-bet and GATA-3 master regulators of Th1 and Th2 conversion, respectively [209]. Despite the evidence that TGF-β is essential for the maintenance of peripheral tolerance, the mechanism by which TGF-β acts remains unclear. TGF-β may directly be important for the induction of peripheral tolerance by downregulating the differentiation and function of auto-reactive effector T cells, as described above. Furthermore, TGF-β is the key molecule that regulates Treg cell differentiation from naïve cells and their trans-differentiation from Th17 cells. In both of these processes, as further discussed below, MDSCs seem to play an important role mediated, at least partially, by their capacity to secrete TGF-β [209], [210].

6.1.2 L-Arginine Metabolism

Many of the inhibitory pathways involved in MDSC-mediated immune suppression are related to the metabolism of the amino acid L-arginine (L-Arg) (reviewed in [126] and discussed by A. Ochoa in another chapter of this book). L-Arg is metabolized mainly by two enzymes: NOS, which oxidizes L-Arg in two steps that generate NO and citrulline, and ARG, which converts L-Arg into urea and L-ornithine [211], [212].

ARG-dependent suppression. MDSCs infiltrating a mouse lung carcinoma expressed high levels of ARG1 and the L-Arg transporter CAT2B [213]. These myeloid cells readily consumed L-Arg and inhibited reexpression of the ζ-chain of CD3 complex in T lymphocytes, thereby impairing their function. The CD3 ζ-chain is the main signal-transduction component of the TCR complex and is required for the

correct assembly of the receptor, and altered expression has been detected in peripheral blood T cells in patients with cancer, chronic infections, and autoimmune diseases [214]. This mechanism of T cell inactivation by ARG-induced deregulation of CD3 ζ-chain was shown to be relevant for tumor escape *in vivo*, because injection of the ARG inhibitor *N*-hydroxy-nor-L-arginine (nor-NOHA) delayed the growth of transplantable lung carcinoma in a dose-dependent manner [213]. Although the decreased CD3 ζ expression might partially explain the MDSC-induced T cell anergy, other mechanisms seem to be involved, since T cells cultured in the absence of L-Arg had an increased production of IL-2 and expressed early activation markers [215]. Indeed, L-Arg starvation arrested T cells in the G_0–G_1 phase of the cell cycle, by failing to upregulate cyclin D3 and cdk4 and increasing cdk6 expression [216]. The decreased expression of cyclin D3 and cdk4 in T cells seems to be mediated by a HUR-dependent decreased mRNA stability and diminished translational rate [217]. Moreover, under L-Arg starvation, T cells accumulate empty aminoacyl tRNAs. This accumulation activates GCN2 kinase which phosphorylates the translation initiation factor eIF2α. The phosphorylated form of eIF2α binds with high affinity to eIF2B, blocking its ability to exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP), which inhibits the binding of the eIF2 complex to methionine aminoacyl tRNA resulting in a decreased initiation of global protein synthesis [215].

NOS-dependent suppression. The ability of NOS inhibitors to reverse MDSC-induced immunosuppression, both *in vivo* and *in vitro*, confirms the immunoregulatory role of NO [126], [212]. NO-mediated suppression of T cell activation is not associated with the early events triggered by TCR recognition but, instead, with the signaling cascade downstream of the IL-2 receptor [142]. NO is known to negatively regulate intracellular-signaling proteins either directly by S-nitrosylation of crucial cysteine residues or indirectly by activation of soluble guanylate cyclase and cyclic-guanosine monophosphate (GMP)-dependent protein kinases [218]–[220]. In T cells, the phosphorylation and thus the activation of important signaling proteins in the IL-2-receptor pathway, including JAK1, JAK3, STAT5, extracellular-signal-regulated kinase (ERK) and AKT, is blocked by NO [142], [220]. Persistent release of NO by MDSCs might also be associated with the transcriptional loss of STAT5A and STAT5B in T and B cells, observed in mice bearing large mammary carcinomas and in individuals infected with HIV [221], [222] and might, therefore, be responsible for the impaired T cell function observed under these conditions. A direct pro-apoptotic effect has also been observed in T cells exposed to high concentrations of NO likely mediated by numerous factors such as the accumulation of the tumor-suppressor protein p53, signaling through CD95 (also known as Fas) or TNF-receptor family members, or signaling through caspase-independent pathways [223], [224].

ARG and NOS cooperation in suppression. Synergism between these enzymes in MDSCs was difficult to envision considering reports indicating that ARG activation limits the availability of L-Arg as a substrate for NOS and thus negatively regulates its enzymatic activity [225]. However, many reports also showed that these two enzymes can be co-expressed within the same population or microenvironment [27], [28], [30], [226]–[231]. When these two enzymes are co-expressed, ARG1, by

lowering the L-Arg concentration in the local environment, operates to switch NOS2 activity, shifting its function from the production of NO to O_2^- [232]–[234]. When L-Arg concentrations are suboptimal, the reductase and oxygenase domains of NOS2 transfer electrons to the co-substrate O_2 and produce O_2^-, which reacts with other molecules, thereby generating several RNIs, such as peroxynitrite, and ROS, such as hydrogen peroxide (H_2O_2). These species have multiple inhibitory effects on T cells. In addition, low levels of NO induce nitrosylation of cysteine residues of ARG1, which increases the biological activity of the enzyme, further reducing L-Arg concentration in the environment [235]. The combined activity of ARG and NOS was recently shown to be important for the suppressive activity of tumor-infiltrating $CD11b^+$ myeloid cells [27], [193] and splenic $CD11b^+Gr\text{-}1^+$ cells from mice bearing subcutaneous tumors or in models of chronic helminthic infections [228], [234].

6.1.3 Cysteine Starvation

Cysteine is an essential amino acid for T cell activation because T cells lack cystathionase, which converts methionine to cysteine, and because they do not have an intact x_c^- transporter [236], [237] and therefore cannot import cystine and reduce it intracellularly to cysteine. Thus, T cells depend on APCs, such as macrophages and DCs, to export cysteine, which is then imported by T cells via their ASC neutral amino acid transporter [238], [239]. MDSCs do express the x_c^- transporter and import cystine but they do not express the ASC transporter and, thus, cannot export cysteine [240]. It was thus advanced that MDSCs compete with APCs for extracellular cysteine, limiting the extracellular pool of cysteine and thus depriving T cells of the cysteine they require for activation and function [240].

6.1.4 Reactive Oxygen Species

In addition to amino acid starvation, MDSCs can block T cell function through the production of highly oxidative ROS, as previously mentioned. H_2O_2 production by macrophages infiltrating metastatic melanoma induced the loss of CD3 ζ-chain in naïve T cells [241]–[243]. Moreover, an increase in $CD11b^+CD15^+$ granulocytes was observed in patients with pancreatic cancer [40]. These cells reduced CD3 ζ expression and decreased cytokine production in T cells through a H_2O_2-mediated mechanism [40]. As discussed above, MDSCs can be divided into granulocytic- and monocytic-like subsets on the base of their morphology and phenotype. It appears that g-MDSCs have substantially higher levels of ROS and myeloperoxidase and reduced phagocytosis compared with m-MDSC [244], [245].

Moreover, MDSCs freshly isolated from tumor-bearing mice, but not their control counterparts, were able to inhibit antigen-specific response of $CD8^+$ T cells [246]. These MDSCs obtained from tumor-bearing mice had significantly higher levels of ROS than $Gr\text{-}1^+$ cell isolated from tumor-free animals. Since ROS production could be blocked by ARG inhibitors, these data suggest that ARG could be involved in

the mechanisms of T cell inhibition through generation of ROS, and may link ARG1 to T cell dysfunction observed at the tumor site [246]. In myeloid cells, NOX is the primary producer of ROS by catalyzing the one-electron reduction of oxygen to superoxide anion using electrons supplied by NADPH [247]. The NOX protein family is composed of seven isoenzymes: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 [248]. The phagocyte NADPH oxidase NOX2/gp91^{phox} was found to be significantly upregulated on MDSCs by STAT3-mediated signaling [41]. Additional mechanisms underlying the preferential ROS generation by MDSCs seem to imply the co-expression, within the tumor microenvironment, of ARG and NOS2. Under conditions of limited availability of L-Arg, not only NOS2 but also NOS1 (also called nNOS) and NOS3 (also called eNOS) produce O_2^- [249]. The only major difference between the NOS isoforms in terms of the reactions performed lies in the rate of this NADPH-dependent oxidation, termed the "uncoupled reaction." Under these conditions, NOS1 continues to transfer electrons to the heme and hence oxidize NADPH at a high rate, whereas in NOS3 and NOS2 this reaction occurs at a much slower rate [249]. While NOS2 at low concentration of L-Arg produce both NO and O_2^- [249], NOS1 in the same condition produce O_2^- and H_2O_2 [250], but not NO [251]. These data, which await to be confirmed in MDSCs, suggest a scenario where NOS isoform expression determines the final molecular mediator of ARG-dependent suppression. One of the most common molecules that reacts with O_2^- is NO, a key biological messenger in mammals. This leads to the formation of the free radical peroxynitrite $ONOO^-$ that can nitrate/nitrosylate tyrosine, cysteine, methionine, and tryptophan in different proteins and enzymes, thus changing their biological functions [252]. For example, peroxynitrite can nitrate/nitrosylate the TCRs and CD8 molecules on the surface of T cells. Following this modification, the TCR lost the ability to recognize specific peptide/MHC (pMHC) complexes and CTLs were thus rendered incompetent in performing their antitumor activity [253]. More recently, we demonstrated that peroxynitrite can nitrate/nitrosylate chemokines within the tumor microenvironment [99]. Modified CCL2 chemokine, in particular, failed to attract T cells to the tumor microenvironment while it was still able to promote the MDSC trafficking to the tumor [99]. Finally, peroxynitrite inhibits the binding of processed peptides to tumor cell MHC, and, as a result, tumor cells become invisible and resistant to antigen-specific CTLs [254].

6.2 Indirect Mechanism of Immune Suppression: Regulation of CD4$^+$CD25$^+$ Treg Cell Homeostasis

In the past years, considerable interest was raised by the hypothesis about a link between MDSCs and CD4$^+$CD25$^+$ Treg cells. MDSCs, in fact, share many features with immature DCs (e.g., low expression of MHC class II, CD80 expression, antigen uptake capacity, etc.) that have often been proposed to be associated with either T cell tolerization or Treg cell expansion. Mahnke et al. [255] demonstrated that specific *in vivo* targeting of immature DCs with the mAb anti-DEC-205 coupled with various antigens resulted in the presentation of the antigens in a tolerizing context. Using

ovalbumin (OVA) as a model antigen, the initial expansion of OVA-specific T cells was followed by anergy and appearance of T cells expressing CD25 and CTLA-4. Functional analysis of this T cell population revealed that $CD25^+$ T cells from the anti-DEC–OVA complex-injected animals suppressed proliferation and IL-2 production of conventional $CD4^+$ T cells in a cell-contact-dependent way. Depletion of $CD25^+$ T cells from bulk T cell cultures restored T cell proliferation [255]. The first evidence of a connection between MDSCs and Treg cells was provided in a model of allogeneic BM transplantation [256]. $CD11b^+$/Gr-1^+ MDSCs, expanded *in vivo* by Progenipoietin-1 (a synthetic G-CSF/Flt-3 ligand molecule) administration, were found to suppress the initiation of graft-versus-host disease (GVHD) after allogeneic BM transplantation by inducing a population of MHC class II-restricted Treg cells producing IL-10 [256]. Moreover, since either plasmocytoid or myeloid DCs, expanded with the same molecules, were unable to affect GVHD, these experiments suggested a prominent role of MDSCs in Treg cell induction and unveiled a new role of MDSCs in regulating peripheral tolerance. The importance of tumor-conditioned infiltrating cells in controlling Treg cell homeostasis was also shown in a melanoma mouse model and a colon carcinoma rat model. Ghiringhelli et al. [257] reported that, during tumor progression, Treg cells accumulate in tumors and secondary lymphoid organs through a mechanism that mainly required the proliferation of preexisting natural Treg cells in the draining lymph nodes and in the tumor bed. In both rodent models, this proliferation was dependent on the accumulation of TGF-β-secreting $CD11b^+CD11c^+$ MHC class II^{low} cells in the tumor draining lymph nodes [257]. This proliferation was significantly reduced in TGF-β $RII^{-/-}$ animals underscoring the importance of the TGF-β pathway in Treg cell proliferation and tumor-tolerance induction [257]. Importantly, the tolerogenic TGF-β-secreting APCs could be recreated *in vitro* by incubating CD11c from tumor-free mice with the supernatant of tumor-conditioned media, suggesting that these tolerogenic APCs might be related to MDSCs [257].

By using the colon carcinoma mouse model MCA stably transformed with the influenza hemagglutinin (HA) antigen, Huang et al. [75] showed that MDSCs from tumor-bearing mice could suppress the expansion of effector HA-specific $CD25^-CD4^+$ T cell through a NOS2-mediated mechanism and also generate or expand the pool of $CD4^+CD25^+Foxp3^+$ Treg cells [75]. *In vitro* experiments showed, in fact, that while HA-specific $CD4^+CD25^-$ T cells cultured with MDSCs failed to proliferate to the cognate antigen, the percentage of HA-specific $CD4^+CD25^+Foxp3^+$ T cells in culture were significantly augmented. The *in vivo* experiments clearly showed that adoptive co-transfer of MDSCs and HA-specific T cells into irradiated tumor-bearing mice resulted in an increase in the number of $CD4^+$, $Foxp3^+$, HA-specific T cells with regulatory capacity [75]. This increase was mediated by TGF-β and IL-10 production by MDSCs as well MDSC activation by IFN-γ [75].

Using the A20 B-cell lymphoma model, we identified in the MDSCs the tolerogenic APCs capable of antigen uptake and presentation to tumor-specific Treg cells. MDSC-mediated Treg cell induction requires ARG but is TGF-β independent [258]. *In vitro* and *in vivo* inhibition of MDSC function, with either NOHA or sildenafil, abrogates Treg cell proliferation and tumor-induced tolerance in antigen-specific T

cells [258]. More recently, the expression of the immune stimulatory receptor CD40 on MDSCs was shown to be required to induce T cell tolerance and Treg cell accumulation [259]. While the adoptive transfer of wild-type Gr-1^{+}CD115^{+} m-MDSCs induced Treg cell differentiation, CD40$^{-/-}$ m-MDSCs failed to induce tolerance and Treg cell accumulation *in vivo* [259].

The ability of MDSCs to induce proliferation/conversion of Treg cells was recently confirmed in human setting: CD14^{+}HLA-DR$^{-/low}$ m-MDSCs isolated from peripheral blood mononuclear cells (PBMCs) of patients with hepatocellular carcinoma were shown to induce IL-10-producing CD4^{+}CD25^{+}Foxp3^{+} Treg cells when co-cultured with autologous CD3/CD28-stimulated T cells [260]. These m-MDSCs expressed a high level of ARG1 that is required for their suppressive activity [260]. The same group later demonstrated that human CD14^{+} HLA-DR$^{-/low}$ m-MDSCs induce Foxp3^{+} Treg cells, whereas CD14^{+}HLA-DR^{high} monocytes promote generation of Th17 cells [261]. Furthermore, MDSCs were shown not only to modulate the de novo induction of Treg cells from CD4^{+} T cells but also to catalyze the transdifferentiation of Foxp3^{+} Treg cells from Th17 cells through a mechanism that is dependent on MDSC-derived TGF-β and retinoic acid [261]. However, the role of MDSC-derived TGF-β in Treg cell conversion/expansion has been recently challenged by others [210]. By using both the 4T1 mammary carcinoma and the LLC models, g-MDSCs were found to block the TGF-β-driven natural Treg cell proliferation and inducible Treg cell differentiation from naïve CD4^{+} T cells. This process required inhibition of early T cell activation and did not require ARG1, NOS2, NO, cystine/cysteine depletion, PD-1 and PD-L1 signaling, or COX-2 [210]. Interestingly, inhibition of inducible Treg cell generation by MDSCs appeared to depend on ROS generation and indoleamine-pyrrole 2,3-dioxygenase (IDO) enzyme, since MDSCs from IDO$^{-/-}$ mice failed to induce Treg cells in the presence of the free radical scavenger N-acetyl-cysteine [210]. However, it is not clear whether IDO is directly involved in MDSC-mediated inhibition of inducible Treg cell differentiation or whether MDSCs raised in IDO$^{-/-}$ mice are defective.

Despite this last provocative report, a clear indication of a role of MDSCs in promoting Treg cells is emerging and may suggest a physiological role for MDSCs, not only in restraining an excessive inflammation but also in the generation of an "infectious tolerance" through the expansion of antigen-specific Treg cells.

7 MDSC Interactions with Other Immune Populations

Besides inhibiting the effector function of CD8^{+} and CD4^{+} T cells, a complex network between MDSCs and other populations is emerging.

7.1 Cross Talk with Treg Cells

The relationship between MDSCs and Treg cells is not limited to the homeostatic control of the CD4^{+} regulatory population. Yang et al. [262] showed that a mouse ovarian carcinoma (MOSEC line 1D8) triggered the accumulation of MDSCs and

$CD4^+CD25^+$ Treg cells in spleen, ascites, and tumor tissue. Since genetic ablation and antibody blockade of either CD80 or its ligand CD152 significantly retarded tumor growth [262], the authors suggested that tumor-mediated CD80 upregulation on MDSC was important for immune evasion and tumor progression. Interestingly, *in vitro* experiments examining the suppressive activity of Treg cells and MDSCs revealed that both populations were simultaneously necessary to inhibit IFN-γ production from antigen-specific T cells stimulated with the cognate peptide. Moreover, CD80 neutralization experiments showed that the engagement of CD80 on MDSC with CD152 expressed by Treg cells was required for MDSC–Treg cell cooperation in inducing IFN-γ suppression. Since binding of CD152-Ig to DCs was shown to induce T cell anergy by upregulating the expression of IDO [263], binding of CD80 and CD152 may also activate MDSC suppressive program, suggesting that in some cases MDSCs and not Treg cells are the final effectors of immune suppression.

In accordance with this hypothesis, Fujimura et al. recently showed that Treg cells can promote B7-H1 expression and IL-10 secretion by MDSCs, molecules by which MDSCs inhibit T cell immunity either directly or by influencing DC biology and function [264].

Besides inducing Treg cells and being activated by Treg cells, MDSCs can affect the trafficking of Treg cells at the tumor site. Tumor-infiltrating m-MDSCs from melanoma-bearing mice were found to express high levels of the CCR5 binding chemokines CCL3, CCL4, and CCL5. Concurrently, Treg cells were found highly positive for CCR5. Genetic inhibition of CCR5 drastically reduced tumor-infiltrating Treg cells suggesting that MDSCs actively recruit Treg cells at the tumor site [265].

7.2 Cross Talk with Other Cell Subsets

Besides Treg cells in the past years, MDSCs have been shown to cross talk with effector T cells, macrophage, and NKT cells. These interactions are mediated not only by soluble factors (i.e., cytokines and chemokines) but also by contact-dependent mechanism. Indeed, as discussed above, MDSCs are activated by IFN-γ, IL-13 [227], and GM-CSF [114] that are produced by activated effector T cells and NKT cells [266]. MDSCs exert their suppressive activity mostly by contact-dependent mechanisms. Recently, a scenario is emerging in which MDSCs can also be activated by contact-dependent mechanisms, as, for example, in the cross talk between MDSC and macrophages. Upon contact with macrophages and IL-6 stimulation, MDSCs increase their production of IL-10 that represses IL-12 secretion and MHC class II expression on macrophages and DCs [121], [267]. Another example of cross talk that requires a physical contact between MDSCs was recently in the MC38 colon carcinoma model [268]. In this model, antigen-specific $CD4^+$ T cells can increase MDSC suppressive activity via a retrograde MHC class II signaling that increases the expression of COX2 and PGE_2 production in MDSCs [268].

8 Human MDSCs

MDSCs have been described in patients affected by different tumors. As in the case of mouse MDSCs, however, the phenotype of these cells is not fully defined and both immature and mature myeloid cells have been described. In a seminal work in head and neck cancer patients, for example, the release of GM-CSF and the tumor infiltration with $CD34^+$ were determined to be negative prognostic factors because both events were associated with an increased rate of tumor and metastasis recurrence [269]. Moreover, the increased number of $CD34^+$ cells in the PBMCs of these patients was associated with the suppression of the anamnestic responses to recall antigens, a frequent characteristic in head and neck cancer patients [270]. Interestingly, exposure of $CD34^+$ suppressors to the cytokine combination GM-CSF+IL-4 induced the maturation of the immature suppressor cells into DCs, with a parallel reversal of their immunosuppressive properties [271]. A more extensive study identified human MDSCs in the peripheral blood of patients with squamous cell carcinoma, head and neck cancer, breast cancer, and non-small cell lung cancer. In these cases, MDSCs were described as immature cells positive for the markers CD34, CD33, and CD13, but negative for the myelomonocytic marker CD15 and for the lineage markers (CD3, -CD14, -CD19, and -CD57) [272]. The variable expression of HLA-DR and CD11c molecules allowed the identification of two main populations: One third of the cells were immature monocyte/DCs and the remaining cells encompassed earlier myeloid differentiation stages. Like mouse MDSCs, human immature cells caused suppression of antigen- and mitogen-induced T-lymphocyte proliferation, and the combination of GM-CSF and IL-4 drove their differentiation to mature DCs [272]. This phenotypic characterization, however, has not been confirmed in other malignancies or in different disease stages. Analysis of PBMCs, from patients affected by metastatic adeno-carcinomas of the pancreas, colon, and breast cancer, revealed an increase in the oxidative activity of $CD15^+$ granulocytes that resulted in an elevated ROS production. Granulocyte activation correlated with the inhibition of TCR ζ-chain expression and cytokine production [40].

A subsequent study prospectively evaluated MDSCs in patients ($n = 123$) with newly diagnosed solid tumors (mostly breast and gastrointestinal tumors, but also including melanomas and other cancers), clinical stages I–IV [39]. MDSCs were evaluated in fresh blood samples and defined as $CD33^+$, $CD11b^+$, $Lin1^{-/low}$(lin-cocktail-contained antibodies to CD3, CD14, CD16, CD19, CD20, and CD56). MDSC levels were found to be significantly higher in cancer patients relative to a smaller cohort of matched healthy controls ($p < 0.0001$) and their concentration was proportional to clinical cancer stage. MDSCs with similar immature phenotype, i.e., $Lin^-HLA\text{-}DR^-CD33^+CD11b^+$, share markers and granule content in common with promyelocytes (even though normal promyelocytes are not immune suppressive) [273] and are increased in the blood of patients with different tumors including glioblastoma and breast, colon, lung, and kidney cancers (reviewed in [274]). Interestingly, recent data point out that this immature fraction might be indicative of the overall tumor burden and their increased circulating levels correlate with worse prognosis and radiographic progression in breast cancer and CRC patients [273], [275].

Also, human MDSCs can be divided into the granulocytic-like and the monocytic-like subsets. Human g-MDSCs are generally described as $CD11b^+CD15^+$ cells that are negative for the monocytic marker CD14, the costimulatory molecules CD11a, CD80, CD86, and CD83, and for HLA-DR. Human g-MDSCs are cryosensitive [276], and thus cannot be studied from frozen sample but only from fresh PBMCs. This characteristic has allowed their functional characterization only in few studies, although the markers associated with g-MDSCs are often used for immune-monitoring purposes. In a large study performed in metastatic RCC, Zea et al. [277] evaluated PBMCs from 123 patients and detected an increase in ARG activity that was associated with the downregulation of the CD3 ζ-chain expression and reduction of IL-2 and IFN-γ production by anti-CD3/anti-CD28-stimulated PBMCs [277]. Cell fractionation studies revealed that ARG activity was limited to $CD11b^+CD15^+CD14^-$ g-MDSCs and depletion of $CD11b^+$ cells from PBMCs was sufficient to restore ζ-chain expression, cytokine production, and proliferation of otherwise anergic T cells present among PBMCs [277]. Furthermore, the percentage of g-MDSCs was statistically higher in cancer patients compared to healthy donors [277]. However, it must be noted that in this study cryopreserved samples were used, and thus it is possible that a particular population was selected and that the observed difference in g-MDSC numbers could have been different (likely higher) if fresh PBMCs were analyzed. In a follow-up study [39], the same group evaluated g-MDSCs in fresh samples, and they confirmed the cellular phenotype and showed that these g-MDSCs also expressed the granulocytic marker CD66b and the vascular epidermal growth factor receptor 1 (VEGFR1), but had low levels of CD16 and CD62 L [39]. Interestingly, the authors presented evidence that, in contrast to murine MDSC, human g-MDSCs do not deplete L-Arg by increasing its cell uptake, but instead release ARG1 into the circulation [39].

The initial reports that pioneered the studies on MDSCs in human were forced to use only a limited number of colors for the flow cytometric analysis and hence the use of the lineage cocktail marker. Even though these studies boosted the interest on human MDSCs as predictive markers and possible pharmacological targets, the use of the lineage cocktail marker, which often includes CD14, together with the few reports that functionally evaluated the suppressive activity of MDSCs, has somehow biased the studies of the m-MDSCs in humans. Indeed, m-MDSCs are generally defined as $CD14^+$, $CD11b^+$, $CD15^{low/neg}HLA\text{-}DR^{low/neg}$. This population has been described in melanoma as $CD14^+HLA\text{-}DR^{-/low}$[278], $CD14^+IL4Ra^+$[279], $CD14^+HLA\text{-}DR^{+/low}B7\text{-}H^+$, or $CD14^+HLA\text{-}DR^{-/low}STAT3^{high}$[280]; in HNSCC and multiple myeloma as $CD14^+ARG^+$ [28]; in colon carcinoma as $CD14^+IL4R\alpha^+$ [279]; and in bladder cancer as $CD14^+$, $CD15^+$, $CD33^+HLA\text{-}DR^-$. In a large study on hepatocellular carcinoma patients, increased levels of ARG-expressing m-MDSCs ($CD14^+HLA\text{-}DR^{-/low}$), capable of suppressing T cell proliferation, were found [281]. In multiple myeloma and HNSCC, depletion or pharmacological inhibition of m-MDSC was sufficient to restore the otherwise anergic phenotype of PBMCs [28]. It must be noted, however, that in these studies frozen specimens were used, and thus it is possible that g-MDSCs were depleted by the freezing and thawing steps. Similar findings were shown in a clinical trial in which stage IV melanoma

patients were vaccinated with the heat shock protein gp96, with or without GM-CSF as adjuvant to better prime the immune response [108]. Similarly to what we reported in mice, where high doses of GM-CSF-secreting vaccine restrained the immune response through the recruitment of MDSCs [107], GM-CSF was shown to lower instead of increasing the frequency of melanoma antigen-specific T cells, as well as their capacity to secrete IFN-γ [108]. Increased frequency of immature $CD14^+HLA\text{-}DR^-$ TGF-β-producing myeloid cells was found in the PBMCs of GM-CSF-treated patients and was correlated with the lack of antimelanoma T cell response [108].

Similar suppressive activities were also found in melanoma and colon carcinoma patients in the $CD14^+IL4R\alpha^+$ cells [279]. In this latter study, fresh blood samples were used to compare the suppressive activity of both m-MDSCs and g-MDSCs. While IL4Rα expression correlated with the suppressive activity of m-MDSC, g-MDSCs were able to suppress an allogeneic response independently of the IL4Rα expression, suggesting that, as observed in mice, different mechanisms of immunosuppression may be operative in specific subsets [279].

A more extensive analysis evaluated the granulocytic and monocytic subsets of MDSCs in the peripheral blood and in the tumor of patients with bladder cancer [282]. PBMCs from bladder cancer patients contain both MDSC subsets: the $CD15^{high}CD33^+CD114^+$, $CD117^+$ granulocyte-like and the monocyte subset defined as $CD15^{low}CD33^{high}CD14^+$, $CD115^+$, $CD116^+$, and $CCR2^+$ [282]. The number of circulating granulocytic but not monocytic myeloid cells in cancer patients was markedly increased when compared to healthy individuals. However, both myeloid cell subsets from cancer patients were highly activated and produced substantial amounts of proinflammatory chemokines/cytokines including CCL2, CCL3, CCL4, G-CSF, IL-8, and IL-6. Interestingly, g-MDSCs were able to inhibit *in vitro* T cell proliferation through induction of $CD4^+Foxp3^+$Treg cells [282]. Analysis of bladder cancer tissues revealed that the myeloid infiltrate was composed of a majority (60–70 %) of $CD11b^+HLA\text{-}DR^+$ monocytes–macrophages and only 30 % of $CD11b^+HLA\text{-}DR^-$ granulocytic myeloid cells, a situation that mirrors the peripheral blood composition [282].

Recently, phase I/II clinical trials showed that vaccines based on tumor-associated peptides could prolong survival in patients with RCC and CRC who showed signs of a multipeptide-specific immunization [45], [283]. Moreover, positive and negative predictors of clinical responses could be found in the blood among leukocyte subsets (Treg cells and MDSCs) and serum proteins (chemokines and apolipoproteins) [45], [283]. In this study, a panel of antibodies was developed to identify six MDSC phenotypes in a single multicolor staining. Levels of all MDSC subsets, except one, were significantly increased in the blood of patients with RCC, suggesting a global modification of myelopoiesis in these patients. However, in a retrospective analysis, only two MDSC phenotypes were significantly negatively associated with survival: $CD14^+HLA\text{-}DR^{-/lo}$ and $CD11b^+CD14^-CD15^+$[283]. Interestingly, in RCC and CRC patients, the prevaccination serum levels of the chemokine CCL2 inversely correlated with the clinical response to the cancer vaccine in subjects responding to multiple peptides present in the vaccine formulation [45]. CCL2 cytokine released by mesenchymal and myeloid cells in the spleen of tumor-bearing mice was found

to be essential for the formation of a tolerogenic environment in the marginal zone of spleen; in this specific compartment of the white pulp, peculiar Ly6C$^+$ monocytic cells were attracted by the chemokine, cross-presented tumor antigen to CD8$^+$ T cells inhibiting their activity, and likely formed a pool of precursors for other MDSC subsets [45].

Taken together, the existing data on human MDSCs indicate that these cells share many of the functional properties found in mice. However, it is still very problematic to associate a unique panel of markers with human MDSCs. This difficulty can depend on the great plasticity and accepted heterogeneity that characterize MDSCs. Phenotypic differences in MDSCs can, in fact, reflect intrinsic differences in human cancers such as tumor stage, patient's age, and therapeutic history or simply the genetic variation, which is much higher in humans than in laboratory mouse strains. Nevertheless, differences in sample preparation (frozen vs. fresh, Ficoll-purified vs. red cell lysis) or in the antibody (i.e., clones, fluorochromes) used can explain, in some instances, the phenotypic differences found between laboratories. Because of the importance MDSCs are assuming in the prognosis and in the treatment of cancer, antibody panels and procedures should be optimized by the scientific community.

9 Targeting MDSCs in Cancer Patients

Important achievements in understanding the molecular mechanisms that regulate the differentiation, recruitment, and suppressive functions of mouse MDSCs have been reached in the past decade. Indeed, MDSCs are emerging as key players that regulate tumor progression, not only because of their capacity to restrain tumor immunity but also for their support to tumor angiogenesis, invasiveness, and metastatic spreading.

Despite the lack of a common consensus for the phenotypic definition of human MDSCs, the main mechanisms controlling the biology of mouse and human MDSCs appear to be conserved across the species and, thus, their therapeutic targeting in human malignancy represent a unique and novel therapeutic opportunity. Different clinical trials have been designed to target MDSCs with the aim to modulate tumor immunity and restrain tumor progression. A comprehensive list of therapeutic approaches targeting human MDSCs was recently published [284]. In this section, we will discuss some novel clinical data and emerging concepts.

One of the first attempts to modulate MDSC activity is based on 1α,25-dihydroxyvitamin D_3. This well-tolerated vitamin was previously shown to induce, *in vitro*, the maturation of immune suppressive CD34$^+$ MDSCs of HNSCC patients into immune stimulatory DCs [271], [285]. Further studies demonstrated that patients treated with 1α,25-dihydroxyvitamin $D_3$for 21 days before surgery had reduced intratumoral levels of MDSCs, an increased level of mature DCs, and a higher number of effector CD4$^+$ and CD8$^+$ T cells infiltrating the tumor and expressing the early activator marker CD69. More importantly, this short presurgical treatment was sufficient to double the time of HNSCC recurrence in the treated patients [286]. Interestingly, these antitumor effects of 1α,25-dihydroxyvitamin D_3 were

characterized by a profound modulation of the cytokine concentration in the plasma and in the tumor specimen [287]. Although induction of MDSCs' differentiation into immune stimulatory DCs may be one of the mechanisms that promotes the immunomodulatory activity of 1α,25-dihydroxyvitamin D_3, other actions might be involved. Indeed, 1α,25-dihydroxyvitamin D_3has been shown to inhibit tumor angiogenesis *in vivo* and the production of VEGF and HIF1α in many human tumor cell lines [288]. Since both VEGF and HIF1α are implicated in the induction of MDSCs by the tumor, it is possible that by modulating the transcriptome profile of neoplastic cells, 1α,25-dihydroxyvitamin D_3deprives tumors of multiple elements that sustain their escape from immune surveillance.

As discussed above, VEGF, PGE_2, GM-CSF, IL-6, and other tumor-derived factors activate aberrant intracellular pathways (i.e., IL6st, STAT3, etc.) in the myeloid lineage, expand the pool of MDSCs, and prevent DC maturation. Thus, inhibition of these intracellular pathways can be a strategy to revert the suppressive tumor macroenvironment and restore effective immune surveillance. Sunitinib, for example, is a small molecule that inhibits multiple tyrosine kinases (VEGFR-1, VEGFR-2, VEGFR-3, PDGFR, c-kit, ret, and STAT3) and that has shown potent effects against MDSCs in both animal models and human studies [289]. Clinical studies in advanced RCC unveiled a reversal of MDSC accumulation in addition to tumor cell apoptosis in sunitinib-treated patients [290], [291]. In particular, patients with metastatic RCC received, after resection of the primary tumor, sunitinib monotherapy for 28 days, followed by 14 days of rest, comprising one 6-week cycle. $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ MDSCs and $CD15^{high}CD14^{-}$ MDSCs significantly decreased starting with the first cycle of sunitinib [289] while circulating m-MDSCs, which were already present in low numbers in these patients, did not seem to be affected. The decrease in g-MDSC and immature myeloid cells correlated with an increased reactivity of PBMCs, after CD3/CD28 stimulation, as assessed by IFN-γ intracellular staining [290]. However, white blood cell counts and the percentage of neutrophils also significantly declined with treatment, suggesting that sunitinib affected total myelopoiesis [290]. In agreement with this hypothesis, a clinical trial using sunitinib in HNSCC demonstrated important hematological toxicities (i.e., lymphopenia, neutropenia, and thrombocytopenia) or bleeding complications in many patients [292]. In addition, a poor therapeutic efficacy was found in this latter trial [293], [294]. Considering the capacity of this molecule to inhibit multiple tyrosine kinases and the fact that some of these pathways need to be transiently activated during normal myelopoiesis and lymphopoiesis, these results are not completely surprising. Nevertheless, positive antitumor results were obtained when sunitinib was administered in conjunction with image-guided radiotherapy for the treatment of patients with oligometastases [295]. These studies indicate that, although sunitinib promotes a reduction of circulating MDSCs, not always does this reduction translate into a therapeutic efficacy. Similarly, sunitinib was shown to prevent MDSC accumulation and restored normal T cell function in the spleens of tumor-bearing mice, independent from the sunitinib's ability to inhibit tumor progression [296]. In the 4T1 mammary carcinoma, both m-MDSC and g-MDSC subsets were shown to be highly sensitive to sunitinib in the periphery but not at the tumor site [296]. As discussed above, this resistance was

conferred by the GM-CSF-driven STAT5 activation that seems to redirect g-MDSC toward a resistant monocytic phenotype [296]. Preliminary analysis from freshly isolated specimens of human RCC suggests that tumors can produce sufficient amounts of GM-CSF to induce STAT5 activation and protect MDSCs *in vitro* from sunitinib-induced apoptosis [297]. Thus, despite its intrinsic toxicity, sunitinib may yet maintain its therapeutic promise as a component of a possible multimodal therapy or in situations of minimal residual disease.

MDSC differentiation into functional APCs by cytokines or small molecules can be an intriguing strategy. In fact, this differentiation would not only remove MDSC suppressive mechanisms but also provide tumor-antigen-loaded APCs, which should potentiate the immune response against the malignancies, since tumor-associated MDSCs can uptake tumor antigens [193], [298]. *In vivo* administration of all-*trans*-retinoic acid (ATRA) reduced the presence of MDSCs in different tumor models and this effect was not a consequence of a direct ATRA-antitumor effect [31].

ATRA effect on MDSCs has been further explored in patients with metastatic RCC [299]. Lin^-/HLA-DR^-/$CD33^+$ MDSCs were evaluated during the treatment with different escalating doses of ATRA and subcutaneous IL-2. The numbers of circulating MDSCs significantly decreased, which correlated with improved antigen-specific T cell responses as measured by recall stimulation with tetanus toxoid [299].

Another agent that has been used to reverse MDSC-mediated immune suppression belongs to the class of COX2 inhibitors. COX2 expression can be detected both in the neoplastic cells and surrounding stroma [300], and, by producing PGE_2, this enzyme activates the suppressive phenotype in the MDSCs and facilitates the differentiation of MDSCs from hematopoietic precursors [148]. Thus, COX2 inhibition can block MDSC differentiation and immune suppression by inhibiting ARG1 [301] at the tumor site. The specific COX2 inhibitor Celecoxib demonstrated antitumor activity in advanced HNSCC when used in combination with Erlotinib (a specific inhibitor of epidermal growth factor receptor, EGFR) and radiotherapy. In combination with Erlotinib, 25 % of the treated patients with unresectable recurrent locoregional and/or distant metastatic HNSCC showed a partial response and a low toxicity profile [302]. Instead, when the same treatment was used in combination with local irradiation on patients with previously irradiated HNSCC, 60 % of the patients showed locoregional control and 37 % progression-free survival at 1 year [303]. Celecoxib unfortunately was also found to be associated with a dose-dependent cardiovascular morbidity, which can limit its dosage and prevents its long-term use in reversing tumor-induced immunosuppression [304]–[306].

In addition to the use of drugs to restrain MDSC differentiation, other strategies have been developed targeting specifically the suppressive mechanisms by which these cells inhibit antitumor immunity. In preclinical models, we previously demonstrated that PDE5 contribute to maintain MDSC suppression [28]. By controlling the intracellular concentration of cGMP, this enzyme directly controls the expression of NOS2, IL4Rα, and ARG1 in MDSCs [28], thereby controlling their suppressive action. Indeed, pharmacologic inhibition of PDE5 using sildenafil or tadalafil (Food and Drug Administration (FDA) approved drugs for the treatment of erectile dysfunction) was sufficient to restrain tumor-induced immune suppression, prime a

spontaneous antitumor immune response, and drastically reduce tumor progression in murine models of breast and colon cancers [28]. Furthermore, in a lymphoma model of tumor-induced tolerance, PDE5 was sufficient to restrain the MDSC-mediated expansion of tumor-specific Treg cells [258]. Finally, and more importantly, when sildenafil was added to anti-CD3/anti-CD28-stimulated PBMCs from patients with HNSCC and multiple myeloma, PDE5 blockade was sufficient to restore the otherwise repressed T cell proliferation [258]. Based on these results two independent clinical trials (at Johns Hopkins University and at the University of Miami) are being conducted in HNSCC patients to test the immune modulatory capacity of tadalafil daily administration before surgical resection of the primary tumor. Interim analyses in both clinical trials seem to suggest that PDE5 blockade lowers MDSC and Treg cell numbers in the blood and at the tumor site, promotes the tumor infiltration of activated ($CD69^+$) $CD4^+$ and $CD8^+$ T cells, and expands the systemic pool of tumor-specific T cells (Serafini, Weed unpublished observations).

10 Conclusions

Despite the bulk of evidence that described the importance and function of MDSC, several issues concerning MDSC biology are not completely defined and will require further studies: (1) The *in vivo* MDSC antigen-specific tolerogenic ability is in sharp contrast with the antigen-independent suppression described in many (if not all) *in vitro* assays; this dichotomy, common also for other regulatory cells of the immune response such as Treg cells, needs to be further investigated; (2) MDSCs employ different mechanisms to suppress T lymphocytes in different tumor models suggesting a tumor type-related influence on the biology and/or activity of these cells. However, the very molecular factors responsible for these functional differences need to be clarified. (3) Cell-specific knockout for genes essential for either MDSC development/activation or effector function needs to be generated to better understand MDSC physiological functions not only in tumor but also in healthy condition. (4) The interplay between the granulocytic and the monocytic components of the $CD11b^+$/Gr-1^+ cells needs to be unveiled; moreover, the *in vitro* culture conditions to differentiate mouse and human MDSCs need to be optimized to maintain in culture a phenotype and a functional activity similar to the tumor-derived MDSCs. (5) Finally, since the phenotype and the prevalence of MDSCs in human cancer remain uncertain, efforts need to be taken to identify functional phenotypic markers that, most likely, are the ones more conserved between species and cancer types. It is important to stress that incongruence in this field likely reflects the multifaceted alteration of myelopoiesis underlying the MDSC appearance. MDSCs are not terminally differentiated myelomonocytic cells and preserve a degree of plasticity that makes them more susceptible than other cell types to the influence of the experimental settings [284]. Furthermore, it must be pointed out that the definition of subsets among MDSCs has just begun, differently from what happened for other cell types such as DCs that have been extensively investigated in the past.

In contrast with these uncertainties, preclinical evidences strongly support the beneficial effect of approaches targeting MDSCs on both the spontaneous antitumor immune responses and the ones elicited by active or passive immunotherapy.

The clarification of the role of MDSC in tumors of different derivation as well as in various stages of cancer progression will not only unveil the biology of these cells but also define new modulators that, hopefully, will significantly improve the efficiency of the antitumor immune intervention.

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Chapter 5
Immunobiology of Dendritic Cells in Cancer

Michael R. Shurin, Anton A. Keskinov and Gurkamal S. Chatta

Abstract Dendritic cells (DCs) play a crucial role in initiating immune responses against both foreign pathogens as well as tumors. DCs also control the type, potency, and extent of T-cell responses, contribute to natural killer (NK) and natural killer T-cell (NKT cell) antitumoral activity, as well as to B-cell-mediated immunity. However, antitumor immune responses are often deficient or suboptimal since tumor cells are able to exploit the functional roles of DCs for tumor progression. Suppression, dysfunction, and repolarization of DC function in cancer patients all contribute to the failure of antitumor immune responses and consequent disease progression. Subversion of tumor immunity by altering the tumor immunoenvironment and DC subset distribution and function is mediated by various malignant cell-derived and tumor stroma-derived factors, many of which remain to be identified. Molecular mechanisms of tumor-mediated dysfunction and repolarization of the DC system are under investigation, and several signaling pathways responsible for DC malfunction in cancer have been already described. Here, we summarize findings in the field of DC biology in cancer and discuss the importance of these data for designing novel DC-based vaccination strategies, as well as their applicability for combinatorial therapeutic approaches.

Keywords Dendritic cells · Dendropoiesis · Immunosuppression · Regulatory dendritic cells · Immunomodulation · Chemoimmunomodulation

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1 Introduction

Tumor development and progression are associated with suppression and malfunction of the immune system, of which dendritic cells (DCs) possess many key regulatory functions, especially those related to cytokine production, antigen presentation to naive T cells, and polarization and balancing T-helper subsets. DCs are professional antigen-presenting cells, strategically positioned for bridging innate and adaptive immunity. DCs can initiate T-cell responses against tumors due to their capacity to process and present tumor antigens and stimulate naive T cells. However, less is known about DC differentiation, behavior, and polarization in vivo in tumor-bearing hosts. Although neglected for many years, the importance of the tumor microenvironment in regulating immunology of DC is becoming more defined, as the dual role of DC in cancer was shown to play an important role in cancer progression [1].

Although alterations in DCs in the setting of cancer were described almost two decades ago, characterization of tumor-derived factors responsible for DC dysfunction and the molecular mechanisms of abnormal DC differentiation and activation are still not well understood. An understanding of how the tumor environment regulates the DC system and how it impacts the efficacy of DC vaccines and other immunotherapeutic approaches is far from complete and clinical trials focusing on the protection of DC from the detrimental effects of the tumor microenvironment are constantly being tested. In addition to the tumor/stromal cells and their interactions, the other factors impacting vaccine efficacy in cancer include (a) the psychological stress of both a potentially fatal disease as well as the stress associated with the treatment of the disease (Fig. 5.1) and (b) aging immune system, since more than 60 % of cancer arises in people older than 65 years of age. Thus, in patients with cancer, the DC system functions under the multidirectorial influences of various local and systemic tumor-derived and tumor stroma-derived factors, acute and chronic stress hormones, therapeutic agents and factors, as well as multifaceted conditions associated with aging, infections, autoimmune diseases, and other acute and chronic disorders (Fig. 5.1).

Modulation of DC generation and function by some of the above-mentioned factors or conditions has been partly described. However, a comprehensive and systematic analysis of the DC system in the tumor environment has not been reported. For instance, both tumor-derived factors (reviewed in [2], [3]) as well as nonmalignant cells in the tumor milieu (reviewed in [4]) have been reported to suppress DC maturation, function, and longevity. Psychological and physical stressors may affect the functional activity of DCs through a variety of hormones, neuromediators, and neuropeptides [5]–[7]. Indeed, modulation of DC maturation and function by glucocorticoids, neuropeptides, and biogenic amines has been described. Glucocorticoid-treated DCs show higher endocytic activity, lower antigen-presenting function, and a lower capacity to secrete cytokines [8]. Norepinephrine can impede interleukin-12 (IL-12) and stimulate IL-10 production in DCs, thus inhibiting their antigen-presenting capability and hampering their motility and chemotaxis [9], [10]. DCs also express receptors for and respond to calcitonin gene-related peptide, neuropeptide Y, opioid peptides, prolactin, bombesin-like peptides, substance P, and

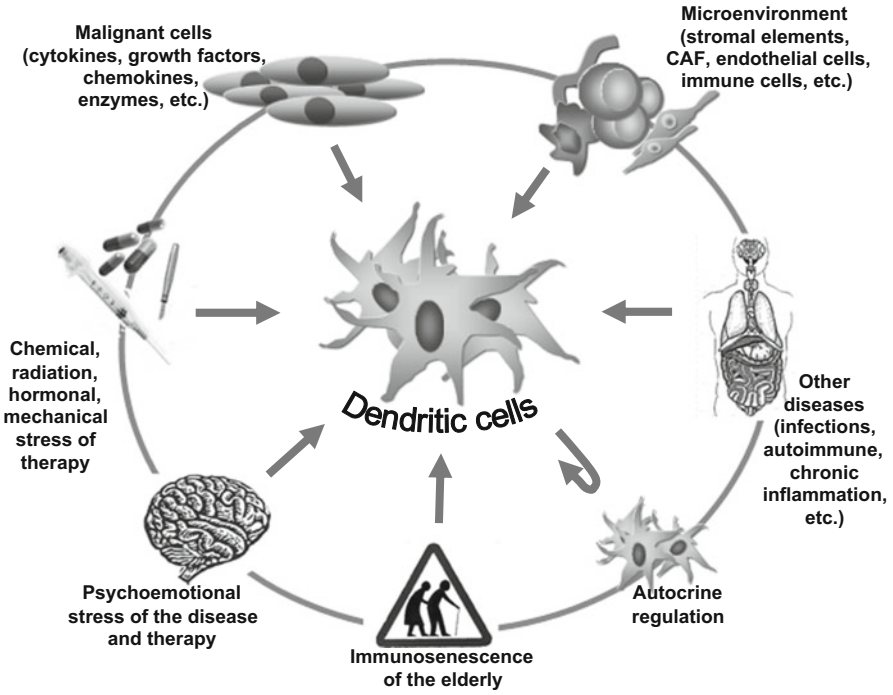

Fig. 5.1 Intrinsic and extrinsic mechanisms of dendritic cell regulation in cancer patients. DC generation, differentiation, polarization, and function are under constant, dynamic, and variable influences and regulatory pathways operating in patients with cancer. This includes numerous tumor-derived factors that affect all stages of DC development and may be represented by cytokines, chemokines, growth factors, prostaglandins, gangliosides, neuropeptides, and many other soluble and membrane-bound molecules on different cell types in the tumor microenvironment. Additional modulation of DC function in cancer may result from psychological stressors associated both with the diagnosis, as well as the effects of the treatment of a potentially fatal illness. Finally, DCs produce different factors, which may modulate cells in an autocrine and paracrine manner, and may also change DC responses to other molecules in the local environment. Thus, an understanding of the complex environmental conditions associated with DC function in cancer is necessary for harnessing the antitumor potential of these unique immunostimulatory and immunoregulatory cells

other neuropeptides, all of which may be involved in stress-related modulation of immunity [11]–[16].

As shown in Fig. 5.1, surgery, radiation, chemotherapeutic agents, and hormonal therapy might alter DC function and survival [17]–[20]. For example, many chemotherapeutic agents are known to suppress DC activity in therapeutic doses, but they may indirectly or directly upregulate DC maturation and function when used in low- or ultralow doses [21]–[25]. Interestingly, certain factors in the common environment, e.g., nanoparticles, may directly affect DC function in the lung or alter homing and function of other immune cells leading to dysfunction of antitumor immunity and tumor progression. For instance, it has been recently reported that in vivo exposure to single-walled carbon nanotubes (SWCNTs) modifies systemic immunity by

modulating DC function [26]. Furthermore, nanomaterials internalized by DCs differently affect their abilities to present antigens to T cells: While C(60)-fullerenes stimulated the antigen-specific major histocompatibility complex (MHC) class I-restricted T-cell response, graphene oxide (GO) impaired the stimulatory potential of DCs [27]. In contrast to C(60)-fullerenes, GO decreased the intracellular levels of low molecular mass polypeptide 7 (LMP7) immunoproteasome subunits required for processing of protein antigens. Interestingly, recent studies show that metastatic establishment and growth of lung carcinoma could be promoted by exposure to SWCNTs [28].

Furthermore, age-related alterations of DC maturity, function, longevity, and subpopulation composition also play a significant role in the ability of the DC system to interact with tumor cells and T cells and induce and maintain an antitumor immune response in patients with cancer (reviewed in [29]). For instance, increased levels of IL-6 and IL-10 repeatedly reported in old individuals might have a direct effect on dendropoiesis (i.e., DC generation) and maturation of DCs and, thus, on their motility and ability to process and present tumor antigens. Finally, exposure to different stimuli induces DCs to produce various endogenous mediators, including arachidonic acid-derived eicosanoids, cytokines, regulatory peptides, and small molecules like nitric oxide (NO). Many secreted products of DCs can act in an autocrine manner and modulate cell function; for instance, autocrine IL-10 can prevent maturation of DCs [30]. Interestingly, aging has been associated with immunological changes (immunosenescence) that mimic changes observed in the setting of chronic stress as well as changes seen with cancer [31], [32]. Thus, there may be common mechanisms of immune alterations in the DC system in cancer, aging, and chronic stress and numerous factors and agents can be involved in abnormal function of DCs in patients with cancer. These and many other issues related to differentiation, function, and clinical application of DCs in cancer have been discussed in the book *Dendritic Cells in Cancer* [33].

2 Alterations of DCs in Cancer

Regardless of the pathways and mechanisms responsible for tumor-associated changes of DCs, *functionally* three basic subtypes of DC can be seen in tumor-bearing hosts (Fig. 5.2): *normal* "unaltered" conventional DCs (cDCs) that can initiate and maintain immune responses, including antitumor responses; *functionally deficient* DCs with suppressed or blocked motility, antigen uptake/processing/presentation, or cytokine production or expression of costimulatory molecules; and *regulatory* or tolerogenic DCs that inhibit T-cell-mediated immune responses by different means. Because this classification is based strictly on DC function, there are no specific phenotypic markers to distinguish all functional subsets of DC seen in the tumor immunoenvironment [34]. In addition, DC function might be dynamically altered by the local microenvironment and surrounding cells. Functional plasticity of DC is a well-known phenomenon and different functional subsets of DC were repeatedly described in patients with cancer.

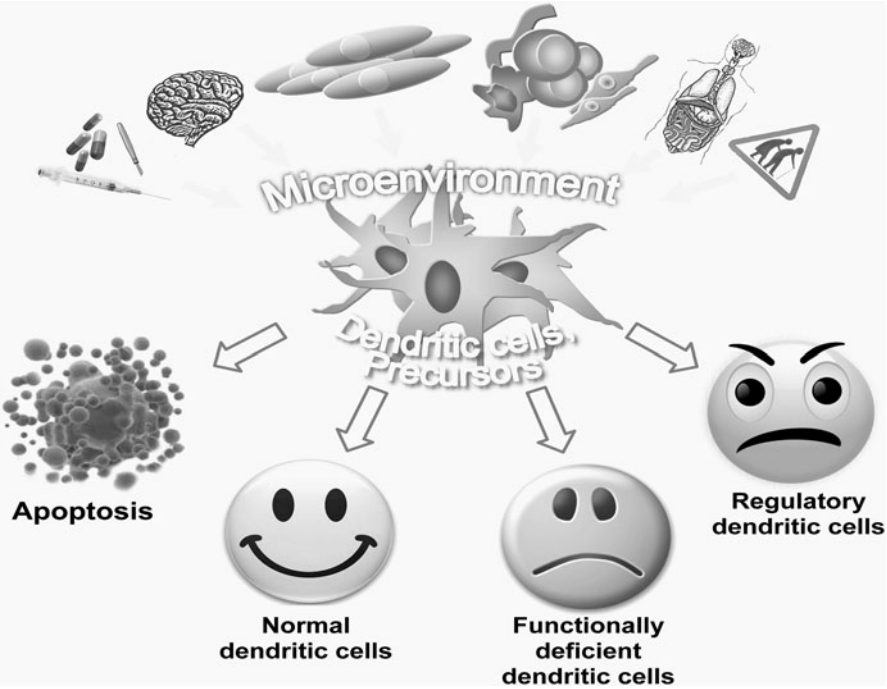

Fig. 5.2 Functional subsets of dendritic cells in cancer patients. The presence of numerous cells and factors affecting differentiation of DC precursors and activity of immature and mature DCs in the tumor environment results in formation of three basic functional subsets of DCs: normal "unaltered" immunostimulatory DCs, non-functional or functionally deficient DCs, and protumorigenic regulatory DCs. These DCs might belong to different or similar DC subpopulations (e.g., conventional or plasmacytoid), be on similar or different stages of maturation (e.g., immature, semi-mature, or mature), and express identical or different phenotypic markers (e.g., high or low MHC, CD80, CD86, CD40), but they act as inducers or suppressors of antitumor immune responses depending on the local and systemic environment

From the mechanistic point of view, most of the pathways that are responsible for altered functionality of DC in cancer can be also grouped in four categories: (1) *elimination* of functional DCs by blocking their production/differentiation/maturation or inducing apoptosis in DC or DC precursors; (2) *inhibition* of critical function of DCs; (3) *polarization* of DC subpopulations toward immunosuppressive or tolerogenic DC subsets; and (4) *avoidance* of the tumor contact with DCs by downregulating the expression of DC-attracting chemokines.

In 1988, Stene et al. revealed that melanoma-associated skin DCs (Langerhans cells) declined in number as melanoma progressed [35]. In 1989, Alcalay et al. described a decreased number and altered morphology of Langerhans cells in squamous cell carcinomas of the skin [36] and showed later (1991) that the antigen-presenting capacity of lymph node cells might be impaired during tumorigenesis [37]. Halliday et al. in 1991 demonstrated that tumor may regulate DC attraction and homing at

the tumor site and suggested that yet-unknown factors may inhibit DC function and thus induction of antitumor immunity [38], [39]. In 1992, Becker speculated that outcome of a primary tumor in patients depends on the ability of DCs to enter into tumors and that tumors might be different in their capacity to destroy or prevent DCs from entering the tumor site [40]. He also hypothesized that DCs and tumor cells interacted by releasing cytokines, which abrogate tumor cells or DCs, respectively [41]. In 1993, Tas et al. showed that DCs are functionally abnormal in patients with cancer [42]. Colasante et al. in 1995 studied the role of cytokines in the distribution and differentiation of DC lineage in primary lung carcinomas in humans and concluded on the potential role for granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), IL-1α, and IL-1β in DC modulation [43]. In 1996, Gabrilovich et al. reported that DCs isolated from tumor-bearing mice showed a significantly reduced ability to induce syngeneic tumor-specific cytotoxic T lymphocyte (CTL) and stimulate allogeneic T cells [44] and Chaux et al. revealed that tumor-associated DCs express low levels of costimulatory molecules [45]. Enk et al. in 1997 showed that melanoma-derived factors converted DC antigen-presenting function to tolerance induction against tumor tissue [46].

Following these initial findings, other teams demonstrated functional suppression in preparation of human CD34-derived and CD14-derived DCs, as well as murine bone marrow-derived DCs by both identified and unidentified tumor-derived factors (Table 5.1). For example, Ninomiya et al. (1999) reported that DCs propagated from patients with hepatocellular carcinoma expressed significantly lower levels of human leukocyte antigen-DR (HLA-DR), had significantly lower capacity to stimulate allogeneic T cells, and produced decreased amounts of IL-12 [47]. In vivo, Lissoni et al. (1999) revealed that the number of circulating DCs in the peripheral blood of cancer patients was also significantly decreased [48], and these results were confirmed by others, e.g., in patients with squamous cell carcinoma of the head and neck (HNSCC) [49], leukemia [50], hepatocellular carcinoma [51], lung cancer [52], and invasive breast cancer [53]. Metastasis development decreased the number of circulating DCs even further [18]. Furthermore, blood monocytes isolated from both patients with glioblastoma and intracranial metastases had significantly reduced expression of granulocyte macrophage colony-stimulating factor receptor (GM-CSFR) and showed a reduced capacity to differentiate into mature DCs [54]. Similar data were reported for other cancers [55]–[58]. Thus, local (at the tumor site) and systemic levels of DC might be markedly lower in cancer patients due to the inhibited or abnormal dendropoiesis [59], i.e., DC generation and differentiation.

Elimination of functional DCs in cancer may be also associated with the killing of DCs or acceleration of their turnover. Induction of apoptosis in DCs by tumor-derived factors was first reported by Esche et al. in 1999 [60] and confirmed by others [61]–[63]. Furthermore, the results were confirmed by documenting the presence of a significantly higher proportion of apoptotic blood DCs in patients with early-stage breast cancer compared to healthy volunteers [64]. Similarly, tumor-mediated cell death of DC precursors [65] and accelerated early apoptosis of DCs [58], [61], [66] were also reported.

Table 5.1 Abnormalities of the DC system in cancer

DC characteristics		Notes	Reference
I. *Elimination*	Quantitative inhibition of DC generation	From both $CD34^+$ hematopoietic precursors and $CD14^+$ macrophages in humans and bone marrow-derived precursors in mice	[54], [67], [166]–[168]
	Immaturity of DC at the tumor site	Low CD83, CD80, and CD86 expression	[157], [169], [170]
	Decreased number of circulating DCs	Low levels of DC1 in blood	[49], [85], [171]
	Low expression of CD40	In vitro and in vivo data	[55], [103]
	Downregulation of CCR7 on DCs	DCs prepared from tumor-bearing mice express low levels of CCR7 protein and mRNA	[172]
	Induction of DC apoptosis and acceleration of DC rate	Tumor-derived factors induced apoptotic death of DCs and increased rate of spontaneous apoptosis both in vitro and in vivo	[58], [60], [61], [64], [173]
	Apoptotic death of DC precursors	Tumor cells may kill DC precursors and decrease numbers of CFU-DCs	[65]
	Retention of DC inside the tumor lesions	Tumor-derived IL-8 might prevent DC emigration	[95]
II. *Inhibition*	Inhibition of DC motility	IL-8 and TGF-β1 as well as low CCR7 expression might prevent DC emigration from tumor lesions	[95], [174], [175]
	Inhibition of endocytic activity of DCs	Phagocytosis and receptor-mediated endocytosis were inhibited in DCs by different tumor cell lines	[58], [111], [131]
	Inhibition of antigen processing in DCs	Tumor suppress expression of MHC class I antigen-processing machinery proteins in DCs	[111]
	Inhibition of antigen presentation by DCs	Due to low MHC class I and II and costimulatory molecule expression	[58], [111], [176]
	Suppression of DC–T cell contact interactions	Decreased ability of DC to form clusters (rosettes) with T cells	[42] (Shurin, unpublished data)
	Low expression of costimulatory molecules on DCs	Downregulation of expression of B7 molecules	[123], [177]

Table 5.1 (continued)

DC characteristics		Notes	Reference
	Suppression of DC adhesiveness	Decreased adhesion of DCs to matrix protein-covered slides and tumor cell monolayers in vitro	(Shurin, unpublished data)
	Downregulation of cytokine production in DCs	For example, low IL-12 production by blood DCs in patients with breast cancer	[53]
III. *Polarization*	Dysbalance between cDC and regDC subpopulations	Decreased numbers of myeloid DCs (mDCs) in the blood Low mDC/pDC ratio in blood Decreased numbers of mDCs and increased numbers of lymphoid DCs (lDC) in the tumor milieu (e.g., peritoneal fluid in ovarian cancer patients)	[49], [53], [82], [85], [87], [105], [139], [178]
	Dysbalance between DCs, macrophages, and MDSCs	Tumor-associated increase in the levels of M2 macrophages and myeloid-derived suppressor cells	[179]–[181]
	Polarization of cDCs into regDCs	Tumor-induced conversion of proimmunogenic cDCs into pro-tumorigenic regDCs	[147], [160]
	Transdifferentiation of bone marrow-derived DCs into endothelial-like cells by tumor	Process of DC endothelialization is VEGF-dependent	[150]
IV. *Avoidance*	Loss of DC-attracting chemokines at the tumor site	Loss or downregulation of CXCL14 expression in tumor cells	[92], [182]

The second type of DC abnormalities in cancer includes the functional deficiency of DCs when compared to cells derived from healthy age-matched controls (Table 5.1). Decreased ability of DCs obtained from cancer patients' blood or lymph nodes, or DCs cocultured with malignant cells to stimulate allogeneic T cells, uptake, process, and present antigen(s), provide costimulatory signals, migrate toward specific chemokines, and produce IL-12 were repeatedly described for prostate, breast, renal, liver, lung cancer, HNSCC, melanoma, myeloma, leukemia, glioma, neuroblastoma, and other tumor types [65]–[74]. These and other results were also reviewed in [2], [3], [75]–[79] and therefore are not detailed here.

Polarization of DC subtypes represents the third type of the DC aberration in cancer (Table 5.1). For instance, there are substantial numbers of tumor-promoting functional plasmacytoid DCs (pDCs or lymphoid DCs by some classifications, but not cDCs or myeloid DCs) accumulated in tumor ascites in patients with ovarian carcinomas [80]. Similarly, estimating conventional and plasmacytoid subpopulations of DCs in the peritoneal fluid of women with ovarian tumors, Wertel et al. reported that the percentage of pDCs was higher in patients with ovarian cancer than in women with serous cystadenoma [81]. They also reported that the percentage of the peritoneal fluid myeloid DCs was significantly lower in patients with ovarian cancer in comparison to the group of nonmalignant ovarian tumors, while the percentage of the peritoneal fluid lymphoid DCs was higher in patients with ovarian cancer than in the reference group [82]. The presence of pDCs within primary breast tumors correlated with an unfavorable prognosis for patients [83]. Using fresh human breast tumor biopsies, the authors observed increased tumor-associated pDC rates in aggressive breast tumors and showed that these pDCs produced very low amounts of IFN-α. Interestingly, within breast tumors, pDCs colocalized with regulatory T cells (Treg cells); the selective suppression of IFN-α production endowed pDCs with the unique capacity to sustain $Foxp3^+$ Treg expansion [83]. The same team has recently identified transforming growth factor- β (TGF-β) and TNF-α as major soluble factors involved in pDC functional alteration in cancer [84]. These findings indicate that IFN-α-deficient tumor-associated pDCs accumulating in aggressive tumors are involved in the expansion of tumor-associated Treg cells in vivo, contributing to tumor immune tolerance and poor clinical outcome.

The levels of myeloid or cDC subsets in circulation may also be significantly lower, while the number of lymphoid or pDC subsets might vary, as was repetitively reported for patients with different tumor types compared to healthy donors [52], [85], [86]. Interestingly, these alterations were reverted by surgical resection of the tumor or by chemoradiotherapy [53], [85], [87], [88] suggesting that tumor-derived factors are responsible for redirecting DC differentiation (dendropoiesis) in the bone marrow, i.e., systemically. Indeed, microvesicles isolated from plasma of advanced melanoma patients, but not from healthy donors, mediated the effect of tumor on $CD14^+$ monocytes and skewed their differentiation from DCs toward $CD14^+$HLA-DR^{low} cells with TGF-β-mediated suppressive activity on T-cell functions [89]. A subset of these TGF-β-secreting $CD14^+$HLA-DR^{low} cells was found to be significantly expanded in the peripheral blood of melanoma patients compared with healthy donors.

Tumor-promoted redirection of dendropoiesis and its repolarization are also associated with increased numbers of immature DCs and the appearance of other related immature cells of myeloid progeny. For example, in addition to having fewer levels of DCs in the peripheral blood, patients with breast and prostate cancer as well as patients with malignant glioma showed significant accumulation of abnormal population of HLA-DR$^+$ immature cells (DR$^+$ICs), which in spite of HLA-DR, CD40, and CD86 expression had reduced capacity to capture antigens and elicited poor proliferation and IFN-γ secretion by T lymphocytes [86]. Immature DCs fail to provide an appropriate costimulatory signal to T cells and might induce tolerance through abortive proliferation or anergy of antigen-specific CD4$^+$ and CD8$^+$ T cells or through the generation of Treg cells that suppress immune responses by producing IL-10 and TGF-β [90]. Immature DCs were found at high levels within tumor-infiltrating leukocytes and increased circulating levels of immature DCs have also been observed in the peripheral blood of patients with lung, breast, head and neck, and esophageal cancer [91]. Immature myeloid precursors of DCs may also suppress T-cell activation as part of a population of myeloid-derived suppressor cells (MDSCs), a heterogeneous population of myeloid cells that comprises immature macrophages, granulocytes, DCs, and myeloid cells at early stages of differentiation, discussed in detail in other chapters of this monograph.

Finally, the last mechanism of decreased number of active DCs associated with the tumor progression is the loss of expression of DC-attracting chemokines at the tumor site (Table 5.1). For instance, it has been demonstrated that HNSCC cells do not express CXCL14 protein and messenger RNA (mRNA), a potent DC-attracting chemokine [92]. This resulted in low chemoattraction of DCs to the tumor bed, low numbers of tumor-associated DCs, and deficient induction of antitumor immunity; however, transduction of CXCL14-negative tumor cells with the CXCL14 gene was associated with increased DC infiltration, an antitumor immune response, and inhibition of tumor growth in vivo. Investigation of the mechanisms of loss of CXCL14 in prostate cancer cells revealed direct evidence for epigenetic regulation of chemokine expression in tumor cells [93]. Interestingly, melanoma cells might utilize an opposite approach and can effectively chemoattract DCs, modulate their phenotype, and, eventually, severely damage DC mobility: Melanoma-conditioned DCs exhibited an increased adhesion capacity to a melanoma cell line in vitro and did not migrate in response to DC chemokines [94]. The explanation for abnormal DC retention inside some human malignant lesions may come from another study where it was found that tumors from patients with hepatocellular carcinoma, colorectal cancer, or pancreatic cancer were producing IL-8 and that this chemokine attracted DCs that uniformly express both IL-8 receptors, CXCR1 and CXCR2 [95].

In summary, abnormal dendropoiesis, DC longevity and function, and DC migration toward or from the tumor site are the key characteristics of the local and systemic DC dysfunction in tumor-bearing hosts that have a crucial role in immune nonresponsiveness to tumors and tumor escape [34].

3 Mechanisms of Dendritic Cell Dysfunction in Cancer

3.1 Factors

Tumors exploit several strategies to evade immune recognition, including the production of a variety of immunosuppressive/immunomodulating factors, which might specifically block or redirect DC maturation, suppress DC survival, and impair function of DC in the vicinity of tumors [4] (Table 5.2). Historically, the first tumor-derived factor inhibiting DC differentiation in cancer was identified as vascular endothelial growth factor (VEGF) [96]. For instance, in patients affected by colorectal cancer, DC numbers inversely correlated with VEGF serum levels, suggesting a possible effect of this cytokine on the DC compartment. In cultures, the exposure of monocyte-derived DCs to VEGF produced a dramatic alteration of DC differentiation by induction of apoptosis, alteration of DC phenotypic profile, and increased CXCR4 expression [97]. VEGF blocks the functional maturation of DCs from hematopoietic progenitor cells by blocking nuclear factor kappa-B (NF-κB) transcription. The family of VEGF molecules also plays a key role in recruiting immature myeloid cells and immature DCS from the bone marrow to enrich the tumor microenvironment [98].

Tumor-derived TGF-β and IL-10 were shown to be responsible for downregulating CD80 expression on blood DCS in myeloma patients [68]. DC maturation, antigen presentation, and IL-12 production induced by inflammatory cytokines IL-1 and TNF-α or by lipopolysaccharide (LPS) might be inhibited by TGF-β [99]. TGF-β might also induce apoptosis in DCs [100]. Increased levels of IL-10 in serum from patients with hepatocellular carcinoma and tumor progression were shown to correlate with profound numerical deficiencies and immature phenotype of circulating DC subsets [101]. Murine bone marrow-derived DCs that were propagated in IL-10 and TGF-β (so-called alternatively activated DC) expressed low levels of Toll-like receptor 4 (TLR4), MHC class II, CD40, CD80, CD86, IL-12p70, and programmed death-ligand 2 (B7-DC; CD273) and were resistant to maturation [102]. They secreted much higher levels of IL-10 and efficiently expanded functional $CD4^+CD25^+Foxp3^+$ Treg cells. We have shown earlier that murine colon adenocarcinoma cells produce IL-10 and that IL-10 causes downregulation of CD40 expression on DCs and is responsible for inhibited CD40-dependent IL-12 production by DCs [103]. These and other studies also revealed the tumor-associated in vivo effects of IL-10 on DC function in eliciting a type 1 immune response in both allogeneic and tumor-specific responses [104]. Furthermore, analyzing pancreatic cancer-derived cytokines responsible for inhibition of DC differentiation, Bellone et al. (2006) reported that IL-10, TGF-β, and IL-6, but not VEGF, cooperatively affect DC precursors in a manner consistent with ineffective antitumor immune responses [105]. However, lung squamous cell carcinoma and adenocarcinoma have been shown to use different mediators to induce comparable phenotypic and functional changes in DCs: IL-6 versus $IL\text{-}10^+IL\text{-}6^+$ prostanoids, respectively [106]. Renal cell carcinoma (RCC)-derived IL-6 and VEGF were shown to block the ability of tumor antigen-loaded DCs to induce CTL in the autologous system [107].

Table 5.2 Mechanisms of DC dysfunction in cancer

Mechanisms and factors		Examples	Reference
Tumor/stroma-derived factors	Cytokines or their combinations	VEGF, M-CSF, IL-6, IL-10, TGF-β, and IL-8	[96], [103]–[105], [183]
	CCL2	Mediates the migration of myeloid suppressors to tumors	[184]
	CCL20/MIP3α	Involved in immature DCs and their precursors attraction	[158]
	Stromal-derived factor-1 and β-defensins	Attract proangiogenic DC subset	[80], [151]
	Prostanoids and prostaglandins	Regulate DC maturation	[109]
	Gangliosides	Suppress dendropoiesis and DC longevity	[63], [69], [111]
	Neuropeptides	Bombesin-like peptides (gastrin-releasing peptide (GRP) and neuromedin B (NMB))	[15]
	Tumor antigens	PSA is a serine protease; MUC1 subverts DC function	[113], [115]
	Other molecules	Lactic acid, hyaluronan, NO, and spermine	[53], [62], [112], [185]
	HLA-G	HLA-G might induce tolerogenic DCs by disruption of the MHC class II presentation pathway	[119], [146]
	Reactive oxygen species	Hydrogen peroxide may activate p38 and JNK in DC and induce apoptosis	[137]
	Tumor-derived microvesicles	Abnormal differentiation of monocytes into IL-6/TNF-α/TGF-β-producing cells	[89]
Affected signaling pathways in DCs	Upregulation of Bax and downregulation of Bcl-2 and Bcl-X_L	Both extrinsic and intrinsic pathways are involved in tumor-induced apoptosis of DCs	[60], [132], [133], [138]
	Ceramide	Mediates tumor-induced DC apoptosis by downregulation of the PI3K pathway	[134]
	Small Rho GTPases	Cdc42 and Rac 1 mediate tumor-induced dysfunction of DCs	[131]

Table 5.2 (continued)

Mechanisms and factors		Examples	Reference
	STAT3	Tumor-mediated induction of STAT3 in DCs results in reduced expression of IL-12, MHC class II, and CD40	[123], [156], [186], [187]
	SOCS1	SOCS1 functions as an antigen-presentation attenuator by controlling the tolerogenic state of DC and the magnitude of antigen presentation	[127]
	p38 MAPK	Tumor activates p38 MAPK and JNK but inhibits ERK in DCs	[129]
	H1 expression	Tumor-derived factors inhibit *h1* expression in DC precursors causing defective DC differentiation	[188]
	PKCβII	Tumor-mediated inhibition of DC differentiation is mediated by downregulation of PKCβII expression	[130]
	Epigenetic regulation of the CIITA type I promoter	Tumor-mediated downregulation of MHC class II in DC development is attributable to the epigenetic control of the CIITA type I promoter	[128]

To define the pathways limiting DC function in the tumor microenvironment, Sharma et al. assessed the impact of tumor cyclooxygenase (COX)-2 expression on DC activities and reported that inhibition of tumor COX-2 expression or activity could prevent tumor-induced suppression of DC capacity to process and present antigens and secrete IL-12 [108]. COX-1- and COX-2-regulated prostanoids and IL-6 were found to be solely responsible for the hampered differentiation of monocyte-derived and $CD34^+$-precursor-derived DCs by freshly excised solid human tumors (colon, breast, RCC, and melanoma) [109]. An important role for the EP2 receptor in prostaglandin E2 (PGE_2)-induced inhibition of DC differentiation and function and the diminished antitumor cellular immune responses in vivo has been also reported [110]. Finally, PGE_2 suppressed differentiation of DCs, it is a potent inducer of IL-10 in bone marrow-derived DCs, and PGE_2-induced IL-10 is a key regulator of the DC pro-inflammatory phenotype [109].

In addition to these "classic" tumor-derived antidendropoietic factors, other molecules were implicated in tumor-mediated dysfunction of the DC system (Table 5.2). Melanoma, neuroblastoma, RCC, and lung cancer were shown to produce and shed various gangliosides, which may suppress dendropoiesis, inhibit DC function, or induce apoptosis in DCs [63], [69], [111]. Tumor-derived lactic acid is also an important factor modulating the DC phenotype in the tumor environment, which may critically contribute to tumor escape mechanisms [112]. Interestingly, several tumor antigens were recently found to display antidendropoietic properties. Prostate specific antigen (PSA), which is a serine protease, was able to inhibit generation and maturation of DCs from $CD34^+$ hematopoietic precursors, assessed by the levels of expression of CD83, CD80, CD86, and HLA-DR, as well as the ability of DC to induce T-cell proliferation [113]. When cultured with the MUC1 glycoprotein, human monocyte-derived DCs displayed decreased expression of CD86, CD40, CD1d, HLA-DR, and CD83 and were defective in the ability to induce immune responses in both allogeneic and autologous settings. The modified phenotype of MUC1-treated DCs corresponded to an altered balance in IL-12/IL-10 cytokine production with a failure to make IL-12 and induce Th1 responses [114], [115]. Finally, human chorionic gonadotropin (hCG), which serves as an important tumor marker for trophoblastic disease, has been recently shown to upregulate expression of indoleamine-2,3-dioxygenase (IDO) in DCs [116].

HLA-G molecules, which are normally expressed in cytotrophoblasts and play a key role in maintaining immune tolerance at the maternal–fetal interface, were also reported to be expressed on malignant cells and they can be regulated by hypoxia [117], [118]. As DCs express immunoglobulin-like transcript 4 (ILT4), an inhibitory receptor capable of interacting with HLA-G, they may be tolerized by HLA-G through inhibitory receptor interactions. Indeed, the HLA-G–ILT4 interaction leads to development of tolerogenic DCs with the induction of anergic and immunosuppressive T cells [119].

Finally, human tumors constitutively release endosome-derived microvesicles, transporting a broad array of biologically active molecules with potential modulatory effects on different immune cells. The first evidence that tumor-released microvesicles alter myeloid cell function by impairing monocyte differentiation into DCs and

promoting the generation of a myeloid immunosuppressive cell subset was probably published by Valenti et al. [89], [120] and then confirmed by other teams [121], [122].

3.2 Signaling Pathways

Many immunosuppressive factors produced by tumor cells induce signal transducer and activator of transcription 3 (STAT3) activation in DCs, blocking their normal functioning. For instance, treatment of DCs with melanoma-conditioned medium resulted in reduced expression of IL-12, MHC class II, and CD40 due to the increased induction of STAT3 [123]. The immunosuppressive effects of tumor-derived factors on DC differentiation were abrogated in cells from STAT3 knockout mice or by the treatment of DC precursors with a phosphopeptide that binds the STAT3 Src homology 2 (SH2) domain and blocks downstream STAT activation. Furthermore, IL-6-mediated suppression of DC maturation was also abrogated in STAT3-deficient DC precursors, indicating the significance of STAT3 in IL-6-mediated suppression of DC maturation and function [123]. Furthermore, constitutive STAT3 activation in tumor cells was shown to inhibit DC function by the increased induction of STAT3 in immature DCs. Thus, immunosuppression mediated by tumor cells results from a circuit of STAT3 signaling that begins in tumor cells and eventually activates inhibitory STAT3 signaling in DCs in part due to the production of cytokines that increase STAT3 activation in DCs (epidermal growth factor (EGF), VEGF, IL-6, IL-10, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and GM-CSF) [123]. In addition, STAT3 phosphorylation in DCs was regulated by IL-6 in vivo, and STAT3 was necessary for the IL-6 suppression of DC activation/maturation [124]. Interestingly, $CD4^+CD25^+Foxp3^+$ Treg cells from tumor-bearing animals may also impede DC function by activating STAT3 and inducing the Smad signaling pathway [125]. The suppression mechanism was also associated with downregulation of activation of the transcription factor NF-κB, required TGF-beta and IL-10, and resulted in strong inhibition of expression of the costimulatory molecules CD80, CD86, and CD40 and the production of TNF-α, IL-12, and chemokine (C-C motif) ligand 5 (CCL5 or RANTES—regulated on activation, normal T cell expressed and secreted) by DCs.

Many STAT family members are developmentally regulated and play a role in DC differentiation and maturation. For instance, the STAT6 signaling pathway is constitutively activated in immature DCs and declines as they differentiate into mature DCs. Downregulation of the STAT6 pathway is accompanied by dramatic induction of suppressors of cytokine signaling 1 (SOCS1), SOCS2, SOCS3, and cytokine-induced SH2-containing protein expression [126]. In contrast, STAT1 signaling is most robust in mature DCs. Thus, it is likely that cytokine-induced maturation of DCs is under feedback regulation by SOCS proteins and that the switch from constitutive activation of the STAT6 pathway in immature DCs to predominant use of STAT1 signals in mature DCs is mediated in part by STAT1-induced SOCS expression [126]. Recent studies also demonstrate that SOCS1 functions as an antigen-presentation

attenuator by controlling the tolerogenic state of DCs and the magnitude of antigen presentation [127]. Since SOCS1 restricts DCs' ability to break self-tolerance and induce antitumor immunity by regulating IL-12 production and signaling, it is quite possible that some products of tumor cells or other cells within the tumor milieu might induce SOCS1 expression in DCs. Although not proven experimentally, this pathway may operate in the tumor microenvironment limiting the ability of DCs to process and present tumor antigens and secrete IL-12.

Another interesting mechanism responsible for tumor-induced downregulation of MHC class II expression in DCs was reported by Choi et al. They found that STAT5 bound to the CIITA pI locus during DC differentiation and that the binding was markedly attenuated by a tumor-conditioned medium or by IL-10 [128]. IL-10 inhibited the expression of type I CIITA during DC differentiation: GM-CSF-mediated histone (H3 and H4) acetylation at the type I promoter (pI) locus of the CIITA gene was markedly increased during DC differentiation and this increase was blocked by IL-10. This suggests that IL-10-mediated MHC class II downregulation results from the inhibition of type I CIITA expression. This inhibition is most likely due to blocking of the STAT5-associated epigenetic modifications of the CIITA pI locus during the entire period of DC differentiation from bone marrow-derived precursors, as opposed to a simple inhibition of MHC class II expression at the immature/mature DC stage.

Wang et al. too speculated that tumor-induced p38 mitogen-activated protein kinase (MAPK) activation and extracellular signal-regulated kinase (ERK) inhibition in DCs might be a new mechanism of tumor evasion [129]. They showed that tumor supernatant-treated DCs were inferior to normal DCs at priming tumor-specific immune responses, but inhibiting p38 MAPK restored the phenotype, cytokine secretion, and function of tumor-treated DCs. Tumor-derived factors activated p38 MAPK and Janus kinase (JNK) but inhibited ERK in DCs. Interestingly, Farrent et al. have recently reported that tumor-mediated myeloid dysregulation may be mediated by Stat3-induced protein kinase C isoform βII (PKCβII) downregulation: they showed that tumors mediate both Stat3 activation and PKCβII downregulation in DC progenitor cells, a process mimicked by the expression of a constitutive active Stat3 mutant [130].

Since many functions of DCs, such as endocytosis, exocytosis, adhesiveness, and motility, depend on actin polymerization and membrane rearrangements, Tourkova et al. analyzed whether small Rho guanosine triphosphatases (GTPases: Cdc42, RhoA, and Rac1/2), which are primarily involved in regulating these functions in DCs [111], might be affected by tumor-derived factors. They found that impaired endocytic activity of DC cocultured with tumor cells was associated with decreased levels of active Cdc42 and Rac1. Transduction of DCs with the dominant negative Cdc42 and Rac1 genes also led to reduced phagocytosis and receptor-mediated endocytosis, while transduction of DCs with the constitutively active Cdc42 and Rac1 genes restored the endocytic activity of DCs that was inhibited by the tumors [131].

Less is known about signaling pathways that control DC longevity and DC sensitivity to tumor-induced cell death. Early studies showed that Bcl-X_L, Bcl-2, and mitochondrial cytochrome c release mediate resistance of DCs to tumor-induced

apoptosis [132], [133]. Other data demonstrated that downregulation of phosphoinositide 3-kinase (PI3K) is the major facet of tumor-induced DC apoptosis [134]. Interestingly, it is known that some cancer cells have increased production of hydrogen peroxide (H_2O_2) [135], [136] and, in DCs, hydrogen peroxide activates two key MAPKs, p38 and JNK. Activation of JNK, which is associated with inhibition of tyrosine phosphatases in DCs, is linked to the induction of DC apoptosis [137]. By targeting different antiapoptotic molecules, including FLICE-like inhibitory protein (FLIP), X-linked inhibitor of apoptosis protein or human IAP-like protein (XIAP/hILP), procaspase-9, and heat shock protein 70 (HSP70), Balkir et al. demonstrated that antiapoptotic molecules other than the Bcl-2 family of proteins were involved in tumor-induced apoptosis in DCs [138]. This suggests that tumor-induced apoptosis of DCs is not limited to the mitochondrial pathway of cell death and that both extrinsic and intrinsic apoptotic pathways play a role in DC survival in the tumor microenvironment.

4 Role of DCs in Tumor Escape Mechanisms

A growing body of evidence clearly demonstrates that different subsets of DC are directly and indirectly involved in controlling tumor growth and progression. However, with the realization that the DC lineage represents a varied collection of distinct populations, a question has arisen as to whether certain types of DC are dysregulated in tumor-bearing hosts, or whether the nature of immunological challenge and state of DC maturation define particular facets of innate/acquired/tolerogenic responses in the tumor environment. Numerous studies have revealed that specific DC subsets might be linked to immunological unresponsiveness and/or tolerance to tumor antigens. For instance, the clinical outcome of children with cancer has been shown to correlate with circulating pDC count: Children with high pDC counts at diagnosis showed significantly worse survival than those with low counts and the development of cancer was associated with low number of cDCs [139]. Elevated levels of pDC have been observed as breast cancer disseminates to the bone. The selective depletion of pDCs in mice led to a total abrogation of bone metastasis as well as to an increase in the T_H1 antitumor response [140]. Thus, tumor-associated pDCs contribute to the tumor immunosuppressive network. Tumor ascites pDCs induced IL-10^+CCR7^+CD45RO^+C$D8^+$ Treg cells, which significantly suppress myeloid DC-mediated tumor-associated antigen-specific T-cell effector functions through IL-10 [141]. pDCs in tumor-draining lymph nodes might create a local microenvironment that is potently suppressive of host antitumor T-cell responses and this mechanism may be mediated by immunosuppressive IDO.

IDO degrades tryptophan to kynurenine, which is further metabolized to 3-hydroxyanthranilic acid and thus initiates the immunosuppressive pathway of tryptophan catabolism (see Chap. 6 in this monograph). Emerging evidence suggests that Treg cells may be generated de novo against specific tumor-derived antigens, and thus they arise as a direct consequence of antigen presentation in the tumor-draining

lymph nodes [142]. IDO can also be expressed within the tumor itself, by tumor cells, or by host stromal cells, where it can inhibit the effector phase of the immune response [143]. Kynurenine pathway enzymes downstream of IDO can initiate tolerogenesis by DCs independently of tryptophan deprivation, as tolerogenic DCs can confer suppressive ability on otherwise immunogenic DCs in an IDO-dependent fashion [144]. IDO, i.e., tryptophan, kynurenine, or 3-hydroxyanthranilic acid, could also induce expression of the tolerogenic molecule HLA-G in DCs [145]. Thus, IDO and HLA-G can cooperate in the immune suppression, since HLA-G-expressing DCs might suppress or alter effector T cells as well. Indeed, activated $CD4^+$ and $CD8^+$ T cells could efficiently acquire immunosuppressive HLA-G from antigen-presenting cells through membrane transfers (a process called trogocytosis) and acquisition of HLA-G immediately reversed T-cell function from effectors to regulatory cells. These Treg cells were able to inhibit proliferative responses through HLA-G that they acquired [146]. Targeting IDO in regulatory DCs (regDCs) may represent a new approach for harnessing DCs in the tumor microenvironment [147].

In support of the concept that certain DC subpopulations play crucial roles in tumor escape, it was recently reported that tumor expansion could stimulate Treg cells via a specific DC subset. During tumor progression, a subset of DC exhibiting a myeloid immature phenotype may be recruited to draining lymph nodes and selectively promote proliferation of Treg cells in a TGF-β-dependent manner [148]. Importantly, tumor cells are necessary and sufficient to convert DCs into regulatory cells that secrete TGF-β and stimulate Treg cell proliferation. Regulatory DCs in cancer may directly and indirectly maintain antigen-specific and nonspecific T-cell unresponsiveness by controlling T-cell polarization, MDSC and Treg differentiation and activity, and affecting specific microenvironmental conditions in premalignant niches [149].

Another subset of DCs might contribute to neovascularization at the tumor site. Recently, Conejo-Garcia et al. reported that within 3 weeks of culture with tumor cell-conditioned medium, bone marrow-derived DCs could be transdifferentiated into endothelial-like cells in vitro [150]. They also identified a novel leukocyte subset within ovarian carcinoma that coexpressed endothelial and DC markers which may play a role in the formation of blood vessels [151]. Curiel et al. observed high numbers of pDC in malignant ascites of patients with untreated ovarian carcinoma and showed that tumor-associated pDC induced angiogenesis in vivo through production of TNF-α and IL-8 [152]. By contrast, cDCs, which might suppress angiogenesis in vivo through production of IL-12, were absent from malignant ascites. Thus, the tumor may attract pDCs to augment neovascularization while excluding myeloid DC to prevent angiogenesis inhibition.

Thus, one mechanism contributing to immunologic unresponsiveness toward tumors may be presentation of tumor antigens by tolerogenic/regulatory host DCs. Indeed, using bone marrow chimeras in transgenic mice, Mihalyo et al. have recently reported that DCs, but not $CD4^+CD25^+$ Treg cells, play a critical role in programming CD4 cell responses to tumor antigens during tumorigenesis [153]. Regulatory DCs could be produced from bone marrow precursors in the presence of GM-CSF, IL-10, TGF-β1, and LPS or TNF-α and they retained their T-cell regulatory

property in vitro and in vivo even under inflammatory conditions [154]. Another minor subpopulation of regulatory DCs has been recently described in murine spleen. These splenic $CD19^+$ DCs that did not express the pDC marker acquired potent IDO-dependent T-cell suppressive functions [155].

However, proponents of the "maturation" hypothesis suggest that the maturation state of the DC in the premalignant/inflammatory milieu or in the newly formed tumor setting predicts the development of an antitumor immune response or tumor tolerance. An increased proportion of immature DCs with reduced expression of costimulatory molecules was seen or isolated from tumor mass of patients with RCC, prostate cancer, basal-cell carcinoma, and melanoma or was found in the peripheral blood of patients with breast, head and neck, lung, or esophageal cancer [156]. Similar data have been obtained using several mouse tumor models. The maturation hypothesis was also bolstered by studies showing that in tumor tissues, immature DCs resided within the tumor, whereas mature DCs were located in peritumoral areas [157]. Immature DCs cannot induce antitumor immune responses and, most importantly, immature DCs can induce T-cell tolerance or anergy. Thomachot et al. showed that breast carcinoma cells produce soluble factors (chemokine (C-C motif) ligand 20 (CCL20) and TGF-β), which attract DC precursors in vivo and promote their differentiation into immature DCs with altered functional capacities, and that these altered DCs may contribute to the impaired immune response against the tumor [158]. Similarly, a medium conditioned by human pancreatic carcinoma cells induced monocyte-derived immature DCs with inhibited proliferation, expression of costimulatory molecules (CD80 and CD40) and HLA-DR, and functional activity as assessed by T-cell activation and IL-12p70 production [105]. Immature DCs generated from pancreatic carcinoma patients in advanced stages of the disease similarly showed decreased levels of HLA-DR expression and reduced ability to stimulate T cells. Direct ex vivo flow cytometric analysis of various DC subpopulations in peripheral blood from hepatocellular carcinoma patients revealed an immature phenotype of circulating DCs that was associated with increased IL-10 concentrations in serum and with tumor progression [47], [101].

To evaluate whether and to what extent the capacity of tumor-infiltrating DCs to drive immunization can be turned off by tumor cells, leading to tumor-specific tolerance rather than immunization, Perrot et al. have characterized the DCs isolated from human non-small cell lung cancer based on the expression of CD11c. All isolated DCs, including $CD11c^{high}$ myeloid DC, $CD11c^-$ pDC, and a third DC subset expressing an intermediate level of CD11c, were immature and displayed poor antigen-presenting function even after TLR stimulation and reduced migratory response toward CCL21 and SDF-1 [159]. Interestingly, $CD11c^{int}$ myeloid DCs, which represented approximately 25 % of total DC in tumor and peritumor tissues, expressed low levels of costimulatory molecules contrasting with high levels of the immunoinhibitory molecule B7-H1. These data suggest that immature tumor-associated DCs have an ability to compromise the tumor-specific immune response in draining lymph nodes in vivo. However, our data demonstrate that immature bone-marrow-derived DCs cannot suppress proliferation of pre-activated T cells without pretreatment with tumor-derived factors. Our recent data also reveal that different

tumor cell lines produce soluble factors that induce polarization of cDCs into regulatory DCs, both in vitro and in vivo. These regulatory DCs can suppress proliferation of pre-activated T cells and are phenotypically and functionally different from their precursors as well as the classical immature cDC [160]. Understanding the biology of regDCs and the mechanisms of their formation in the tumor immunoenvironment will provide a new therapeutic target for repolarizing protumorigenic immunoregulatory cells into proimmunogenic effector cells able to induce and support effective antitumor immunity.

In spite of multiple evidence supporting both "subpopulation"-based and "maturation"-based explanations of how the DC system is involved in tumor escape (Table 5.3), additional data suggest that the real situation might be significantly more complex. The first layer of complexity arrives from the results showing that DC subsets may induce both tolerogenic and immunogenic responses depending on the environmental stimuli. For example, although the general thought is that pDCs are usually tolerogenic, it appears that the functional role of pDCs in cancer immunity depends on cytokines that affect the balance between immunity and tolerance in the tumor and lymphoid organ microenvironment. In an analysis of draining lymph nodes in breast cancer, pDCs with a relative increase in IL-12 and interferon-γ (IFN-γ) were associated with a good prognosis, whereas pDCs with a relative increase in IL-10 and IL-4 were associated with a poor prognosis [161]. In confirmation of this conclusion, Kim et al. have reported that although pDCs recruited to the tumor site are implicated in facilitating tumor growth via immune suppression, they can be released from the tumor as a result of cell death caused by primary systemic chemotherapy and can then be activated through TLR9 [162]. Thus, synergistically with cDCs, pDCs may also play a crucial role in mediating cancer immunity. In fact, new results from a recent clinical trial indicate that vaccination with naturally occurring pDCs is feasible, with minimal toxicity, and that in patients with metastatic melanoma, it induces favorable immune responses [163]. Thus, we can conclude that pDCs, as well as myeloid cDCs, have a dual role not only in initiating immune responses but also in inducing tolerance to tumor antigens.

An additional layer of complexity of the DC subset versus the DC maturation problem in cancer comes from the data revealing different maturation patterns of different DC subsets and its differential regulation by other immune cells. For example, analysis of the maturation of human blood-derived cDCs and pDCs activated with TLR ligands in the presence of Treg cells revealed that pre-activated Treg cells strongly suppressed TLR-triggered cDC maturation, as judged by the blocking of costimulatory molecule upregulation and the inhibition of pro-inflammatory cytokine secretion that resulted in poor antigen presentation capacity. Although IL-10 played a prominent role in inhibiting cytokine secretion, suppression of phenotypic maturation required cell–cell contact and was independent of TGF-β and CTLA-4. In contrast, the acquisition of maturation markers and production of cytokines by pDCs triggered by TLR ligands were insensitive to Treg cells[164]. Therefore, human Treg may enlist conventional DCs, but not pDCs for the initiation and the amplification of tolerance in vivo by restraining their maturation after TLR stimulation.

Table 5.3 DC-mediated mechanisms of tumor escape

Mechanisms	Notes	Reference
Inability to present tumor antigens to T cells and induce tumor-specific CTL	Tumor-mediated suppression of critical functions of DCs	[2], [44], [107]
Immaturity of DC in the tumor environment	High levels of immature DCs can actively induce T-cell tolerance to tumor antigens and promote cancer progression	[47], [101], [105], [158]
Expression of IL-10	IL-10-producing DCs efficiently expand functional $CD4^+CD25^+$Foxp3 Treg cells	
Expression of TGF-β	DCs exhibiting a myeloid immature phenotype may promote proliferation of Treg cells in a TGF-β-dependent manner	[148]
Low IL-12 production	DCs isolated from tumor-bearing animals or cancer patients produce low levels of spontaneous and inducible IL-12	[71], [103], [105]
Low IL-18 expression	Melanoma might block IL-18 synthesis in DCs and thus prevent activation of NK cells by DCs	[189]
IDO-mediated mechanisms	Tolerogenic DCs can confer suppressive ability on otherwise immunogenic DCs in an IDO-dependent fashion	[143], [190]
Immunoglobulin-like transcript 3 (ILT3) and ILT4 (human) Paired immunoglobulin-like inhibitory receptor (PIR-B) (murine)	The HLA-G-ILT4 (immunoglobulin-like transcript) interaction leads to development of tolerogenic DCs with the induction of anergic and immunosuppressive T cells	[191]
Expression of HLA-G molecules	HLA-G on DCs induces immune suppression and tolerance	[145], [146]
Expression of B7-H1	Upregulation of B7-H1 on DCs in the tumor microenvironment downregulates T-cell immunity	[159], [192]
Attraction and protection of pDC precursors	Accumulation of pDCs in peritoneal fluid, ascites in ovarian cancer	[80], [81], [140]
Protumorigenic function of pDC	Depletion of pDCs in mice with breast cancer led to a total abrogation of bone metastasis and increase in Th1 antitumor response	
Stimulation of angiogenesis by tumor-associated pDC and DC precursors	Tumor pDCs produce high levels of TNF-α and IL-8 and induce neovascularization in vivo	[150], [152]
Induction of Treg	Tumor ascites pDCs induce $IL\text{-}10^+CCR7^+CD45RO^+CD8^+$ Treg	[141], [142]

Table 5.3 (continued)

Mechanisms	Notes	Reference
Induction of regDC	Tumor might induce a subpopulation of DCs which secrete TGF-β and support proliferation of Treg cells	[148], [154], [155], [160]
Inhibition of IKDC subset (?)	IFN-producing killer DCs (IKDCs) are $B220^{+}NK1.1^{+}Gr1^{-}$ DCs that kill tumor cells through the TRAIL pathway	[193], [194]
Impairment of DC migration toward tumor site or lymphoid tissue	Decreased attraction of increased retention of DCs at the tumor site	[92], [95]
Anti-inflammatory and tolerogenic properties of tumor-induced immature DCs	Decreased production of IL-1, IL-6, and TNF-α and increased expression of IL-10 and TGF-β after capturing apoptotic tumor cells	[90]

In another study, evidence was provided that maturing cDCs and pDCs express different sets of molecules that drive distinct types of T-cell responses [165]. Although both maturing cDCs and pDCs upregulate the expression of CD80 and CD86, only pDCs upregulate the expression of inducible costimulatory ligand (ICOS-L) and maintain high expression levels upon differentiation into mature DCs. High ICOS-L expression endows maturing pDCs with the ability to induce the differentiation of naive CD4 T cells to produce IL-10 but not the Th2 cytokines IL-4, IL-5, and IL-13. These IL-10-producing T cells are Treg cells, and their generation by ICOS-L is independent of pDC-driven Th1 and Th2 differentiation. Thus, in contrast to cDCs, pDCs are poised to express ICOS-L upon maturation, which leads to the generation of IL-10-producing Treg cells [165].

As such, there is still a confusion in the field as to whether certain DC subpopulations have evolved to fulfill unique immunological roles in cancer (Th1/Th2/Th3/Th17 polarization, Treg induction, tolerance, etc.), or whether distinct DC subsets exist to uniquely respond to tumor-derived stimuli but each participates in maintaining tolerance or immunity in the immature or mature state. It is also somewhat undecided whether some of the diversity in the DC lineage as determined by cell surface-molecule expression represents genuine distinct DC subsets or particular developmental/activation states of the same DC subtype. However, collectively, an emerging view in the field is that DCs control the course of tumor immunity/tolerance on at least three levels: (1) the developmental repertoire of DC lineage populations which can dictate the nature of DC response to a particular stimulus in the tumor microenvironment, (2) the maturation stage of DCs when cells interact with other immune cells or respond to immunological signals (i.e., cytokines, chemokines, and TLR ligands), and (3) the environment within which DCs encounter tumor antigens, as defined by the tissue type, infiltrating leukocytes, and an inflammatory cytokine milieu.

Therefore, DCs in the tumor microenvironment serve as a double-edged sword and, in addition to initiating potent antitumor immune responses, may mediate genomic damage, support neovascularization, block antitumor immunity, and stimulate cancerous cell growth and spreading [149]. The importance of these issues and mechanisms controlling them is significant, as efforts to harness the power of DCs in vaccination strategies against tumors would ultimately aim to identify the correct type of DC for a particular approach and insure that these cells are appropriately activated or protected from tumor influence to elicit the desired response.

5 Concluding Remarks

Numerous experimental and clinical observations discussed above suggest that tumor-induced apoptosis or altered differentiation and function of DCs as well as accumulation of immature DCs or DC precursors with inhibitory and tolerogenic function could impair antitumor immune responses. For patients with cancer, the resulting dysfunction of the DC system would result in marked deficiency in

the induction of antitumor immunity, tumor progression, and probably, low response to immunotherapy [34]. This is really important for understanding tumor immunopathology as well as reevaluating tumor immunotherapeutic strategies since DCs prepared from patients with cancer are being evaluated as a cellular vaccine in multiple clinical trials worldwide. However, to date, DC-based immunotherapies have met with limited success for several reasons, including the restricted longevity and efficacy of administered DCs in a suppressive tumor environment. Therefore, alternative approaches, including protection of DC longevity, blockade of tumor-mediated inhibitory pathways, and prevention of DC dysfunction/polarization ex vivo, should be evaluated to potentiate the efficacy of DC-based cancer vaccines. Given that endogenous DCs might be important for fulfilling the potential of various cellular vaccines, gained knowledge in the area of DC immunobiology in cancer may help to find new drugs to selectively block suppressive pathways and restore the original function of DCs.

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Chapter 6
Macrophages and Tumor Development

Suzanne Ostrand-Rosenberg

Abstract Macrophages are malleable cells that can adapt varying phenotypes and functions. They are critical components of the innate immune system that have the potential to eliminate intracellular pathogens, to facilitate wound healing, and to activate T cells and natural killer (NK) cells. Their specific phenotype and functions are regulated by the cytokines and chemokines in their microenvironment. Although macrophages have the potential to be tumoricidal, the inflammatory milieu of the tumor microenvironment co-opts macrophages and renders them tumor promoting. Tumor-associated macrophages (TAMs) are present in most solid tumors where they facilitate tumor progression by enhancing angiogenesis, promoting tumor cell invasion and metastasis, protecting tumor cells from chemotherapy, and inhibiting antitumor immunity. Macrophage infiltrates in solid tumors have long been recognized as indicators of poor prognosis. However, current studies are exploring novel strategies for reprogramming TAMs and converting them into cells that facilitate tumor rejection.

Keywords M1 · M2 · Tumor-associated macrophages · Immune suppression · Angiogenesis · Tumor invasion and metastasis · Polarization · Inflammation

1 Introduction

Macrophages are an exceptionally diverse population of cells. They are critical players in both innate and adaptive immunity and their functions impact responses to pathogens, to self-antigens (autoimmunity), and to cancer cells. Macrophages were first identified in the late 1800s by Elie Metchnikoff, a Ukrainian pathologist. He observed that if starfish were injected with dye particles, cells within the starfish would engulf the particles. He called these cells "phagocytes" based on the Greek words

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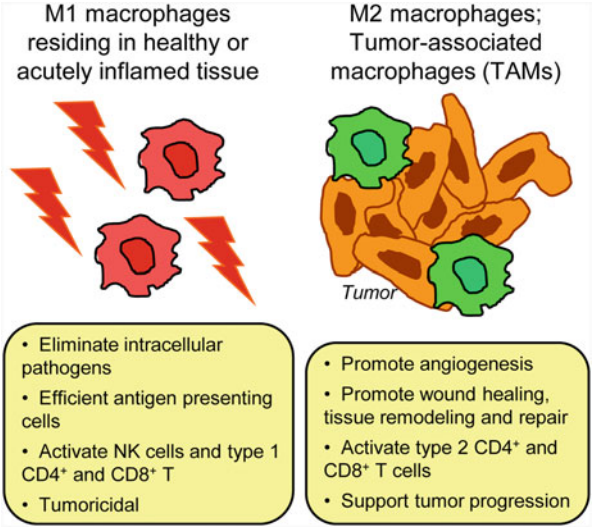

Fig. 6.1 Macrophages residing in healthy or pathogen-infected tissues (M1 macrophages) and tumor-associated macrophages (TAMs, M2 macrophages) have distinct functions

macros (large) and *phagein* (to eat). He subsequently observed similar cells in human blood, and in 1892 he proposed his "cellular (phagocytic) theory of immunity," stating that white blood cells are the key elements of the immune system that protect against pathogens [1], [2]. At that time, this theory was both groundbreaking and controversial because it contradicted the prevailing concept that humoral immunity, mediated by soluble proteins (antitoxins) and serum, was responsible for immune protection. It was not until the 1940s that immunologists appreciated the dual role of humoral and cellular immunity, despite the recognition of both cellular and humoral immunity by the awarding of a shared Nobel Prize to Metchnikoff and Paul Ehrlich, the discoverer of antitoxins (antibodies), in 1908.

The phagocytic cells seen by Metchnikoff were actually a mixture of multiple cell types, including what we now know as dendritic cells (DC), neutrophils, and macrophages. In addition to phagocytosis, macrophages also mediate their effects through their production of soluble factors including cytokines and chemokines, and by direct cell-to-cell contact with their cellular targets. Macrophages are now recognized as central players in the immune system and as having extensive plasticity. Their role and function depend largely on their anatomical location and microenvironment since their plasticity is largely driven by factors produced by surrounding cells. In healthy individuals, macrophages play an important role in facilitating wound healing, in regulating adaptive immunity, in eliminating infectious agents, and in regulating metabolism (reviewed in [3]–[6]). Macrophages have been categorized as "M1-like" and "M2-like" to reflect this apparent dichotomy of function (Fig. 6.1). M1-like macrophages typically reside in healthy tissue or at sites of acute inflammation. They eliminate intracellular pathogens, activate type 1 cluster of differentiation 4 ($CD4^+$) and $CD8^+$ T cells by functioning as antigen-presenting cells (APC), and may be cytotoxic for tumor cells. In contrast, M2-like macrophages (also referred to as "alternatively activated macrophages") mediate wound healing,

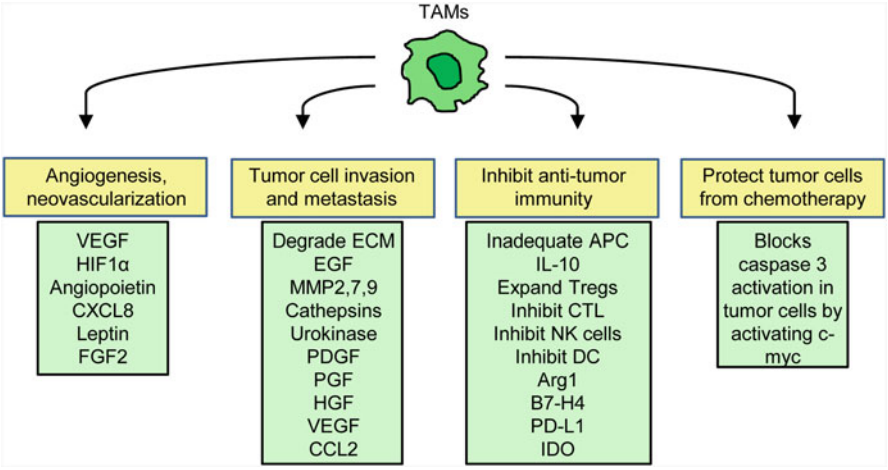

Fig. 6.2 Tumor-associated macrophages (*TAMs*) facilitate tumor progression through diverse mechanisms. Tumor-promoting mechanisms are listed in *yellow boxes*; factors mediating the mechanisms are listed in *green boxes*

promote angiogenesis, and drive type 2 immunity. The conditions and factors that drive the development of M1 and M2 macrophages are described in Sect. 5. In individuals with cancer, macrophages are usually co-opted by tumor-secreted factors and facilitate the development and progression of tumors. These tumor-associated macrophages (TAMs) are immune suppressive and share many characteristics with M2-like macrophages.

2 Macrophages Promote Tumor Progression Through Multiple Mechanisms

It is well established that macrophages localize to tumor sites and many studies demonstrate a strong correlation between quantity of macrophages and poor prognosis [7], [8]. TAMs are recruited to solid tumors because of the hypoxic and inflammatory tumor microenvironment. These conditions retain macrophages within tumors while additional factors modulate macrophage phenotype and enable them to promote tumor progression [9], [10]. TAMs facilitate tumor progression through diverse mechanisms. Figure 6.2 summarizes these mechanisms which are described in the following sections.

2.1 TAMs Drive Angiogenesis and Neovascularization

As solid tumors enlarge, they become too large to obtain their nutrients and oxygen through simple diffusion and must induce neovascularization and establish a vascular network. This process, known as the "angiogenic switch," as well as continued

neovascularization of growing tumors, is mediated by macrophages through their production of vascular endothelial growth factor A (VEGF-A) and other pro-angiogenesis factors [11]–[14]. Macrophages expressing Tie2, the receptor for angiopoietin-2 (ANG2), are particularly associated with neovascularization [12]. Induction of VEGF in macrphages is partially regulated by hypoxia [15], [16]. Poorly vascularized regions of solid tumors become hypoxic and chemoattract macrophages [17]. The hypoxic environment turns on the transcription factors, hypoxia-inducible factors (HIF) 1α and 2α, which in turn upregulate VEGF synthesis. VEGF then chemoattracts more macrophages [18], [19]. Macrophages also produce matrix metalloproteinases (MMP) and other proteases that degrade the extracellular matrix (ECM) causing the release of additional pro-angiogenic factors [20].

Inflammation through the action of the pro-inflammatory cytokine interleukin-1 beta (IL-1β) also induces VEGF synthesis by stabilizing HIF1α. This upregulation occurs in both normoxic [21] and hypoxic [22] conditions and involves nuclear factor kappa B (NF-κB) upregulation of cyclooxygenase-2 [21]. Other factors also contribute to macrophage-induced angiogenesis. Urokinase-type plasminogen activator and its receptor, molecules that increase vascularization, are upregulated on TAMs [23]. Macrophage-induced angiogenesis has also been attributed to fibroblast growth factor-2, angiopoietin, chemokine (C–X–C motif) ligand 1 (CXCL8 or IL-8), and leptin [24], as well as more than 30 other genes that are upregulated by hypoxia [25].

2.2 TAMs Promote Tumor Cell Invasion and Metastasis

Tumor cell invasion and metastasis are complex processes that require tumor cells to leave their primary site, invade their surrounding normal tissue and ECM, intravasate into and traffic via either the circulatory or lymphatic system, extravasate at a distant location, and acquire nutrients and oxygen at their final destination. Macrophages facilitate several of these steps. Early experiments using knockout mice deficient for colony-stimulating factor 1 (CSF-1), a factor required for macrophage differentiation, demonstrated that macrophages in the local microenvironment are essential for tumor cell invasion and metastasis [26]. Elegant multiphoton microscopy studies demonstrated that TAMs facilitate tumor cell intravasation into surrounding normal tissue and blood vessels [27]. In vivo studies demonstrated that resistance to established murine mammary carcinoma metastatic disease requires macrophage depletion or polarization of macrophages towards a tumor-rejecting M1-like phenotype [28], [29]. Several factors produced by macrophages contribute to these processes. In addition to CSF-1 serving as a differentiation factor, it also stimulates macrophage production of epidermal growth factor (EGF) which is a chemoattractant for tumor cells and promotes tumor cell invasiveness [30]. The release of macrophage inhibitory factor (MIF) also promotes tumor cell trafficking [31]. TAMs also synthesize and secrete multiple MMP (MMP-9, -2, and -7) [32], [33], cathepsins [34], [35], urokinase [36], and other proteases [37] which degrade the ECM and basement membrane, thereby allowing tumor cells to migrate to distant sites. Macrophages

also produce platelet-derived growth factor, placental growth factor (PIGF), and hepatocyte growth factor which directly support tumor cell proliferation [25]. Recent studies in multiple mouse tumor systems and in immune-deficient mice with human tumors definitively identified $CD11b^+$ $F4/80^+$ macrophages as essential for promoting tumor cell extravasation and for "conditioning" a metastatic niche prior to arrival of migrating tumor cells [38]. Subsequent studies revealed that the progenitors of these macrophages are $Gr1^+$ inflammatory monocytes that express C–C chemokine receptor type 2 (CCR2) and are chemoattracted by tumor and stromal cells producing chemokine (C–C motif) ligand 2 (CCL2), and that extravasation involves monocyte and macrophage production of VEGF. These studies demonstrate the link between inflammation and cancer in the metastatic process and provide a mechanistic explanation for the poor prognosis of patients with high levels of CCL2 and tumor-infiltrating macrophages [39].

2.3 TAMs Inhibit Antitumor Immunity

TAMs inhibit both innate and adaptive antitumor immunity and are themselves impacted by the immune system of tumor-bearing individuals. Unlike M1-like macrophages which have high levels of major histocompatibility complex (MHC) class II molecules and are effective APC, TAMs are poor APC because they have reduced levels of MHC II molecules as well as low levels of the costimulatory molecules CD80 and CD86. Hypoxia contributes to these decreased levels [40], [41], as does interaction with myeloid-derived suppressor cells (MDSC) [42].

TAMs are characterized by their high production of IL-10 and their minimal production of IL-12. IL-10 polarizes immunity towards a type 2 response whereas IL-12 polarizes towards a type 1 response. Type 1 responses favor tumor rejection while type 2 responses promote tumor progression. IL-10 production drives $CD4^+$ Th2 cells at the expense of $CD4^+$ Th1. Optimal activation of potentially tumoricidal $CD8^+$ T cells requires help from $CD4^+$ Th1 cells, so the absence of Th1 cells usually results in sub-optimally activated cytotoxic $CD8^+$ T cells. IL-10 also inhibits $CD8^+$ T-cell function by inducing the expansion of natural T regulatory cells (Tregs) and the development of induced Tregs. The Tregs, in turn, directly inhibit $CD8^+$ cytotoxic activity [43]. Since IL-12 contributes to the activation and efficacy of both natural killer (NK) cells [44] and DC [45], TAMs also impact antitumor immunity by diminishing NK-mediated cytotoxicity and antigen presentation by DC.

TAMs directly impact T-cell activation and proliferation through several mechanisms. They produce high levels of arginase 1, an enzyme that degrades arginine. Arginine degradation not only deprives T cells of L-arginine needed for protein synthesis but also results in the generation of toxic catabolic products including oxygen and nitrogen radicals [46], [47].

TAMs also suppress T-cell activation through their expression of immune suppressive co-inhibitory molecules. B7-H4 is a member of the B7 family of genes and is induced in macrophages by IL-6 and IL-10. A subset of TAMs from ovarian

cancer patients express B7-H4. When B7-H4 interacts with its receptor on T cells, T cells fail to progress through the cell cycle and therefore neither proliferate nor produce the cytokines essential for tumor cell killing [48]. Some TAMs also express the co-inhibitory molecule programmed cell death ligand-1 (PD-L1, also called B7-H1). PD-L1 causes T-cell apoptosis when it binds to its receptor, PD-1, which is expressed on activated T cells. IL-10 and tumor necrosis factor alpha (TNFα) in the tumor microenvironment of hepatocellular carcinoma patients induce expression of cell surface PD-L1 in TAMs, and these macrophages suppress antitumor immunity [49].

The enzyme indoleamine 2,3-dioxygenase (IDO) is an immune suppressive molecule produced by macrophages and other cells of myeloid origin. IDO degrades tryptophan and therefore depletes the microenvironment of tryptophan making surrounding cells unable to progress through the cell cycle [137]. Tumor cells themselves secrete IDO which to some extent reduces tumor growth. However, DC and macrophages also produce IDO, and this IDO suppresses T-cell activation and outweighs the direct effects of IDO on tumor cells. As a result, lymphocytes in the lymph nodes draining solid tumors are tolerized or nonresponsive to tumor antigens, thereby eliminating adaptive antitumor immunity [138], [139]. Recent studies in mouse models have established that the immune suppressive effects of IDO significantly contribute to the progression of both primary and metastatic lung and mammary tumors [50].

2.4 TAMs Protect Tumor Cells from Chemotherapy

Macrophages also promote tumor growth by interfering with chemotherapy [51]. In patients with multiple myeloma, large quantities of macrophages are present in the bone marrow. Chemotherapy of multiple myeloma with dexamethasone and melphalan induces apoptosis of tumor cells by cleaving and activating caspase 3. Cell-to-cell contact between macrophages and myeloma cells prevents caspase 3 activation and therefore protects tumor cells from apoptosis [52]. Interactions between myeloma cells and macrophages depend on binding of myeloma cell-expressed P-selectin glycoprotein-1 and ICAM-1/CD18 to their respective partners on macrophages (selectins and CD11b, respectively). Binding of these ligand–receptor pairs activates the c-myc pathway and kinases Src and Erk1/2 in myeloma cells, rendering them resistant to caspase activation [53].

3 Origin and Identification of Macrophages

Macrophages are a type of white blood cell (leukocyte) that reside in both lymphoid and non-lymphoid tissues. In the mouse, macrophages are identified by their expression of the cell surface markers CD11b (a subunit of Mac-1) and F4/80, a G protein-coupled receptor. CD11b is also expressed by macrophages in humans,

along with the cell surface protein CD68. Mouse and human macrophages are also characterized by their expression of other markers which are acquired in response to their microenvironment (see the following sections on macrophage polarization and inflammation).

Macrophages may have specialized names depending on their location. For example, macrophages within the central nervous system are called microglia while those in the lung are termed alveolar macrophages. Most macrophages are derived from pluripotent hematopoietic stem cells (HSC) that originate in the bone marrow (Fig. 6.3). HSC give rise to a common myeloid progenitor (CMP) that in turn gives rise to a granulocyte/macrophage progenitor (GMP) which serves as the progenitor for multiple myeloid cells including monocytes that circulate in the blood, bone marrow, and spleen [54]. Circulating monocytes become macrophages when they migrate into and become resident in tissues. Recent studies have demonstrated that some tissue-resident macrophages are present and proliferate in the absence of HSC and have identified a second lineage of macrophages that arises from the yolk sac in 9.5–10.5-day-old mouse embryos. The transcription factor Myb was found to be essential for the development of classical hematopoietic-derived macrophages, but was not necessary for the development of microglia or macrophages in the liver [55]. Although studies have not specifically been conducted on the origin of tumor-infiltrating macrophages, it is likely that these cells are of hematopoietic origin since they are derived from circulating monocytes.

4 Inflammation Drives Malignant Transformation and Tumor Progression and Recruits TAMs

Inflammation is now appreciated as one of the hallmarks of cancer [56]–[58]. Inflammation in the tumor microenvironment facilitates the growth of established tumors, and approximately one quarter of cancers are thought to be the result of chronic inflammatory conditions [59]–[64]. The tumor microenvironment is a highly complex milieu of pro-inflammatory cytokines, chemokines, and bioactive lipids that are produced by tumor cells themselves, as well as by infiltrating host cells, including macrophages [65]. Pro-inflammatory cytokines such as IL-6 and TNFα produced by many tumor cells are key drivers of inflammation in the tumor microenvironment [9], [63], [66]–[68]. In addition to their inherent inflammatory properties, TNFα and IL-6 stimulate production of pro-inflammatory chemokines such as CCL2, CXCL1, CXCL8, and CXCL12, as well as MMP [65]. These cytokines and chemokines establish a setting that not only maintains inflammation but also recruits macrophages, other immune cells, and stromal cells that produce inflammatory mediators. The result is a network of autocrine factors that sustain an inflammatory tumor microenvironment.

The transcription factor NF-κB is the major intracellular regulator of the inflammatory program and is activated in macrophages and in tumor cells through a Toll-like receptor-myeloid differentiation primary response gene (88) (TLR-MyD88)

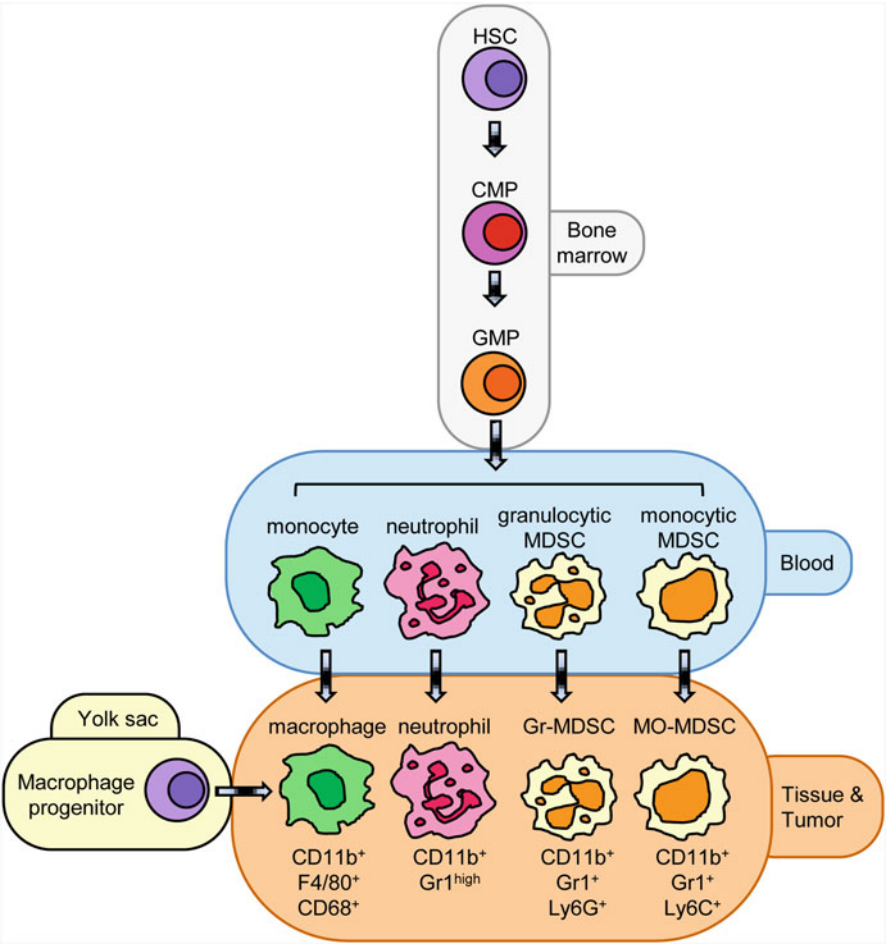

Fig. 6.3 Macrophages differentiate from hematopoietic stem cells in bone marrow or from yolk sac. Hematopoietic stem cells (*HSC*) in the bone marrow give rise to a common myeloid progenitor (*CMP*) which gives rise to a granulocyte/macrophage progenitor (*GMP*). The *GMP* differentiates into monocytes, neutrophils, and monocytic and granulocytic myeloid-derived suppressor cells (*MDSC*) which circulate in the blood. When monocytes move into tissue or tumor, they become macrophages. Tissue macrophages also differentiate from a progenitor cell that arises in the yolk sac during embryogenesis. Cell surface markers for neutrophils, granulocytic MDSC (*Gr-MDSC*), and monocytic MDSC (*MO-MDSC*) are for murine cells

pathway. Ligands include bacterial products, cellular debris, IL-1β, and TNFα [56]. In tumor cells, it induces a more aggressive phenotype and regulates the production of chemokines and cytokines that chemoattract macrophages that in turn promote tumor growth [69]. The role of NF-κB in macrophages is less well defined. In agreement with its pro-inflammatory role in malignant cells, inhibition of NF-κB in macrophages of mice that spontaneously develop tumors results in delayed tumor

onset [70], as well as in mice with chronic inflammation that progresses to malignancy [62], [64], [71]. However, in late stage tumors, TAMs have defective NF-κB and restoring NF-κB activity in these TAMs delays tumor growth [72]. Therefore, whether activated NF-κB is pro-tumor or antitumor may depend on the stage of disease and the type of cancer.

5 Macrophage Polarization and Phenotype: M1-like Macrophages have Antitumor Activity While M2-like Macrophages Facilitate Tumor Progression

Depending on environmental cues, macrophages can either facilitate the destruction of tumor cells or promote the progression of tumor growth. This dichotomy of function is the result of the extreme plasticity of macrophages and their phenotypic adaptation to their environment. Similar to the Th1 vs. Th2 paradigm for $CD4^+$ T lymphocytes, Mills proposed that macrophages also polarize into two categories based on their phenotype and function [73]. This nomenclature was further developed by Mantovani and colleagues and has now become the accepted jargon to describe macrophages [3], [74]–[76]. M1-like macrophages (also known as "classically activated" macrophages) are activated by bacterial products such as lipopolysaccharides (LPS) in combination with interferon gamma (IFNγ). M1 polarization has also been attributed to TNFα. These cells produce high levels of IL-12 and low levels of IL-10 and are able to lyse tumor cells. They also synthesize and contain inducible nitric oxide synthase (iNOS or NOS2) and typically express high levels of cell surface CD86 and MHC class II molecules. In contrast, M2-like macrophages are activated by IL-4, IL-13, IL-10, and glucocorticoid hormones. They produce low levels of IL-12, high levels of IL-10, and support tumor growth. They also synthesize and contain high levels of arginase 1 and express low levels of MHC class II and high levels of the cell surface molecules FIZZ1 and Ym1. Their high level of expression of IL-4Rα facilitates their polarization towards an M2 phenotype by the binding of IL-4 and IL-13 [6].

M1 macrophages eliminate intracellular pathogens and mediate tumor cell destruction by their production of a variety of reactive intermediate species that result from the production of nitric oxide (NO) and its reaction with molecular oxygen [77]. M1 macrophages also contribute to tumor destruction through their skewing of immunity towards a type 1 response that activates tumor-reactive T cells. Their production of the chemokines Mig (CXCL9) and IP-10 (CXCL10) chemoattract Th1 cells [65], [78]. Type 1 immunity is further facilitated by their production of pro-inflammatory cytokines including IL-1β, IL-6, IL-23, and TNFα combined with their ability to function as APC to activate Th1 and Tc1 $CD4^+$ and $CD8^+$ T cells, respectively [79].

In contrast, M2 macrophages are predominately anti-inflammatory, although they secrete some pro-inflammatory molecules. They promote type 2 immunity that facilitates the elimination of parasites, promotes wound healing, and drives tumor

progression. TAMs are polarized towards an M2 phenotype. The production of CCL22 by M2 macrophages chemoattracts Tregs which inhibit T-cell activation [80]. M2 macrophages also secrete prostaglandin E2 and transforming growth factor beta (TGFβ), both of which are immunosuppressive [81], and they express PD-L1 which binds to its receptor PD-1 on activated T cells and causes T-cell apoptosis [49]. Their production of IL-10 and their lack of IL-1β production due to their high expression of IL-4 receptor antagonist and decoy type II receptor promote their anti-inflammatory effects [82]. M2 macrophages also stimulate angiogenesis which facilitates tumor growth [74] and they produce high levels of arginase 1 (Arg1) which prevents T-cell proliferation by depriving T cells of arginine [83], [84].

The metabolism of the amino acid arginine by M1 vs. M2 macrophages is a major distinguishing characteristic of the two macrophage phenotypes. M1 macrophages predominantly metabolize arginine via the NO pathway, while M2 macrophages use the arginase pathway [85]. iNOS or NOS2 is upregulated in M1 macrophages by IFNγ and TNFα. The NO that is produced is cytotoxic and destroys target cells by inhibiting cell proliferation [86], blocking mitochondrial respiration [87], and inducing apoptosis [88], [89]. NO also reacts with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$) [88], [90]. In contrast, Arg1 is upregulated in M2 macrophages by IL-4, IL-13, TGFβ, and IL-10 [91], [92]. Arg1 converts arginine to ornithine which is subsequently converted to polyamines. Ornithine is a precursor for proline, a major constituent of collagen which is necessary for tumor infrastructure. Polyamines promote tumor cell proliferation because they are required for deoxyribonucleic acid (DNA) replication [93]. Figure 6.4 schematically illustrates the two pathways. MDSC metabolize arginase through the same two pathways with monocytic MDSC (MO-MDSC) using the iNOS pathway while granulocytic MDSC use the Arg1 pathway [47]. Interestingly, MDSC production of iNOS does not result in tumoricidal activity as it does for M1 macrophages.

The preceding description of macrophage polarization applies to murine macrophages. Human macrophages are more enigmatic in terms of polarization and distinct classes of macrophages have not been conclusively identified. Whether this is due to a lack of adequate markers or a different program of differentiation is unclear at present.

Macrophages are characterized by their expression of the specific markers discussed above. However, some of these markers are also expressed by other cell types and not all macrophages express all of these markers. For example, murine macrophages are often characterized by their expression of CD11b; however, CD11b is also expressed by other myeloid cells. Likewise, MHC II serves as a marker for macrophages; however, DC also express MHC II. Similarly, F4/80 is expressed by macrophages as well as by eosinophils and Langerhans cells. Expression of functional molecules such as iNOS and Arg1 is also not restricted to macrophages in that subpopulations of MDSC also express these molecules. There are many fewer cell surface markers for human macrophages. CD68 expression is typically used, although it is also expressed by some fibroblasts. Therefore, macrophage identification by marker expression is not always unambiguous and straightforward, although a combination of cell surface markers and functional markers can typically give a reliable identification.

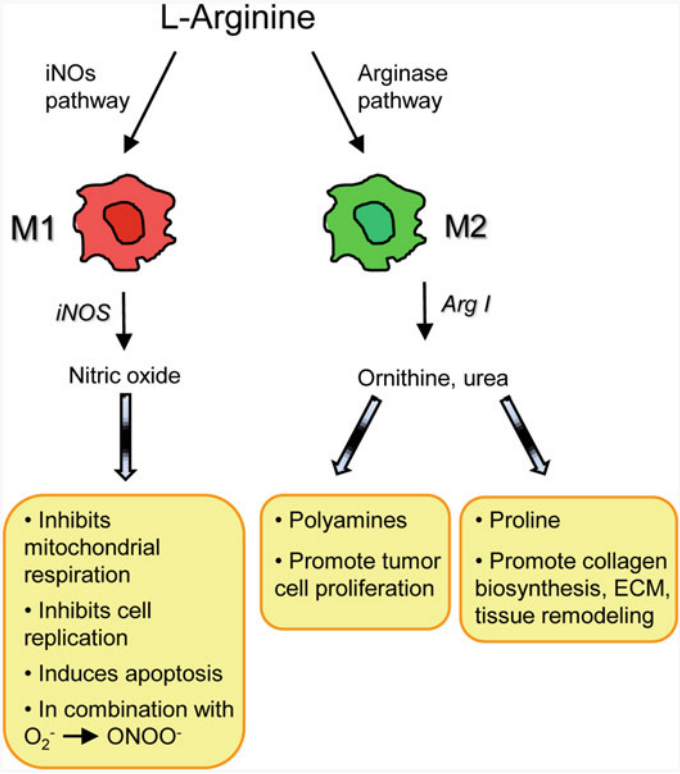

Fig. 6.4 *M1* and *M2* macrophages metabolize arginine through different pathways. *M1* macrophages metabolize arginine through the *iNOS* pathway which converts arginine to nitric oxide. *M2* macrophages metabolize arginine through the arginase pathway and produce polyamines and proline

Figure 6.5 summarizes the phenotypes and functions of M1- and M2-polarized macrophages.

6 Macrophages are a Heterogeneous Mixture of Myeloid Cells

The M1–M2 paradigm is a convenient nomenclature for categorizing macrophages. However, most macrophages do not conveniently fit into one or the other of these categories, and the M1 and M2 states represent the extremes of a continuum of macrophage phenotypes that are governed by the local environment. Since tumors include a diversity of microenvironments, it is not unusual that different regions of solid tumors will have macrophages with differing and intermediate phenotypes. Since the microenvironments within solid tumors are generated by factors secreted by tumor cells, and different types of cancers produce different factors, macrophage populations between tumors can vary significantly. Likewise, as solid tumors evolve

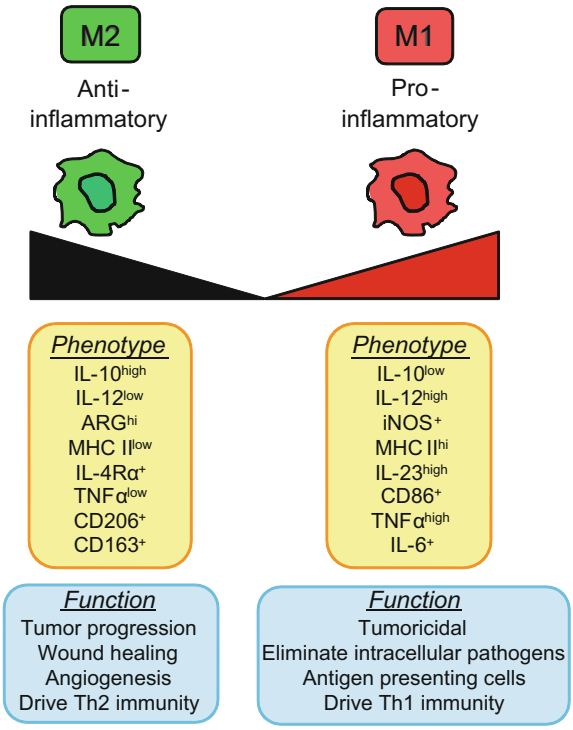

Fig. 6.5 Macrophages display a continuum of phenotypes and functions, with *M1*-like and *M2*-like cells representing the extremes. *M2*-like and *M1*-like macrophages have distinct phenotypes (shown in *yellow boxes*) and functions (shown in *blue boxes*). *M2*-like macrophages are anti-inflammatory and facilitate tumor progression, while *M1*-like macrophages are pro-inflammatory and facilitate tumor rejection

in individuals through the process of immunoediting and selection, tumor-secreted factors are likely to change such that macrophage populations will also evolve with tumor progression.

Macrophages also differ significantly within a single tumor. Movahedi and colleagues have identified seven distinct subsets of macrophages in mammary carcinoma and lung adenocarcinoma [94]. These subsets are distinguished by their level of expression of MHC II, the monocyte marker Ly6C, the homing receptor L-selectin (CD62L), and the chemokine receptors CX_3CR1 and CCR2. The different subsets had varying half-lives as well as different differentiation kinetics. Monocytes with a $Ly6C^{hi}$ phenotype were observed to be precursors of all TAMs. Gene expression profiles have been performed on macrophages from multiple different types of tumors [95]–[97]. These studies revealed that overall TAMs are predominantly of an M2 phenotype. Additional profiling studies on macrophage subsets from three different mouse tumors revealed that macrophages with an MHC II^{low} phenotype were the dominant TAM population and that these cells express high levels of M2 genes including *Arg1*, *IL4Rα*, and *Il10*. MHC II^{low} TAMs were also present, but at a lower level and expressed high levels of M1 genes including *Cox2* and *IL-1β*. RNA levels roughly correlated with protein levels for most genes; however, *NOS2* mRNA was highest in MHC II^{low} macrophages, while NOS2 protein was highest in MHC II^{high} macrophages [94]. These studies also noted that MHC II^{low} macrophages were most

frequently present in hypoxic regions of the tumor, while MHC II^{high} cells localized to normoxic areas. Hypoxia has also been shown to drive expression of the angiopoietin receptor Tie2 on a subset of macrophages (TEMs) that are pro-angiogenic [98]. Another subset of TAMs identified by their motility, invasiveness, and wound-healing properties was characterized by their high content of molecules associated with the Wnt signaling pathway [99].

7 The Tumor Microenvironment Regulates Macrophage Polarization

In addition to TAMs and tumor cells, the tumor microenvironment includes a complex milieu of host cells such as CD4$^+$ and CD8$^+$ T lymphocytes, Tregs, B lymphocytes, DC, mast cells, MDSC, NK cells, natural killer T (NKT) cells, neutrophils, and cancer-associated fibroblasts (CAFs). Many of these host cells are induced by tumor-secreted products to secrete factors that drive the polarization of macrophages towards an M2 phenotype. For example, both solid and ascites human ovarian cancer cells produce soluble mediators that upregulate macrophage production of IL-10 and CSF-1. These cells also produce many chemokines characteristic of the M2 phenotype. TNFα, a cytokine produced in abundance by ovarian cancer cells, upregulates hemoglobin scavenger receptor A (CD163), a marker characteristic of TAMs [100].

CD4$^+$ T cells also impact macrophage polarization. In vitro coculture studies demonstrated that CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs divert macrophages towards an M2 phenotype by increasing macrophage expression of CD163 and CCL18, and by increasing macrophage phagocytic activity through their production of IL-10. IL-4, IL-13, and IL-10 produced by Tregs downregulate macrophage production of pro-inflammatory mediators produced in response to LPS. Tregs also reduced macrophage expression of MHC class II molecules [101]. Studies in transgenic mice that spontaneously develop mammary carcinoma (*PyMT* mice) confirmed an in vivo role for CD4$^+$ T cells in polarizing macrophages and further demonstrated that the altered macrophages promote metastatic disease by activating EGF receptor signaling in the mammary epithelial cells. These latter effects were due to CD4$^+$ T effector cells and not Tregs, indicating that CD4$^+$ T cells alter macrophage phenotype through diverse mechanisms [102].

Macrophages are also impacted by B lymphocytes and by CAFs. Coculture and in vivo experiments using B1 lymphocytes (B220^{low}IgM^{high}CD11b$^+$) increased macrophage production of IL-10 and decreased macrophage production of M1-type molecules including TNFα, IL-1β, and CCL3. M2 markers FIZZ1 and Ym1 were also increased [103]. In a chemically induced two-stage skin carcinogenesis system, large quantities of fibroblasts accumulate at the site of carcinogen application. The fibroblasts produce high levels of monocyte chemotactic protein 1 (MCP-1) which chemoattracts macrophages to the carcinogenic locale and promotes papilloma progression [104].

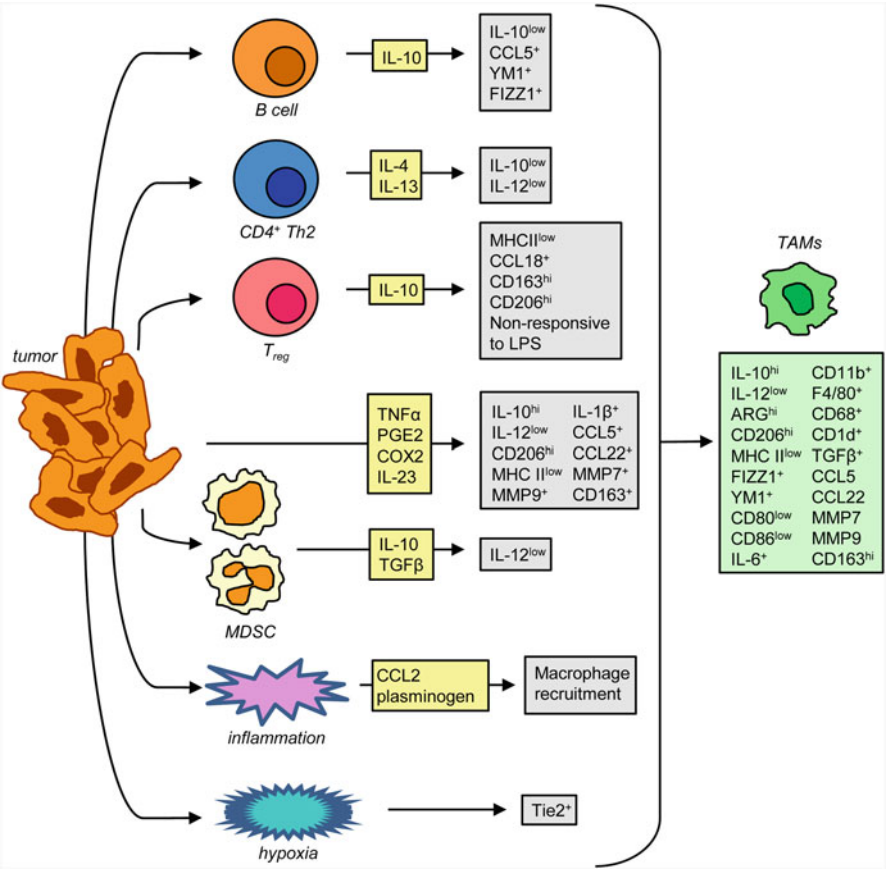

Fig. 6.6 Tumors produce multiple factors that condition their environment and surrounding cells and mold macrophage phenotype. Tumor-produced factors polarize immunity towards a type 2 response and induce *B cells*, *CD4*$^+$ *Th2* cells, T regulatory cells (*Tregs*), and myeloid-derived suppressor cells (MDSC) to produce cytokines and chemokines that polarize macrophages towards an M2 tumor-promoting phenotype. Tumor-secreted factors also induce an inflammatory and hypoxic environment that favors the development of *TAMs*. Cytokines and inflammatory mediators (in *yellow boxes*) are produced by tumor cells, the indicated *B cells*, *CD4*$^+$ *Th2* cells, *Tregs*, *MDSC*, inflammation, and/or hypoxia and induce specific markers and molecules in *TAMs* (in *gray boxes*). TAMs may express all or a subset of the markers/molecules shown in the *green box*

Crosstalk with MDSC also drives macrophage phenotype. In the presence of MDSC, macrophage production of IL-12 and IL-6 and expression of MHC II are reduced. Macrophages, in turn, increase MDSC production of IL-10 via an IL-6-dependent mechanism [42], [105]. This crosstalk is regulated by signaling through the TLR4 pathway in MDSC, involves upregulation of CD14 on MDSC, and is exacerbated by inflammation [106].

Figure 6.6 summarizes how tumor-secreted factors and host cells impacted by tumor-secreted factors drive macrophages towards a tumor-promoting phenotype.

8 Multiple Regulatory Elements Control Macrophage Polarization

Macrophage polarization has been attributed to multiple genes and regulatory elements. Deletion of the suppressor of cytokine signaling 3 (*SOCS3*) gene in myeloid cells generated M2-like macrophages, while deletion of the SOCS2 gene yielded phenotypically M1 macrophages. Targeted deletion of SOCS in macrophages also altered macrophage function. SOCS3-deficient macrophages were more potent recruiters of Tregs, while SOCS2-deficient macrophages were not. Polarization towards an M2 phenotype yielded increased activation via signal transducer and activator of transcription 6 (STAT6), while polarization towards an M1 phenotype increased activation through STAT1 [107]. These changes in signal transduction preference correlate with the known role of STAT1 in transmitting signals from IFNγ, a known inducer of M1 phenotype cells, and STAT6 in transmitting signals from IL-4 and IL-13, known inducers of M2 macrophages.

MicroRNAs (miRNA) have also been shown to regulate macrophage polarization. The mannose receptor (CD206), encoded by the *MRC1* gene, is a marker of TAMs and facilitates macrophage phagocytosis of microbial and host glycoproteins [108]. The *MRC1* gene encodes miR-511-3p. This miRNA is constitutively expressed at high levels in TAMs; however, increasing expression inhibits tumor growth and reduces the pro-tumor phenotype of TAMs. Therefore, miR-511-3p levels appear to be important for maintaining TAM phenotype, but overexpression skews macrophages away from an M2 state [109].

Tumor antagonizing/malignancy suppressor genes (TAG/MSG) are a family of genes that suppress tumorigenicity in vivo but have no apparent in vitro effects. *RNASET2*, an extracellular RNAse, is a member of this family. Ovarian cancer cells expressing wild type or catalytically inactive *RNASET2* grow much more slowly than cells containing mutated *RNASET2*. Reduced tumor progression is due to the infiltration of $iNOS^+$ M1 macrophages since supernatants of cancer cells containing wild-type *RNASET2* polarized macrophages towards an M1 phenotype [110]. Thus, *RNASET2* is another gene that regulates macrophage polarization and its ability to polarize is independent of its catalytic RNAse activity.

Histone-rich glycoprotein (HRG) is an anti-angiogenic and immunomodulatory factor present in serum and produced by platelets that regulates blood vessel formation. The tumor microenvironment typically contains less HRG than tumor-free corresponding normal tissue. Overexpression of HRG in several mouse tumor cell lines prevented the development of disorganized blood vessel formation associated with wild-type tumor, and delayed tumor progression. HRG overexpression reduced the number of M2-like TAMs within tumors by half and increased the number of M1-like cells. These were direct effects of HRG on macrophages since similar skewing was observed when macrophages were cultured in the presence of HRG. HRG mediates these effects by downregulating PIGF, a known driver of angiogenesis and homolog of VEGF-A. Depletion of TAMs from HRG overexpressing tumors restored blood vessel abnormalities and increased tumor growth, indicating that HRG regulates tumor progression via macrophage polarization [111].

Macrophage polarization is also regulated epigenetically by chromosome remodeling. Proteins containing a Jumonji-C (JmjC) domain, including Jmjd3, are histone demethylases. Many genes that are activated by LPS are targets for Jmjd3. TLR stimulation activates Jmjd3 in macrophages via an NF-κB-dependent mechanism that controls the expression of the *Bmp2* and *Hox* genes. In a parasite model, macrophages attain an M1 phenotype in the absence of Jmjd3 activation, whereas Jmjd3 activation is essential for the generation of M2 macrophages [112], [113]. Jmjd3 is also activated in macrophages by IL-4 through a STAT6-dependent mechanism. Activated STAT6 increases transcription of Jmjd3 which subsequently demethylates M2 marker genes, polarizing macrophages towards an M2 phenotype [114].

9 Macrophages as Prognostic Indicators of Tumor Progression

Macrophage infiltrates in solid tumors have long been recognized as indicators of poor prognosis. A recent clinical study in Hodgkin's lymphoma patients cemented this correlation. Gene profiling and immunohistochemistry revealed that high levels of $CD68^+$ macrophages predicted poor outcome after primary and secondary therapy. In contrast, low levels of tumor-associated $CD68^+$ macrophages were associated with a subgroup of patients that had 100 % long-term disease-free survival [115]. In contrast to most cancers, levels of peritumoral $CD68^+$ macrophages correlate with a good prognosis in colorectal cancer patients [116], [117]. This apparent inconsistency is because most macrophages in colorectal cancer are M1-like macrophages. As for other cancers, high levels of M2-like macrophages correlate with poor prognosis [118]. Expression of CD40 by HLA-DR^+ $CD80^+$ $CD86^+$ M1-like macrophages has also been identified as an indicator of favorable prognosis in colorectal cancer patients [119]. Experimental studies in mice indicate that activation of macrophages by antibodies to CD40 in combination with IL-2 therapy induces macrophage production of NO and reduces metastatic disease [120]. High levels of peritumoral macrophages also correlate with good prognosis for patients with high-grade osteosarcoma. These tumors contain both M1-like and M2-like macrophages; however, there was no correlation between macrophage phenotype and patient outcome [121].

These studies suggest that although most tumors induce tumor-promoting M2-like macrophages, some tumors induce M1-like macrophages which may contribute to tumor regression. A better understanding of the conditions that drive M1 vs. M2 polarization is essential to eliminate pro-tumor M2-like macrophages and induce antitumor M1-like macrophages.

10 TAMs Can be Reprogrammed Toward an M1-like Phenotype

Given the potent antitumor activity of M1 macrophages, considerable attention has been devoted to strategies to repolarize TAMs. Several studies have identified IL-12 as a key molecule for converting M2 macrophages to an M1 phenotype. Treatment

with IL-12 decreases TAM expression of the tumor-promoting factors IL-10, MCP-1, and TGF-β and induces expression of TNFα, IL-15, and IL-18, factors that facilitate tumor rejection [122]. TAMs are also repolarized by IL-12 through treatment with a chimeric antigen receptor (CAR) that redirects cytotoxic T lymphocytes (CTL) to release IL-12 at the tumor site [123]. Delivery of IL-12 by engineered tumor-specific $CD8^+$ T cells reprograms much of the myelomonocytic compartment of tumor stoma by converting immune suppressive macrophages, DC and MDSC into cells that sustain T-cell activation and promote debulking of large tumors [124]. This latter effect involves IFNγ and is consistent with previous findings that IFNγ repolarizes TAMs from an immune suppressive M2 phenotype to an antitumor M1 phenotype [125].

Other conditions also reeducate TAMs. Administration of IL-2 in combination with antibodies to CD40 converts TAMs to an M1 phenotype of high NO production [120]. In the inflammatory tumor microenvironment, macrophages acquire an M2 phenotype by signaling through the IL-1R and activating NF-κB. Inhibition of NF-κB signaling "reeducates" TAMs to an MHC II^{high}, $IL\text{-}12^{high}$, ARG^{low} phenotype. Inhibition of NF-κB also renders them tumoricidal and generates antitumor activity through the recruitment of IL-12-dependent NK cells [126]. Whether inhibition of NF-κB will be generally applicable, however, is unclear because microarray studies demonstrated that TAMs induced by some tumors are constitutively defective in NF-κB signaling [95]. The Notch pathway has also been invoked in macrophage polarization. In mouse tumor models, TAMs have lower levels of Notch pathway activation compared to M1 macrophages. Macrophages with defective Notch signaling or TAMs treated with Notch signaling inhibitors acquire an M2 phenotype [127].

Additional cytokines and growth factors have also been identified as potential reagents for reprogramming TAMs. Restoration of host-produced histidine-rich protein (HRP) converts TAMs to M1 macrophages by downregulating PIGF [111]. TAMs, as well as DC, are repolarized by treatment with anti-IL-10R antibodies plus the TLR9 ligand, CpG [128]. In another study, IL-4 and IL-10 were identified as critical factors for driving macrophage polarization towards an M2 phentoype, and inhibition of the IL-4Rα in mice with VEGF-induced skin carcinogenesis prevented M2 polarization of macrophages [129], [130].

Macrophage polarization is also regulated by the transcription factor STAT6 since IL-4 and IL-13 drive M2 polarization by binding to the IL-4Rα and signaling through the JAK2/STAT6 pathway. As a result, macrophages in STAT6-deficient mice default develop into M1 cells and these cells contribute to the rejection of established metastatic mammary carcinoma [28]. The nonclassical MHC class I CD1d gene also regulates macrophage polarization. $CD1^{-/-}$ mice are IL-13-deficient because they lack NKT cells which produce IL-13. The absence of IL-13 causes macrophages to default to an M1 phenotype. These mice also reject established metastatic mammary carcinoma [29] and are resistant to recurring fibrosarcomas [131].

These reprogramming studies have been done in mouse tumor models and have resulted in innate antitumor immunity and delayed tumor progression and/or reduction of metastasis. Given the heterogeneity of TAMs, and the lack of well-defined markers for M1 and M2 phenotypes in human cancer, it remains to be demonstrated whether the findings in mice will be applicable to human systems. Table 6.1 summarizes

Table 6.1 Strategies for reprogramming TAMs

Effect on TAMs	Effect on tumor	Strategy	Tumor	Reference
Decrease TAM production of IL-10, MCP-1, TGFβ; increase TNFα, IL-15, IL-18; increase NK and CTL		IL-12 in microspheres	Murine 3LL lung	[122]
Increase quantity of tumor-infiltrating macrophages that expressed CD80 and CD86 and produced TNFα (M1 phenotype)	Reduce primary tumor growth	CAR-redirected CTL releases IL-12 at tumor site	Murine MC38 colon carcinoma	[123]
Reprogram TAMs, DC to type 1		IL-12-engineered CTL	Murine B16 melanoma	[124]
Convert macrophages to M1 phenotype	Reduce primary tumor growth	IL-2 plus mAbs to CD40	Murine Renca renal cell carcinoma under kidney capsule	[134]
Convert macrophages to M1 phenotype that secrete high levels of NO; downregulated MMP2, MMP9	Reduce metastasis	IL-2 plus mAbs to CD40	Murine Renca renal cell carcinoma i.v. and intrasplenic	[120]
Convert macrophages to MHC $II^{high}IL\text{-}12^{high}ARG^{low}$; improve APC function; recruit NK cells	Reduce primary tumor growth	Inhibit NF-κB by targeting IκB kinase	Murine ID8 ovarian carcinoma	[126]
Convert macrophages to M1 by upregulating CXCL9, IFNβ, IL-12; downregulating CCL17, IL-10, MRC1	Reduce primary and metastatic tumor growth	Restore histidine-rich protein which downregulates PGF	Murine T241 fibrosarcoma, Panc02 pancreatic tumor, 4T1 mammary carcinoma; demonstrated HRP decreased in multiple human tumors	[111]
Convert macrophages (and DC) to type 1 that secrete IL-12, TNFα	Reject primary tumor and metastasis	Adenovirus delivery of CCL16 plus CpG plus Ab to IL-10R	Murine TSA and 4T1 mammary carcinomas, MCa38 colon carcinoma	[128]
Convert macrophages to M1 that secrete IL-12	Reduce primary tumor growth	Activate notch pathway	Murine B16 melanoma, 3LL lung carcinoma	[127]
Convert macrophages to M1 phenotype	Reduce primary tumor growth	Inhibit NF-κB by overexpressing p50 NF-κB inhibitory homodimer	Murine MN/MCA1 fibrosarcoma, B16 melanoma	[72]

Table 6.1 (continued)

Effect on TAMs	Effect on tumor	Strategy	Tumor	Reference
Convert macrophages to tumoricidal M1 phenotype by inducing NO secretion, reducing ARG secretion and are tumoricidal	Reject metastatic disease; confer survival	Delete STAT6 gene	Murine 4T1 mammary carcinoma	[28]
Convert macrophages to tumoricidal M1 phenotype by inducing NO secretion, reducing ARG secretion and are tumoricidal	Reject metastatic disease; confer survival	Delete CD1 or IL-4Rα gene which eliminates IL-13 or IL-13 responsiveness, respectively	Murine 4T1 mammary carcinoma	[29]
Drive macrophages to an ARG^{low}, NO^{high} M1 phenotype		Increase SHIP expression		[135]
Convert TAMs from ovarian ascites to IL-10^{low}IL-$12^{high}$$CD86^{+}$ M1 phenotype		IFNγ	Human ovarian cancer	[125]
Convert TAMs to IL-$10^{low}$$VEGF^{low}$NF-κB-activated M1 phenotype	Reduce primary tumor growth; extend survival time	Zoledronic acid	Murine TUBO mammary carcinoma	[136]

the therapeutic approaches that have been developed to reprogram M2 to M1 macrophages. Several recent reviews include additional information [9], [79], [132], [133].

11 Conclusions

Macrophages can either enhance tumor progression or facilitate tumor rejection. Their ultimate phenotype and function are determined by their tissue microenvironment. Virtually all solid tumors chemoattract monocytes and polarize them to M2-like tumor-promoting macrophages. These TAMs are a major contributor to disease progression through their direct promotion of tumor cell growth, and their indirect effects of suppressing antitumor immunity, promoting angiogenesis, and facilitating tumor cell invasion and metastasis. Strategies for eliminating TAMs are being actively pursued in animal models. However, given the extreme plasticity of macrophages and the tumoricidal properties of M1-like macrophages, therapies that repolarize TAMs towards a tumor-rejecting M1 phenotype may be more beneficial.

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Chapter 7
Angiogenesis and Immune Suppression in Cancer

Greg T. Motz and George Coukos

Abstract Angiogenesis and immune suppression share common cellular and molecular mediators, and these processes are linked in diverse biological processes such as wound healing, pregnancy, and cancer. This shared program exists under physiological conditions and is co-opted under pathological conditions. Here, we focus on these connections in the context of tumor biology, emphasizing the role of vascular endothelial growth factor (VEGF) and that of the angiogenic tumor endothelium as an immune regulator.

Keywords Angiogenesis · Immune suppression · Tumor endothelium · Vascular endothelial growth factor · Tumor infiltrating lymphocytes · T regulatory cells

1 Introduction

There is an interconnected and reciprocal biological program of angiogenesis and immune suppression that is activated in response to diverse biochemical and biophysical stimuli. Stimuli such as injury, hypoxia, and oxidative stress are drivers underpinning the processes of wound healing, infection, and even pregnancy. This program likely evolved to ensure stabilized physiology and tissue homeostasis following a biologic crisis. This coordinated response is the likely result of evolutionary pressure to temper an excessive inflammatory response and host autoreactivity, while promoting the regeneration of damaged, stressed, or infected tissues through increased blood supply and tissue rebuilding with as few resources as possible. The list of cell types and molecular mediators that can promote both immune suppression and angiogenesis is extensive and supports our hypothesis of such a program.

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Sustained angiogenesis and immune suppression are hallmarks of cancer, and tumors capitalize on the existence of this program, promoting their dissemination. Here, we focus on the roles of this program in tumor development, highlighting particularly relevant cellular and molecular mediators involved in tumors. We place particular emphasis on the roles of vascular endothelial growth factor (VEGF) and the tumor endothelium as primary examples of the activation of this program in tumor biology.

2 Tumor Angiogenesis, Immune Suppression, and Tolerance

The vascular system develops through the coordinated actions of both vasculogenesis and angiogenesis. While vasculogenesis gives rise to *de novo* blood vessels, angiogenesis is the sprouting of new vessels from preexisting ones. Physiological angiogenesis, occurring during development and wound healing, proceeds through vessel destabilization, sprouting, endothelial migration, and proliferation and is followed by resolution and stabilization of the new vessel. Pathological angiogenesis shares many of the same processes, but is characterized by a failure of the resolution phase and the generation of a highly disorganized vascular network. Pathological angiogenesis is a key feature of tumor biology, but is also involved in a broad spectrum of inflammatory diseases [1]. It is our view that this program is intimately integrated with and co-regulated by fundamental immune processes.

A key component, and newly recognized hallmark of cancer [2], is immune subversion by tumors. Local immune suppression, leading to antigenic tolerance or ignorance, is a key feature of tumor biology orchestrated by an overwhelmingly complex set of factors leading to overall tumor progression [3]. Whether tumors truly escape constant immune surveillance or simply adapt to avoid antitumor immune responses is still somewhat controversial [4]. However, for the purposes of this discussion we refer to immune suppression as any process that limits the functions of antitumor immune responses leading to the promotion of tumor growth, regardless of the mechanism.

3 Physiological Stimuli Link Immune Suppression and Angiogenesis

Within the tumor microenvironment, conditions of low oxygen saturation are characterized by hypoxia, nutrient and metabolite deficiencies, and anaerobic metabolism. The biological crosstalk activated by these changes in physiology is well known to promote angiogenesis, but it is also becoming apparent that these changes promote a tolerizing immune outcome [5]. Among the best studied of these stimuli, particularly in the context of tumors, is hypoxia. It is established that hypoxia plays a key role in the regulation of angiogenesis that is required for invasive tumor growth

and metastasis [6]. As tumors grow, the distances to existing vasculature become too great and the lack of oxygen diffusion induces a hypoxic stress response in tumor cells [7], [8]. Hypoxia leads to the stabilization and activation of hypoxia-inducible factors (HIFs) that activate a wide range of target genes involved in a number of pathways important for tumorigenesis including those with established roles in angiogenesis (*VEGF*; platelet-derived growth factor, *PDGF*; phosphatidylinositol glycan anchor biosynthesis class F, *PIGF*), degradation of the extracellular matrix (matrix metalloproteinases, *MMP*s), metastasis (lysyl oxidase, *LOX*; C-X-C chemokine receptor type 4, *CXCR4*; stromal-derived-factor-1, *SDF1*), epithelial-to-mesenchymal transition (*SNAIL*, *SIP*, *ZEB*), and immortalization (human telomerase reverse transcriptase, *hTERT*) [9].

Consistent with our hypothesis, hypoxia also has significant roles in immune suppression. Hypoxia upregulates a number of growth factors like VEGF and CC-chemokine ligand 28 (CCL28), described in more detail below, that have chemotactic functions and recruit immunosuppressive cells to tumors. It has been shown that hypoxia directly increases the potency of T regulatory cells (Tregs) and that hypoxia-exposed Tregs are more effective at suppressing the proliferation of effector T cells, thus limiting antitumor immunity [10]. It has also been shown that exposure of tumor cells to hypoxia inhibits autologous cytotoxic T-lymphocyte (CTL)-mediated lysis through a hypoxia-inducible factor-1α–signal transducer and activator of transcription 3 (HIF-1α–STAT3)-dependent mechanism [11]. Therefore, hypoxia can directly promote both angiogenesis and immune suppression through overlapping and complementary pathways.

4 Immunosuppressive Leukocytes Promote Angiogenesis

A number of leukocytes possess both the capacity for immune suppression and the ability to promote angiogenesis either directly or indirectly. Many of these cells are actively recruited to the tumor microenvironment, while others differentiate locally into suppressor cells. However, many leukocytes, particularly of the myeloid lineage, are likely induced locally into a reversible functional state rather than terminal differentiation. The immunosuppressive functions of these cells are well reviewed; therefore, here we choose to focus on the roles of these cells in angiogenesis.

4.1 Myeloid Cells are Key Regulators of Immune Suppression and Angiogenesis

Myeloid cells are well studied in terms of their ability to promote immune suppression and angiogenesis in tumors. Chief among them are myeloid-derived suppressor cells (MDSCs) (reviewed extensively elsewhere [12]). Their functional immunosuppressive roles are ultimately based on their ability to suppress T-cell- and natural

killer (NK)-cell activation [13], likely through several mechanisms involving nitric oxide (NO), reactive oxidative species (ROS), arginase, interleukin-10 (IL-10), and transforming growth factor-β (TGF-β); but, there are also reports that MDSCs may specifically induce the expansion of Tregs [14].

MDSC numbers are significantly increased in both tumor-bearing mice and cancer patients, with large percentages of total leukocytes reported [12]. MDSCs can directly promote angiogenesis [15], and in tumor-bearing mice treated with a neutralizing anti-BV8 (also referred to as prokinectin 2) antibody, which reduces the number of MDSCs, angiogenesis is significantly reduced, strongly implicating them in this process [16]. MDSCs can secrete the pro-angiogenic factors VEGF and MMP9, which are required for their pro-angiogenic function [15]. Importantly, the pro-angiogenic function of MDSCs can render tumors refractory to angiogenic blockade by VEGF-specific antibodies, through the secretion of alternative pro-angiogenic factors, such as protease BV8, which is upregulated by granulocyte colony-stimulating factor [16].

Additional myeloid cell subsets contribute to angiogenesis, including myeloid and plasmacytoid dendritic cells (DCs), tumor-associated macrophages (TAMs), TIE2-expressing monocytes (TEMs), mast cells, and neutrophils (extensively reviewed elsewhere [17]). Often, such cells are polarized by the tumor microenvironment and following their recruitment to tumors, chemokines or antimicrobial peptides can alter their phenotype [18]. For example, myeloid cells such as immature DCs and TAMs can acquire a pro-angiogenic profile characterized by the secretion or expression of VEGF, basic fibroblast growth factor (bFGF; also known as FGF2), CXC-chemokine ligand 8 (CXCL8), and cyclooxygenase 2 (COX2), while their immunostimulatory functions are downregulated (e.g., by downregulating IL-12 expression due to autocrine IL-10 production [17], [19]–[21]). Monocyte populations have also been described to structurally integrate into tumor blood vessels either at luminal or pericyte locations [18], where they could play a supporting role in the angiogenic process but also establish an important immunosuppressive dialogue with extravasating leukocytes.

4.2 Lymphocyte Populations

Early reports indicated that T cells may play substantial roles in angiogenesis. T cells exposed to hypoxia express VEGF, and it was also found that T cells within tumors express VEGF [22]. Later, support for a role of T cells came from the observation that CD4-deficient mice have an impaired angiogenesis response following ischemia [23]. However, neither of these studies addressed the roles of specific T-cell subsets or their specific mechanisms in angiogenesis.

$CD4^+CD25^+Foxp3^+$ Tregs are perhaps the most well-known cells contributing to both immune suppression and angiogenesis. Tregs are crucial mediators of immune suppression and tolerance in tumors [24], and increased numbers of Tregs in tumors are associated with poor survival in many solid tumors including in breast cancer [25], gastric cancer [26], and ovarian cancer [27], [28]. In ovarian cancer, a low

abundance of tumor-infiltrating Tregs can translate into years of added survival, adding emphasis to the importance of these cells in tumor progression [27]. It has been observed that Tregs at tumor sites correlate with evidence of accelerated angiogenesis such as VEGF overexpression and increased microvessel density in endometrial [29] and breast cancers [30]. A large body of evidence exists demonstrating that Tregs contribute to tumor angiogenesis through indirect mechanisms. Tregs promote tumor angiogenesis by specifically inhibiting tumor-reactive T cells [31], and it is likely that the suppression of the T helper 1 (Th1) effector T cells and their cytokines is the predominant mechanism. Th1 cytokines like tumor necrosis factor α (TNFα) and interferon-γ (IFN-γ) as well as IFN-induced chemokines such as CXCL9, 10, and 11 have well-established roles in angiostasis and directly inhibit the activities of endothelial cells [32], [33].

Recently, our group has demonstrated that Tregs can also make significant direct contributions to the promotion of tumor angiogenesis [34]. We showed that hypoxic ovarian tumor cells specifically upregulate expression of CCL28, leading to the preferential recruitment of Tregs through ligation of the cognate receptor C-C chemokine receptor type 10 (CCR10) expressed on Tregs. CCL28 expression was linked to shorter survival of patients with ovarian cancer, and artificial overexpression resulted in rapid ovarian tumor growth in mice. In mice, enhanced tumor growth was due to increased recruitment of Tregs, which established a local immunosuppressive tumor microenvironment rich in VEGF and associated increased angiogenesis. We demonstrated that $CD4^+CD25^+$ Tregs secreted higher amounts of VEGF at the steady state as well as under hypoxic conditions when compared with $CD4^+CD25^-$ T cells. Further, media conditioned by Tregs in hypoxia promoted capillary tube formation in vitro, an effect dependent on VEGF signaling. Using an entirely cell-free Matrigel implant, we showed that supernatants of hypoxic Tregs were able to significantly promote angiogenesis in vivo [34]. Importantly, depletion of $CD25^+$ cells or $CCR10^+$ cells eliminated Tregs from the tumor microenvironment and significantly suppressed tumor VEGF expression and angiogenesis [34]. Thus, we established a new mechanism whereby tumor hypoxia recruits Tregs to tumor sites that leads to significant direct contributions to the pro-angiogenic tumor microenvironment.

A role for Th2 T cells in tumor angiogenesis is yet unclear. Indirect data from humans suggest that protective antitumor immunity is associated with the development of Th1 immunity, while tumor promotion occurred in patients who developed Th2-skewed immune responses [35], [36]. These findings are in agreement with various experimental data from mice indicating that Th1 cells are capable of a strong antitumor immune response, while Th2 responses fail to protect [32], [33], [37] or even promote tumor progression [38]. However, these studies failed to address any connections between Th2 immunity and tumor angiogenesis. Although little work has been done on the role of Th2 in tumor angiogenesis, there is much known on the role of Th2 cells in angiogenesis in asthma. The lungs of asthma patients are characterized by excessive, pathological angiogenesis [39] and Th2 cells can mediate the angiogenic switch [40]. The Th2 cytokine IL-25 can directly promote angiogenesis through effects on $IL\text{-}25R^+$ endothelial cells in asthmatics [41], and Th2 cytokines may synergize with hypoxia to induce angiogenic growth factors like VEGF [42].

In addition to Th2 cells, it is also possible that Th17 cells may contribute to tumor angiogenesis. The expression of IL-17 has been associated with microvessel density in cancer [43], and as a result, it may promote tumor progression in some cancers [44]. Experimentally, T-cell-derived IL-17 can promote tumor progression and angiogenesis in some mouse tumor models [45], [46]. IL-17 can directly induce angiogenesis through actions on endothelial cells [45], but it can also indirectly promote angiogenesis by inducing VEGF from myeloid cells [47], [48]. However, the role of Th17 in the overall promotion or inhibition of angiogenesis is controversial as it has been shown that an increased number of Th17 cells in tumors is associated with a better clinical outcome [49], and experimentally, adoptive transfer of tumor-reactive Th17 cells is highly effective at eliminating tumor by promoting Th1 immunity [50]. It is likely that the net overall outcome on angiogenesis and tumor growth depends on the balance of Th17 with respect to additional immunosuppressive cells like Tregs.

Although the relevance is unknown, it has been demonstrated that activated $CD4^+$ T cells can acquire neuropilin-1 (NRP-1), a co-receptor that binds VEGF, from DCs upon interaction through a process known as trogocytosis [51]. NRP-1 expressed on the membrane of DCs is transferred to and becomes incorporated into the membrane of recipient T cells. The acquisition of NRP-1 enables T cells to bind VEGF, potentially converting $CD4^+$ T cells into VEGF shuttling cells [51]. Although activated $CD4^+$ T cells are able to capture NRP-1 from DCs, Tregs constitutively express NRP-1, allowing for the possibility that they could transport additional VEGF to tumor sites following specific recruitment through tumor-derived CCL22 or CCL28 [27], [34], [52].

Additional lymphocyte subsets that have immunosuppressive potential include B cells [53], γδ T cells [54], NK cells [55], [56], and invariant (type I) natural killer T cells (NKT cells) [57] that can express VEGF. Whether these cell types contribute in any significant way to tumor angiogenesis is unknown, but some of these lymphocyte subsets can be quite abundant in various tumors. The exact role of these lymphocyte cells in tumors requires further investigation.

4.3 Stromal Cells

Typically associated with wound healing through the deposition of extracellular matrix, fibroblasts play important roles in both immune modulation and angiogenesis [58]. In the tumor microenvironment, cancer-associated fibroblasts (CAFs) can be activated by TGF-β, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) [58]; and in turn, CAFs may secrete angiogenic growth factors such as FGF2 and VEGF [58], [59], while promoting the recruitment and function of immunosuppressive cells, particularly of the myeloid lineage, like TAMs and MDSCs through the secretion of CCL2 and CXCL12 [17], [60]. In addition, CAFs may suppress T effector cells through secretion of TGF-β [61].

Another adherent stromal cell population, the mesenchymal stem cell (MSC), is derived from the bone marrow. Myeloid-derived MSCs secrete VEGF and promote tumor angiogenesis by differentiating into CAFs or perivascular mural cells

expressing alpha-smooth muscle actin, TIE2, and other pericyte markers [62]. Importantly, MSCs exert important immunosuppressive functions by blocking proliferation and function of T effector cells [63], [64]. MSCs may be part of a homeostatic program that responds to tissue injury and the robust capacity of their immunosuppressive capabilities has been demonstrated from their roles in transplantation tolerance [64], [65]. The full extent of the contributions of fibroblasts and myeloid-derived MSCs of the tumor stroma remains to be fully understood, but it is becoming apparent they likely play integral roles in the establishment of a tumor-promoting microenvironment, supporting both immunosuppression and angiogenesis.

5 VEGF as a Primary Mediator of Angiogenesis and Immune Suppression

The list of cytokines and growth factors that can simultaneously promote immune suppression and angiogenesis either directly or indirectly is extensive [66]. Factors such as adenosine [67], prostaglandin E2[68], TGF-β [69], and endogenous Toll-like receptor ligands (TLRs) [70] have key roles in the proliferation, survival, migration, and vessel formation of endothelial cells [70]. Further, many of these mediators have known functions in the suppression of antigen-presenting cell (APC) activation, maturation, and antigen presentation, or they directly suppress T-cell activation while promoting Treg functions [66]. The repertoire of mediators with dual function is ever expanding, and with new discoveries come new targets for disrupting favorable tumor microenvironmental conditions.

Here, we will focus on the most well-characterized growth factor with this capacity, VEGF, emphasizing its role in immune suppression. The development and maturation of new vessel growth during angiogenesis is multifaceted, requiring the precise and coordinated activation of a multitude of ligands and receptors (e.g., PDGF, Tie1, Tie2), but the most pivotal regulator in both physiologic and pathologic angiogenesis is the VEGF and the VEGF-receptor (VEGF-R) system [71, 72]. VEGF signaling remains a critical rate-limiting agent in angiogenesis with pleiotropic effects controlling a multitude of angiogenic processes [73].

VEGF overexpression is associated with tumorigenesis and a poor prognosis in a multitude of cancers, including gastric carcinoma [74], colorectal carcinoma [75, 76], lung cancer [77], melanoma [78], prostate cancer [79], breast [80], and ovarian carcinoma [81]. VEGF is upregulated in cancer cells in vivo by hypoxia and nutrient starvation [82], but also by oncogene activation, which drives constitutive VEGF overexpression [83]. VEGF directly promotes tumor angiogenesis through multiple mechanisms such as endothelial cell proliferation and survival, endothelial cell migration, vessel destabilization via Tie-2 [84], and enhancement of chemotaxis of bone marrow-derived vascular precursor cells (e.g., endothelial cells, pericytes, vascular leukocytes) [18, 85]. In addition, VEGF promotes tumorigenesis through autocrine signaling, regulating tumor cell functions and driving tumor metastases [85].

DCs are central to the generation of an antitumor response. As professional APCs, they present tumor antigens to T cells, generating an antigen-specific antitumor response. Defective DC function, combined with a failure of DC maturation, is frequently observed in cancer patients and in tumor-bearing mice. These defects occur in DCs found in the blood, tumor tissue, or draining lymph nodes [86]–[89]. The effects of defective DC function (i.e., defective antigen presentation) on the antitumor response are somewhat clear: lack of tumor antigen presentation means lack of effective antitumor response or even worse, active tolerance. Indeed, it has been speculated that immature or incompletely matured DCs may mediate tumor tolerance, inducing T-cell anergy or the expansion of Tregs [90], [91].

The clinical significance of DC dysfunction has been demonstrated in a study of patients with breast, neck/head, and lung cancer [87]; DCs isolated from cancer patients were functionally impaired in a mixed leukocyte reaction, and this functional impairment corresponded to a more severe cancer diagnosis (higher stage) [87]. Further, both the percentage and total number of DCs were significantly reduced in the peripheral blood of cancer patients, and this observation correlated with an increase in the total number of immature hematopoietic cells. The increase of immature cells in the blood was closely correlated to serum VEGF levels, but not to TGF-β, IL-6, or granulocyte macrophage-colony stimulating factor (GM-CSF) levels [87]. Importantly, these aberrations in DCs were somewhat corrected following chemotherapy and anti-VEGF therapy [87].

DC defects can be induced by tumor-derived TGF-β [92] and IL-10 [93]. However, VEGF plays a significant role in the suppression of DC maturation and function. Although DC defects in cancer patients and tumor-bearing mice had been appreciated for several years, Gabrilovich and colleagues were the first to identify a soluble factor, released from tumor cells, that was capable of impairing both DC function and DC maturation from $CD34^+$ hematopoietic precursors [88]. By using neutralizing blocking antibodies, the tumor-derived soluble factor was discovered to be VEGF, and antibodies against IL-10 or TGF-β were unable to reverse the suppression [88]. Similar observations of defective DCs in cancer patients, with a dependence on or association with VEGF, have since been made [94], [95]. Experimentally, these findings have been recapitulated in the mouse, suggesting a common mechanism and inherent role for VEGF in the antitumor response. In particular, Ishida and colleagues demonstrated that tumor-bearing mice displayed defects in DC numbers as well as function and that a VEGF- blocking antibody reversed these defects [96].

Although several mechanisms may be involved in the generation of DC defects, VEGF can exert its immunosuppressive effects through the disruption of normal hematopoiesis. VEGF continually infused in mice, at levels commonly associated with cancer pathology, resulted not only in defects of DC maturation and function but also in widespread changes in the differentiation of multiple hematopoietic lineages. For example, VEGF infusion induced a significant increase in B cells and $Gr\text{-}1^+$ immature myeloid cells [15], [88], [94]–[99]. It has been discovered that VEGF mediates the suppression of DC maturation through the impairment of normal nuclear factor-kappa B (NF-κB) signaling during hematopoiesis [100], mediated through VEGF-R1 signaling on $CD34^+$ hematopoietic progenitor cells [101].

The effects of VEGF on DC maturation and function can be partially reversed through VEGF blockade. Treatment of patients with the VEGF blocking antibody, bevacizumab, has been shown to partially reverse some of the DC defects. In an initial study by Almand et al., cancer patients receiving anti-VEGF antibody demonstrated a reversal of maturation defects of their DCs, and this has also been observed by others [87], [102], [103]. These observations have also been recapitulated experimentally in mouse tumor models [104]–[106]. Therefore, VEGF blockade may be critical to the success of any cancer immunotherapeutic strategy.

VEGF likely exerts effects on the immune system beyond its role in the suppression of hematopoiesis. Programmed death ligand 1 (PDL1)—a major negative regulatory ligand that suppresses T-cell activation through the cognate programmed death-1 (PD1) receptor—is expressed on tumor cells, but it is also highly expressed on tumor-associated myeloid DCs in ovarian cancer patients [107]. Interestingly, incubation of blood myeloid DCs with VEGF induced robust expression of PD-L1 on the cell surface [107]. PD-L1 (B7-H1) is a cell surface protein belonging to the B7 family of co-stimulatory molecules. PD-L1 may inhibit T-cell growth by ligation of the PD-1 receptor, as well as promote programmed cell death of effector T cells through an unknown mechanism [107]. Therefore, expression of PD-L1 is associated with suppression of T-cell effector functions. Thus, VEGF has potential roles in multiple aspects of immunosuppression mediated through DCs.

In the context of cancer immunotherapy, T cells have a well-appreciated role in the antitumor response, and cancer immunotherapies rely on the use of autologous, tumor-reactive T cells to mediate tumor regression [108]. In ovarian cancer, our laboratory has demonstrated that the presence of intratumoral T cells (also called intraepithelial T cells) was significantly associated with an increase in overall survival [109]. This observation is not unique to ovarian cancer as the infiltration of T cells into tumors has been associated with positive clinical outcomes in breast [110], prostate [111], esophageal [112], and colorectal cancers [113]. The effects of VEGF extend to many cell types in the hematopoietic system and are not exclusive to DCs [97], [114]. VEGF-Rs are expressed on many additional cell types, notably T cells. Interestingly, we observed that ovarian tumors expressing high levels of VEGF were rarely associated with intratumoral T cells [109]. Whether this observation is mediated by VEGF through direct or indirect action on T cells remains to be determined.

Thymic atrophy is a common characteristic of cancer patients [115]. Although most cancer patients tend to be older, premature thymic atrophy occurs in many childhood cancers, which is partially reversible upon treatment [115]. Further, thymic involution occurs in tumor-bearing mice, suggesting a common mechanism [115]. In addition to negative effects on DC maturation, VEGF is also believed to suppress proper T-cell development [114], [115]. Treatment of mice with pathologic levels of VEGF, comparable to that seen in cancer patients, induced a robust thymic atrophy, and a significant reduction in $CD4^+$ T cells and $CD8^+$ T cells [115]. Further, VEGF blockade in tumor-bearing mice partially reversed the thymic atrophy [115]. The immunosuppressive effects of VEGF on T cells occurred on bone marrow precursors, as VEGF did not appreciably disrupt maturation of T cells already present in

the thymus [115]. These effects likely occur through VEGF-R2 signaling on bone marrow precursor cells [114]. Although pathologic levels of VEGF clearly influence the proper development of T cells, the relevance of these findings and their impact on the antitumor response remain undefined.

As described above, Tregs suppress antitumor immunity. NRP-1, a co-receptor that interacts with VEGF-R1 and VEGF-R2 and binds VEGF, has been detected on $CD4^+CD25^+$ Tregs [52]. Enhanced activation of NRP-1 increased $CD4^+CD25^+$ Treg interactions with DCs in preference to Th cells [52]. This suggests a role for VEGF in promoting immune suppression and tumor tolerance through Treg activation. Recently, the importance of NRP-1 on Tregs has been shown to be crucial for their recruitment to the tumor microenvironment in a VEGF-dependent manner [116]. NRP-1 on Tregs was shown to have direct roles in Treg trafficking, and using mice with Tregs deficient in NRP-1, Hansen and colleagues were able to show that tumors grown on these mice were smaller, had much fewer tumor-infiltrating Tregs, and had enhanced activation of $CD8^+$ T cells [116]. This phenotype was reversible by adoptive transfer of NRP-1^+ Tregs from wild-type mice [116].

A few studies have addressed the direct role of VEGF on effector T-cell function. Treatment of mouse T cells with VEGF has been demonstrated to induce IL-10 production from T cells while suppressing IFN-γ production [117]. This immunosuppressive effect was attributed to VEGF-R1 expressed on T cells [118]. In humans, all T cells derived from the peripheral blood of normal donors or from peripheral blood or ascites of ovarian cancer patients upregulate VEGF-R2 following activation [119], [120]. Importantly, treatment of activated T cells from patients and normal donors suppresses T-cell proliferation and cytotoxicity [119], [120]. Thus, tumor-derived VEGF may also directly suppress the functions of antitumor T cells.

Although the direct effects of VEGF on T-cell functions are not yet fully elucidated, insights into the roles of VEGF on the T-cell antitumor response, either direct or indirect, can be gleaned from studies using VEGF blocking antibodies. In one single-arm clinical trial of a tumor vaccine combined with anti-VEGF therapy (bevacizumab), it has been shown that the combination is associated with a high rate of T-cell-specific immune response, characterized by increased IFN-γ levels and T-cell proliferation following stimulation with antigen [121]. Supporting this observation, VEGF-R2 blockade in mice using an anti-VEGF-R2 antibody has been demonstrated to induce a *de novo* T-cell-mediated antitumor response [122]. VEGF-R2 blockade resulted in spontaneous infiltration of $CD4^+$ T cells and $CD8^+$ T cells that produced IFN-γ, and VEGF-R2 blockade protected against subsequent tumor challenge in a tumor vaccine model [122]. However, VEGF-R2 blockade resulted in a substantial increase in serum VEGF levels. Therefore, it is unknown whether the antitumor T-cell response was generated through blockade of tumorigenic angiogenesis, or whether increased serum VEGF enhanced activation of T cells through VEGF-R1 signaling. However, consistent with a role for VEGF signaling in $CD4^+CD25^+$ Tregs, VEGF-R2 blockade in this study enhanced T-cell effector functions in a tolerized mouse tumor model system [122]. This observation is supported by the demonstration that anti-VEGF treatment in mice reduced the number of Tregs, decreased Foxp3 expression, enhanced CTL induction, and increased tumor vaccine efficacy [123]. In conclusion,

VEGF or VEGF-R blockade predominantly enhances T cell antitumor immunity, an effect most consistent with the concept that VEGF has direct immunosuppressive functions on T cells.

6 Roles of VEGF on the Tumor Vasculature in Immune Suppression

The tumor vascular endothelium is a substantial obstacle to the success of immune therapies, as it establishes a physical barrier through which tumor-reactive T cells must pass in order to recognize and eliminate their tumor targets. In many T-cell immune therapies that have been conducted, it has been noted that while activated T cells could be found in the periphery, they often failed to infiltrate the tumor itself [124]–[126]. Thus, successful transmigration through the tumor endothelial barrier is required for optimal tumor regression. The prohibitive nature of the tumor endothelium is maintained by locally expressed cytokines, growth factors, and the nature and quantity of adhesion molecules expressed by the endothelium [4], but precisely how the tumor vasculature establishes immune privilege is not well known. However, VEGF signaling on endothelial cells does play a pivotal role in reducing leukocyte homing and extravasation through the vascular endothelium, making VEGF a mediator of tumor immune privilege.

T cells extravasate through the endothelium to the tumor in a multistep process that includes binding to adhesion molecules expressed on endothelial cells, followed by diapedesis. VEGF has been demonstrated to increase the expression of many endothelial cell adhesion molecules (CAMs), particularly in the context of angiogenesis (reviewed in detail by Francavilla and colleagues [127]). In agreement with this observation, VEGF-induced enhancement of CAM expression has been associated with increased leukocyte adhesion both in vitro and in vivo [128], [129]. However, understanding the role of VEGF in leukocyte adhesion is complicated by reports which demonstrate that VEGF may actually inhibit adhesion molecule expression on endothelial cells [128]–[133].

Although the role of VEGF signaling and leukocyte adhesion may be difficult to discern, in the context of a proinflammatory environment, the emerging concept is that angiogenic growth factors impair immune cell adhesion [130]–[132]. For example, Griffioen and colleagues demonstrated reduced expression of adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), after treatment of TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) with bFGF or VEGF [131]. In a similar manner, Bouzin and colleagues observed reductions in ICAM-1 and VCAM-1 expression in TNF-α stimulated HUVECs as early as 2 hours after VEGF addition [132]. Although these effects on adhesion molecule expression were transient, longer treatment times demonstrated a disruption of adhesion molecule organization and clustering on the cell surface [132]. This response was associated with a perturbation of the spatial

organization and clustering of ICAM-1 and was dependent on caveolin-1 and NO [132].

The interaction between endothelin and VEGF regulates multiple aspects of angiogenesis including endothelial cell proliferation, migration, invasion, vessel formation, and neovascularization [134]. We have recently demonstrated an additional endothelial cell-associated mechanism of regulation of T-cell infiltration into tumors through activation of the endothelin-B receptor (ET_BR, also known as EDNRB) [135]. Tumor endothelial ET_BR was found to be upregulated in ovarian tumors lacking tumor-infiltrating lymphocytes (TILs) [135] and, similar to the absence of TILs [109], ET_BR overexpression was associated with poor survival. Endothelin 1 (ET1) signaling through ET_BR was found to block T-cell adhesion to the endothelium through suppression of ICAM1 clustering on endothelial cell membranes, an effect mediated by NO [135]. Importantly, ET1 is overexpressed in ovarian cancer, among other cancers [136], establishing a tumor cell–endothelium ET1–ET_BR paracrine axis, which is upregulated specifically in the tumor microenvironment and suppresses T-cell adhesion to the endothelium even in the presence of TNF-α. This is important, as it reconciles the coexistence of inflammation (TNF is commonly overexpressed in the tumor microenvironment) and a quiescent tumor endothelium phenotype that does not support homing of T cells.

An open question that remains is whether the endothelium may be able to distinguish among leukocyte subsets, selectively allowing trafficking of only certain immune subsets according to their "polarization" (Th1 versus Th2, Th17, or Treg), phenotype, or activation status. Both CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1) and CD166 have been suggested to control Treg migration through the endothelium in the liver [137] and in pancreatic cancer [138], respectively. In particular, CLEVER-1 is synergistically regulated by angiogenic growth factors hepatocyte growth factor (HGF) and VEGF. Thus, it is possible that in tumors, under the influence of the local angiogenic milieu, the tumor endothelium could allow immunosuppressive cells to pass, while blocking access to tumor-reactive effector T cells and NK cells establishing a selective immune barrier.

7 Immunoregulation by Tumor Vasculature

An active role for the tumor vasculature in regulation of immune responses is becoming a greater area of research, and endothelial cells can express a number of mediators that suppress the actions of effector lymphocytes such as Fas ligand (FasL) [139] and TNF-related apoptosis-inducing ligand (TRAIL) [140] and possibly even CD31, a classical endothelial cell marker [141]. Endothelial cells can also express numerous soluble mediators that suppress immune responses such as IL-6, IL-10, TGF-β, and prostaglandin E2 (PGE2) [142], [143]. Additionally, endothelial cells can express a number of important molecules that may be involved in the direct stimulation of T cells, such as inducible T-cell co-stimulator ligand (ICOS-L), CD137, CD58, CD40, CD80, CD86, and major histocompatibility complex (MHC) class I

and class II molecules, many of which are upregulated by angiostatic, Th1-associated cytokines [144]. Whether the expression and/or function of these immunostimulatory molecules are influenced by angiogenic mechanisms is largely unknown, but it is possible that downregulation or inhibition of their expression on endothelial cells occurs during angiogenesis. It is likely that the overall outcome however, in tumor endothelial cells is largely immunosuppressive; and support for this hypothesis comes from the observation that tumor cell supernatants can induce an endothelial suppressor phenotype [145], [146].

Endothelial cells express a number of molecules from the B7 family of immune regulatory molecules. B7 family members either co-activate or inhibit T-cell responses, and their functions are often context dependent. PD-L1 and PD-L2 are members of the B7 family and engagement of the receptor PD-1 expressed on T cells transduces a signal that inhibits T-cell proliferation, cytokine production, and cytolytic function [147]. Both PD-L1 and PD-L2 have been detected on endothelial cells, and their endothelial expression has been shown to limit T cell immune responses in infection and autoimmunity [148]–[150]. A role for PD-L1/2 expression on the tumor endothelium has not yet been defined.

An additional B7 family member, B7-H3, is not well characterized with regard to function, but its expression is often associated with aggressive tumor progression and immune inhibition (see [151] and references within). B7-H3 has been detected on the tumor endothelium in ovarian [152] and endometrial cancers [151]. Importantly, vascular expression of B7-H3 is linked to a poor clinical outcome and advanced tumor stage for both types of cancers. In endometrial cancer, expression of tumoral B7-H3 was also associated with a lack of tumor-infiltrating CD8 T cells, although no association was found for endothelial expression [151].

Tumor endothelial cells may also limit T-cell responses through the production of soluble mediators. Tumor-derived VEGF and IL-1 have been shown to enhance production of PGE2 by the tumor endothelium [153], [154]. PGE2 is a potent suppressor of T-cell activation, and its production by the tumor endothelium was demonstrated to inhibit T cell effector functions. [154]

Very recently, the lymphoma endothelium was shown to express T-cell immunoglobulin domain and mucin domain 3 (TIM3), a protein that contributes to immune suppression through the activation of the interleukin-6–signal transducer and activator of transcription 3 (IL-6–STAT3) pathway in endothelial cells [155]. TIM3-expressing endothelial cells promoted the onset, growth, and dissemination of lymphoma by inhibiting activation of $CD4^+$ T cells and Th1 polarization.

Another interesting molecule in this interface is indoleamine 2,3-dioxygenase (IDO), which can be expressed by tumor endothelium [156], [157] and has also been detected in the human placental vascular endothelium during development [158]. IDO is largely believed to suppress T-cell activation through the depletion of tryptophan, but additional mechanisms also may play a role [159]. Artificial expression of IDO in ovarian cancer cells promotes angiogenesis [160], while IDO inhibition can delay tumor growth independently of adaptive immune mechanisms (in immunodeficient mice). Further, expression of IDO in endothelial cells promotes survival and resistance to oxidative stress in vivo [161], and ectopic expression of IDO by

the corneal endothelium promotes graft survival [162]. Kynurenine, a major metabolite of IDO, has significant roles in regulating vascular tone and endothelial dilation [163] and may directly regulate angiogenesis [164]. However, kynurenine likely regulates vascular tone additively with NO through cyclic guanosine monophosphate (cGMP) pathways, suggesting that together, they promote vasodilation [163]. Thus, expression of IDO is potentially crucial to endothelial cell biology and may link angiogenesis and immune suppression [66].

Similar to tumor-associated myeloid cells, endothelial cells have been found to express arginase I [165]. Expression of arginase I by the endothelium prevented T-cell proliferation in vitro, and arginase inhibition promoted survival of corneal allografts in vivo. It is believed that arginase enzyme activity depletes T cells of essential arginine, and thus starves them of an essential nutrient similar to the effects of IDO expression.

7.1 Supporting Vascular Cells

Surrounding the endothelial cells but integral to the vasculature are mural cells, which are endowed with plasticity and can acquire phenotypes of pericytes or vascular smooth muscle cells [62]. Mural cells play significant roles in angiogenesis and have also been demonstrated to have immunosuppressive functions [62]. Various myeloid populations have been described to participate in the process of blood vessel formation in tissues undergoing post-infarction repair [62], and myeloid cells including MDSCs and tolerogenic DCs may also incorporate into the tumor endothelium [15], [167], [168]. We have found that monocytes with a DC phenotype may associate with vasculature in ovarian tumors [167], and these cells exhibit high expression of IL-10 and can be recruited by antimicrobial peptides to tumors. Ovarian tumors overexpressing VEGF and β-defensin-29 coordinately incorporated tolerogenic DCs into the vasculature [18]. β-Defensin-29 behaved as a chemoattractant through CCR6, while VEGF enabled the incorporation and migration of DCs into developing vessels [18]. Thus, we believe that incorporation of these various immunosuppressive cells of myeloid origin in and around the vasculature may represent an additional mechanism whereby blood vessel development and immune suppression are co-implemented.

8 Implications for Cancer Therapy

Cancer immune therapies that have relied heavily on promoting strong antitumor immune responses often meet with limited success in the clinic, perhaps due in part to lack of recruitment of immune cells through the tumor endothelial barrier [169]. Future strategies for the elimination of tumors should contain complementary strategies to block mediators capable of promoting both angiogenesis and immune suppression, such as PGE2 and VEGF, while simultaneously using a strong antitumor immune

response. For example, combinatorial approaches using cancer vaccines and ET_BR blockade [135], cancer vaccines with COX2 inhibitors [170] or VEGF-specific antibody and adoptive T-cell therapy [171] have ultimately been more successful than any strategy alone in pre-clinical models. Additionally, strategies to eliminate the tumor endothelium itself have also been met with recent success [172]. Alternatively, strategies aimed at eliminating immunosuppressive cells like Treg cells, in combination with VEGF blockade, are also currently underway [173]. It is important to note that strong Th1 cytokines known for their roles in tumor elimination, like IL-12, IFN-γ, and IFN-inducible chemokines such as monokine induced by IFN-γ (MIG, also known as CXCL9) and IFN-γ-induced protein 10 (IP-10, also known as CXCL10), can exert potent angiostatic effects through direct action on endothelial cells [174]–[176]. Thus, complete tumor eradication will ultimately require tipping the balance in favor of an immunostimulatory and angiostatic microenvironment. The open question remains as to whether there exists a central regulatory cell type or central mediator that, when blocked, can relieve immune suppression and angiogenesis, thereby promoting an antitumor immune response leading to elimination of the tumor.

Here, we have presented parallels between cancer and other biological processes that lead to the unifying hypothesis that angiogenesis and immune suppression operate hand in hand and are truly two sides of the same homeostatic tissue repair program. The challenges remaining are to discover targeted approaches to utilize this understanding to regulate the homeostatic tissue repair program to eliminate cancer, expedite wound healing, promote transplant tolerance, and relieve symptoms of autoimmunity.

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Chapter 8
Tim-3 Regulation of Cancer Immunity

Kaori Sakuishi and Ana C. Anderson

Abstract Chronic unrelenting immune responses can lead to immunopathology that can be fatal. Consequently, the immune system has evolved both molecular and cellular mechanisms that serve to contract active immune responses and restore immune homeostasis. Molecular mechanisms include the upregulation of inhibitory or immune checkpoint receptors on T cells post activation. Cellular mechanisms include regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) that suppress active T-cell responses. Unfortunately, all of these mechanisms have been co-opted in cancer to suppress the generation of productive antitumor T-cell responses. In tumor-bearing hosts, the sustained expression of immune checkpoint receptors on T cells results in T-cell dysfunction or exhaustion. Moreover, MDSCs expand to large numbers in tumor-bearing hosts and the tumor microenvironment promotes Tregs. The inhibitory receptor T-cell immunoglobulin and mucin domain 3 (Tim-3) has a role in each of these mechanisms of immune suppression, thus highlighting the value of Tim-3 as a target for anticancer immunotherapy. Here, we discuss the role of Tim-3 in each of these mechanisms and the implications for the development of agents that target Tim-3 for cancer treatment.

Keywords Regulatory T cells (Tregs) · Checkpoint receptor · T-cell exhaustion · Immunotherapy

1 Introduction: Tim-3 is an Inhibitory Molecule

T-cell immunoglobulin and mucin domain 3 (Tim-3) was first identified 10 years ago as a molecule selectively expressed on both $CD4^+$ and $CD8^+$ T cells that have differentiated to an interferon-gamma (IFN-γ)-secreting phenotype [42]. Thus, Tim-3 marks effector $CD4^+$ T helper 1 (Th1) and $CD8^+$ T cytotoxic 1 (Tc1) cells that mediate immunity against viruses and intracellular pathogens. Initial experiments employing anti-Tim-3 antibodies showed exacerbation of experimental autoimmune

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encephalomyelitis (EAE), an autoimmune disease of the central nervous system where IFN-γ-secreting Th1 cells play a major role [42]. Animals treated with anti-Tim-3 antibody developed hyperacute disease that was accompanied by uncontrolled macrophage activation. These data provided the first indication that Tim-3 may function as an inhibitory molecule that serves to contract inflammation driven by IFN-γ-secreting Th1 and Tc1 cells. Indeed, the inhibitory function of Tim-3 became clear upon the identification of the C-type lectin galectin-9 as a Tim-3 ligand. It was shown that galectin-9 triggering of Tim-3 induced cell death in Tim-3^+ T cells and ameliorated EAE [73]. Other studies further showed that both Tim-3-deficient mice and mice treated with a Tim-3-Ig fusion protein exhibited defects in the induction of antigen-specific tolerance [52]. Lastly, Tim-3 has been recently identified as a critical determinant of the dysfunctional or exhausted phenotype that develops in $CD8^+$ T cells in chronic diseases, such as chronic viral infection [24], [31], [32], [55] and cancer [20], [45], [53], [72] in both humans and experimental models of disease. Thus, the major role of Tim-3 is to dampen or contract Th1/Tc1 T-cell responses.

As a consequence of its inhibitory function, Tim-3 is now classed with other T-cell inhibitory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death-1 (PD-1), and lymphocyte-activation gene 3 (Lag-3) that are commonly referred to as immune checkpoint molecules. Immune checkpoint molecules are currently at the forefront of anticancer immunotherapy. The role of Tim-3 in shaping antitumor immunity, the potential interplay of Tim-3 with other checkpoint molecules, and the features that distinguish Tim-3 from other checkpoint molecules will be discussed below.

2 Tim-3 in T-cell Exhaustion

2.1 T-cell Exhaustion and Chronic Viral Infection

T-cell exhaustion describes a state of T-cell dysfunction wherein T cells are incapable of eliciting the robust effector functions typically observed in the effector and memory T-cell populations. T-cell exhaustion was first described in experimental chronic viral infection in mice (reviewed in [63]). It was observed that virus-specific T cells in chronic lymphocytic choriomeningitis virus (LCMV) infection exhibited functional unresponsiveness to antigen re-challenge, unlike the rigorous effector responses observed in memory cells arising from acute LCMV infection. It was later found that exhaustion develops in a hierarchical manner such that the proliferative response, cytotoxic function, and interleukin-2 (IL-2) production are lost at the early stage. This is then followed by a loss of tumor necrosis factor (TNF) production and finally IFN-γ production [68]. At the latest stage, exhausted T cells are deleted. Contrary to the loss of effector cytokines, an increase in the levels of suppressive cytokines such as IL10 and transforming growth factor beta (TGFβ) has been observed in exhausted T cells [7], thus raising the possibility that exhausted T cells may actively suppress the local immune response.

Since the initial discovery of exhausted T cells in mice chronically infected with LCMV, exhausted virus-specific $CD8^+$ T cells have been identified in a variety of chronic infections such as human immunodeficiency virus (HIV) [16], [49], [59], hepatitis C virus (HCV) [50], [60], hepatitis B virus (HBV) [8], polyoma virus [65], adenovirus [37], and Friend virus [55]. Thus, T-cell exhaustion exists in both mouse and man. This evolutionary conservation indicates that exhaustion likely evolved as a host defense mechanism aimed at protecting the host from the tissue pathology that would ensue from chronic unrelenting immune responses.

The predominance of exhausted T cells among different chronic viral infections together with their dysfunctional phenotype makes exhausted T cells a significant barrier to successful viral clearance. Accordingly, a considerable amount of effort has gone into elucidating the factors that promote the development of T-cell exhaustion. In this regard, the duration or chronicity of antigen exposure has been identified as a critical determinant of T-cell exhaustion [43]. This has clear implications for cancer where long-term exposure to tumor antigens occurs. Interestingly, it has also been noted that polyfunctional T cells, those that exhibit a wide array of effector functions, are the first to become functionally inactivated or exhausted [68]. Indeed, exhausted T cells express high levels of CD69 and low levels of CD62 L, a profile typically associated with effector T cells and not central memory T cells. These observations indicate that an exhausted T cell emerges from a pool of robust effector T cells and suggest that re-functionalization rather than elimination of exhausted T cells is desirable clinically.

In order to target exhausted T cells therapeutically, a means for specifically identifying them is required. Genome-wide expression profiling of exhausted T cells from mice chronically infected with LCMV identified the immune checkpoint receptor PD-1 as a benchmark for exhausted T cells [5]. Indeed, it is now known that exhausted T cells express not only PD-1 but also a whole array of checkpoint receptors that include CTLA-4, Lag-3, and Tim-3 [6]. Interestingly, it has been noted that the most severely or deeply exhausted T cells also express the widest array of checkpoint receptors [6]. More importantly, accumulating evidence points to the fact that exhausted T cells can be successfully re-functionalized by blocking these checkpoint receptors. Indeed, when the PD-1/PD-L1 pathway is blocked by anti-PD-L1 antibody in vivo during chronic LCMV infection, robust expansion of virus-specific T cells and restoration of effector function in $PD\text{-}1^+$ cells are observed, resulting in better viral control [5]. In contrast, anti-PD-L1 antibody did not have any effect on acute LCMV infection. Interestingly, although CTLA-4 is also expressed on virus-specific exhausted $CD8^+$ T cells, anti-CTLA-4 antibody treatment did not improve either virus-specific T-cell responses or viral control. These observations indicate that while checkpoint blockade can be an effective therapeutic approach in chronic disease, not every checkpoint molecule is an ideal target. The differences among various checkpoint receptors and the implications of those differences for cancer immunotherapy are discussed below.

2.2 Role of Tim-3 in T-cell Exhaustion in Chronic Viral Infection

The role of the Tim-3/Tim-3L pathway in T-cell exhaustion was first reported in HIV patients. In HIV, Tim-3 marks virus-specific $CD8^+$ T cells that exhibit impaired IFN-γ secretion. Interestingly, the Tim-3^+ T cells in HIV patients do not express PD-1 [32], indicating that PD-1 is not always a reliable marker of T-cell exhaustion. Moreover, the presence of Tim-3^+ T cells in HIV appears to be clinically significant as their presence positively correlates with increasing viral load and reduced Tim-3 expression on T cells correlates with highly active antiretroviral therapy. Furthermore, Tim-3 blockade has been shown to successfully restore proliferation and cytokine production in response to HIV-1 peptides in both $CD4^+$ and $CD8^+$ T cells.

Dysfunctional T cells expressing Tim-3 are also found in patients with chronic HCV infection [24]. Unlike in HIV, co-expression of Tim-3 and PD-1 marks the most dysfunctional population of HCV-specific $CD8^+$ T cells. Moreover, in HCV, the presence of dual Tim-3 and PD-1-expressing cells precedes the development of viral persistence [41]; patients who resolved HCV infection demonstrated low frequencies of Tim-3^+ PD-1^+ HCV-specific cytotoxic T lymphocytes (CTLs) at all time points, whereas patients who developed viral persistence exhibited high frequencies of Tim-3^+ PD-1^+ HCV-specific CTLs. Furthermore, it was shown that $CD4^+$ T cells expressing Tim-3 are impaired in secreting IL-2 and that the patients who cleared HCV did not demonstrate increased expression of Tim-3 on $CD4^+$ T cells. These observations indicate that Tim-3 expression also marks a dysfunctional group of $CD4^+$ T cells that may not be able to provide adequate T-cell help to $CD8^+$ T cells in the early stages of infection. Together these observations indicate that as in HIV the presence of Tim-3^+ T cells has prognostic value in HCV infection. Also, as observed in HIV, Tim-3 blockade restores cytotoxic function to HCV-specific $CD8^+$ T cells.

Tim-3^+ PD-1^+ $CD8^+$ T cells have also been identified as the T cells in the deepest state of exhaustion in the chronic LCMV infection model [31]. Interestingly, these LCMV-specific $CD8^+$ T cells exhibit impaired proliferative function and inflammatory cytokine production but produce significantly more of the suppressive cytokine IL-10 than other $CD8^+$ T cells that are present, including Tim-3^- PD-1^+ cells. Thus, in addition to being functionally impaired, exhausted T cells may also actively suppress the activity of neighboring T cells via IL-10. Although blockade of Tim-3 signaling alone with Tim-3-Ig fusion protein exhibits a small effect in chronic LCMV, Tim-3-Ig potently synergizes with anti-PD-L1 antibody to restore cytotoxic function to virus-specific $CD8^+$ T cells and decrease viral titer.

As in HCV and LCMV, Tim3^+ PD1^+ $CD8^+$ T cells have similarly been identified as the most dysfunctional population of $CD8^+$ T cells in HBV [33] and Friend virus [55] infection. That Tim-3 expression together with PD-1 and not PD-1 expression alone marks the most dysfunctional T cells in a wide array of chronic viral infections underscores the importance of the Tim-3 pathway in the mechanism of T-cell exhaustion. More importantly, these observations suggest that although immune checkpoint molecules do co-exist in dysfunctional T cells, their function is not necessarily redundant and some may play a more significant role than others in certain aspects of exhaustion.

2.3 Role of Tim-3 in T-cell Exhaustion in Cancer

Cancer and chronic infection share common aspects. In both cases, the host is persistently exposed to high antigen load for a prolonged period of time and this persistent exposure is ensured by the immunosuppressive environment. However, there is a major difference in the T-cell repertoire. Unlike virus-specific T cells, which recognize exogenous antigen, tumor-reactive T cells primarily recognize endogenously derived tumor antigens. Since most tumor antigens are derived from self and highly self-reactive T cells are purged from the T-cell repertoire during negative selection in the thymus, tumor antigen-specific T cells are present in much lower frequency and are of much lower avidity compared to virus-specific T cells. Yet, despite this difference, it has become increasingly evident that tumor-specific T cells undergo phenotypic alterations that are very similar to the T-cell exhaustion that develops in virus-specific T cells in chronic infection.

As with exhausted virus-specific T cells, Tim-3 is expressed on $CD8^+$ exhausted T cells in solid tumors. $CD8^+$ $Tim\text{-}3^+$ T cells comprise approximately two-thirds of the $CD8^+$ T cells within tumor-infiltrating lymphocytes (TILs) in CT26 colon carcinoma and 4T1 mammary carcinoma and approximately one-third of $CD8^+$ TILs in B16F10 melanoma [53]. Tim-3 is also found in up to 30 % of $CD8^+$ TILs in methylcholanthrene (MCA)-induced fibrosarcomas [45]. Reminiscent to what has been observed in exhausted T cells in chronic infection, these $Tim\text{-}3^+$ $CD8^+$ TILs are highly dysfunctional and co-express PD-1.

Similar observations have been made in cancer patients. In patients with advanced melanoma, approximately 30 % of New York esophageal squamous cell carcinoma-1 (NY-ESO-1)-specific $CD8^+$ T cells co-express Tim-3 and PD-1 [20]. In non-small cell lung cancer (NSCLC) patients, approximately one-third of TILs express Tim-3 with the majority of these TILs co-expressing PD-1. In patients with follicular B cell non-Hodgkin's lymphoma (FL) [67], approximately one-third of T cells in lymph node biopsies co-express Tim-3 and PD-1. In all three cancer types, Tim-3 expression marks dysfunctional $CD8^+$ T cells. Interestingly, as was observed in both HIV and HCV, the presence of $Tim\text{-}3^+$ T cells correlates with poor clinical prognosis in both NSCLC and FL patients. This will be discussed in more detail below.

Tim-3 expression is also associated with T-cell dysfunction in hematologic malignancies. Dysfunctional $CD8^+$ T cells co-expressing Tim-3 and PD-1 are found in the liver, which is an initial site of metastasis in mice with advanced acute myelogenous leukemia (AML) [72]. Interestingly, while PD-1 deficiency confers partial resistance to AML, the $Tim\text{-}3^+$ $CD8^+$ T cells from PD-1-deficient mice bearing AML still exhibit reduced cytokine production. In contrast, galectin-9 deficiency confers resistance to AML and the T cells in galectin-9-deficient mice exhibit a more modest induction of both PD-1 and Tim-3 relative to that observed in wild-type mice. Together these observations indicate that the role of the Tim-3 pathway in exhaustion may be largely independent of the PD-1/PD-L1 pathway.

Another similarity between cancer and chronic viral infection is that in both cases the $CD8^+$ $Tim\text{-}3^+$ $PD\text{-}1^+$ cells exhibit a surface phenotype that is consistent with that

of effector/memory T cells. In FL patients, $CD8^+$ Tim-3^+ PD-1^+ cells are $CD45RO^+$ $CCR7^-$ [67]. In mice with colon carcinoma, $CD8^+$ Tim-3^+ PD-1^+ TILs are $CD62L^-$ [53]. Thus, the $CD8^+$ Tim-3^+ PD-1^+ T cells in cancer are derived from a pool of antigen-experienced cells that could be effective against tumor if their function could be restored.

A major difference between viral infection and tumor is the localized presence, and presumably induction, of exhausted T cells in tumor tissue. In a study of patients with metastatic melanoma, it was clearly shown that T cells found in tumor-infiltrated tissue exhibit a deeply exhausted phenotype, while tumor-specific T cells circulating in the peripheral blood display intact effector function [4]. In line with this observation, it was found that the inhibitory receptors Tim-3, Lag-3, CTLA-4, and PD-1 were more highly expressed in the Melan-A/MART-1-specific $CD8^+$ T cells present in tumor-infiltrated lymph nodes than in the T cells present in the peripheral blood. Similarly, in NSCLC, Tim-3^+ T cells that exhibit dysfunctional phenotype are only found in tumor tissue [23]. This selective presence of $CD8^+$ Tim-3^+ PD-1^+-exhausted T cells in tumor tissue is also observed in experimental models of cancer [53]. Given these observations, it is interesting to speculate that driving factors exist within the tumor microenvironment to induce and/or keep tumor-specific T cells functionally impaired. Indeed, galetin-9 and IL-12 may be two important players in driving the Tim-3-mediated T-cell dysfunction in tumors [67], [72]. However, other factors likely exist.

Whether the dysfunctional antigen-specific T cells in tumors are exhausted or anergic is currently a matter of debate [35]. Anergy and exhaustion both describe a state of T-cell unresponsiveness. However, anergy and exhaustion arise by two very different means, lack of T-cell co-stimulation and chronic antigen exposure, respectively. Both of these mechanisms could be operative in cancer. Anergy may be operational early on as tumors often lack expression of co-stimulatory molecules, while exhaustion may develop over time in the face of antigen persistence. Thus far, genome-wide expression profiling has been performed on exhausted $CD8^+$ T cells from LCMV-infected mice [64] and on anergic T cells obtained from various sources [71]. These data suggest that while there may be some significant overlap, there are some important features that distinguish anergy from exhaustion, namely the role of E3 ligases such as Grail and transcription factors of the early growth response (egr) family, namely egr2 and egr3 [35]. Now that Tim-3 can be used to more precisely identify exhausted $CD8^+$ T cells in both chronic viral infection and cancer, these studies can be revisited and should yield more definitive answers as to the differences in the molecular programs that drive T-cell anergy versus T-cell exhaustion.

3 Tim-3 in Regulatory T Cells

Recently, a few studies have reported that Tim-3 is up-regulated on the $FoxP3^+$ regulatory T cells (Tregs) that are found in tissue sites in different pathological settings, including cancer. One study reported that in a model of allograft rejection,

up to 40 % of graft-infiltrating FoxP3$^+$ cells express Tim-3 [25]. These Tim-3$^+$ Tregs are donor-derived and arise from Tim-3$^-$ FoxP3$^+$ cells both in vitro and in vivo. Moreover, Tim-3$^+$ Tregs express more CD25, CD39, and IL-10 and have superior suppressive function when compared to Tim-3$^-$ Tregs in vitro. However, rather contradictory to this finding, adoptive transfer of Tim-3$^+$ Tregs is less efficient at prolonging graft survival than transfer of Tim-3$^-$ Tregs. Interestingly, the frequency of Annexin V$^+$ cells is higher in the Tim-3$^+$ Tregs than in the Tim-3$^-$ Tregs present in allografts. Based on this finding, the authors claim that Tim-3$^+$ Tregs are a highly suppressive Treg population that is quickly purged from the repertoire as a means of preventing prolonged immunosuppression. Whether the short life span of the Tim-3$^+$ Tregs that arise in the acute inflammatory environment associated with allograft rejection is also a feature of the Tim-3$^+$ Treg that arise in the chronic inflammatory environment associated with cancer remains to be determined.

Tim-3$^+$ Tregs also have been described in a cancer setting [23]. In patients with NSCLC, approximately 60 % of CD4$^+$ FoxP3$^+$ TILs express Tim-3. Interestingly, these Tim-3$^+$ Tregs are infrequent in the peripheral blood of NSCLC patients. As observed in CD8$^+$ TILs, the Tim-3$^+$ Tregs present in TILs also express PD-1. Moreover, the presence of Tim-3$^+$ Tregs appears to correlate with poor clinical parameters such as the presence of nodal metastases and advanced cancer stage. Although no characterization of the suppressor function of the Tim-3$^+$ Treg in TILs in NSCLC was done, the correlation of their presence with poor prognosis is in line with their being a highly suppressive population of tissue of Tregs. Collectively, the observation of Tim-3$^+$ Tregs in NSCLC opens a new area of investigation in the biology of Tim-3 in T cells in cancer and adds to the potential mechanisms by which Tim-3 can have an impact on antitumor immunity.

At this point, there are many unanswered questions as to the role of Tim-3$^+$ Tregs in cancer. First, it still has to be clarified whether the presence of Tim-3$^+$ Tregs is a common feature across tumors of different tissue origin. Second, it remains to be shown whether intra-tumoral Tim-3$^+$ Tregs are indeed more suppressive than Tim-3$^-$ Tregs. In support of Tim-3$^+$ Tregs being more highly suppressive than Tim-3$^-$ Tregs is the observation that the Tim-3$^+$ Tregs in NSCLC also express PD-1. Indeed, the expression of PD-1 as well as other checkpoint receptors, namely CTLA-4 and Lag-3, has been described on Tregs and shown to promote Treg suppressor function [28], [54], [21]. While this promotion, of Treg suppressor function by checkpoint receptors seems paradoxical to the role of checkpoint receptors in dampening CD8$^+$ effector T cells and promoting the development of T-cell exhaustion, the overall outcome of checkpoint receptor activity on the immune response is still the same, namely inhibition. Whether the expression of multiple checkpoint receptors on Treg is indeed associated with enhanced Treg suppressor function remains an open question. In line with this, it will be interesting to determine whether there are differences in the expression of CTLA-4 and Lag-3 in intra-tumoral Tim-3$^+$ Treg versus Tim-3$^-$ Treg.

As mentioned above, the Tim-3$^+$ Tregs that arise during allograft rejection are short-lived [23]. This is not surprising given that galectin-9 triggering of Tim-3 has been shown to induce cell death in Tim-3$^+$ effector T cells during acute inflammatory responses [73]. As was mentioned earlier, this may not be the case

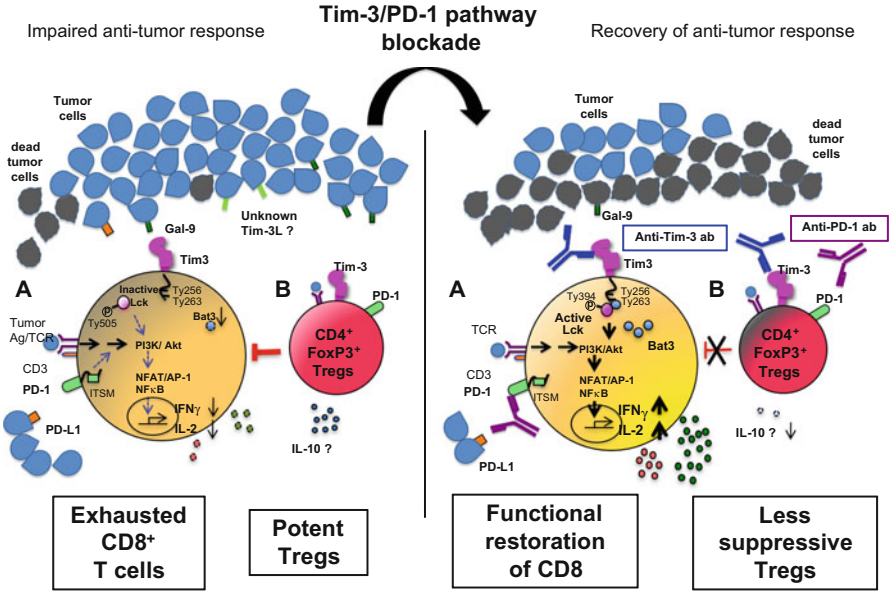

Fig. 8.1 Model for the effects of Tim-3/PD-1 pathway blockade on exhausted $CD8^+$ T cells and $FoxP3^+$ Treg in cancer. **a** Exhausted $CD8^+$ T cells in the tumor microenvironment express Tim-3 and PD-1. Triggering of Tim-3 by galectin-9 (*Gal-9*) on tumor cells results in decreased expression of Bat3 and accumulation of inactive Lck. Triggering of PD-1 by PD-L1 expressed on tumor cells triggers the inhibition of the phosphatidylinositol-3-kinase (*PI3K*)/Akt pathway via the immunoreceptor tyrosine-based switch motif (*ITSM*) of PD-1. Blockade of the Tim-3 and PD-1 pathways with antibodies restores T-cell receptor (*TCR*) signaling and effector function in exhausted T cells. **b** Similarly, Tim-3^+ FoxP3 Tregs are present within tumor and their presence is correlated with poor prognosis. Tim-3^+ Tregs are reported to be more potent suppressors than Tim-3^- Tregs and co-express PD-1. Tim-3/PD-1 pathway blockade may decrease the suppressive capacity of these cells

in cancer as the chronic inflammatory environment present in tumors is highly immunosuppressive and different from the acute inflammatory environment present during allograft rejection. The examination of Tim-3^+ Tregs over the course of tumor progression should resolve this issue.

From a clinical standpoint, the single most important outstanding question regarding Tim-3^+ Tregs in cancer is how Tim-3^+ Tregs are affected by Tim-3 blockade. If Tim-3 functions in a similar manner as other checkpoint receptors that support Treg suppressor function, Tim-3 blockade would be expected to abrogate Treg suppressor function. Indeed, the remarkable efficacy of Tim-3/PD-1 blockade in controlling tumor growth in preclinical models of cancer could be due to the combined effects of dual blockade on restoring function to exhausted T cells and abrogating Treg-mediated suppression (Fig. 8.1).

The observation that Tim-3^+ Tregs in NSCLC patients are found uniquely in tumor tissue indicates that Tim-3^+ Tregs represent a specialized population of Tregs that arise from the circulating Treg pool in response to cues present in the tumor

microenvironment. This concept of specialized subsets of Tregs that have differentiated to suppress specific classes of immune responses at tissue sites is an emerging one. Indeed, distinct subsets of FoxP3$^+$ Tregs that express different transcription factors and suppress specific classes of effector T cells have been identified [10]. For example, Tregs that express T-bet suppress Th1 cells [36], while Tregs that express interferon regulatory factor-4 (IRF4) suppress Th2 cells [70]. Furthermore, a recent study has also shown that the Tregs present in a specific anatomical compartment, such as adipose tissue, exhibit a distinct phenotype from the Tregs present in peripheral lymphoid tissues [19]. The adipose tissue Tregs are distinct from the Th1 and Th2 Tregs mentioned above and are dependent on peroxisome proliferator-activated receptor-gamma (PPAR-γ) for their unique functional phenotype [13]. Collectively, these observations raise the question of how distinct are Tim-3$^+$ Tregs from the Tregs found in peripheral lymphoid tissues and what is the factor(s) that determines the phenotype of Tim-3$^+$ Tregs.

4 Tim-3 in Innate Cells

4.1 Tim-3 Promotes Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are known to be major inhibitors of the anticancer immune response. MDSCs are a heterogeneous population of immature myeloid cells that exhibits features of both granulocytes and monocytes. MDSCs are distinct from tumor-associated macrophages (TAMs) as they lack expression of the mature macrophage marker F4/80. In mice, MDSCs express CD11b and high levels of the granulocyte marker Gr-1. MDSCs expand in a variety of pathological conditions but most notably in cancer where they expand to large numbers in tumor-bearing hosts and are potent suppressors of T-cell responses. Indeed, the presence of MDSC has been correlated with poor clinical prognosis in cancer. Consequently, MDSC represent an important therapeutic target in cancer, and much effort is being invested in understanding the mechanisms by which they arise and how they suppress T-cell responses (reviewed in [22]).

Transgenic overexpression of Tim-3 on T cells has been shown to promote expansion of CD11b$^+$ Gr-1$^+$ cells [15]. Accordingly, tumor growth is accelerated in mice that harbor Tim-3 overexpressing T cells, and Tim-3 antibody blockade reverses MDSC expansion and slows tumor growth in Tim-3 transgenic mice. Interestingly, galectin-9-transgenic mice also exhibit expansion of MDSCs, and the introduction of Tim-3 deficiency reverses this expansion. Lastly, Tim-3-deficient mice bearing 4T1 mammary carcinoma exhibit less MDSC expansion than wild-type controls. Collectively, these observations support the fact that the Tim-3/galectin-9 pathway has an important role in promoting MDSCs in cancer. These observations further raise the possibility that the Tim-3$^+$-exhausted T cells and Tim-3$^+$ Tregs present in tumor tissue may further contribute to the immunosuppressive tumor microenvironment by promoting MDSCs. Given the effect of anti-Tim-3 on MDSC expansion together with

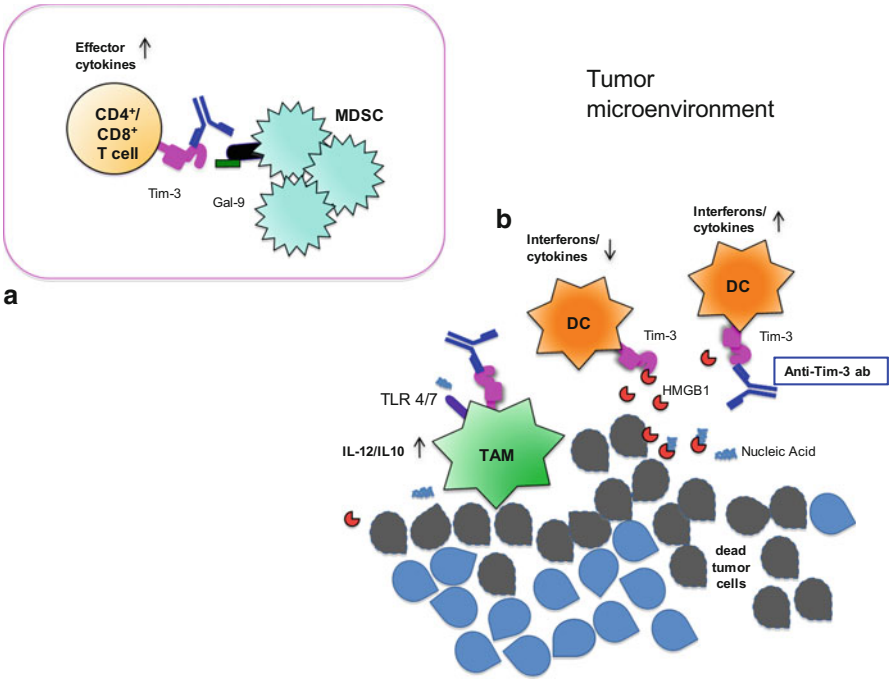

Fig. 8.2 Role of the Tim-3/Tim-3L pathway in innate cells in cancer. **a** Tim-3 expression on T cells promotes MDSC expansion. Blockade of the Tim-3/galectin-9 (*Gal-9*) interaction by Tim-3 antibody limits MDSCs, thereby improving the T-cell response. **b** Tim-3 is expressed on TAMs and tumor-associated dendritic cells (*TADCs*). Tim-3 has an inhibitory role on both of these cell populations, albeit through different mechanisms. Blockade of Tim-3 on TAM improves responsiveness to TLR signals and promotes cytokine secretion. Blockade of Tim-3 on TADCs interferes with high mobility group protein B1 (*HMGB1*) binding. The increased free HMGB1 is now available to bind nucleic acid released by tumor cells undergoing necrotic cell death and trigger DC activation

the fact that anti-PD-1 has been shown to reduce MDSC in mice bearing melanoma [14], it is interesting to speculate whether the clinical efficacy of combined blockade of the Tim-3 and PD-1 pathways is also due in part to reduced MDSC expansion (Fig. 8.2a).

4.2 Role of Tim-3 in TAMs

In addition to its role in promotion of MDSCs, Tim-3 may also have a role in TAMs. Recent studies suggest that Tim-3 has a direct inhibitory role in human monocytes [69]. Activation of monocytes through Toll-like receptors (TLRs) 4 and 7 results in the downregulation of Tim-3 that is concomitant with increased IL-12 production. Furthermore, blockade of Tim-3 signaling either with anti-Tim-3 antibody or via Tim-3 knockdown with small-interfering RNA increases the production of both

IL-12 and IL-10 in monocytes after stimulation through TLR4/7. Interestingly, this also decreases the expression of PD-1, which also functions to negatively regulate monocyte activation (reviewed in [9]). Thus, reduction of Tim-3 signaling in monocytes results in increased activation, indicating that Tim-3 also functions as an inhibitory receptor on monocytes.

This work has clear implications for tumor immunology. While the expression of Tim-3 and its role in TAMs have been little explored, it is possible that constitutive expression of Tim-3 in TAMs underlies their failure to adequately prime the immune response locally within tumor tissue. If this is the case, blockade of Tim-3 with antibody would be expected to alter the function of TAMs, increasing their antigen presenting function and ability to prime antitumor immune responses (Fig. 8.2b).

4.3 Tim-3 as Regulator of Innate Sensing in the Tumor Environment

Tim-3 is highly expressed on dendritic cells (DCs) in both mouse and humans [1]; however, whether Tim-3 expression by DCs has a role in the context of antitumor immunity has been largely unexplored. A recent study by Chiba et al. [12] suggests a role for Tim-3$^+$ DCs in the regulation of innate-sensing pathways specifically in the tumor microenvironment. This study found that tumor-associated DCs (TADCs) express very high levels of Tim-3, whereas peripheral DCs express little, if any, Tim-3. This is in opposition to a previous report that found very high expression of Tim-3 in DCs in human peripheral blood and also in the spleens of unmanipulated mice [1]. Indeed, Chiba et al. provide some data showing that tumor-derived factors such as vascular endothelial growth factor (VEGF) upregulate Tim-3 on DCs. While the reasons for these discrepancies are unclear at present, it will be important to resolve this issue through further research.

Regardless, Chiba et al. found that Tim-3$^+$ DCs exhibit a reduced capacity to respond to stimulation with certain TLRs, particularly those that recognize nucleic acids: TLR3, TLR7, and TLR9. Interestingly, the response to stimulation through TLR2 and TLR4, which recognize peptidoglycan and lipopolysaccharide, was not altered in Tim-3$^+$ DCs. This led the authors to hypothesize that Tim-3$^+$ DCs have a role in regulating antitumor immunity through regulation of DNA-sensing pathways in innate cells. Indeed, the authors find that Tim-3 blockade improves IFN-γ and IL-12 production by DCs in response to stimulation with CpG or DNA. Accordingly, they find that Tim-3 blockade synergizes with DNA vaccine to reduce tumor growth in vivo. Interestingly, the two known Tim-3 ligands, galectin-9 and phosphatidyl serine, did not participate in the Tim-3-mediated suppression of nucleic acid-driven inflammation. This led to the identification of high mobility group protein B1 (HMGB1) as a third Tim-3 ligand.

HMGB1 is an intracellular protein that translocates to the nucleus where it can promote gene transcription by bending DNA. It is also released from stressed cells undergoing necrotic cell death. Extracellular HMGB1 can bind to several receptors,

including receptor for advanced glycation end products (RAGE) and TLRs (2,4, and 9). Extracellular HMGB1 acts as an alarmin. It binds DNA from dying cells and facilitates delivery to innate cells, thereby triggering activation and release of proinflammatory cytokines. The binding of Tim-3 to HMGB1 effectively reduces the amount of free HMGB1 available to bind to RAGE or TLRs, thereby interfering with activation of the innate immune response via this pathway (Fig. 8.2b).

The implications of this work for cancer therapy are numerous. First, it suggests that Tim-3 blockade would promote the activation of the innate immune system that is elicited by dying tumor cells in a normal cancer setting. Second, it raises the possibility of utilizing Tim-3 blockade to improve the efficiency of DNA-based tumor vaccines. Lastly, it also suggests that Tim-3 blockade may improve responsiveness to chemotherapy. In this regard, the authors examine the response to cisplatin in the presence or absence of Tim-3 blockade and find that anti-Tim-3 improves both the production of inflammatory cytokines by DCs (IL-12 and IFN-β1) and control of tumor growth in cisplatin-treated mice. Interestingly, cisplatin is known to trigger release of HMGB1 as does oxaliplatin [56]. However, oxaliplatin, but not cisplatin, has been shown to trigger the release of calreticulin and thus oxaliplatin, but not cisplatin, triggers "immunogenic" cell death. Unfortunately, the authors did not examine how Tim-3 blockade affects responses to oxaliplatin. Thus, it remains to be addressed whether the manner in which cells die affects the outcome of Tim-3 blockade in mice treated with different chemotherapeutic agents.

5 Other Roles of Tim-3 in Cancer

5.1 Tim-3 in Tumor Endothelium

A couple of recent studies have reported expression of Tim-3 on tumor endothelium. The first study reported Tim-3 expression in B-cell lymphoma-derived endothelial cells [29]. Interestingly, the level of Tim-3 in tumor endothelium was closely correlated with dissemination and poor prognosis. The same study further confirmed that Tim-3 is also expressed in the microvessels of breast cancer patients. Moreover, Tim-3$^+$ endothelial cells were found to suppress the activation of CD4$^+$ T cells in vitro. A second study reported that the expression of Tim-3 on endothelial cells facilitates the metastasis of melanoma via activation of the NF-κB pathway [66]. Unfortunately, the relationship of Tim-3 expression on TILs and expression of Tim-3 on endothelial cells was not explored in either of these two studies.

A recent study of NSCLC reports Tim-3 expression on tumor cells themselves [74]. In this study, Tim-3 expression is observed on tumor cells in 87 % of NSCLC patient samples and, like ectopic expression on endothelial cells, this expression positively correlates with poor clinical prognosis. This study also reported Tim-3 staining on TAMs and TILs. The expression of Tim-3 on tumor cells raises the issue of what the effect of Tim-3 blockade on tumor cells might be. Notwithstanding this issue, the expression of Tim-3 on endothelial cells indicates the potential of

modulating the vascular component of tumors to enhance antitumor immunity and control metastasis through targeting the Tim-3 pathway.

5.2 Tim-3 in Cancer Stem Cells

Two recent studies have also revealed differential expression of Tim-3 on leukemic stem cells (LSCs) versus hematopoietic stem cells (HSCs) in patients with AML [30], [34]. Tim-3 was identified as a surface molecule specifically expressed on LSCs but not on normal HSCs in most types of AML with the exception of acute promyelocytic leukemia [30]. Tim-3^+ but not Tim-3^- AML cells reconstitute human AML in immunodeficient mice, suggesting that the Tim-3^+ population contains functional LSCs [34]. Anti-Tim-3 antibody was successful in blocking the engraftment of AML after xeno-transplantation and eliminating the reconstitution of human AML by LSCs in secondary recipients while not interfering with reconstitution of normal human HSCs [34]. These observations raise the possibility of targeting Tim-3 for the selective elimination of LSCs in AML patients. Whether Tim-3 is expressed in cancer stem cells of other hematologic and non-hematologic cancers remains to be further explored.

6 Tim-3 as a Target for Immunotherapy

6.1 Current State of Anticancer Immunotherapy

Blockade of immune checkpoint molecules is at the leading edge of anticancer immunotherapy. This is due to the clinical success of anti-CTLA-4 antibody (ipilimumab) in prolonging survival in patients with advanced metastatic melanoma. The clinical efficacy of ipilimumab revolutionized the field of anticancer immunotherapy by legitimizing a treatment modality that had been met with skepticism for many years. Unfortunately, the response rate to ipilimumab is still at best only 20 % and is not without consequences as approximately 60 % of patients experience autoimmune-like toxicity, with 10–15 % of the patients experiencing serious grade 3–4 toxicities such as colitis and hypophysitis [27]. These drawbacks have catalyzed effort into the development of therapies that target other immune checkpoint molecules such as PD-1. At present, there are over 20 registered clinical trials examining antibodies that block the PD-1 receptor or the PD-1 ligand (PD-L1) in different cancer indications. Results from a phase 1 trial of anti-PD-1 antibody in multiple different cancer indications show a response rate of about 25 %, with 14 % of patients treated with anti-PD-1 antibody exhibiting autoimmune-like toxicities that are similar to those observed in patients treated with ipilimumab, with the notable exception of pneumonitis [58]. Collectively, these data indicate that blockade of PD-1 is more efficacious than blockade of CTLA-4 while maintaining a similar toxicity profile. However, the reported response is still only marginally better than

that of ipilimumab. This leaves the door open for therapies that target other immune checkpoint molecules, such as Tim-3.

6.2 Studies of Tim-3 Blockade in Preclinical Models of Cancer

The Tim-3/Tim-3L pathway has attracted considerable attention as the next wave of checkpoint blockade for cancer immunotherapy. In the WT3 sarcoma model and transgenic adenocarcinoma of mouse prostate-C1 (TRAMP-C1) prostate cancer model, anti-Tim-3 antibody therapy was clearly effective when administered alone and the effect was dose-dependent. In preclinical models of colon carcinoma, such as CT26 and MC38, monotherapy with anti-Tim-3 antibody was shown to be partially effective, similar to PD-1/PD-L1 pathway blockade alone [45]. However, a clear synergistic effect was observed when both the Tim-3 and PD-1 pathways were blocked, such that complete tumor regression was observed in nearly half of the mice [53]. This synergistic effect of Tim-3 and PD-1 pathway blockade can be demonstrated even in B16F10 melanoma, which is known to be poorly immunogenic and resistant to treatment [45]. Importantly, significant effects were observed when the anti-Tim-3 and anti-PD-1 antibodies were administered to mice with established CT26 or B16F10 tumors. Moreover, a clear synergistic effect was confirmed in MCA-induced fibrosarcoma, a well-characterized model of de novo carcinogenesis [45]. Similarly, survival in mice with AML was not increased in mice treated with Tim-3-Ig fusion protein alone, but co-administration with anti-PD-L1 antibody significantly prolonged survival to a higher degree than that observed with anti-PD-L1 antibody therapy alone [72]. Together these data strongly support the potential of Tim-3 pathway blockade for the treatment of various cancer types and further suggest that combinatorial therapy may be more effective in the clinical setting.

The mechanisms responsible for the effects of Tim-3 blockade either alone or together with PD-1 blockade need to be delineated in further detail. We have shown that Tim-3/PD-1 blockade is clearly successful in recovering the antitumor response [51]. Mark Smyth's group has shown that anti-Tim-3 is largely ineffective in the absence of $CD4^+$ or $CD8^+$ T cells, but effective in the absence of mature B cells [45]. Moreover, anti-Tim-3 is ineffective in the absence of $CD8^+$ T cells capable of secreting IFN-γ. Whether the $CD4^+$ T cells are capable of secreting IFN-γ does not matter so long as the $CD8^+$ are IFN-γ sufficient. Together these data support that IFN-γ-producing $CD8^+$ T cells have the key role in conferring antitumor activity in mice treated with anti-Tim-3.

6.3 Tim-3 in Human Cancer

Increasing data support the relevance of targeting Tim-3 in human cancer. Tim-3 is expressed in approximately 30 % of NY-ESO-1-specific $CD8^+$ T cells in patients. These melanoma-specific T cells exhibit dysfunctional or exhausted phenotype and

Tim-3 pathway blockade restores IFN-γ and TNF-α production and proliferative responses to tumor antigen stimulation in these T cells [20]. Simultaneous blockade of the Tim-3 and PD-1 pathways further restores IL-2 production in these T cells. Thus, the synergy of Tim-3 and PD-1 pathway blockade in restoring T-cell function is also observed in T cells isolated from cancer patients.

Two recent studies in patients with NSCLC further substantiate the value of Tim-3 as a target for anticancer immunotherapy [23], [74]. Both studies report Tim-3 expression on TILs in NSCLC. Interestingly, Tim-3 expression is specific to the $CD4^+$ and $CD8^+$ T cells that infiltrate tumor as it is not observed in the T cells present in the peripheral blood or in the adjacent non-tumor lung tissue of NSCLC patients [23]. Moreover, Tim-3 expression is not observed on T cells present in the lung tissue of patients with bronchodilation [74]. Together these data support the fact that Tim-3 expression is specific to the T cells present in tumor tissue. Indeed, this tumor tissue-specific pattern of Tim-3 expression on T cells is similar to what has been observed in preclinical cancer models [53].

As mentioned above, the expression of Tim-3 on $CD4^+$ TILs in NSCLC patients is primarily on $FoxP3^+$ Tregs and the presence of these Tim-3^+ Tregs appears to correlate with poor prognosis. In the $CD8^+$ T-cell compartment, the majority of Tim-3^+ TILs in NSCLC patients co-express PD-1 and fail to produce IFN-γ upon stimulation [23]. Thus, as is the case in melanoma patients, co-expression of Tim-3 and PD-1 on $CD8^+$ T cells marks cells with exhausted phenotype. In addition to lymphocytes, one study further reports Tim-3 expression on TAMs in NSCLC patients. As is the case with T cells, the expression of Tim-3 on macrophages is specific to tumor tissue, as it is not observed in macrophages present in normal lung tissue [74]. As mentioned above, exploring the function of Tim-3 on these TAMs is an open area of investigation.

As mentioned earlier, Tim-3 has been reported on tumor cells in NSCLC patients. This is the first report of Tim-3 expression on malignant lung cells. The relevance of this Tim-3 expression is suggested by the correlation of moderate to high Tim-3 staining on tumor cells with a poor 5-year survival rate [74]. Given the reported expression of Tim-3 on cancer stem cells in AML, it is interesting to speculate whether Tim-3 marks cancer stem cells in NSCLC.

As mentioned earlier, Tim-3 expression has also been reported on T cells in patients with FL [67]. In FL patients, IL-12 treatment has been explored as a therapy but has either shown no clinical benefit [18] or shown to be detrimental if used in combination with anti-CD20 (rituximab) [2]. Given the association of IL-12 with induction of Tim-3 and the increasing association of Tim-3 with T-cell dysfunction, Yang and colleagues investigated a potential connection between IL-12 treatment, Tim-3 expression, and T-cell exhaustion in FL patients [67]. Yang and colleagues show that IL-12 levels are elevated in the serum of FL patients and that elevated serum IL-12 correlates with a shorter progression-free survival. They further show that the frequency of Tim-3-expressing $CD4^+$ and $CD8^+$ T cells is higher in the peripheral blood of FL patients relative to healthy controls and that the frequency of Tim-3^+ T cells is highest in the tumor tissue of FL patients. Moreover, the majority of Tim-3^+ $CD4^+$ and $CD8^+$ T cells co-express PD-1. Lastly, the Tim-3^+ $CD8^+$ T cells in FL patients exhibit exhausted phenotype. The enrichment of Tim-3-expressing

T cells in tumor tissue, co-expression with PD-1, and exhausted phenotype are all in line with what has been observed in patients with melanoma and NSCLC and in preclinical models of cancer.

In contrast to NSCLC, the $CD4^+$ Tim-3^+ T cells in FL patients do not appear to be $FoxP3^+$ Tregs; instead, they appear to be exhausted $CD4^+$ T cells, which have been described in chronic viral infections [63]. Whether the fact that the $CD4^+$ Tim-3^+ T cells in FL cancer patients fall into the effector $CD4^+$ T-cell compartment while the $CD4^+$ Tim-3^+ T cells in NSCLC cancer patients fall into the Treg compartment reflects a difference between lymphoma and carcinoma remains to be addressed through further examination of other cancers of similar tissue type. Notwithstanding this difference, Tim-3 blockade restores function to both exhausted $CD4^+$ and $CD8^+$ T cells in FL patients, thereby further supporting its value for anticancer immunotherapy [67].

Interestingly, although Tim-3 and PD-1 are co-expressed on both $CD4^+$ and $CD8^+$ T cells, a head-to-head comparison of the relationship of Tim-3^+ versus PD-1^+ T cells to patient survival in FL patients demonstrated that Tim-3^+ but not PD-1^+ T cells correlate with poor patient survival [67]. Indeed, it is the presence of $CD4^+$ Tim-3^+ T cells that correlates most significantly with poor survival. This is reminiscent of the correlation of $CD4^+$ Tim-3^+ T cells with poor prognosis in NSCLC patients, albeit, in NSCLC, the $CD4^+$ Tim-3^+ are $FoxP3^+$ Tregs.

Collectively, the few studies examining Tim-3 in human cancer support its role in negatively regulating both $CD4^+$ and $CD8^+$ T-cell function, and potentially innate cells, in multiple different cancers. In addition, recent studies highlight the potential value of Tim-3 expression on T cells, on tumor cells, and on tumor-associated endothelium as prognostic indicators. Together with the mounting data showing the clinical effect of Tim-3 blockade in multiple experimental models of cancer, these data strongly support that the Tim-3/Tim-3L pathway is a promising target for anticancer immunotherapy.

6.4 Distinguishing Features of Tim-3

As plans move forward to target Tim-3 for anticancer immunotherapy, one has to consider how Tim-3 differs from other checkpoint receptors that are currently being targeted. One very important consideration is the pattern of expression. This is of importance because blockade of checkpoint receptors could trigger autoimmunity. Indeed, this is best exemplified by the fact that CTLA-4-deficient mice exhibit massive lymphoproliferation, multi-organ lymphocyte infiltration, splenomegaly, and lymphadenopathy and die by 4 weeks of age [57], [62]. Indeed, as mentioned above, autoimmune-like toxicities are commonly observed in patients treated with anti-CTLA-4. The fact that CTLA-4 is upregulated on all activated T cells in order to promote the resolution of effector T-cell responses underlies these observations. PD-1 is similarly upregulated on all activated T cells and PD-1-deficient mice also develop spontaneous autoimmunity [46], [47], although with lower incidence and at

a later age than CTLA-4-deficient mice. In this regard, targeting Tim-3 is advantageous since Tim-3 is not expressed on all T cells but rather is selectively expressed on T cells that have differentiated towards an IFN-γ-producing phenotype [42], and in cancer patients, it seems to be expressed primarily in intra-tumoral T cells [20], [23], [53], [67]. Thus, interfering with the negative regulatory function of Tim-3 is less likely to have untoward global effects than interference with either CTLA-4 or PD-1. The fact that Tim-3-deficient mice do not exhibit any autoimmunity [52] and that tumor-bearing mice treated with anti-Tim-3 antibody do not exhibit any overt autoimmunity [45] support the fact that targeting Tim-3 may be safer than targeting either CTLA-4 or PD-1.

Another important consideration is the mechanisms of action by which checkpoint receptors inhibit T-cell responses. At the cell surface, CTLA-4 inhibits T-cell responses by outcompeting CD28 for binding to the shared co-stimulatory ligands, B7-1 and B7-2. CTLA-4 also functions intracellularly to suppress T-cell responses. Ligation of CTLA-4 inhibits phosphorylation of the serine/threonine kinase Akt, which is important for cytokine synthesis, glucose metabolism, and cell survival. This CTLA-4-mediated inhibition of Akt is dependent on the serine/threonine phosphatase PP2A [48]. Thus, CTLA-4 promotes the termination of T-cell responses by preventing continued T-cell co-stimulation and interfering with proximal T-cell receptor (TCR) signaling.

PD-1 has a well-characterized immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail. The ITSM motif recruits the Src homology region 2 domain containing phosphatases SHP-1 and SHP-2 [11], resulting in the dephosphorylation of phosphatidylinositol-3-kinase (PI3K), a membrane proximal component of the TCR signaling complex that is upstream of Akt. Thus, ligation of PD-1 also inhibits Akt, but through a different pathway than ligation of CTLA-4 [48].

Unlike PD-1, the intracellular tail of Tim-3 does not contain any inhibitory signaling motifs. It has six tyrosines in its cytoplasmic tail and it has been recently found that two of these tyrosines can couple with the TCR signaling pathway and augment TCR-dependent T-cell activation [38]. The Src family tyrosine kinases Fyn and Lck can directly bind to Tim-3 and phosphorylate tyrosines in the cytoplasmic tail. The phosphorylation of tyrosine in turn promotes the recruitment of SH2 domain-containing proteins to Tim-3, such as the p85 adaptor of PI3K, phospholipase C-gamma1 (PLC-γ1), and Ras GTPase activating protein 1 (RasGAP1), which effect increases in nuclear factor of activated T cells/activator protein 1 (NFAT/AP-1) and NF-κB activity. These data indicate that Tim-3 promotes rather than inhibits TCR signaling. This seems paradoxical to the role of Tim-3 as an inhibitory molecule and its role in T-cell exhaustion. In this regard, it has been postulated that Tim-3 may act to enhance T-cell activation and thus accelerate the acquisition of exhausted phenotype. The fact that gene expression profiling data indicate that exhausted T cells are more similar to effector T cells than memory T cells [64] supports this hypothesis. The one major caveat to the study demonstrating Tim-3 promotion of TCR signaling is that it primarily examines ligand-independent Tim-3 signaling [38], thus calling into question the translation of these observations to a physiological context.

More recently, our group reported that human leukocyte antigen B-associated transcript 3 (Bat3) binds to the cytoplasmic tail of Tim-3 and that its loss can negatively affect TCR signaling [51] (Figure 8.1). During T-cell activation, Tim-3 is recruited to the supra-molecular activation cluster and forms an intracellular molecular complex with Bat3 and the catalytically active form of Lck, thus promoting T-cell activation. Interestingly, we found that loss of Bat3 resulted in the accumulation of the catalytically inactive form of Lck and was associated with defective IL-2 and IFN-γ production. These data gave the first indication that Bat3 may function as a key regulator of Tim-3, preventing its inhibitory effects on T-cell signaling and activation. In support of this hypothesis, we found that Bat3-deficient T cells exhibit increased Lag3, prdm1, and pbx3, all of which have been associated with T-cell exhaustion. We further found that exhausted $CD8^+$ $Tim\text{-}3^+$ $PD\text{-}1^+$ TILs also exhibit decreased Bat3 expression. While much more work is required to fully elucidate the Tim-3 signaling pathway, it is clear at this time that Tim-3 affects the signaling pathway downstream of TCR activation in a very different way from PD-1 and CTLA-4.

The utilization of distinct and potentially synergistic inhibitory mechanisms by different checkpoint receptors forms a basis that can be exploited therapeutically. The fact that both CTLA-4- and PD-1-mediated inhibition target Akt, albeit through different pathways, suggests that the combination of Tim-3 blockade with either CTLA-4 or PD-1 blockade may be more advantageous. Indeed, this could partly underlie the remarkable clinical efficacy of Tim-3/PD-1 co-blockade that has been observed in preclinical models of cancer.

6.5 Strategies for Targeting Tim-3

Currently, anti-CTLA-4 monotherapy is in clinical use for advanced melanoma. As for Tim-3, the clinical efficacy of anti-Tim-3 monotherapy seems to be limited in preclinical cancer models. However, significant effects of anti-Tim-3 antibody are consistently observed in in vitro assays using T cells from melanoma [20] and lymphoma [67] patients. Also, given that Tim-3 is expressed in innate cells, adaptive cells, tumor-associated endothelium, and cancer stem cells, it might be worthwhile to first consider treating patients with anti-Tim-3 alone.

Without a doubt, the combination of targeting the Tim-3 pathway together with other checkpoint molecule pathways will probably be most effective. As has been shown in multiple animal models and in tumor-specific T cells from patients, the most promising target to be combined with Tim-3 is the PD-1/PD-L1 pathway. Combined treatment with anti-CTLA-4 antibody has been attempted in animal models but has not been shown to be as effective as the anti-Tim-3/PD-1 combination [45]. Whereas triple targeting of Tim-3, PD-1, and CTLA4 does seem to present the strongest synergistic effect, the greater adverse effect is of concern. Meanwhile, the combination of anti-Tim-3 and anti-LAG3 antibody is another tempting combination to be explored in the future.

Another therapeutic approach where anti-Tim-3 antibody may be beneficial is tumor vaccination. One study has shown anti-Tim3 antibody can augment the effect of vaccination by irradiated Flt4L-secreting B16 (FVAX) by promoting greater cytotoxic activity in intra-tumoral lymphocytes [3]. Another group demonstrated that 3LL tumor cells expressing Tim-3-hIg as a blocking agent can be used as a prophylactic tumor vaccine [39]. Whether anti-Tim-3 antibody can enhance the efficacy of other forms of vaccination like GVAX, a vaccine consisting of tumor cells that are retrovirally transduced to secret granulocyte–macrophage colony-stimulating factor (GM-CSF) for enhancing DC recruitment and cross priming, remains to be seen. Considering that both anti-CTLA-4 [26], [61] and anti-PD-1 [40] enhance the effectiveness of GVAX, Tim-3 blockade is likely to similarly potentiate the effects of GVAX. Lastly, as mentioned earlier, anti-Tim-3 may improve the efficacy of DNA-based antitumor vaccines by increasing the amount of available HMGB1 to activate the innate immune response [12].

6.6 Targeting Tim-3 Ligands

Interestingly, immunohistochemical analyses of the tumor tissue from patients in the anti-PD-1 trial showed that all of the patients whose tumors lacked PD-L1 expression were nonresponders [58]. This observation underscores the relevance of checkpoint ligand expression in the target tissue and its potential use as a predictive biomarker for patients who respond to immunotherapy. It further suggests that targeting the ligands for checkpoint receptors may be advantageous. With regard to Tim-3, at least three ligands have been described: galectin-9 [73], phosphatidylserine [17], [44], and HMGB1 [12]. It is known that many tumors express galectin-9 and that galectin-9 is upregulated by IFN-γ [75]. Phosphatidylserine is predicted to be present in abundance in the tumor environment where cell death is a continual process. Likewise, HMGB1 is one of the damage-associated molecules that comprises the inflammatory milieu. Indeed, HMGB1 is reported to be more highly expressed in tumor tissue relative to normal tissue [12]. One caveat to Tim-3 ligand blockade is that there are multiple Tim-3 ligands and each ligand has the potential to interact with multiple receptors besides Tim-3. At this time, we do not know which ligand(s) has the dominant role in the different biologies associated with Tim-3 in tumor immunity.

7 Concluding Remarks

Tim-3 has emerged as a master inhibitor of the immune response, employing multiple mechanisms to dampen the immune response. How and when these mechanisms operate in different cancer types and the receptor ligand relationships and cell types involved in these various mechanisms will require further experimentation. Accumulating evidence points towards a beneficial effect of blocking the Tim-3 pathway for eliciting potent antitumor immunity. Although Tim-3 is expressed on a wide array of

cell populations in tumors, the studies discussed herein all point towards anti-Tim-3 treatment enhancing the antitumor response. The next important step towards harnessing Tim-3 for anticancer immunotherapy will be to elucidate the signals, both extracellular and intracellular, that drive the upregulation of Tim-3 expression and to unravel the many roles of Tim-3 in cancer. This will aid in determining the most effective combination of approaches to obtain the best clinical outcome in different cancer indications.

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Chapter 9
Transcriptional Regulation of Dendritic Cells in the Tumor Microenvironment

Aimin Jiang, Katherine E. Stagliano, Steven M. Cuss, Ashley Triplett, Chunmei Fu and Arthur A. Hurwitz

Abstract Dendritic cells (DCs) play a critical role in the initiation of adaptive T cell-mediated, immune responses. In addition, they also respond to inflammatory signals as part of the innate immune response. DCs that infiltrate tumors are usually defective in their ability to elicit both adaptive and innate immune responses and often promote immune suppression. This suppressive activity is mediated by many different mechanisms and may be due to the varied transcriptional profile of these cells. This chapter discusses the different transcription factors that may contribute to the immunosuppressive function of tumor-associated DCs. Past and current studies reveal a complex and diverse network of transcription factors contributing to the inhibitory activity of tumor DCs. By understanding these regulatory pathways, novel targets for improving immunity to cancer may be identified.

Keywords Dendritic cell · Tumor · Microenvironment · Transcription factor · Immune suppression

Dendritic cells (DCs) are a critical component to the generation of adaptive immune responses. They function as antigen-presenting cells (APCs) that initiate T cell responses. In addition, DCs also contribute to innate immunity, recognizing a variety of pathogens via pattern recognition receptors (PRRs), resulting in cytokine and chemokine expression. As such, they are a diverse population of cells which plays key roles in immune homeostasis and response to infection and serves as a sentinel for the immune system.

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1 Dendritic Cell Biology

Development of DCs is tightly regulated and is specific for different types of DCs and their associated functions (reviewed in [1]). Conventional or "classical" DCs (cDCs) are appropriately equipped to process and present antigen to T cells and can be classified as tissue-resident (but migratory) or lymphoid-resident. They express elevated levels of major histocompatibility complex (MHC) class II and co-stimulatory ligands required to elicit T cell responses. cDCs can be broadly categorized based on expression of CD8 or CD11b; $CD8^+$ DCs are known to effectively cross-present (exogenous) MHC class I-restricted antigens to T cells, whereas $CD11b^+$ ($CD8^-$) myeloid DCs are most proficient at priming $CD4^+$ T cells. Expression of the αE integrin, CD103, also identifies many nonlymphoid (often migratory) DC populations which are either $CD11b^+$ or $CD11b^-$ but are generally $CD8^-$. While these phenotypic markers classify murine DCs, human DCs are not as well characterized, but presumably similar populations exist; for example, recent studies suggest that BDCA-3^+ human DCs are efficient at cross-presenting exogenous antigen to $CD8^+$ T cells [2–5]. Langerhans cells (LCs) are another population of migratory DCs which localize to the skin and play a strong role in immune surveillance. They are marked by the expression of CD205 (DEC205) and CD207 (Langerin) and arise through a unique, IL-34-dependent mechanism [6].

Plasmacytoid DCs (pDCs) represent another population of DCs which contributes to host responses. Originally described as type-I interferon (IFN)-producing cells, (reviewed in [7]) and later unified to include "plasmacytoid monocytes," pre-DC2 cells, and natural IFN-producing cells, pDCs are a rare population of cells with a broad range of functions. They naturally express low levels of MHC class II and co-stimulatory ligands. In humans, they express BDCA-2 and ILT7 and in mice, they are marked by the expression of B220, SiglecH, and BST2. pDCs respond to inflammatory stimuli such as Toll-like receptor (TLR) ligands (principally activating TLRs 7 and 9) and their activity is regulated by other receptors such as BST2 and SiglecH. Both activating and inhibitory functions have been attributed to pDCs in adaptive immunity. Presumably, the context in which they present antigen to T cells, specifically the microenvironment of the lymphoid tissue, parenchymal tissue, or tumor, will dictate the outcome of T cell priming as generating stimulatory or tolerogenic function.

2 Dendritic Cell Development

Given the complexity and heterogeneity of DC populations, it is not surprising that the differentiation and development of DCs are equally as complex. DCs probably share a developmental pathway with other myeloid cells, specifically macrophages, and form the mononuclear phagocyte system [8]. Common lymphoid and myeloid progenitors can give rise to DC precursors termed as common DC progenitors (CDPs) which, in turn, give rise to pDCs or cDC progenitors. CDPs are $Flt3^+Csf1R^+$, and

therefore, both pDCs and cDCs are Flt3- and Csf-1 (M-CSF) dependent. Pre-cDC precursors arise in the bone marrow and can be found in lymph node, thymus, spleen, and some parenchymal tissues (e.g., gut and skin). LCs develop from a different myelo-monocytic precursor, apparently early on during embryonic development [9].

Genetic programming of DC development relies on cues from the tissue environment, many of which remain incompletely understood. Recently, however, several studies have demonstrated that DC subset development is tightly associated with transcription factor (TF) expression. Most populations of DCs, including cDCs and pDCs, rely on expression of interferon regulatory factor (IRF) 8 [10] and PU.1 [11]. BATF3, an AP1 family member, is required for development of $CD8^+$ DCs, although it is also expressed by some $CD4^+$ DCs [12]. *Batf3*-deficient mice are only lacking mature $CD8^+$ DCs, not their precursors. cDC populations are also dependent on a variety of TFs, including ID2 [13], [14] and IRF4 [10], [15]. $CD103^+$ migratory DCs also rely on expression of BATF3 and ID2 [13], [14], [16], [17]. Spi-B and PU.1, both *E-twenty-six* (ETS) family members, are required for pDC development [10], [18]. In addition, E2-2 (encoded by *Tcf4*) was also reported to be critical for pDC maturation [19], [20]. E2-2 is a basic helix–loop–helix TF which was demonstrated to control the expression of Spi-B and IRF-8, among other pDC-associated genes [19]. Interestingly, a human disease (Pitt–Hopkins syndrome) characterized by, among other symptoms, low interferon responses is associated with haploinsufficiency of the *TCF4* locus [19], [21]–[23].

3 Dendritic Cells and Cancer

During tumor development, a microenvironment develops that promotes growth and metastasis. This tumor microenvironment (TME) is comprised of tumor cells, inflammatory cells, and other stromal cells such as fibroblasts and pericytes. The complexity of the TME differs among tumors, presumably due to anatomic location, original tissue composition, state of differentiation of the tumor, growth factors expressed within the TME, and other factors which are poorly understood. Among the inflammatory cells that infiltrate the tumor are both lymphoid and myeloid cells. T cells are a major component of the infiltrating leukocytes, and while some of the tumor-infiltrating lymphocytes (TILs) are effector T cells, most are either hyporesponsive (tolerant) or exert suppressive activity, including regulatory T (Treg) cells. This may include naturally occurring Treg cells or effector T cells which are induced to become suppressive within the TME. The suppressive activity may be mediated by both cell contact-dependent and -independent mechanisms. The TME also contains tumor-associated macrophages (TAMs) which have a phenotype consistent with alternatively activated or M2 macrophages. TAMs often exert their suppressive activity on T cells through expression of enzymes which catabolizes or sequester amino acids critical for maintaining durable T cell responses. A close relative of the TAM and neutrophil, the myeloid-derived suppressor cell (MDSC), may also accumulate within the tumor or in the peripheral lymphoid tissues. MDSCs

are poorly differentiated myeloid cells which suppress T cells via arginine catabolism and elaboration of reactive oxygen and nitrogen species (reviewed in this volume by Bronte et al.).

DCs which infiltrate tumors are generally perceived to be immature or immune suppressive. While DCs may initially infiltrate the tumor as a means of immune surveillance, their functional capacity is usually restricted. Cytokines that are produced in the TME, such as interleukin-6 (IL-6), TGF-β, vascular endothelial growth factor (VEGF), and IL-10, along with prostaglandins, maintain or induce an immature phenotype and suppressive function. While one study suggested that cDCs that have a mature phenotype are prognostic of a more favorable outcome [24], another study suggested that accumulation of pDCs conferred poorer prognostic value [25].

Tumor-associated DCs (TADCs) are capable of suppressing T cell immunity (reviewed in this volume by Shurin et al.). Some TADCs, specifically those with a pDC phenotype, suppress T cell immunity through expression of indoleamine-2,3-dioxygenase (IDO), an enzyme which catabolizes tryptophan, an amino acid critical for T cell responses and produces kynurenine, which exerts immune-suppressive activity, as well [26]. Expression of IDO by pDCs in sentinel (tumor-draining) lymph nodes was shown to be associated with a poor prognosis [27], underscoring the impact of this immune-suppressive mechanism. Other TADCs express arginase, which catabolizes arginine which, in turn, directs downregulation of the T cell antigen receptor-associated CD3−ζ chain, crippling T cells [28]. Finally, TADCs may suppress T cell immunity through expression of ligands which bind inhibitory receptor on T cells. This includes ligands for PD-1 (PD-L1 and PD-L2), which inhibit T cell responses and may induce T cell "exhaustion" [29]. Triggering of LAG-3 [30], [31] and TIM-3 ([32], reviewed in another chapter in this volume) by TADCs may also contribute to immune suppression and reduce immunity to tumor antigens.

4 NF-κB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that has a wide range of target genes, and hence functions, in many cell types. Not surprisingly, NF-κB is an important TF for DC biology and has effects on DC survival and function as related to their ability to elicit antitumor immune responses. Therefore, effects of the TME on the activation of NF-κB expressed by DCs can have major effects on evasion of the immune response by a tumor.

There are five proteins of the NF-κB family: NF-κB1, NF-κB2, RelA, RelB, and c-Rel [33]. All of these proteins have a Rel homology domain (RHD), which contains the regions responsible for DNA binding, dimerization, and nuclear localization [33]. NF-κB exists as either a homo- or heterodimer, and, when inactive, is localized to the cytoplasm. This cytoplasmic restriction is due to interactions with inhibitor of κB proteins (IκB), which form a complex that masks the nuclear localization signal. During one type of activation, IκB Kinase can phosphorylate the IκB proteins, causing their degradation, and hence exposing the NF-κB nuclear localization signal.

NF-κB can then enter the nucleus and is able to affect gene expression. An alternative type of activation (noncanonical) involves the processing of NF-κB2, which is synthesized as a larger precursor protein, into the active subunit. This involves activation of NF-κB inducing kinase (NIK), which phosphorylates NF-κB2, causing its processing.

4.1 NF-κB and DC Development/Function

NF-κB plays an important role in the development of DCs, and RelB-deficient mice have an altered repertoire of DCs [34]. NF-κB is also central for the DC maturation process [35], which enhances their ability to stimulate T cell responses; during this process, MHC II and co-stimulatory molecules are upregulated, DCs gain the ability to migrate to the lymphoid organs, and they express pro-inflammatory cytokines, such as IL-12 [36]. Hence, activation of NF-κB enables DCs to stimulate antitumor T cell responses. This was directly demonstrated by Labeur et al. in a study which examined bone marrow-derived dendritic cells (BMDCs) stimulated in vitro to varying maturation states and demonstrated that when transferred to tumor-bearing mice, the more mature DCs were the most effective at inducing antitumor immunity [37]. In support of this, another study boosted DC function by genetically modifying the cells to have enhanced NF-κB signaling through overexpression of a truncated RelA. These DCs had higher levels of maturation markers and increased cytokine production, and so they were more effective at inducing antitumor immunity [38].

Due to these immune stimulatory effects of NF-κB signaling in DCs, it could be beneficial for tumor growth if NF-κB were inhibited. This appears to be an immune evasion strategy utilized by tumors, as DCs in the TME usually display an immature phenotype, which is indicative of low NF-κB activation [39]. One mechanism which could inhibit DC activation of NF-κB is the production of vascular endothelial growth factor VEGF by tumor cells [40]. Studies have demonstrated a role for VEGF in blocking the maturation of stem cells to DCs via inhibition of NF-κB. As a consequence of VEGF-induced signals, a systemic reduction in DCs was also noted [39]–[41]. More recent studies extended those findings and reported that immature DCs loaded with myeloma lysates show defective maturation, leading to a reduced capacity to stimulate T cell responses; strikingly, these defects were not seen if VEGF was neutralized [42]. VEGF may mediate this effect by activating signal transducer and activator of transcription 3 (STAT3) (Fig. 9.1), which can inhibit NF-κB [42], [43]. The mechanism of NF-κB inhibition by STAT3 may be due to direct interaction and inhibition [44], [45]. Other tumor-generated cytokines may also mediate similar effects through STAT3 activation. Accordingly, tumors can express IL-6 and IL-10, which both activate STAT3 [43]. While IL-6 mediated STAT3 activation is known to affect DC maturation [46], IL-6 can activate NF-κB in some situations [47], and so the cross talk between these pathways is complex.

Foxp3$^+$ Treg cells are an immunosuppressive population known to infiltrate tumors. Production of IL-10 by Treg cells (among others) may activate STAT3 in DCs

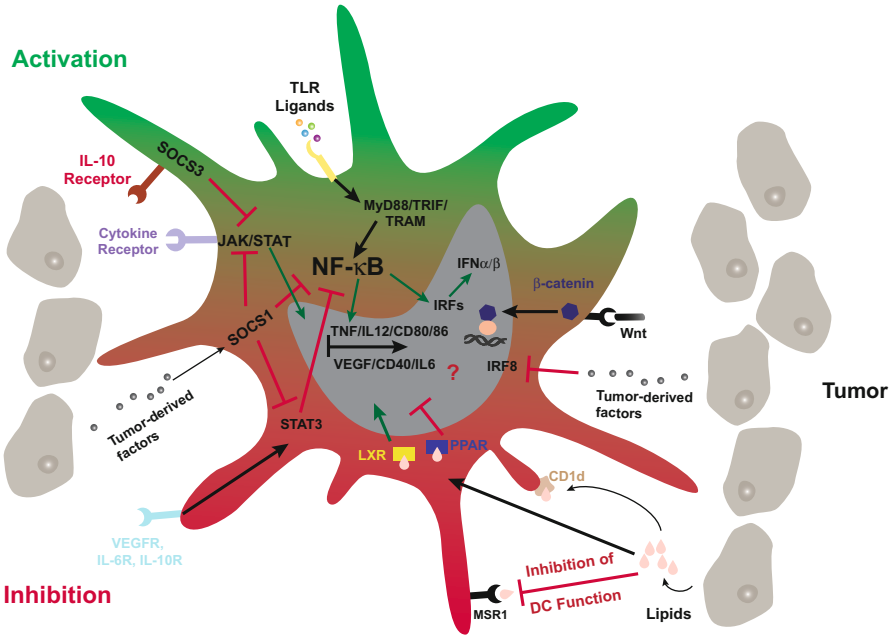

Fig. 9.1 Dendritic cell expression of transcription factors is influenced by the tumor microenvironment. Extracellular signals such as cytokines, TLR ligands, and lipids can deliver signals which induce expression of transcription factors. In addition, as-yet undefined tumor-derived "factors" can also influence transcription factor expression. The complex signaling pathways that are induced can either acitvate DCs to become stimulatory to the immune system, or become suppressive

and hence inhibit NF-κB, leading to suppression of DC function [48]. These findings demonstrate that, while tumor cells may affect NF-κB activation within DCs directly, other cells within the TME can also contribute to inhibiting DCs, and it is likely that any cells generating IL-10 or IL-6 could exert this effect.

Despite a clear role for NF-κB activation in DC development and maturation, it has also been demonstrated that the production of some pro-inflammatory cytokines by DCs can aid tumor growth and that these cytokines are produced in response to NF-κB activity. Therefore, in this context, NF-κB activation in DCs is beneficial for tumor growth. IL-6 is an example of one of these cytokines, as it has a positive effect on tumor growth and is produced in response to NF-κB signaling [49], [50]. Although this role appears to be at odds with the other mechanisms discussed, it may be more important for inflammation-induced cancers, such as colitis-associated gastrointestinal cancers [50].

5 STATs

STATs are a family of transcription factors composed of seven recognized members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, which share structural homology [51]. These powerful transcription factors integrate signals from

cytokines and growth factors resulting in the regulation of genes associated with cellular differentiation, proliferation, immune function, and apoptosis [52]. STATs are thought to affect DC function in a variety of ways, including development, maturation, expression of co-stimulatory molecules, and antigen cross-presentation [53]–[57].

STATs exist in latent, inactive forms in the cytoplasm until they are activated by phosphorylation by upstream tyrosine kinases, such as Janus kinases (JAKs) [51]. Typically, JAK-mediated phosphorylation is triggered by the binding of cytokines or growth factors to their receptors which subsequently results in the activation of the JAKs via dimerization or, in some cases, oligomerization [58]. There are certain growth factor receptors, such as the epidermal growth factor (EGF) receptor, that utilize intrinsic receptor tyrosine kinase activity to directly activate STATs. Other mechanisms for stimulating STAT activity have also been documented, including a variety of tyrosine kinases that are not associated with receptors, for example, Src and Abl [59], [60]. Upon activation, STATs dimerize and translocate to the nucleus where they bind specific DNA sequences to regulate gene expression [51], [61]. In the past 15 years, growing evidence has implicated some individual STATs with cancer development. In particular, substantial evidence implicates STAT3 as playing a role in both tumor development as well as altering DC function in cancer.

5.1 STAT1

STAT1 is best known for its ability to transmit signals from interferons, both type I and type II, however, a variety of other cytokines can also activate STAT1 [62]. STAT1 signaling is important in the maturation of DCs and is needed to maintain expression of CD40 and CD11c [63]. Complete activation of STAT1 requires both IL-4 and granulocyte-macrophage colony-stimulating factor (GMCSF) in the context of DC maturation [63]. Interestingly, inhibition or downregulation of STAT1 was associated with a tolerogenic or regulatory phenotype in DCs characterized by $CD40^{lo}$ IL-10^{+} [64]. Although STAT1 signaling is not well described in DCs in the tumor setting, it can be postulated that enforcing STAT1 expression in DCs would be useful in maintaining a mature phenotype in DCs. The longer that mature stimulatory DCs can be sustained in the TME, the longer they can provide support for endogenous and therapeutic antitumor immune responses.

5.2 STAT3

STAT3 is a major mediator of IL-6 signaling, as well as IL-2, IL-10, G-CSF, and GM-CSF [65], [66]. STAT3 upregulates genes involved with proliferation (cyclin D1 and Myc), suppression of apoptosis (Bcl2, MCL1, bcl-xl, survivin, and Hsp70) and angiogenesis (VEGF) [67]–[71]

Studies in patients with hyper immunoglobulin E (IgE) syndrome revealed evidence linking STAT3 to IL-10 signal transduction in DCs. These patients harbor

STAT3 mutations which affect STAT3 signaling and DNA-binding capacity. DCs from these patients had a decreased ability to respond to IL-10 and were less likely to become tolerogenic [72]. Applying this concept to cancer immunotherapy, blockade of IL-10 in the TME would relieve tolerogenic pressure on DCs, allowing them to respond in a more immunogenic fashion. Early studies from the laboratories of Trinchieri and Colombo revealed that blockade of IL-10 enhanced tumor immunity [73], [74]. More recently, Liu et al. investigated this question directly by treating DC cultures with exogenous IL-10 which resulted in decreased IL-12 production as well as downregulation of markers of activation such as CD80 and CD86 [57]. In contrast, blocking IL-10 through the use of a neutralizing antibody increased IL-12 production as well as upregulated activation markers.

STAT3 is required for the differentiation of cDCs and transduces survival signals in myeloid cells [75], [76]. pDC development is also dependent, in part, on STAT3 [77]. Onai et al. (2006) showed that forced expression of STAT3 in DC progenitor cells guides their differentiation into pDCs and cDCs [53]. Although required for DC development, STAT3 activation also plays a role in maintaining DCs in an immature state.

Immature DCs reinforce the tolerogenic milieu of the TME by their inability to initiate an effective immune response. Nefedova et al. (2004) demonstrated that treatment of DCs with tumor-derived M-CSF, VEGF, and IL-10 results in STAT3 activation in immature DCs [78]. Other groups have shown that STAT3 activation can inhibit the maturation of DCs and IL-12 secretion [57]. It is known that IL-6 signaling through STAT3 blocks DC maturation, thereby, maintaining DCs in an immature state [46]. These studies and others draw a direct line between STAT3 activation in DCs and the maintenance of the immature DC phenotype in the tumor setting (Fig. 9.1). Blocking tumor-derived factors, such as IL-6 or IL-10, which trigger STAT3 activation, may provide a means to allow DCs to develop a more immunogenic phenotype.

STAT3 plays a role in blocking DC activation through its influence on the NF-κB pathway [79]. Interestingly, Cheng et al. (2010) suggest that Notch signaling may trigger activation of STAT3 which in turn regulates NF-κB [55]. TMEs which cultivate Notch ligands thus have an avenue to inhibit DC activation, and targeting Notch ligands may provide a novel therapeutic means to reduce STAT3-mediated signals as well as to lessen the tolerogenic influence of the TME.

Early studies by Sotomayor et al. demonstrated that *Stat3*-deficient APCs were not only non-tolerogenic, but were capable of reversing T cell anergy [80]. More recent studies demonstrated that APCs expressing defective STAT3 are better able to prime cytotoxic T lymphocytes (CTLs) with tumor-associated antigens and have enhanced cross-presentation to $CD8^+$ T cells compared to wild-type (WT) APCs [56]. The $CD8^+$ T cells in turn proliferate more and express increased quantities of IFN-γ and tumor necrosis factor-α (TNF-α). Interestingly, Wolfle et al. (2011) revealed evidence that STAT3 may directly induce a tolerogenic phenotype in DCs by upregulating expression of PD-L1 [81]. Since PD-L1 is a key inducer of T cell dysfunction, disruption of STAT3 signals might reduce tolerogenic burden by lowering PD-L1 levels. Taken together, these studies reveal STAT3 as a powerful target for enhancing immunity to tumor antigens.

Pioneering efforts by Yu and colleagues have led to the development of an effective molecular platform to silence STAT3 in DCs in vivo. Their system employs a STAT3 siRNA which was conjugated to the TLR9 agonist, CpG [82]. When TLR9 is triggered along with ablation of STAT3 in DCs, they observed an increase in DC immunogenicity, as measured by increased effector function of adoptively transferred $CD8^+$ T cells in tumor models. T cells in these treatment groups showed increased killing, tumor infiltration, as well as upregulation of effector molecules such as IFN-γ [83]. A variety of other STAT3 inhibitors have been developed and are being tested both in vitro and in vivo [84]. Although *Stat3*-deficient mice are nonviable, and STAT3 is critical for hematopoiesis, nonmalignant cells do not rely solely on STAT3 for survival, using other pathways to provide compensatory signals they need to survive. Therefore, targeting of STAT3 may not seriously harm normal cells. Additional methods for STAT3 depletion exist and work well in cell culture and animal models, thereby, further supporting the plausibility of targeting STAT3 in cancer therapy.

5.3 STAT4

A role for STAT4 in DCs in tumor settings is not well described; however, some established features of this TF do suggest potential STAT4-based interventions for cancer therapy. STAT4 signaling in DCs is essential for the production of IL-12 which in turn guides a Th1 immune response [85], [86]. Clinical responsiveness to cancer immunotherapy is hampered, in part, by the predominance of Th2 over Th1 immunity in the TME. Enhancing STAT4 activation in DCs would enhance IL-12 production, which could encourage the development of a Th1 environment, which has been suggested by some investigators [85], [87].

6 SOCS

The suppressor of cytokine signaling (SOCS) genes encode a family of eight proteins that are able to negatively regulate cellular responses to cytokines. All members of this family contain an SH2 domain and a C-terminal SOCS box domain, which allow binding to phosphotyrosines and deliver ubiquitin ligase activity, respectively [88]. Mechanisms used by the SOCS proteins to regulate inflammatory gene expression include acting as a pseudo-substrate for JAK, ubiquitinating proteins in the JAK–STAT signaling pathway, hence promoting their degradation or binding and inhibiting cytokine receptors directly. For instance, the SOCS family member cytokine-inducible SH2 protein-3 (CIP) can bind and inhibit the erythropoietin receptor (EPOR) [88]–[91].

The SOCS proteins are expressed in DCs; SOCS1, 2, and 3 are induced as DCs mature [92]. As the SOCS family restricts cytokine signaling, its members have a

negative effect on the ability of DCs to stimulate immune responses. SOCS1-deficient mice die within 3 weeks due to excessive T cell activation, and specifically silencing SOCS1 in DCs makes them better at stimulating CD8 T cell responses, both in vivo and in vitro [93], [94]. These SOCS1-silenced DCs were more mature and were overreactive to IFN-γ or IL-4 stimulation [93].

SOCS1 expression by DCs may be a regulator of antitumor immunity. Transfer of antigen-loaded DCs is a common method of inducing antitumor immunity in tumor models, and silencing SOCS1 in DCs was reported to enhance tumor vaccine efficacy resulting in reduced growth of tumors [95]. This was tested both with tumors expressing the model antigen ovalbumin (OVA) and with mice treated with a DC vaccine presenting a physiological antigen specific for melanoma [95]. These findings demonstrate that upregulation of SOCS1 in DCs within the TME may be a mechanism of immune evasion by tumors. Although the precise mechanism by which SOCS1 restricts DC priming is unclear, it is interesting that SOCS1 has been reported to affect the NF-κB pathway directly [96]. For instance, SOCS1 has E3 ubiquitin ligase activity that enables it to promote the degradation of RelA [96]. In support of this proposed ability to directly affect RelA, SOCS1 has been demonstrated to bind to RelA as part of a larger multimeric protein complex [97]. Thus, while SOCS1 is not a transcriptional regulator itself, its inherent activity may alter gene expression in DCs associated with tumors.

DCs can have a regulatory phenotype, and skewing towards this state would be beneficial for the tumor. The SOCS proteins have a potential role in this process as SOCS3 was found to be fundamental for the generation of a regulatory DC subset [98]. This was demonstrated by the silencing of SOCS3 in progenitor cells, which resulted in the development of fewer regulatory DCs [98]. SOCS3 was shown to be activated by IL-10, which caused signal transduction by the JAK–STAT pathway and epigenetic modifications that increased SOCS3 expression, causing the DCs to diverge into a suppressive phenotype [98]. Upon in vivo induction of these regulatory DCs, inflammatory responses were suppressed [98], and so the IL-10-rich environment of the tumor could lead to the production of these immune-suppressive DCs and hence a suppression of antitumor immunity. SOCS3 expression by tumor-associated DCs has been described as well [99], [100], thereby implicating this pathway in the induction of similar regulatory properties for DCs in tumors.

7 Lipids and DCs

Lipids are primarily known for their role in cell membrane integrity and energy storage. However, they also play a vital role in signaling events that affect cellular differentiation and function [101]. DCs are often exposed to large amounts of lipids, such as host- and pathogen-derived lipoproteins, lipids released by apoptotic cells, as well as dietary lipids, fatty acids, retinoids, and cholesterol in the gut-associated lymphoid tissue [101], [102]. These lipid derivatives are detected within the microenvironment by several mechanisms. TLR have been shown to sense lipids, such as the recognition of lipopolysaccharide (LPS) by TLR4, resulting in cytokine production by DCs

[103], [104]. Interestingly, CD1 on DCs not only recognizes extracellular lipids, but presents them to T cells, as well [105]. There is also a well-documented role of nuclear hormone receptors, particularly the peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), in detection of lipids in the environment [106]. Upon binding of lipids, these receptors are then able to recruit co-activator or co-repressor complexes to regulate the transcription of target genes [107].

7.1 DC Regulation by PPARs and LXRs

PPARγ activation in DCs by fatty acids or synthetic ligands, such as rosiglitazone or troglitazone, leads to a reduction in pro-inflammatory cytokine production (including IL-12, TNF-α, IL-15, and IL-6), diminished CD80 expression with enhanced CD86 levels, reduced IκB and NF-κB activity, and favored T_H2 differentiation [102], [108]–[110]. PPARγ activation also leads to decreased CD40 and CD83 expression in DCs, thus rendering the cells less stimulatory [109] (Fig. 9.1). Furthermore, a reduction in the cluster of CD1 proteins, which are capable of presenting lipid antigen derived from host or pathogenic microbes and are essential for the expansion of invariant natural killer T cells (iNKT), was observed following activation of DC by PPARγ [108], [111]. Metabolically, PPARγ activation induces CD36 and adenosine triphosphate (ATP) binding cassette-1 (ABCA-1) expression, which is involved in lipid and cholesterol efflux, as well as increased LXR, which can also regulate cholesterol trafficking, and although less understood in DC function, increased fatty acid binding protein 4 (FABP4) expression has been observed [108], [112].

LXRs, particularly LXRα, have also been shown to be expressed by mouse and human DCs. Under physiological conditions, activation of LXRs via ligation of agonists, such as cholesterol, leads to an upregulation in CD80/CD86 expression and increased pro-inflammatory cytokine production (including IL-12, TNF-α, IL-6, and IL-8), resulting in enhanced T cell activation [113]. LXR also has a key role in cancer. Recent reports demonstrated the release of cholesterol metabolites from various mouse and human cancer cell lines, which upregulated and activated LXR on DCs, inhibited DC migration into lymphoid organs via a reduction in CCR7 expression [114]. This will undoubtedly have a pronounced effect on T cell priming to tumor antigens. Previous studies also confirmed that PPARγ activation downregulated CCR7 on DCs, a phenomenon that may be, in part, due to PPARγ-mediated upregulation of LXR [115]. Furthermore, reconstitution of WT mice with LXRα deficient bone marrow, or the blockade of LXRα signaling, slowed tumor growth significantly due to the reacquisition of DC migration and inflammation. However, this phenomenon did not occur in immunodeficient mice, thus demonstrating the imperative role of DCs and T cells in this mechanism [114].

7.2 Lipids and DC Dysfunction

Only recently has the role of lipids in tumor-infiltrating DC function been examined. Studies using experimental mammary and colon cancer models (CT26), as well as

models of thymic lymphoma (EL4), have demonstrated that DCs located within the tumor bed, draining lymph node, or at distant sites, such as the spleen and peripheral blood, have an increase in intracellular lipid concentration when compared to DCs of non-tumor-bearing mice [116]. Both $CD11c^{+}CD11b^{+}B220^{-}$ DCs and $CD11c^{+}CD8\alpha^{+}$ DCs were reported to have higher levels of triglyceride accumulation in tumor-bearing mice. In contrast, pDCs did not accumulate unusually high levels of lipids. Additionally, DCs from patients with non-small-cell lung, head and neck, and renal cell carcinomas displayed an increase in intracellular lipid concentration [116]. BMDCs that were treated with EL4, CT26, or B16F10 tumor supernatants showed a sixfold increase in lipid accumulation, specifically in triacylglycerol levels, which was associated with a significant upregulation of the scavenger receptor macrophage scavenger receptor 1 (MSR1, also known as SRA or CD204) on BMDCs. Tumor-bearing mice displayed elevated MSR1 on DCs from peripheral blood, spleen, and draining lymph node, along with an even higher expression level in DCs isolated from the tumor. MSR1 plays a vital role in the intracellular transport of lipids and is also capable of inhibiting immunostimulatory signals mediated by TLR-4 [117] (Fig. 9.1). Treatment of DCs with fucoidan, a soluble ligand that inhibits binding of natural ligands to scavenger receptors, and blockade of MSR1 with a neutralizing antibody inhibited the uptake and accumulation of lipids in tumor supernatant-treated DCs. These data suggest that upregulation of MSR1 on DCs is crucial for increased intracellular lipid concentrations; however, the tumor-derived factors that induce MSR1 expression remain elusive.

Herber et al. further investigated antigen recognition, processing, and presentation in lipid-laden DCs, concluding that lipid accumulation rendered DCs incapable of processing and loading antigen onto MHC complexes, and thus making them inefficient at stimulating tumor-specific T cell responses [116]. Exactly how elevated intracellular lipids interfere with these functions remains to be determined. However, an inhibitor of acetyl-CoA carboxylase, 5-(tetradecycloxy)-2-furoic acid (TOFA), which blocks the synthesis of triacylglycerol despite the increased lipid uptake, was tested [116]. DCs that were cultured with tumor supernatant in the presence of TOFA showed reduced lipid accumulation and an improved ability to stimulate T cells. The same effects were observed in EL-4 tumor-bearing mice treated with TOFA. Furthermore, combination of TOFA with tumor antigen-pulsed DCs as a vaccine resulted in a marked decrease in tumor growth, compared to TOFA or vaccine alone.

These data suggest that inhibition of lipid uptake and triacylglycerol synthesis in DCs using TOFA could yield effective and potent antitumor T cell responses. In addition to TOFA's direct effect on DCs, studies have shown that acetyl-CoA inhibitors have direct cytotoxic effects on tumor cells, which can stimulate the release of tumor antigens and further enhance antitumor T cell responses [116], [118]. Taken together, this recent work has demonstrated how inhibition of metabolic enzymes and lipid accumulation in DCs may reverse tumor-induced immune suppression.

8 Interferon Regulatory Factors and DCs

Regulation of cytokine gene expression is carefully regulated in most cell types. The interferon regulatory factors (IRFs) are a family of transcription factors encoded by nine mammalian genes which recognize a canonical sequence in the promoter of a variety of genes associated with inflammatory and immune responses (reviewed comprehensively in [119]). The DNA binding domains contain a conserved tryptophan repeat sequence which binds to a consensus DNA sequence with high homology to the interferon-stimulated response element (ISRE). IRFs mediate diverse inflammatory signals, both stimulatory and inhibitory, by activating or repressing gene expression, respectively. For example, signals delivered by viral infections, PRRs like TLRs, and cytokines induce IRFs that, in turn, induce expression of a variety of inflammatory mediators. In contrast, some cytokine-regulated signals can induce IRF-1 expression which, in turn, represses IL-4 [120] and FoxP3 expression [121].

IRFs are also critical for the development of various DC subsets. Development of cDCs is dependent on IRF-1 [122], IRF-4 [15], and, in part, IRF-8 [123]. Both IRF-1 and IRF-8 are required for $CD8\alpha^+$ DCs [122], [124]. In contrast, IRF-1 inhibits pDC development [122], which, like $CD8\alpha^+$ DC development, is also dependent on IRF-8 [124]. Interestingly, cDCs which mature in the absence of IRF-1 develop a tolerogenic phenotype [122], expressing elevated levels of IDO, IL-10, and TGF-β. IRF-2, along with IRF-4, is required for the $CD4^+$ DC subset [125].

Not surprisingly, IRFs are critical for DC activation in response to pathogens. cDCs and pDCs express TLRs which signal through the adaptor protein myeloid differentiation primary response gene (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), and/or TRIF-related adapter molecule (TRAM), which results in activation of NF-κB, MAP Kinase, and IRF expression, in turn leading to cytokine gene expression. TLRs 3 and 4 activate IRF-3 and -7; TLR4 signals in a TRAM/TRIF-dependent activation of the classical NF-κB pathway. TLRs 7, 8, and 9 activate IRF-4 using the MyD88 pathway. In pDCS, IRF-7 is the principal mediator of TLR9-induced type I interferon expression [126], [127] (Fig. 9.1). TLR9 signals also activate IRF-8, which can produce both IL-12 and type I interferon [128], [129]. IRF-1 also contributes to TLR 9-induced expression of type I interferon through direct binding to MyD88 [130], [131]. IRF-4, and -5 are both associated with interferon expression following MyD88 activation, although IRF-4 and -5 may compete for MyD88 binding and cross-regulate each other to regulate cytokine gene expression [132]. The complexity of these signals and the counterregulatory mechanisms underscore how important this family of genes is in regulating inflammation and immunity.

While TLRs and cytokines lead to activation of immune responses, the TME generally promotes immune suppression. The infiltration and accumulation of DCs in the TME are a common feature of many cancers. As described above, tumor-associated DCs (TADCs) display altered stimulatory capacity and are capable of tolerizing T cells. It remains unclear how TADCs acquire this suppressive function,

but given their critical role in DC development and function, altered expression of IRFs is a logical mechanism.

In one study, Shurin et al. studied the effect of tumor cells on DC function [133]. Using a model where DCs prepared from bone marrow were treated with a culture supernatant from a murine prostate tumor cell line (referred to as tsDC), the authors reported that the loss of T cell stimulation by tsDC was associated with reduced expression of genes associated with antigen processing and antigen presentation by class I MHC. Similarly, reduced expression of IRF-8 was noted in tsDC (Fig. 9.1). More strikingly, silencing IRF-8 by siRNA led to reduced T cell priming and diminished levels of genes associated with antigen processing and presentation, consistent with the tsDC phenotype and function.

As mentioned above, IRF-8 is important for DC development [10]. Interestingly, IRF-8 is an interferon gamma-induced transcription factor known to promote expression of inflammatory cytokines and MHC expression [134], [135]. Clearly, based on these observations, down regulation of IRF-8 would presumably lead to DCs which have reduced inflammatory properties and might lead to T cell dysfunction. However, another study also reported that loss of IRF-8 led to reduced IDO expression and reversal of a tolerogenic phenotype in DCs, although in that report, DCs were not studied in the context of tumor development [136]. Therefore, the role of IRF-8 in controlling TADC function may be more complex than originally reported.

More recently, another group reported a different role for IRF-8 in control of tumor growth [137]. Melanomas implanted into *Irf8*-deficient hosts grew faster and were more lethal than those implanted into WT hosts. This was associated with reduced infiltration of both DCs and T cells, and an increase in accumulation of MDSCs. While neither DC-specific deficiency nor DC function were addressed, the findings reported do confirm that stromal expression of IRF-8 is important to maintain a strong inflammatory response that can contribute to immune surveillance of tumors. Of course, the results need to be interpreted carefully because, as described above, *Irf8*-deficient mice have defects in DC development [10], [138].

9 Wnt-β-Catenin Axis

The Wnt signaling pathways have been conserved throughout evolution and play crucial roles in embryonic development, cell fate determination, and tissue homeostasis [139]–[142]. As a result, mutations or dysregulation of components of the Wnt signaling pathway have been linked to a wide range of human diseases including cancer [143]. The best-studied Wnt pathway is the canonical Wnt/β-catenin-T cell factor (TCF) signaling pathway, which activates the function of β−catenin as a transcriptional co-activator in the nucleus that, in turn, controls key developmental gene expression programs, in addition to its role in regulating cadherin-dependent cell adhesion [144]. The majority of β-catenin is localized to the cell membrane in resting cells, for example, bound to the cytoplasmic domain of E-cadherin in epithelial cells [145]–[148]. In the absence of Wnt, the cytosolic pool of β-catenin protein

dissociated from the E-cadherin/β-catenin complex is further attenuated by its phosphorylation by glycogen synthase kinase 3β (GSK-3β) and casein kinase Iα (CK Iα), leading to its ubiquitination and subsequent proteasomal degradation [146], [149]. Upon binding of Wnt proteins to their receptors and co-receptors, phosphorylation of β-catenin is inhibited, leading to accumulation and translocation of β-catenin into the nucleus where it forms complexes with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to activate target genes, such as Myc and cyclin D. As β−catenin serves as an essential component in both the canonical Wnt pathway and E-cadherin-mediated adhesion [150], it is not surprising that activation of β-catenin has been reported in a variety of human tumors including prostate carcinoma, melanoma, ovarian carcinoma, pancreatic and colorectal carcinoma [151]. Activation of β-catenin in tumor cells could suppress antitumor immunity under certain conditions, as activation of β-catenin in human melanoma cells has been shown to promote IL-10 expression which impairs CTL function of melanoma-specific $CD8^+$ T cells [152].

9.1 β-Catenin in the Immune System: DC Development and Function

In the immune system, the canonical Wnt/β-catenin-TCF signaling pathway plays a critical role in lymphopoiesis and hematopoiesis and in the self-renewal of hematopoietic stem cells [153]–[155]. For T cells, recent studies have shown that the β-catenin-TCF pathway plays a critical role in regulating the function of mature $CD8^+$ and $CD4^+$ T cells besides its well-documented role in T cell development [156]–[158]. Activation of β-catenin was implicated in promoting tolerance in $CD4^+$ T cells, as constitutively active β-catenin has been shown to enhance the function and survival of regulatory $CD4^+$T cells [159]. In DCs, expression of β-catenin was previously reported in LCs, as a structural component of adherens junctions (AJ) [160], [161] and has been shown to play a role in DC development [162].

The role of β-catenin activation in regulating DC function in tolerance versus immunity was not elucidated until recently. We have surprisingly found that β-catenin and E-cadherin are highly expressed in both human and murine-cultured DCs [163], suggesting that expression of E-cadherin/β-catenin system is not limited to LCs (see reference [164] for review on E-cadherin/β-catenin in DCs). Indeed, E-cadherin expression was recently shown in populations of DCs from spleen, blood, and lymph nodes and marks a subset of colitogenic DCs [165]. Consistent with earlier studies showing that disruption of LC–LC interactions triggers maturation of these DCs [166]–[168], disruption of E-cadherin-mediated adhesion activates β-catenin to induce DC maturation. However, DCs matured through E-cadherin/β-catenin signaling exhibit a transcriptional profile distinct from DCs matured by TLR ligation, leading to increased expression of multiple β-catenin-TCF target genes. More importantly, TCF-dependent transcription is required for β-catenin-induced DC maturation, as

overexpression of constitutively active β-catenin, but not a truncated form lacking its C-terminal transcriptional transactivation domain, induces DC maturation.

Interestingly, these matured DCs fail to produce immunostimulatory cytokines and elicit an entirely different T cell response in vivo, generating T cells with a regulatory as opposed to an effector phenotype. DCs matured through β-catenin activation induce $CD4^+$ T cell tolerance in both an OVA sensitization model and the murine model of experimental autoimmune encephalomyelitis (EAE), suggesting that β-catenin activation in DCs may serve as a signal to tolerize DCs [163]. Consistent with this notion, targeted deletion of the β-catenin gene in DCs results in a reduction of tolerance and susceptibility to chronic inflammation [169]. Of note, both studies confirmed the expression of key elements of the Wnt signaling pathway including Wnt ligands and their receptors [163], [169]. Other studies have also confirmed β-catenin as a distinct maturation signal to generate tolerogenic DCs [170]. Thus, activation of β-catenin-TCF pathway in DCs could program DCs to become tolerogenic to suppress T cell immunity. Given that tumors often induced immunosuppression, it is conceivable that tumors might suppress antitumor immunity by modulating β-catenin-TCF pathway in DCs.

9.2 β-Catenin Expression and Tolerogenic DCs in Cancer

As these studies primarily examined the impact on function of $CD4^+$ T cells, it remains unclear whether β-catenin/TCF signaling in DCs functions similarly to regulate $CD8^+$ T cell immunity that is critical for antitumor immunity. Perhaps more importantly, an unanswered question was whether tumors suppress $CD8^+$ T cell immunity by activating β-catenin signaling in DCs, and if so how activation of β-catenin in DCs suppresses antitumor $CD8^+$ immunity. We have found elevated protein expression of β-catenin and increased TCF-dependent transcription in DCs in the lymphoid organs from mice bearing B16 melanoma (Jiang and Fu, unpublished observations). We further found that antigen-specific $CD8^+$T cells primed in these mice resulted in dampened $CD8^+$ memory responses (Jiang and Fu, unpublished observations). Taken together, our unpublished data suggest that β-catenin-mediated inhibition of cross-priming represents a new and potentially general mechanism that tumors employ to achieve immunosuppression of $CD8^+$ T cell-mediated immunity. Studies are underway in our laboratory to examine whether the $CD4^+$ T cell-mediated immunity is also suppressed by tumor-induced β-catenin activation.

How tumors activate β-catenin in DCs is not currently known. In vitro treatment with conditioned media from multiple tumor cells results in increased protein levels of β-catenin in DCs and activates TCF-dependent transcription in DCs expressing the TOP-GFP (GFP under the control of six copies of a TCF/LEF response element) reporter (unpublished observations), suggesting that a soluble factor or factors likely mediate tumor-induced activation of β-catenin. Previous studies have suggested Wnt ligands, cytokines, and E-cadherin signaling as potential candidates [163], [171], [172]. Wnt ligands, however, are probably not the factors responsible

for the B16-mediated activation of β-catenin that we have observed, as previous studies have shown that B16-conditioned media did not contain active Wnt ligands that lead to activation of β-catenin/TCF-dependent transcription [173]. TGF-β1 has recently been shown to activate β-catenin to promote the differentiation of epidermal Langerhans DCs [171]. Most likely, different tumors might utilize multiple different factors including Wnts and TGF-β to activate β-catenin in DCs to suppress host T cell immunity.

9.3 *Mechanisms of β-Catenin-Mediated Regulation of DC Function*

Although previous studies have shown a role for β-catenin in regulating DC maturation and cytokine production, another unaddressed but important question is how β-catenin inhibits the ability of DCs to cross-prime antigen-specific $CD8^+$ T cells [163], [169], [170], [174]–[176]. As TCF-dependent transcription has been shown to be required for β-catenin-induced DC maturation [163], it will be important to determine whether TCF-dependent transcription is also required for β-catenin-mediated inhibition of cross-priming. The identification of forkhead box O3 (FOXO3) as a factor involved in regulating DC function [177] raises an important possibility, as previous studies have shown an evolutionarily conserved interaction of β-catenin with FOXO3 in response to oxidative stress, which leads to enhanced FOXO3 transcriptional activity [178], [179]. Interestingly, interaction of FOXO3 and β-catenin reduces the binding of β-catenin to TCF, resulting in the inhibition of TCF transcriptional activity [180]–[182]. Thus, cross talk between FOXO3 and β-catenin-TCF in DCs might ultimately determine DC function in tolerance versus immunity. Indeed, DCs from DC-β-catenin active mice exhibit elevated expression of FOXO3 (Jiang and Fu, unpublished observation). On-going studies will investigate whether β-catenin works in concert with FOXO3 to program tolerogenic DCs that function to suppress $CD8^+$ T cell immunity by inhibiting cross-priming.

9.4 *Notch and β-Catenin*

Cross talk between Notch and Wnt signaling pathway could also affect β-catenin-TCF-dependent transcription, as Notch signaling has been shown to negatively regulate β-catenin-dependent transcription in stem cells by its direct association with active β-catenin [183]. Like Wnt, Notch is a highly conserved developmental pathway that functions in multiple developmental processes that govern normal morphogenesis, including proliferation, cell fate specification, differentiation, and survival [184], [185]. Notch signaling pathway has been linked to many cancers and could be either oncogenic or tumor suppressive depending on the cellular context [186]. Notch signaling has also emerged as an important regulator of immune cell

development and function, including generation and maintenance of hematopoietic stem cells, T cell development and function, and DC differentiation [187]–[189].

Cross talk between the Wnt and Notch pathways has been reported in many different systems that regulate multiple processes including vascular remodeling, maintenance of hematopoietic stem cells, and tumorigenesis by diverse mechanisms [190], [191]. The cross talk between Wnt and Notch signaling in DCs was reported as indicating that Notch and Wnt cooperate in regulating DC differentiation [162]. Activation of Notch signaling in hematopoietic progenitor cells promotes differentiation of conventional DCs via activation of canonical Wnt signaling, mainly via the upregulation of Wnt receptors [162]. Importantly, inhibition of β-catenin completely abrogates the effects of Notch signaling on DC differentiation, suggesting that Wnt signaling is downstream of Notch in regulation of DC differentiation [55], [162].

These findings raise the interesting question whether cross talk between Wnt and Notch pathways similarly regulates DC function in tolerance. Indeed, several studies have suggested the involvement of Notch signaling in DC-mediated T cell tolerance [192]–[196]. For example, overexpression of the Notch ligand Jagged 1 in splenic DCs directs $CD4^+$ T cells to a regulatory phenotype [192], and DCs activated by Jagged 1 promote the survival, proliferation, and suppressive capacity of regulatory T cells [196]. In colorectal cancer, cross talk between Notch and Wnt signaling, specifically Notch activation by β-catenin-mediated upregulation of Jagged 1, is required for tumorigenesis [197]. Thus, it would be interesting to determine whether Notch signaling is involved in β-catenin-mediated immunosuppression. Besides FOXO3 and Notch signaling pathway, interaction between β-catenin-TCF and other transcription factors such as AP-1, and cross talk with mechanistic target of rapamycin (mTOR), transforming growth factor-β (TGF-β) and hypoxia-induced signaling pathways have also been shown to regulate β-catenin-TCF-dependent transcription [181], [182], [198], [199]. Therefore, further studies are warranted to elucidate the underlying mechanisms by which Wnt/β-catenin-TCF signaling programs DCs to achieve immunosuppression in the TME, especially in the context of these other transcriptional activators.

10 Conclusions

Tumor development is a complex and heterogeneous process. For many cancers, inflammation is reported to be an integral component of this process [200]. Thus, the recruitment of immune and inflammatory cells contributes to an environment which fosters tumor initiation, growth, and metastasis.

DCs are an integral component of the TME and have a variety of functions for both adaptive and innate immune responses that shape tumor development. As transcription factors (TFs) regulate DC ontogeny and activation, it is not surprising that these TFs may also regulate DC phenotype and function in the TME. A delicate balance exists between stimulatory and suppressive activity of DC exists. Therefore,

TFs may represent critical regulators of this balance. Identification of "master regulators" that control DC function may reveal novel TF targets that could serve to alter tumor initiation or tumor progression.

However, the intricacies of the pathways that regulate gene expression require intense scrutiny. The interaction of multiple TFs that can regulate immune stimulatory and/or immune suppressive pathways adds an additional level of complexity that must be considered. For example, interactions between NF-κB and Wnt/β-Catenin [201], [202], which reciprocally control DC function, may provide unique yet unpredictable levels of regulation that could be targeted for maintaining DC stimulatory capacity. Similarly, TFs that contribute to DC differentiation and plasticity, such as IRFs, may also provide clues for enhancing immunity to tumors. By enforcing signals transduced by IRFs (including IRF-8), activation of a pathway that mimics TLR activation can either convert DC activity from suppressive to stimulatory, or simply maintain DCs in the latter state. Alternatively, cross-regulation of TF expression may also be a relevant consideration. This would include the somewhat ubiquitous role of NF-κB in regulating gene expression during immune and inflammatory responses. NF-κB is an upstream and downstream regulatory of many of the TFs described in this chapter and therefore its pleiotropic functions associated with DC function interact with many different regulatory mechanisms and transcriptional pathways. The downstream effects of NF-κB activation generally result in expansion of T cells and elaboration of pro-inflammatory cytokines.

Finally, the fact that TFs make very difficult druggable targets due to their nuclear localization also adds to the challenge of altering the "master regulator" functions of TFs in DCs. Therefore, a more complete understanding of these pathways, including signals that induce or inhibit TF expression, will permit more efficient ways of generating and maintaining durable antitumor immunity by inhibiting the complex array of suppressive signals in DCs.

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Chapter 10
S100A9, Inflammation, and Regulation of Immune Suppression in Cancer

Thomas Condamine, Indu R. Ramachandran and Dmitry I. Gabrilovich

Abstract Chronic inflammation plays a major role in tumor initiation, promotion of tumor growth, and induction of immune suppression. While inflammation and antigen-specific immune responses can be initiated to target the tumor for eradication, inflammation can also promote cancer development either directly by inducing genetic instability within the cancer cell or indirectly by promoting immune suppression. Such inflammation-induced immune suppression allows tumor cells to avoid immune surveillance. The S100A9 protein is one of the major mediators of inflammation. It belongs to the S100 family of Ca^{2+}-binding proteins and is produced primarily by myeloid cells. It has a pleiotropic effect on myeloid, endothelial, and tumor cells. In this chapter, we will discuss the contribution of myeloid cells as one of the main elements of the progression from inflammation to tumor and the role of S100A9 proteins in the regulation of inflammation and immune responses in cancer.

Keywords Myeloid-derived suppressor cells · $CD8^+$ T cells · $CD4^+$ T cells · S100A9 · S100A8 · Inflammation · Immune suppression · Cancer · Macrophages · Dendritic cells

1 Tumor-Induced Inflammation in the Regulation of Immune Suppression

Inflammation is necessary for the clearance of pathogens or abnormal cells as well as for tissue remodeling. During typical inflammation, innate and adaptive immune responses are initiated and act in collaboration to clear the danger signal, which ultimately leads to the resolution of the inflammation. When the immune system fails to eradicate the danger, inflammation is maintained resulting in chronic inflammation. In cancer, inflammation plays a dual role. While inflammation and

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antigen-specific immune responses can be initiated to target the tumor for eradication, inflammation can also promote cancer development either directly by inducing genetic instability within the cancer cell or indirectly by promoting immune suppression. Such inflammation-induced immune suppression allows tumor cells to avoid immune surveillance. It is now widely accepted that chronic inflammation plays a major role in tumor initiation, promotion of tumor growth, and induction of immune suppression [1], [2].

1.1 Tumor-Associated Inflammation

Chronic inflammation is now considered as one of the hallmarks of tumor development and progression [3], [4]. Tumor infiltration by immune cells has been described since the nineteenth century [5], [6], and, in recent years, a large body of literature has shown that immune cells can directly contribute to tumor growth, angiogenesis, tumor cell survival, and metastasis [7], [8]. Several mechanisms by which inflammation contributes to the initiation and promotion of cancer have been described. They include production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by inflammatory cells and stressed tissues which can induce DNA damage and pathological changes within the cancer cell [9]. Activation of several oncogenes like *ras* and *myc* in the cancer cells and surrounding stromal cells can lead to the increased production of various cytokines and chemokines, which contribute to the establishment of the tumor microenvironment [10], [11]. The tumor microenvironment initiates and sustains the recruitment of a wide array of immune suppressive cells: regulatory T cells [12], regulatory B cells [13], tumor-associated macrophages (TAMs) [14], tolerogenic or immature dendritic cells (DCs) [15], tumor-associated neutrophils (TANs) [16], and myeloid-derived suppressor cells (MDSCs) [17]. All these cells, in turn, also contribute to the formation of a tumor microenvironment by secreting a large amount of various inflammatory factors.

1.2 Myeloid Cells Within the Tumor Microenvironment

The myeloid cells within the tumor microenvironment are highly heterogeneous and include four main groups of cells: TAMs, DCs, TANs, and MDSCs [18]. Activation by interferon-γ (IFN-γ) usually drives the polarization of macrophages into M1 or "classically activated" macrophages, which secrete interleukin-12 (IL-12), drive Th1 polarization of T cells, and are considered tumoricidal. In contrast, TAMs are typically M2 polarized and secrete IL-4 and IL-13, which contribute to tumor progression via various mechanisms [19]. By secreting a large amount of type 2 cytokines, M2 macrophages can drive the differentiation of T helper cells into a Th2 phenotype.

DCs are terminally differentiated cells and are commonly considered professional antigen-presenting cells because of their high capacity to process and present antigens to naïve T cells. DCs in cancer are functionally defective since the tumor microenvironment blocks them in an immature state, thus strongly decreasing their ability to activate T cells [20]. DCs in the tumor microenvironment can also acquire a tolerogenic phenotype and suppress T-cell activation through various mechanisms including upregulation of the FOXO3 transcription factor [21], secretion of transforming growth factor beta (TGF-β) and IL-10, as well as depletion of tryptophan through the enzyme indoleamine-2,3-dioxygenase (IDO) [22].

One report has described two types of TANs: N1 and N2 [23]. N1 neutrophils are suggested to have antitumor activity due to their direct cytotoxic effect mediated through Fas–FasL interaction. N1 cells can additionally recruit and activate $CD8^+$ T cells by releasing chemokine (C–C motif) ligand 3 (CCL3) and IL-12, respectively. N1 cells can shift to an N2 phenotype inside the tumor microenvironment, and this shift is driven by TGF-β [16], [23]. N2 neutrophils are characterized by low levels of tumor necrosis factor alpha (TNF-α) and high expression of Arginase 1 and are suggested to support tumor growth. Neutrophils can promote angiogenesis by secreting matrix metalloproteinase 9 (MMP-9), which, in turn, enhances the production of pro-angiogenic vascular endothelial growth factor (VEGF) [24].

MDSCs are a heterogenic population of immature myeloid cells characterized by their ability to suppress immune responses. MDSCs are now divided into two different subsets [25]. The granulocytic type of cells is commonly termed as polymorphonuclear (PMN)-MDSCs and is characterized by the expression of the Ly6G marker in mice ($CD11b^+Ly6C^{lo}Ly6G^+$) and the CD15 marker in humans ($CD11b^+CD33^+CD14^-CD15^+$). These cells suppress T-cell activation and proliferation mainly through ROS [26]. Monocytic MDSCs (M-MDSCs) express the Ly6C marker in mice ($CD11b^+Ly6C^{hi}Ly6G^-$). In humans, these cells are characterized by two different phenotypes: $CD11b^+CD33^+CD14^+HLA\text{-}DR^{lo}$ or $CD11b^+CD33^+CD14^-CD15^-$. M-MDSCs suppress T-cell activation through different mechanisms that include the ribosome–nascent complex (RNC), arginine and cysteine depletion, and immune suppressive cytokines [27].

1.3 Inflammatory Factors that Affect Immune Suppressive Cells

Many pro-inflammatory factors produced in the tumor microenvironment have been shown to affect immune cell differentiation, accumulation, and function and consequently promote immune suppression. TNF-α is one such factor, which is mainly secreted by the tumor cells themselves, and can lead to increased MDSC accumulation and survival [28], as well as promote TAM activation. MDSCs and TAMs can, in turn, release large amounts of pro-inflammatory factors [29]. IL-1β and IL-6, along with granulocyte macrophage colony-stimulating factor (GM-CSF), G-CSF, and VEGF, can induce Stat-3 activation and promote MDSC expansion. This effect is

associated with the inhibition of DC differentiation and maturation [27], [30]. GM-CSF activation of Stat-5 has also been described to play a role in the accumulation of MDSCs and their survival [31].

Cytokines released by activated T cells, namely, IFN-γ and IL-4/IL-13, have been shown to upregulate inducible nitric oxide synthase (iNOS) and Arginase 1, respectively, in MDSCs. These are two major mechanisms used by MDSCs to inhibit T-cell proliferation and activation [32]–[34]. IL-4 and IL-13 can also drive the polarization of M2 macrophages in a Stat-6-dependent manner [14]. In response to inflammation, TAMs and MDSCs, along with tolerogenic DCs, also produce large amounts of anti-inflammatory factors like TGF-β and IL-10, which can convert naïve T cells into regulatory T cells [35], [36] and drive the polarization of M2 macrophages and induce tolerogenic DCs [14], [37]. In addition, TGF-β can also polarize neutrophils into a tumor-promoting phenotype [23]. Chemokines play a major role in the recruitment of immune suppressive cells. Among other cytokines produced by tumor cells and TAMs, CCL2 [38] and CCL22 [39] can recruit MDSCs and regulatory T cells, respectively.

As was briefly described above, inflammation can affect the accumulation, survival, differentiation, polarization, and function of immune suppressive cells. The S100A9 protein is one of the major mediators of this critical inflammatory process.

2 S100A9 Protein

2.1 Structure and Cellular Localization

S100A9 belongs to the S100 family of Ca^{2+}-binding proteins expressed exclusively in vertebrates. It is also known by several synonyms including the myeloid-related protein of molecular weight of 14 kDa (MRP-14), calgranulin B, and P14 and has been well characterized as a pro-inflammatory molecule. The first member of the S100 family was discovered in 1965 in bovine brain and derived its name due to its solubility in 100 % ammonium sulfate solution [40]. Since then, the S100 family of proteins has grown to 25 members. All family members contain two Ca^{2+}-binding EF-hand domains, which form helix–loop–helix motifs that can participate in protein–protein interaction. Most family members, including S100A8 (calgranulin A) and S100A9, exist as homodimers. However, S100A9 can also form heterodimers and heterotetramers with S100A8. To date, the S100A8/A9 heterodimer, also known as calprotectin, has been shown to mediate most of the known biological functions of S100A9.

S100A9 is primarily an intracellular cytoplasmic protein. Here, S100A9 contributes to cell migration by promoting cytoskeletal reorganization. Ca^{2+}-dependent S00A8/A9 heterotetramers interact with keratin intermediate filaments and tubulin to promote microtubule formation [41], [42]. In addition to its intracellular role, S100A9 can also be secreted. The exact mechanism by which S100A9 is secreted remains unclear. In monocytes and plasmacytoid DCs, IL-10 or binding of immune

complexes has been shown to trigger translocation of S100A9 to the membrane and cell surface, promoting its secretion [43], [44]. Similarly, in some breast cancer cells, Ca^{2+} influx leads to surface expression of membrane-associated S100A8/A9, which was Annexin A6 dependent [45]. The function of secreted S100A9 has been better characterized than its intracellular role.

2.2 Receptors and Signaling

Extracellular S100A9 is able to bind several receptors. Functional interactions between S100A9 and its receptors require divalent cations such as Zn^{2+} and Ca^{2+}. Extracellular S100A9 can undergo conformational changes in the presence of Zn^{2+} and become a ligand for two of its known receptors: receptor for advanced glycation end products (RAGE) and Toll-like receptor 4 (TLR4). RAGE is expressed on leukocytes, endothelial cells, and many types of cancer cells. RAGE is known to be a promiscuous receptor for many ligands including AGE, high-mobility group box 1 (HMGB1), amyloid beta peptide, and several S100 family proteins besides S100A9, including S100A12, S100A13, S100A11, and S100P. S100A9 signaling through RAGE requires MyD88, Toll-IL-1 receptor domain-containing adaptor protein (TIRAP), and TNF receptor-associated factor 6 (TRAF6) and leads to nuclear factor kappa B (NF-κB) activation [46]. In some cell types, S100A9–RAGE signaling can activate mitogen-activated protein kinase (MAPK) [47]. This interaction can induce leukocyte migration to inflammatory sites, as well as tumor cell migration to distant metastatic sites. Signaling from the S100A9–RAGE complex can lead to a feed-forward loop of both receptor and ligand upregulation to promote inflammation and inflammation-induced tumorigenesis. RAGE contains a single variable domain with two N-glycosylation sites. Srikrishna's group showed that S100A9 only binds to a subset of RAGE receptors that have been modified by carboxylated N-glycans. This interaction can be blocked by an antibody specific for N-glycans, as demonstrated by the inhibition of the chemotaxic effect of S100A9 on neutrophils or by the inhibition of NF-κB induction by S100A9 in colon cancer cells [48], [49].

The S100A8/A9 complex also serves as an endogenous ligand for the TLR4–MD2 complex [50], which is a known receptor for the bacteria-derived ligand lipopolysaccharide (LPS). In this interaction, the S100A8 component of the complex is responsible for activating the TLR4 signaling cascade involving MyD88 and IL-1 receptor-associated kinase 1 (IRAK-1). This signaling leads to NF-κB activation resulting in secretion of inflammatory mediators such as TNF-α.

In addition to RAGE and TLR4, S100A9 has been recently reported to bind extracellular matrix metalloprotease inducer, EMMPRIN (CD147), a cancer cell-associated surface molecule [51]. Signaling through EMMPRIN recruits TRAF2 and results in the induction of MMPs. EMMPRIN has been shown to be expressed in the invading edge of melanomas resulting in migration toward S100A9-expressing cells through Cdc42 activation, thus promoting tumor cell migration and metastasis.

S100A8/A9 is also able to bind the scavenger receptor CD36 [52], the major fatty acid transporter of endothelial cells, in complex with arachidonic acid. This process promotes fatty acid uptake and myeloid cell migration.

Finally, S100A9 has also been shown to bind heparin, heparin sulfate proteoglycans, and carboxylated glycans in endothelial cells [53].

2.3 S100A9 Expression in Tissues

The S100A9 protein is frequently co-expressed as a heterodimer with S100A8. Constitutive expression of S100A8/A9 is predominantly found in cells of the myeloid lineage including monocytes, granulocytes, osteoclasts, and MDSCs. These two proteins represent $> 40\,\%$ of the cytosolic proteins of neutrophils. In the bone marrow, promyelocytes differentiating to myelocytes/granulocytes increase the expression of S100A9 [54].

Upon myeloid cell differentiation to macrophages and DCs, they begin to lose S100A9 and A8 expression, with tissue macrophages or DCs showing very little to no S100A9 and A8 expression. However, in the periphery, M1-polarized macrophages do express S100A9 proteins [55].

While cells of the lymphoid lineage do not express S100A8 and S100A9, their expression can be induced in several other cell types such as keratinocytes and epithelial cells, under inflammatory conditions. IL-1-β can induce S100A8/A9 expression in keratinocytes [56]. Many malignant cells overexpress S100A8 or S100A9 proteins, as a result of translocation or duplications in the chromosomal region 1q21, which contains a cluster of the majority of S100 genes. Tumor stroma has also shown to express S100A9 [57], [58].

3 S100A9 Association with Cancer

Upregulation of the expression of the S100A9 gene was found in several types of cancer including breast, cervical, lung, colon, colorectal, liver, and gastric cancers as well as in the stroma of nasopharyngeal and bladder cancers [59]. Elevated serum levels of S100A9 were found in patients with different types of cancer and correlated with predominantly bad prognosis [60]. In most cases, S100A9 expression in tumor cells, or surrounding stroma, correlated with poor prognosis. However, in some cancers, increased S100A9 expression correlated with good prognosis, dependent on the cell type present in the tumor microenvironment. For instance, in gastric cancer, increased S100A9 expression in the stroma and infiltrating myeloid cells correlated with decreased lymph node metastasis [57] and better prognosis [61]. Similarly, an increase in the macrophage component of the tumor stroma correlated with increased survival of patients with non-small cell lung carcinoma. These S100A9-expressing macrophages were shown to be M1 polarized [62]. Overall, with the exception cited

above, S100A9 has been proposed as a marker of bad prognosis in cancer patients. It is now clear that in some situations, polarization of macrophages toward M1 type has antitumor effect. However, the specific factors in the tumor microenvironment, which are involved in such a polarization, as well as the role of S100A9 in this process, are currently not clear and need further investigation.

3.1 S100A9 Effect on Tumor Cells

S100A9 has been shown to promote both tumorigenesis and metastasis of tumor cells. Myeloid cells in the periphery can secrete S100A9, which can attract tumor cells, enhancing their metastatic potential. Subsequently, recruited cancer cells produce chemokine (C–X–C motif) ligand 1 (CXCL1) and CXCL2, which can attract new S100A9-producing myeloid cells, and improve the tumor cell survival [63]. S100A9 produced by the infiltrating myeloid cells can engage RAGE and other carboxylated N-glycans to induce upregulation of genes that promote leukocyte infiltration, tumor cell migration, and angiogenesis and drive the establishment of pre-metastatic niches in distant sites [64]. It was shown that some pro-inflammatory factors (TGF-β, TNF-α, VEGF-A) induce expression of S100A9 in endothelial cells and $CD11b^+$ myeloid cells in the lungs and promote the recruitment of S100A9-responsive tumor cells [65]. Further evidence for the role of S100A9 in cancer metastasis comes from studies that inhibit S100A9–TLR4 interaction with a small molecule Tasquinimod, which inhibits lung and lymph node metastasis in a mouse model of prostate cancer [66].

3.2 S100A9 and MDSC Accumulation

Studies of S100A9-deficient mice have established an important role of S100A9 in MDSC accumulation in several tumor models of lymphoma and colon cancer [64], [67]. In cancer patients, MDSCs have been shown to have increased expression of S100A9, suggesting that S100A9 could be used as a potential MDSC marker in humans. In the blood of colon cancer patients, S100A9-expressing $CD14^+$ cells were all HLA-DR^{low}, representing a population of M-MDSCs that was expanded, when compared to healthy donors [68]. In non-small cell lung carcinoma patients, increase in the presence of $S100A9^+$-immunosuppressive $CD33^+CD15^-CD14^+$HLA-DR^{low} M-MDSC correlated with poor prognosis and poor response to chemotherapy [69].

4 S100A9 and Immune Suppression

The role of S100A9 in the regulation of myeloid cells in cancer is not clear. There is evidence indicating that the role of S100A9 in myeloid cell function depends

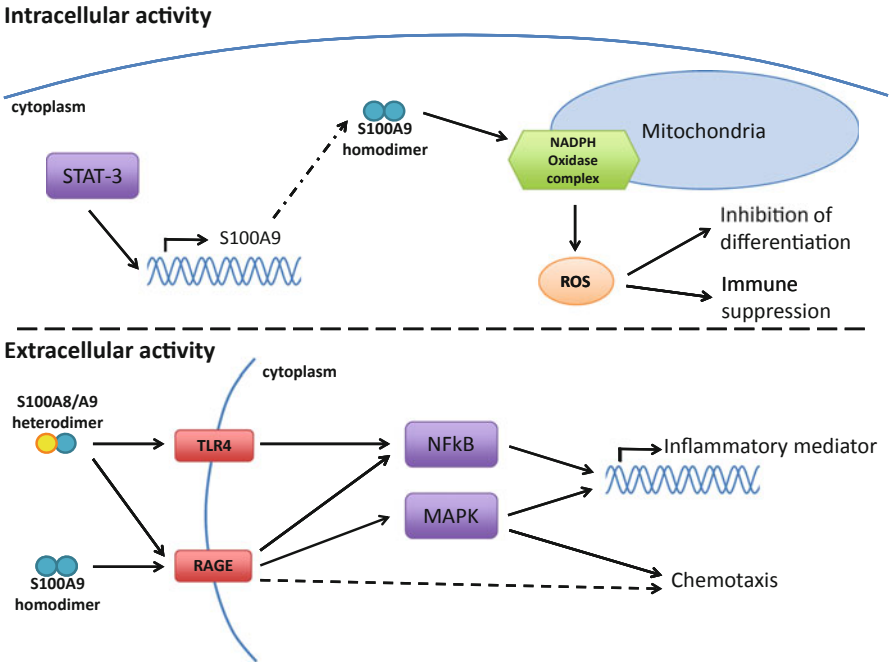

Fig. 10.1 Mechanism of S100A9 activity in myeloid cells. Intracellular (*top panel*) vs. extracellular (*bottom panel*) signaling is shown

on whether S100A9 signals intracellularly or extracellularly by interacting with receptors. Intracellular S100A9 can regulate the differentiation of myeloid cells and activate the NADPH oxidase complex, which leads to production of ROS. On the other hand, extracellular protein can activate NF-κB through TLR4/MD2 or RAGE engagement and recruit myeloid cells into the tumor microenvironment (Fig. 10.1).

4.1 Activation of the NADPH Complex and Production of ROS

It is known that one of the main mechanisms used by MDSCs to inhibit T-cell activation and proliferation is through the production of ROS [25], [70]. The main effect is mediated by peroxynitrite, which is the product of NO and superoxide anion, and inhibits T-cell activation by nitrating their T-cell receptor (TCR) and by altering the recognition of the peptide major histocompatibility (MHC) class I complexes [71]. The first link between S100A9 and the NADPH oxidase complex was reported in the early 2000s [72], [73]. In these two studies, S100A9 was shown to potentiate the activity of the NADPH oxidase complex by delivering arachidonic acid to the complex. The ability of S100A9 to bind and transport arachidonic acid

was previously demonstrated [74], [75]. Later studies clarified the mechanism by showing that the S100A8 and S100A9 components of the S100A8/A9 heterodimeric complex transport arachidonic acid to the NADPH oxidase complex by binding the $p67^{phox}$ and $gp91^{phox}$ subunits, respectively [76]. An increase in the NADPH oxidase complex activity induced by S100A9 has also been reported in keratinocytes [77]. Thus, S100A9 can increase the production of ROS in myeloid cells such as neutrophils and MDSCs and promote their suppressive activity.

4.2 Differentiation of Myeloid Cells

The possible role of S100A9 in myeloid differentiation was first suggested in the 1990s when this protein was shown to be expressed in fetal myeloid progenitors and immature myeloid cells [78]. In the same study, the authors demonstrated that S100A9 expression was lost during terminal macrophage differentiation under normal conditions. On the contrary, macrophages recruited to the site of inflammation continued to express S100A9. During chronic inflammation, S100A8 and S100A9 expression in myeloid cells was increased, and it reflected the deregulation of monocyte/macrophage differentiation [55]. More recently, the overexpression of S100A9 in myeloid progenitors has been attributed to the secretion of IL-6 and IL-8 by myofibroblasts in the context of colorectal cancer [79]. Recently, S100A9 has been shown to inhibit DC maturation. DCs derived from S100A9-deficient mice induced a stronger response of allogeneic T cells. This response was associated with an increased secretion of IL-2 and IFN-γ by T cells [80].

Several studies have linked S100A9 with the accumulation of MSDCs. In one study, Cheng et al. demonstrated that overexpression of S100A9 in myeloid progenitors in S100A9 transgenic mice led to the accumulation of $CD11b^{+}Gr1^{+}$ cells, while inhibiting the differentiation of mature myeloid cells like macrophages and DC [67]. These authors also showed that S100A9 overexpression in tumor-bearing mice was driven by Stat-3 activation, and that myeloid differentiation in S100A9 transgenic mice was regulated via increased ROS production. These data were confirmed by studies in mice deficient in S100A9 (KO). Tumors developed at a slower rate in knockout (KO) mice and were associated with a decrease in the accumulation of MDSCs. On the other hand, tumors grew faster in S100A9 transgenic mice. In the other study, Sinha and colleagues also linked S100A9 with MDSC accumulation in tumor-bearing mice [81]. In this study, S100A9 was shown to activate the NF-κB pathway (known to be involved in MDSC accumulation [30]) by signaling through the RAGE receptor. The authors also showed that inhibition of S100A9 binding to its receptor with RAGE/N-glycan antibody decreased MDSC accumulation in secondary lymphoid organs of mice with metastatic tumors. Collectively, these data suggest that extracellular S100A9 can also induce MDSC accumulation through RAGE/NF-κB activation.

4.3 Chemotaxis and Recruitment of Suppressive Cells in Tumors

As mentioned above, S100A9 is a known chemotactic agent for myeloid cells. The trigger of RAGE by S100A9 promoted the migration of MDSCs [81]. MDSCs migrated toward tumor condition media derived from 4T1 mammary tumor cells, and the blockade of S100A9 with a neutralizing antibody reversed the effect. A separate study showed that S100A9 deficiency or treatment with an antibody targeting N-glycan/RAGE decreased the accumulation of MDSCs in the pre-metastatic sites in a mouse colon cancer model [64]. Overall, the chemotactic function of S100A9 in cancer is bidirectional. Tumor cell and stroma can secrete S100A9 and recruit MDSCs to the tumor site, which in return will secrete more S100A9 and amplify the recruitment of suppressive cells. On the other hand, MDSCs in the periphery, via secretion of S100A9, can recruit tumor cells to distant sites and promote the formation of metastases.

MDSCs are not the only myeloid cells to migrate toward an S100A9 gradient. Neutrophils from tumor-free hosts have also been shown to migrate in response to S100A8 or S100A9. Not only are these proteins chemotactic but they also increase neutrophil adhesion in a CD11b-dependent manner [82]. Intravenous injection of recombinant S100A8, S100A9, or heterodimer of S100A8/A9 was also shown to increase the number of circulating neutrophils [83]. In a model of streptococcal infection, the same group has shown that blocking of S1008/A9 with neutralizing antibody inhibited the migration of phagocytic cells [84], further confirming the chemotactic effect of S100A9 toward neutrophils. However, the exact role of S100A9 in the recruitment of neutrophils in the context of cancer still remains to be demonstrated.

4.4 Engagement of MAPK and NF-κB Signaling

In tumor cells and immune cells, heterodimers of S100A8/A9 can bind to the RAGE receptor or the TLR4/MD2 complex and activate downstream signaling including the MAPK and NF-κB pathways. These two signaling pathways are known to control the transcription of pro-inflammatory factors. So far, it has been thought that S100A9 is responsible for the structural interaction with both receptors whereas S100A8 regulates the ability of the heterodimer to complex with RAGE and TLR4 [59].

Activation of the MAPK pathway by S100A9 in neutrophils has been shown to promote degranulation. Inhibition of p38 MAPK blocks this effect of S100A9, further demonstrating the role of S100A9-induced MAPK activation in neutrophil degranulation [85]. Engagement of the MAPK pathway by S100A9 binding to RAGE in tumor cells has also been shown to promote their proliferation [47].

In macrophages, the S100A8/A9 heterodimer can activate the NF-κB pathway and promote production of TNF-α in a TLR4-dependent manner [50]. In the same study, using S100A9 KO mice, the authors also showed that the S100A8/A9 heterodimer could amplify the effect of LPS by binding to TLR4. Macrophages from the S100A9 KO mice showed a decreased response to LPS stimulation characterized by a reduced

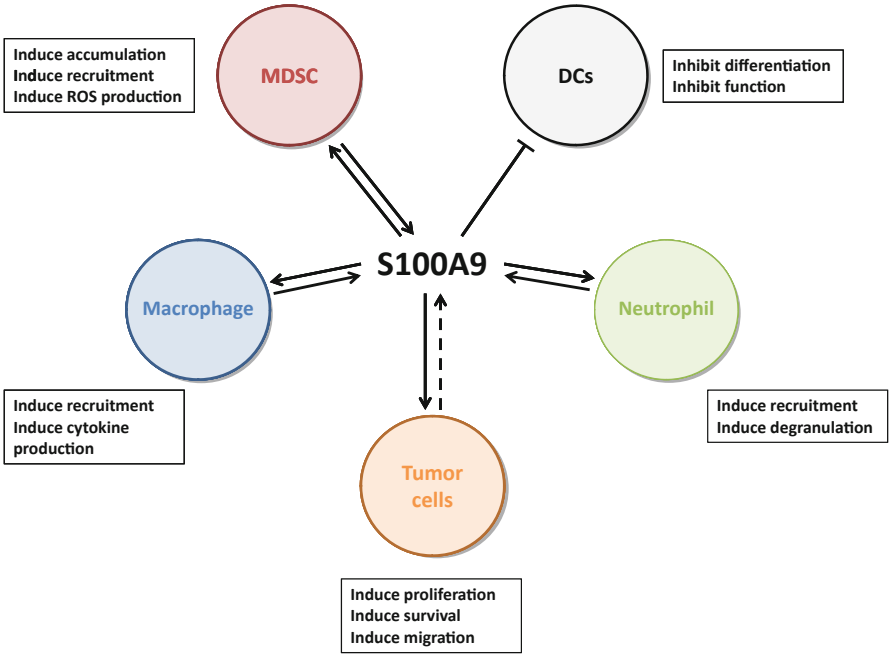

Fig. 10.2 Effect of S100A9 on different myeloid cells

binding of the NF-κB subunits p50 and p65 to the TNF-α promoter. In addition, the response to LPS in S100A9-deficient cells could be restored by adding recombinant protein suggesting that extracellular S100A9 was indeed playing a major role in the cellular response to LPS stimulation.

5 Conclusions

Inflammation stimulates the immune response in order to clear pathogens, infection, and damaged cells. However, in the tumor-bearing host, chronic inflammation contributes to the induction of immunosuppression instead of promoting immune responses. Among the many tumor-induced pro-inflammatory factors shown to be involved in chronic inflammation, S100A9 has gained prominence over the last decade because of its critical role in the accumulation of immune suppressive myeloid cells. S100A9 promotes MDSC accumulation, inhibits the differentiation of mature myeloid cells, and increases ROS production by myeloid cells, which contributes to the ability of MDSC to suppress T-cell activation. S100A9 can also play a role in recruiting other myeloid cells to the tumor site and activating them by stimulating the NF-κB pathway. These effects of S100A9 can promote tumor progression either directly by acting on tumor cells or indirectly by stimulating immune suppression in the myeloid compartment (Fig. 10.2). Taken together, these observations

suggest that targeting S100A9 could be of therapeutic value in cancer treatment. A recent study showed that a small molecule, quinolone-3-carboxamide, also known as Tasquinimod, can block the interaction between S100A9 and TLR4 in a dose-dependent manner [86]. In the same study, the authors showed that in vivo treatment with Tasquinimod inhibited the growth of EL4 lymphoma which was associated with a decreased production of TGF-β. The same small molecule has also been shown to have an antitumor effect in mouse prostate cancer progression and formation of metastases [66], [87]. A phase II clinical trial using Tasquinimod to target S100A9 in prostate cancer patients showed that Tasquinimod slowed the progression of the disease with relatively low adverse effects [88]. Tasquinimod has now entered a phase III clinical trial on patients with prostate cancer. Thus, targeting of S100A9 in cancer may have important therapeutic value.

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Chapter 11
IDO in Inflammatory Programming and Immune Suppression in Cancer

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Abstract Tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO) contributes to immune tolerance and inflammatory programming in a variety of tissue microenvironments. In cancer, IDO is overexpressed in both tumor cells and stromal cells where it promotes malignant development and progression by sustaining supportive inflammatory processes and engendering tolerance to tumor antigens. Genetic and pharmacological studies in mice indicate that IDO activity is crucial for cancer development and progression, particularly in settings where inflammatory drivers are essential. IDO is critical for myeloid suppressor functions that contribute to angiogenesis and metastasis. Mechanistic investigations have defined the aryl hydrocarbon receptor (AhR), the master metabolic regulator mammalian target of rapamycin complex 1 (mTORC1), and the stress kinase general control non-repressed 2 (GCN2) as key effector signaling targets of IDO, which also displays a non-catalytic function in transforming growth factor beta (TGF-β) signaling. Small-molecule inhibitors of IDO exhibit anticancer activity and cooperate with radiotherapy, immunotherapy, or chemotherapy to trigger regression of aggressive tumors otherwise largely resistant to treatment. IDO inhibitors that block catalytic activity or selectively reverse IDO-mediated suppression of mTORC1 are being evaluated now in clinical trials. Interestingly, the dramatic antitumor activity of certain targeted therapeutics such as imatinib can be traced, in part, to IDO downregulation. After presenting a historical background on its discovery and early study, this chapter focuses on work that defines IDO as an important mediator of pathogenic inflammation in cancer and summarizes the development of IDO inhibitors as potential anticancer modalities.

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1 Introduction

Advanced metastatic cancer remains a challenge to chemotherapy and other systemic modalities, which provide only limited benefit to ∼50 % of cancer patients who present with advanced disease at diagnosis in developed countries. Similarly, current regimens ultimately fail patients that relapse with disseminated disease following the initial treatment of primary tumors. Tumors display immunogenic antigens but escape immune rejection, somehow evading, subverting, or perhaps reprogramming the immune system for their own benefit. While it has become clear that immune escape is central to the development of a clinically relevant cancer, the basis of this phenomenon remains relatively poorly understood, in part because its role as a critical trait of cancer was not fully appreciated by cancer geneticists until recently [1]–[3].

While an appropriately activated immune system can eradicate cancer, even when it is aggressive and disseminated, spontaneous occurrences of such events in humans are rare. Cancer immunology is one of the oldest parts of the field of cancer research, and during the past century numerous kinds of molecule and cell-based immunotherapy strategies aimed at stimulating an antitumor immune response have been explored. In recent decades, investigators focused on active immune therapies tested many cytokines, tumor-associated antigen peptide vaccines, dendritic cell (DC) vaccines, or adoptive transfers of tumor antigen-specific effector T cells expanded ex vivo from cancer patients [4]–[10]. In contrast to passive immunotherapies, which mainly involve the administration of targeted antibodies, these active immunotherapies are based conceptually on stimulating components of host immunity to elicit an effective response against cancer cells. Having mainly failed historically to generate broadly effective responses, it has become clear that this type of approach is insufficient to overcome tumoral immune suppression and escape mechanisms, which are based upon the dominance of pathological immune tolerance in cancer patients as proposed [12]. While not all escape mechanisms involve an active principle of immune tolerance, as such mechanisms have been defined, it has become increasingly clear that their disruption is important to license the efficacy of active immunotherapies which have failed over the years. In short, to "get on the gas" of immune activation against tumors, it is clear that it is necessary to "get off the brakes" of tumor-associated immune suppression.

Since 2000, there have been rapid advances in understanding how tumors escape the immune system [11], [13]. Intriguingly, it appears that many immune escape mechanisms are configured as active immune suppression by the tumor or stromal cells under the influence of the tumor, implying that continuous activity from the escape mechanism is required. Further, it has been clear that disrupting these active

mechanisms of immune suppression can de-repress (activate) the immune system, enabling it to attack the tumor. Such mechanisms may offer particularly attractive targets for therapeutic intervention with small-molecule drugs [14], which have distinct advantages over biological agents that are currently the norm for immunotherapeutic strategies. Of the mechanisms which have been described to date, one with considerable practical appeal involves the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) [15].

2 Background on IDO and Its Recently Discovered Relative IDO2

2.1 Historical Perspective

While most early studies of IDO did not relate to cancer, the discovery of this enzyme was rooted in initial observations made in the 1950s in cancer patients where tryptophan catabolism was found to be elevated [16]. Later studies extended these findings with observations that tryptophan catabolites are elevated in the urine of patients with a variety of malignancies including leukemia, Hodgkin's disease, prostate cancer, and breast cancer [17]–[22]. The hepatic enzyme tryptophan dioxygenase (TDO2), which was the first inducible mammalian enzyme ever to be isolated, had been known since the 1930s to initiate the metabolism of dietary tryptophan [23], [24]. However, no increase in TDO2 activity was detected in cancer patients who presented with elevated tryptophan catabolites [25], implying the existence of a second enzyme.

The extrahepatic tryptophan-catabolizing enzyme termed IDO (aka IDO1; originally D-tryptophan pyrrolase) was first isolated in 1963 [26], [27]. Notably, while IDO catalyzes the same reaction as its hepatic relative TDO2—the conversion of tryptophan to N-formyl-kynurenine—these two enzymes are otherwise remarkably dissimilar [28]. Whereas active TDO2 is a homotetramer of 320 kD, IDO is a monomeric enzyme of 41 kD that is antigenically distinct from TDO2 [29] and lacking in amino acid sequence similarity. Additionally, IDO has less stringent substrate specificity, cleaving a number of indole-containing compounds not recognized by the hepatic enzyme. Lastly, while both enzymes contain heme, IDO utilizes superoxide anion for activity whereas TDO2 does not use superoxide to donate oxygen in the tryptophan catabolic reaction.

Structural and enzymological studies have revealed several interesting features about IDO. Enzymological studies indicate that an electron donor such as methylene blue is critical to achieve full activity in vitro, a role that in vivo is thought to be assumed by tetrahydrobiopterin or flavin cofactors. The binding site on the enzyme for the putative cofactor is distinct from the substrate-binding site [30], implying the potential for allosteric regulation and possibly opportunities for developing non-competitive enzymatic inhibitors (in addition to the more classical substrate-competitive inhibitors). Crystallographic studies of human IDO reveal a two-domain structure of alpha-helical domains with the heme group located in

between [31]. Notably, these findings suggest that strict shape requirements in the catalytic site are required, not for substrate binding but instead for abstraction of a proton from the substrate by iron-bound dioxygen in the first step of the reaction [31]. This detail of the reaction mechanism is important because it is distinct from that used by other monooxygenases (e.g., cytochrome P450), filling a gap in understanding of heme chemistry. In terms of small-molecule inhibitor development, the biochemical differences that distinguish IDO from TDO2 and other monooxygenases are useful because they increase the likelihood of identifying IDO-specific inhibitors.

2.2 IDO: Function in Immune Modulation

In contrast to the biochemical and genetic knowledge about IDO that accumulated relatively quickly in the years since its discovery, a precise understanding of its physiological function remained obscure due to the fact that mammals mostly salvage rather than synthesize nicotinamide adenine dinucleotide (NAD) to meet their metabolic needs. Why then was IDO evolutionarily conserved in mammals? Initial clues as to its function were suggested in the late 1970s by findings from Hayaishi and his colleagues that IDO expression was strongly stimulated in the lungs of mice by viral infection, or exposure to bacterial lipopolysaccharide (LPS) or interferon-γ (IFN-γ) [32]. These findings prompted the interpretation that elevated tryptophan catabolism by IDO at sites of inflammation might provide an antimicrobial benefit. Given the antitumor properties of IFN-γ, this concept was extended to encompass the notion that IDO acted functionally in the manner of a tumor suppressor, contributing to the antitumor effects of IFN-γ activity by starving growing tumor cells of tryptophan [33].

It was not until the late 1990s that a conceptual breakthrough emerged from work by Munn, Mellor, and their colleagues, establishing the possibility that IDO might mediate an immunosuppressive function based on the preferential sensitivity of T cells to tryptophan deprivation. In this radical reconceptualization of the biological role of IDO-based metabolic activity, impaired antigen-dependent T-cell activation occurs in microenvironments where IDO activation results in reduced tryptophan levels [34], [35]. The ability of IDO to promote immune tolerance to "foreign" antigens was supported by the evidence that the specific bioactive IDO inhibitor 1-methyl-tryptophan (1MT) [36] could elicit major histocompatibility complex (MHC)-restricted T-cell-mediated rejection of allogeneic mouse concepti [37], [38]. In cancer, these findings implied that IDO could be prooncogenic by limiting the eradication of tumor cells that occurs through immune-based recognition of "foreign" tumor antigens.

In the past few years, the concept that tryptophan catabolism regulates T-cell immunity has now been corroborated in many laboratories, with regulatory functions identified for both tryptophan depletion and the production of downstream catabolites. In particular, there has been a keen focus on the immune regulatory role of IDO expressed in DCs, an important class of "professional" antigen-presenting cells

(APCs). IDO expression in a small minority population of DCs enables them to dominantly suppress the activation of T cells that occurs through antigen presentation [39], [40]. Tryptophan depletion has been shown to promote T-cell anergy by signaling through the integrated stress-response kinase general control non-repressed 2 (GCN2), which is also required for IDO-induced differentiation of $CD4^+$ T cells into T regulatory (Treg) cells [41]. Likewise, tryptophan catabolites can block T-cell activation and trigger T-cell apoptosis while also promoting the emergence of Treg cells through a transforming growth factor beta (TGF-β)-dependent mechanism, and evidence of synergistic consequences of both depleting tryptophan and elevating tryptophan catabolites have been described [42]. IDO has been implicated widely in cancer, chronic viral infections, allergies, and various autoimmune and inflammatory disorders where immune control is disordered [43].

2.3 IDO2: Discovery and Distinctions from IDO

Mammalian genomes include not only the IDO-encoding gene *IDO1* but also a more recently identified relative termed *IDO2* [44], [45]. Human *IDO1*, located at 8p12–11, comprises 10 exons spanning ~15 kb that encode a 403-amino-acid polypeptide of ~41 kD [46], [47]. Mouse *Ido1* is syntenic and similar in its genomic organization; however, the gene diverges somewhat at the primary sequence level from human *IDO1*, sharing only 63 % identity. The likely existence of a related *IDO2* gene became apparent to us while inspecting sequences immediately downstream of *IDO1* in the human genome [45]. At the time, the genome database in that region was erroneously annotated, referring to a set of partial *IDO1*-related sequences by the anonymous nomenclature *LOC169355*. Correction of the erroneous annotation by trial-and-error exon searches revealed the presence of a 420-amino-acid open-reading frame (ORF) that is 44 % identical to IDO at the primary sequence level. The protein encoded by the *IDO2* ORF conserves all the residues in IDO that have been defined as critical for tryptophan binding and catabolism [31]. The IDO2 proteins in mouse and human are more closely conserved than the mouse and human IDO proteins, displaying 73 % identity at the primary sequence level. The presence of the two IDO-related proteins in such close proximity is likely the result of a gene duplication event, and phylogenetic analysis has been interpreted to indicate that *IDO2* may actually be the ancestral gene [48]. As in the human genome, the mouse *Ido2* gene is located immediately downstream of *Ido1*. Expression of *IDO2* message was detected in a more limited range of tissues than *IDO1* [45]. At the cellular level, evaluation of the National Center for Biotechnology Information (NCBI) SAGEmap database identified the top hits for *IDO2* expression to be bone marrow-derived DCs [45], which is intriguing given the evidence that IDO-based activity profoundly influences the immunogenic nature of DCs.

Most of the signaling and mechanistic data surrounding the IDO proteins have come from studies of IDO and not the more recently identified IDO2. Due to the more restricted localization of IDO2 compared to IDO, it is conjectured that these two molecules do not serve a redundant function. This view is supported by a divergence

in signaling between these two molecules through the integrated stress-response pathway. Local tryptophan depletion due to IDO activity engages this pathway resulting in the elevated expression of liver inhibitory protein (LIP) [45], a truncated isoform of the transcription factor nuclear factor interleukin 6/CCAAT-enhancer-binding protein β (NF-IL6/CEBPβ), which alters the expression of key immune modulatory factors including IL-6, TGF-β, and IL-10. Supplementing with additional tryptophan after depletion quickly abolished the LIP response induced by IDO but not by IDO2 [45]. Thus, after IDO2 induction, LIP expression is maintained in a tryptophan-independent manner, indicating a stable effect of tryptophan catabolic signaling unique to IDO2. While the significance of this distinction has yet to be evaluated in vivo, one implication is that IDO2 might differ from IDO in its ability to transmit a stable immune regulatory signal. LIP-mediated signaling initiated by IDO2 could alter distal immunity, since the signal could persist in microenvironments where tryptophan levels are normal. Alternately, IDO2 might produce a stable differentiation signal. Intriguingly, the IDO2 gene is regulated in DCs by activation of the aryl hydrocarbon receptor (AhR) [49], which as discussed further below has been identified recently as a receptor for kynurenine [50], the product of tryptophan catabolism by IDO or IDO2. Along with other evidence linking AhR and kynurenine in immune control [51], these connections hint at a dynamic signaling node that may act to modulate inflammation as well as adaptive immunity.

Another unique aspect of IDO2 is the considerable genetic variability that exists among different individuals for expressing the active enzyme. This variability is due to the presence of two commonly occurring, non-synonymous single-nucleotide polymorphisms in the *IDO2* gene that ablate its enzymatic activity [45]. Indeed, as many as 50 % of individuals of European or Asian descent and 25 % of individuals of African descent appear to lack functional *IDO2* alleles [45]. The frequent occurrence of inactive genetic variants in human populations suggests that there may be some evolutionary benefit to attenuating IDO2 activity, perhaps reflecting competing selective pressures to establish an optimal degree of immunological responsiveness under differing conditions of infection, autoimmunity, and malignancy. In this vein, one clinical study suggests that active IDO2 alleles may be disproportionally represented among younger individuals with aggressive pancreatic cancer [52]. While the relevance of IDO2 function to immune regulation has yet to be directly corroborated, one recent study offers some support for this expectation based on evidence that IDO2 activity can inhibit the proliferation of human $CD4^+$ and $CD8^+$ T cells in vitro, albeit in a manner insensitive to 1MT treatment [53].

2.4 Immune Suppression by Other Tryptophan Catabolic Enzymes TDO2 and TPH

The fundamental role of IDO in immune tolerance was recognized several years before its connections to cancer were discovered, and it is by far the most broadly expressed and studied of the tryptophan-metabolizing enzymes. IDO action leads to

both tryptophan deprivation and Kyn generation, both of which cooperate to inhibit the activation of immune cells known as T cells, through various mechanisms that also affect the activities of other classes of immune cells. In mammals, there are two other enzymes that catabolize tryptophan, but only one of which also generates kynurenine.

TDO2 is a multimeric enzyme that is structurally distinct from IDO and IDO2, probably an example of convergent evolution. Until recently, TDO2 was widely considered to serve in degrading excess tryptophan in the liver, where it is mainly normally expressed, but it has also been implicated now in cancer and immune modulation. In particular, TDO2 is frequently activated in brain cancers and other cancers where IDO is not activated [54]. In initial studies, one unique aspect of TDO2 in cancer appears to be that its activation promotes cancer cell migration, which has not been reported for IDO, suggesting some divergence in function despite the enzymes' shared ability to generate kynurenine. One possibility is that the preference of TDO2 for substrates may differ, perhaps varying the biological functions of TDO2 from IDO or IDO2 to some extent. Whatever the case, TDO2 appears to be upregulated in a variety of cancers [54], [55], and small-molecule inhibitors of TDO2 may be useful to treat these IDO-independent cancers or cancers that might become resistant to IDO inhibitors through TDO2 activation. In support of this likelihood, in a preclinical model TDO2 expression by tumors was found to prevent their rejection by immunized mice, and systemic treatment with a novel TDO2 inhibitor restored the ability of mice to reject TDO2-expressing tumors [55].

Tryptophan hydroxylase 1 (TPH1), the enzyme responsible for seratonin production from tryptophan, also has been implicated recently in immune control, including in cancer [56]. TPH1 does not generate kynurenine, so it is clear that its immunoregulatory role relates solely to tryptophan deprivation. Studies in mice that are genetically deficient in TPH1 have illustrated its role in skin allograft tolerance, tumor growth, and experimental autoimmune encephalomyelitis, where loss of enzymatic activity can mediate allograft tolerance, induce tumor remission, and intensify neuroinflammation, respectively [56]. These effects were all found to be independent of serotonin. Mast cells are a major source of TPH1 expression, and restoring TPH1 in these cells in vivo was sufficient to correct defects in the genetically deficient mice [56]. Thus, these findings introduced an important and previously unappreciated new role for mast cells in inflammatory programming and immune regulation, through their ability to modulate tryptophan degradation.

3 Complex Control of IDO by Immune Regulatory Factors

3.1 Transcriptional Control

Initial clues regarding the involvement of IDO in inflammation originated with the finding that its expression and activity in many cell types is stimulated strongly by the cytokine IFN-γ [57]. IFN-γ is now recognized as a major inducer of IDO, especially

in APCs including macrophages and DCs [58]–[61]. Transcriptional induction of the *IDO1* gene through IFN-γ is mediated through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, in particular JAK1 and STAT1α [62]. STAT1α appears to act to induce *IDO1* gene expression both directly through binding of gamma-activated sequence (GAS) sites within the *IDO1* promoter as well as indirectly through induction of interferon regulatory factor 1 (IRF-1) which binds the *IDO1* promoter at two interferon-stimulated response element (ISRE) sites [63]–[65], [62], [66]. The upregulation of IDO in APCs that occurs in response to IFN-γ, which is produced by activated T cells, suggests that IDO participates in a negative feedback loop that regulates T-cell activation.

The transcription factor NF-κB, which has a central role in directing inflammatory processes, has also been identified as a key factor controlling the induction of IDO. The precise mechanisms for NF-κB-mediated control of *IDO1* expression are not fully elucidated and may be contextually based in various cells, given that both the canonical and non-canonical pathways have been found to be important under different experimental conditions [67]–[69]. IRF-1 may be a common element through which both STAT1α and NF-κB contribute to the induction of *IDO1*, as both IFN-γ and tumor necrosis factor alpha (TNF-α) (which signals through NF-κB) can synergistically induce expression of IRF-1 through a novel composite binding element for both STAT1α and NF-κB in the IRF-1 promoter (termed a GAS/κB element) that combines a GAS element overlapped by a non-consensus site for NF-κB [70].

A recent analysis of functional polymorphisms in the IDO1 gene promoter was conducted which may explain some interindividual variability in IDO expression that has been documented [71]. This study identified a variable nucleotide repeat (VNTR) polymorphism, the presence of which correlated with serum tryptophan concentration in female but not male subjects. Interestingly, this VNTR did not affect basal or cytokine-induced activity of the IDO1 promoter, but it harbored functional binding sites for the transcription factor lymphoid enhancer-binding factor 1 (LEF-1) which is responsible for changes in gene expression mediated by WNT signaling, which is activated in many epithelial cancers, most notably colon cancers. However, the pathogenic role of this polymorphism in promoting cancer progression, if any, will require further analysis.

3.2 IDO Control in Dendritic Cells

In DCs, interferons (both type 1 and type 2) have been found to act at a central interface between IDO and other components of inflammation and immunity. Toll-like receptor 9 (TLR9) ligands such as CpG were found to induce IDO expression in a subset of DCs through a type 1 interferon-dependent signaling pathway [72]. Interactions with immune cells are also implicated in IDO regulation. The first of these interactions to be characterized was an intriguing reverse-signaling mechanism described for the inhibitory T-cell co-receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is constitutively expressed on Treg cells. By binding to B7 ligands (CD80 and CD86) on DCs, CTLA-4 was shown to elicit the IFN-γ-dependent induction of IDO

[73]. The stimulatory T-cell co-receptor CD28 also binds the same B7 ligands but fails to similarly induce IDO because of the concomitant induction of IL-6 which interferes with IFN-γ elicited STAT signaling through upregulation of suppressor of cytokine signaling 3 (SOCS3) [74]. Other cell surface proteins including CD40, CD200 and glucocorticoid-induced TNF receptor family-related gene (GITR) have since been shown to induce IDO through similar reverse-signaling mechanisms all of which appear to share the non-canonical NF-κB pathway as a common point of convergence [75].

TGF-β was initially reported to antagonize IFN-γ-mediated induction of IDO expression [76]. These experiments, carried out in fibroblasts, appear to run counter to immunosuppressive activity ascribed to TGF-β but are consistent with its ability to antagonize positively regulated targets of IFN-γ. More recently, the opposite relationship between IDO and TGF-β has been reported in experiments carried out in DCs suggesting that the regulatory impact of TGF-β on IDO expression may be complex and contextual. In these experiments, autocrine TGF-β sustained the activation of IDO in a tolerogenic subpopulation of $CD8^+$ DCs while exogenous TGF-β could convert immunogenic $CD8^-$ DCs into tolerogenic cells in conjunction with induction of IDO [77]. In this milieu, it was found that even DCs that lack expression of IDO could be rendered tolerogenic by exposure to tryptophan catabolites produced by IDO-expressing cells [78] as part of a feedforward expansion of IDO-elicited immune suppression described as "infectious tolerance" [79].

3.3 COX2 and Prostaglandins in IDO Control

The proinflammatory prostaglandin E-2 (PGE-2), which is frequently elevated during cancer progression as a result of activation of cyclooxygenase-2 (COX-2), has also been implicated as an important inducer of IDO activity. In support of the concept that IDO acts downstream of COX-2, induction of IDO activity can be blocked in vitro by COX-2 inhibitors such as aspirin, indomethacin, and phenylbutazone but not by anti-inflammatory agents that do not affect prostaglandin production [80]. This signaling mechanism may be relevant to the biological activity of upstream regulators of *COX2* expression as well. For instance, hepatocyte growth factor (HGF), which is known to be able to elevate COX2 activity, has been found to also elevate IDO in monocyte-derived DCs [81]. The relationship between PGE-2 and IDO is complicated insofar as IDO activity can affect the ratio of prostaglandin synthesis [82]. The complex interplay between IDO and COX2 in inflammatory processes in cancer and autoimmune and chronic inflammatory diseases has been reviewed in detail elsewhere recently [83]. Interestingly, while PGE-2 is employed widely as an in vitro maturation factor for DCs, treatment of these cells with PGE-2 has been reported to elevate IDO expression ~ 100-fold [84]. Although the induction of IDO enzymatic activity does appear to require an additional signal(s) (i.e., exposure to TNF or agonists of TLRs), these findings raise the concern that such preparations may inadvertently compromise the desired immune stimulatory activity of the DCs used in the setting of cancer vaccines.

3.4 IDO Control by Aryl Hydrocarbon Receptor Signaling

More recently, interconnections identified between IDO and the xenobiotic AhR have been generating particular interest due to a developing appreciation for the importance of AhR in modulating immune function especially at the level of mucosal immunity [85] where IDO may also be particularly relevant. AhR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) resulted in the induction of both IDO1 and IDO2 in vitro [86]. Furthermore, it was shown that TCDD treatment of mouse splenic T cells resulted in increased levels of FoxP3—an effect that was abrogated in *AhR*-null mice, suggesting that AhR is important for the development of Treg cells possibly through the induction of IDO [86]. Conversely, several tryptophan catabolites have been implicated as physiological ligands for AhR including kynurenine [54], [87], produced by the ubiquitous arylformamidase enzyme following the IDO- or TDO2-initiated catabolism of tryptophan. Further biological ramifications can be inferred from studies by DiNatale et al. [88] showing that kynurenic acid, another downstream tryptophan catabolite, induces AhR-mediated induction of IL-6, an important inflammatory cytokine for promoting tumor progression. Interestingly, *Ido1*-nullizygous mice exhibited a marked reduction in IL-6 levels in primary lung tumors and pulmonary metastases which was functionally linked to increased tumor resistance [89]. As noted earlier in this section, IL-6 has been demonstrated to antagonize IDO expression, suggesting its involvement in an important negative regulatory feedback loop that may go awry during the development of cancer.

3.5 Negative Control of IDO by Nitric Oxide

In addition to IL-6, other important negative regulators of IDO have been identified. Inducible nitric oxide synthase (iNOS) and IDO appear to be mutually antagonistic in DC-based studies [90]–[92]. The production of NO by iNOS prevents the IFN-γ-induced expression of IDO [93], interferes directly with its enzymatic activity [93]–[95], and promotes its proteolytic degradation [96]. NO can directly inactivate IDO by binding to the heme iron, which under lowered pH conditions induces iron–His bond rupture and the formation of a 5C NO-bound derivative that is associated with protein conformational changes that may be sufficient to target the protein for ubiquitination and proteosomal degradation [97]. In the non-obese diabetic (NOD) mouse model of diabetes, in vivo evidence suggests that IFN-γ signaling is impaired as the result of nitration of the downstream STAT1 transcription factor by peroxynitrate, which is derived from NO and superoxide. This impairment can be overcome by CTLA-4-Ig treatment, which, by promoting phosphatase and tensin homolog (PTEN) activity, relieves the negative regulation that phosphorylated Akt imposes on FOXO3a-mediated transcription of superoxide dismutase (SOD2) which degrades peroxynitrate [98]. Through this complex route, the blockade to activation of IDO gene expression, to which iNOS contributes through peroxynitrate-mediated nitration of STAT1, is relieved. Two implications of the configuration of this mechanism

are the following. First, NO agonists will tend to reverse immunosuppression at the level of DCs in cancer, which should benefit treatment. Second, small-molecule inhibitors of Akt that are being developed as anticancer therapeutics will tend to heighten immunosuppression by phenocopying this effect of CTLA-4-Ig on IDO expression. Other findings suggest that Akt inhibition may also heighten the invasive capability of cancer cells [99]. Thus, for cancer treatment, the desirable proapoptotic quality of Akt inhibitors may be balanced by their undesirable proinvasive and immunosuppressive properties.

4 IDO Dysregulation in Cancer Pathogenesis

4.1 IDO Upregulation in Cancer Cells Through Attenuation of Tumor Suppressor Gene Bin1

IDO overexpression is associated with poor prognosis in many different cancers [100]. Tumor transplant studies in mice have likewise linked IDO expression with enhanced tumor outgrowth in the context of an active immune system [68], [101], [102]. Upregulated IDO expression occurs commonly in human cancer cells [102]. While the basis for this upregulated expression is not fully understood, studies of the tumor suppressor gene *Bin1* have identified it as a central regulatory event in this process. *Bin1* is among the most frequently attenuated genes in human cancer, due to aberrant RNA splicing patterns that eliminate tumor suppressor function [103]–[107], or due to altered gene methylation patterns that extinguish expression [108]–[112]. Loss of *Bin1* function affects cancer cell proliferation, motility, survival, and immune escape [113]. However, in vivo studies clearly suggest that the most pathogenically significant effect of *Bin1* loss in promoting cancer is through IDO activation and IDO and IDO-mediated immune suppression [113], [114].

Genetic studies in the mouse have established an antagonistic relationship between *Bin1* and *Ido1*, such that functional ablation of *Bin1* causes transcriptional upregulation of *Ido1* with increased responsiveness to IFN-γ [68]. Oncogenic transformation of murine embryo fibroblasts or skin keratinocytes with *c-Myc* + *Ras* indicated that while *Bin1* loss affected cell growth, invasion, and survival [115], more dramatic differences were revealed in the growth of tumors in immunocompetent hosts, where *Bin1*-null cells formed large tumors in contrast to *Bin1*-expressing cells which formed only indolent nodules [114]. This dichotomy reflected different immune responses to the cells, as *Bin1*-expressing cells produced rapidly growing tumors when introduced into T cell-deficient mice. IDO was identified as a key target of *Bin1*-dependent transcriptional repression that was activated in *Bin1*-deficient cells [114], although other genes implicated in immune suppression such as *CD39* and *Arginase-1* were also identified (unpublished observations). Notably, treatment of *Bin1*-deficient cells with small-molecule inhibitors of IDO suppressed the outgrowth of *Bin1*-null tumors in syngeneic mice but not immunocompromised nude mice or

mice that were immunologically deprived of $CD4^+$ T cells [114]. Taken together, these findings established that *Bin1* loss led to IDO upregulation and tumor promotion by enabling IDO-mediated escape from T-cell immunity. Given the relationship between *Bin1* and *IDO* established by genetic studies in the mouse, which causally link Bin1 attenuation to IDO overexpression, it will be important to further evaluate their mechanistic relationship and integrate it with immunometabolic regulatory processes that may be affected by *Bin1*, such as adenosine or arginase signaling.

4.2 IDO is a Crucial Contributor to the Inflammatory Tumor Microenvironment

The tissue microenvironment where a tumor arises poses a huge barrier to its development and progression. In particular, it is clear that the interplay of cancer cells with immune cells is one of the most important determinants for whether an early cancer is destroyed by the immune system, persists in a dormant or slowly growing state (which often makes the tumor localized and treatable), or progresses to an invasive or metastatic state that becomes clinically challenging.

Unlike a conventional proto-oncogene, the role of IDO in cancer is predominantly to create a more hospitable environment for the tumor rather than enhancing malignant properties intrinsic to the tumor cells. Therefore, it is not surprising that normal cells outside the tumor have also been found to be a relevant source of IDO expression. In particular, a subset of DCs, with characteristics indicative of the B-cell lineage [116], expresses high levels of IDO in the proximal lymph nodes of mice with subcutaneous melanoma tumor grafts that exhibited no expression of IDO in the tumor cells themselves [117]. Elevated levels of IDO have also been reported in the tumor-draining lymph nodes (TDLNs) of human cancer patients [117]. Preclinical studies using a classical two-stage model of inflammatory skin carcinogenesis demonstrated that mice lacking the *Ido1* gene encoding IDO were quite resistant to the development of tumors [118]. In this model, tumors are initiated with a single exposure of the *ras*-activating carcinogen 7,12-dimethylbenz [*a*]anthracene (DMBA) followed by multiple exposures to the proinflammatory phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (also known as phorbol 12-myristate 13-acetate or PMA), the latter of which drives a state of chronic inflammation that promotes tumor outgrowth. These studies provided the first direct genetic evidence that IDO is crucial for de novo tumorigenesis. In this model of inflammatory carcinogenesis, but also within a T cell-suppressive population of DCs localized at TDLNs, which had the same characteristics as those observed previously in a melanoma tumor graft model [117], [118]. Notably, in TDLNs in the skin model, TPA strongly upregulated IDO expression [119] and subsequent findings confirmed this proinflammatory stimulus acting through protein kinase C (PKC) stimulates a unique pathway of IDO activation [120]. Together, these findings provided a glimpse of the complexity of interpreting IDO effects in biological systems, given that its expression can be either intrinsic or extrinsic to cancer cells.

Subsequent genetic studies in different mouse models of carcinogenesis have established that IDO contributes a crucial function to the inflammatory tumor microenvironment. Further, they argue that the initial characterization of IDO solely as a modifier of adaptive immune tolerance may oversimplify its role in cancer pathogenesis. Studies in the skin model showed that IDO loss did not exacerbate classical inflammatory responses to TPA but that its induction was integral to the inflammatory tissue microenvironment even in the absence of cancer [120]. In connecting IDO to inflammatory stimuli, it was found that IDO loss had little impact on tumor outgrowth if the carcinogenesis model employed lacked an explicit inflammatory promoter. For example, IDO ablation did not affect induction of skin tumors elicited by multiple topical exposures to DMBA that are sufficient for carcinogenesis in the absence of TPA, nor did IDO ablation affect induction of breast tumors where progesterone was used instead of TPA as a non-inflammatory promoter after a single intraperitoneal exposure to DMBA [120]. Moreover, it is clear that IDO deficiency does not influence the engraftment of established tumor cell lines that have previously developed an effective immunoediting route, unlike transgenic models where the route must be developed and will therefore vary between individual mice. In the context of TPA-driven skin carcinogenesis, where IDO was critical, bone marrow transplant experiments revealed that the most important source of IDO function were radiation-resistant, non-hematopoietic cells in the model, supporting evidence that *Bin1* deficiency in *myc* + *ras*-transformed skin cells was sufficient to facilitate IDO-mediated immune escape by a cell autonomous mechanism [120]. Together, these findings argued that IDO was a key element of "cancer-associated" inflammation that tilts the immune system toward tumor support. More broadly, they prompted the concept that mediators of immune escape and cancer-associated inflammation may be genetically synonymous.

Other observations from *Ido1*-deficient mice strengthen the concept that IDO exerts a proximal influence on inflammation that is too subtle to understand as simply immunosuppressive. If IDO were a solely immunosuppressive enzyme, inflammation might be expected to run rampant in *Ido1*-deficient mice where this presumptive check is no longer in place. However, *Ido1* deficiency does not produce such effects, in contrast to deficiency of an immunosuppressive function like *CTLA-4*. Moreover, the inflammation that develops in *Ido1*-deficient mice treated with TPA is not discernibly different than in wild-type control animals receiving the same treatment [121]. So, rather than IDO simply being an immunosuppressive counterbalance in inflammatory reactions, a more nuanced interpretation for the role of IDO is required in which IDO shapes the pathogenicity of the tissue microenvironment.

The degradation of normal cellular physiology leading to malignancy involves acquisition of the cell-intrinsic traits of immortalization, growth sufficiency, insensitivity to growth inhibitory signals, and resistance to apoptosis, along with the cell-extrinsic traits of angiogenesis, invasive capability, metastatic capacity, and immune escape. In this context, immune escape mechanisms utilized by tumors, such as IDO induction, have been postulated to be a terminal feature of the immunoediting process, which comprises the three distinct phases of elimination, equilibrium, and escape [122]. However, a contrarian argument has also been made that tumoral immune escape is not a late event driven by selective pressure, but instead develops

as an early, integral component of the tumorigenic process [123]. The multistage aspect of the DMBA/TPA carcinogenesis protocol described above provided us with a unique opportunity to investigate this question with regard to the role of IDO induction in the contextual setting of de novo tumor development. The immunoediting postulate would require that there be at least some nascent tumor present for IDO to be induced. Instead, however, TPA treatment alone was sufficient to induce IDO in the proximal lymph nodes [118]. Because these mice were never exposed to DMBA-based tumor initiation, this elevation of IDO occurred in the absence of cancer, as TPA alone is not able to drive the development of neoplasia in the absence of an initiating agent. This outcome, therefore, was more in line with IDO elevation being an early event driven by TPA-elicited inflammation, rather than a late event driven by immune selection.

Other studies extend the notion that IDO acts in a proximal manner to program pathogenic inflammatory processes which then go on to direct antigenic tolerization in the adaptive immune system at a more distal level. In one study, ectopic modulation of IDO in murine breast cancer cells not only influenced T-cell responses in immunocompetent mice but also affected primary tumor growth and metastasis in immunodeficient severe combined immunodeficiency *(scid)/beige* mice which lack T, B, and natural killer (NK) cells [124]. Thus, these pathogenic effects of IDO overexpression could not be readily interpreted as mediated solely by adaptive immunological mechanisms. The conceptual realization that IDO acts as an integral component of the inflammatory milieu is supported additionally by evidence of a role in supporting other pathogenicities associated with chronic inflammation. For example, IDO-mediated tryptophan degradation is elevated in rheumatoid arthritis and systemic lupus erythematosus patients, suggesting a role for increased IDO activity in promoting autoimmune disease [125], [126] that has some direct corroborative support from studies in the K/BxN spontaneous mouse model of arthritis [127]. In the KxB/N model, IDO activity is elevated at disease onset, and administration of the IDO inhibitor 1MT resulted in alleviation of joint inflammation, with 1MT-treated animals exhibiting minimal synovial expansion and fewer infiltrating inflammatory cells [128]. In this setting, 1MT treatment did not affect levels of Treg cells or T helper type 1 (Th1)/Th2/Th17 cytokines, but it did greatly diminish the autoreactive B-cell response, indicative of a role for IDO upregulation in supporting the development of autoimmune disease by supporting the activation of autoreactive B cells. In conjunction with results from cancer models, these results argue strongly that IDO contributes to pathogenic forms of chronic inflammation in a manner that is more complex than simply acting as an immunosuppressive brake.

4.3 IDO Activation Is a Critical Contributor to Tumor Angiogenesis and Metastasis

Our most recent findings further elucidate how IDO contributes to cancer development by altering the inflammatory milieu. *Ido1*-deficient mice exhibit a reduced tumor burden in a K-RasV12-induced model of lung adenocarcinoma and a reduced

susceptibility to development of pulmonary metastases in the 4T1 model of breast cancer, in both settings displaying improved survival [129]. Notably, IL-6 levels were attenuated by *Ido1* deficiency in each model, leading to an impairment of myeloid-derived suppressor cell (MDSC)-mediated suppression of T cells. The importance of these findings to pulmonary tumor development was demonstrated in the metastatic model where restoration of IL-6 overcame the MDSC impairment and allowed metastatic disease to progress at the rate observed in *Ido1*-competent mice [129]. The implication that IL-6 serves as a key regulator of tumor growth downstream of IDO has therapeutic value as increased IL-6 levels are associated with recurring tumors in patients [130]. In yet another clue to the role of IDO beyond adaptive immune control, *Ido1*-deficient mice were found to display an angiogenic defect in lungs even in the absence of tumors. Together, these studies highlight a more complex and nuanced interpretation of what tryptophan catabolism means to a developing tumor, extending beyond adaptive immunoregulation to inflammatory programming, metastasis, and angiogenesis.

5 IDO Effector Pathways in Cancer Pathogenesis

5.1 Kynurenine Activates the Aryl Hydrocarbon Receptor to Modulate Inflammation

Kynurenine production resulting from IDO-mediated tryptophan catabolism is widely recognized as one of the elements which mediate the immunosuppressive effects of IDO [131], [132]. How kynurenine may contribute to inflammatory programming by IDO has been less clear, but an important perspective has opened up on this aspect with the identification of AhR as the physiological receptor for kynurenine [54]. This connection links the fields of toxicology, immunology, and cancer biology, and it may help explain why tryptophan consumption assists pathogenic inflammatory programming and drives malignant progression. In activating AhR, kynurenine not only mediates an effector signaling pathway from IDO but also TDO2 in driving cancer growth [54]. Kynurenine binding to AhR is essential to generate Treg cells that suppress adaptive immunity [133]. In binding AhR, kynurenine triggers nuclear translocation of this receptor, licensing activation of its target genes. A broad literature implicates AhR in immune regulation, inflammation, and carcinogenesis [134] in the same vein that IDO has been implicated [135]. Elevated levels of AhR correspond with poor prognosis in cancer patients [54]. The discovery that kynurenine is an endogenous ligand for AhR helps explain why there is a selection for tryptophan consumption mediated by IDO or TDO2 during tumor development, because the kynurenine that is produced binds AhR to help tumors program a pathogenic inflammation in their microenvironment that can tilt it from an antagonist to a facilitator role (i.e., from immunosurveillance toward immune escape). By connecting tryptophan consumption to AhR activation, this discovery also helps explain why immune escape and tryptophan consumption are so integrally connected in cancer [135].

5.2 IDO Activation Stimulates Stress Kinase GCN2 and Elevates IL-6 Synthesis

In a nutrient-deprived tissue microenvironment, such as that which occurs within tumors, tryptophan degradation by IDO (or IDO2 or TDO2) may cause a local tryptophan deficiency that leads to the accumulation of uncharged tryptophan-transfer RNA (tryptophan-tRNA). In this way, IDO activity may lead to activation of GCN2, a stress-response kinase that is simulated by elevations in uncharged tRNA and that limits or alters protein translation in response to this condition. Notably, T cells where GCN2 is genetically disrupted are not susceptible to IDO-mediated suppression of proliferation in vitro or in vivo, and these T cells cannot be anergized by IDO-expressing DCs [136]. Further, IDO-expressing DCs can induce the production of immunosuppressive Treg cells, but this effect is abolished by genetic disruption of GCN2. Thus, one critical downstream effector pathway for IDO to blunt T cell-mediated tumor immunity appears to involve GCN2 activation in T cells, which allows them to respond to IDO activity manifested in a local tissue microenvironment.

GCN2 functions to blunt protein translation by phosphorylating the initiation factor eukaryotic initiation factor 2 alpha (eIF-2α) which blunts its activity and prevents the readout of most RNA transcripts. However, under such conditions, some RNA transcripts actually become preferentially translated, including LIP, an isoform of the immunoregulatory transcription factor NF-IL6 (also known as CEBP-β), which then goes on to activate the expression of certain immunoregulatory cytokines such as IL-6 [137]. The relevance of this pathway is documented in vivo in tumor-bearing animals, where IDO genetic deficiency leads to reduced IL-6 production, a factor that is causally related to tumor outgrowth and metastasis [129]. The consequences of GCN2 activation by IDO in this regard may differ between cell types, since the effect of IDO on IL-6 production through this pathway can be repressive or inductive [129], [136]. Nevertheless, in experiments conducted in at least two mouse models of cancer, it appears that IDO supports IL-6 production and that this production is critical for MDSC function and malignant progression [129].

5.3 IDO Activation Inhibits mTORC1 and Stimulates Autophagy

While GCN2 is recognized as an important effector of the IDO pathway, studies in our laboratory suggested that its role in detecting tryptophan deprivation and regulating IL-6 may be insufficient for the manifestation of inflammation-driven cancers. In particular, in the mouse model of DMBA + TPA-induced inflammatory skin carcinogenesis, we found that genetic ablation of *GCN2* did not promote resistance to papilloma tumor development in the same manner that IDO did [119], [138]. This difference implied the existence of additional cancer-relevant pathways that operate downstream of IDO. In considering effector mechanisms beyond GCN2 activation, we hypothesized that IDO may suppress the master metabolic regulator mammalian

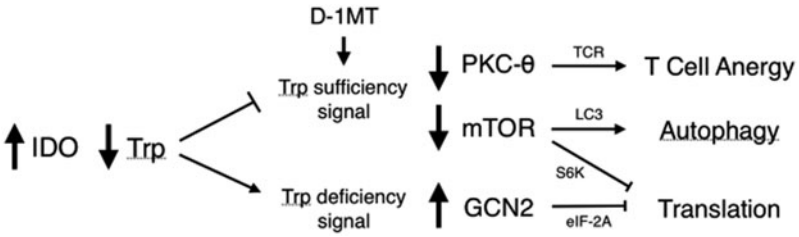

Fig. 11.1 Trp deprivation caused by IDO generates signals sensed by distinct amino acid sufficiency and deficiency pathways. Trp deficiency is sensed by the integrated stress kinase GCN2 that inhibits eIF-2α and blocks translation. Through a distinct pathway, the lack of Trp sufficiency causes mTOR to be inactivated, leading to autophagy via LC3 de-repression and translational blockade via S6 kinase inactivation. D-1MT acts as a mimetic of Trp in the sufficiency pathway, thereby functionally reversing the effects of IDO on mTOR. (The figure and legend are taken from [138])

target of rapamycin complex 1 (mTORC1), which is known to monitor not only energy status through adenosine monophosphate-activated protein kinase (AMPK) but also essential amino acid status [139], [140]. Indeed, in a set of experiments employing cells harboring an inducible IDO gene, we demonstrated [138] that IDO-mediated catabolism of tryptophan inhibits mTORC1 as well as the T-cell receptor (TCR) regulatory kinase PKC-θ, both of which are regulatory targets of a master amino acid-sensing kinase glucokinase 1 (GLK1) acting upstream of mTORC1 (also known as mitogen-activated protein kinase kinase kinase kinase 3, MAP4K3) [141]. Our findings suggest that tryptophan deprival resulting from IDO activation is read out in two distinct effector pathways, one of which is activated by tryptophan insufficiency (GCN2) and the other suppressed by tryptophan insufficiency (mTOR/PKC-θ via presumptive GLK1 blockade). As expected, mTORC1 suppression by IDO triggered autophagy, as measured by light chain 3 (LC3) processing and relocalization in cells, and this effect could be reversed by tryptophan restoration which relieved mTOR blockade [138]. The finding that IDO can regulate mTORC1 and autophagy distinct from GCN2 control may advance understanding of IDO function in the many settings where mTOR acts as a pivotal immune regulator. Further, this work offers a novel conceptual perspective on IDO by suggesting its analogy to the mTOR inhibitor rapamycin and by revealing how IDO can trigger autophagy to anergize T cells in the tumor microenvironment.

5.4 An Integrated Model for Effector Signaling by IDO-Mediated Tryptophan Deprival

Integrating our findings with existing knowledge of IDO signaling, our work supports a model in which IDO coordinately affects pathways of essential amino deficiency and sufficiency via GCN2 and mTOR, respectively, in controlling inflammatory responses and immune tolerance (see Fig. 11.1). Nutrient-sensing processes in

mammalian cells involve a set of master regulatory kinases, including AMPK, which monitors levels of ATP (energy), GCN2, which monitors levels of uncharged tRNA (amino acids), and mTOR, which integrates all nutrient information to control cell growth and autophagy. Studies in yeast [142] and hepatocytes [143] suggest that the GCN2 and mTOR pathways function in concert, for example, by demonstrations that deprivation of an essential amino acid can elevate insulin sensitivity through coordinate GCN2 activation and mTOR repression in settings where AMPK is active (i.e., energy is sufficient) [144], [145]. mTOR receives insulin or other growth factor-derived signaling information via the PI3K/Akt pathway, with Akt directly phosphorylating and activating mTOR in the rapamycin-sensitive mTORC1 complex and directly phosphorylating and inactivating tuberous sclerosis 2 (TSC2) in the mTORC1 repressor complex Rheb/TSC1-2. When activated, mTOR licenses protein synthesis by phosphorylating S6K and other translational regulators, but only if amino acid sufficiency is established by the Ragulator small guanosine triphosphatase (GTPase) complex and other signals needed to recruit mTOR to late-stage autophagosomes where it blocks autophagy (a starvation-induced process for generating amino acids). In this way, mTORC1 licenses protein synthesis if AMPK, PI3K/AKT, and Ragulator signals are all positive.

While it is not yet clear how the mTORC1 complex receives amino acid sufficiency signals, recent work [146] suggests a pivotal role for MAP4K3/GLK1, a kinase that is stimulated by undefined amino acid-binding molecules acting further upstream. MAP4K3/GLK1 would seem to offer a logical effector molecule for IDO acting upstream of mTOR and PKC-θ, based on present evidence of its role in regulating amino acid sufficiency signaling [141], [147]. In considering direct sensors of tryptophan that act further upstream of MAP4K3/GLK, the most logical candidates are the tryptophan-tRNA synthetases WARS1 and WARS2. The candidacy of a WARS molecule or variant as a proximal effector molecule for IDO is based not only upon existing evidence of WARS multifunctionality [148] but also on the recent striking discovery that the leucine-tRNA synthetase LARS senses branched chain amino acid to control mTOR activation status [149]. In future work, it will be important to establish whether WARS and MAP4K3/GLK will complete the connections of IDO to mTOR and PKC-θ to fully define this new IDO effector pathway which influences amino acid sufficiency signaling.

IDO-mediated tryptophan deprival may provide an integrated molecular switch to establish an immunosuppressive environment by amplifying tolerogenic APCs, expanding Treg cells, downregulating cytotoxic T-cell activity, and sustaining other cells that provide critical support to inflammatory carcinogenesis [120], [129]. By analogy to the mTOR inhibitory agent rapamycin, IDO may blunt immune activation and D-1MT may reorient this process by controlling tryptophan sufficiency signals needed to license mTOR activation, relieve immunosuppression, and reestablish proinflammatory states that together limit the progression of cancer or other diseases characterized by disordered inflammation and immunity. Given the implications of all mammalian tryptophan-catabolizing enzymes IDO, IDO2, and TDO in cancer progression [54], [102], [129], [135], [150], [151], further investigation is needed to understand how tryptophan depletion promotes immune escape by supporting the

development of Treg cells and MDSCs that are important to IDO-mediated cancer progression [129]. On the other hand, deprivation of any essential amino acid may be sufficient to reorient naïve $CD4^+$ T helper cells to support Treg generation [140], as the rarest amino acid Trp may assume a special position in modulating local GCN2 and mTOR status in tissue microenvironments. In future work, it will be important to explore in more detail the crosstalk between IDO and the Ragulator, MAP4K3/GLK1, and PKC-θ signals which all exert major physiological and pathophysiological effects on inflammatory programming and immune control. PKC-θ is a notable connection given its predominant function in TCR signaling which has been elucidated most fully only recently [152]. PKC-θ is dispensable for general T-cell development but critical for Treg development [153]. Its activation relies upon T538 phosphorylation [154], which occurs only upon stimulation of the TCR along with co-activator signals such as those provided by CD28 ligation. Notably, the kinase responsible for PKC-θ activation is MAP4K3/GLK [141], which is essential for signaling and differentiation of Th2 cells and IL-17-producing helper cells (Th17 cells), but not for Th1 cells. In summary, our work supports a role for PKC-θ function in IDO effector signaling, perhaps through MAP4K/GLK, as a novel potential mechanism for Treg control by IDO-mediated tryptophan catabolism.

6 IDO Inhibitors in Cancer Therapy

6.1 Therapeutic Prototype 1MT

Many studies have now provided evidence that IDO inhibition with 1MT or other small-molecule inhibitors of the IDO pathway can exert anticancer effects. Initial evidence was offered in 2002 that the IDO inhibitor 1MT could partly retard the growth of mouse lung carcinoma cells engrafted onto a syngeneic host [155]. Similar results were obtained as part of an investigation to assess the ramifications of IDO overexpression, which was detected in a wide range of human tumors [102]. In this study, ectopic overexpression of IDO in an established tumor cell line was shown to be sufficient to enable tumor formation in animals pre-immunized against a specific tumor antigen, and 1MT partially suppressed tumor outgrowth in this context as well. Against established, autochthonous (spontaneously arising) mammary tumors in the mouse mammary tumor virus-neu/human epidermal growth factor receptor 2 (MMTV-neu/HER2) transgenic mouse model of breast cancer, we found that 1MT could likewise retard tumor outgrowth [68]. By itself, however, 1MT was unable to elicit tumor regression in this model, as shown previously in the tumor cell graft models, suggesting that IDO inhibition may produce limited antitumor efficacy when applied as a monotherapy.

In contrast, the delivery of 1MT in combination with a variety of classical cytotoxic chemotherapeutic agents elicited regression of established MMTV-neu/HER2 tumors which responded poorly to any single-agent therapy [68]. In each case, the observed regressions were unlikely to result from a drug–drug interaction, that is,

by 1MT acting to raise the effective dose of the cytotoxic agent, because efficacy was increased in the absence of increased side effects (e.g., neuropathy produced by paclitaxel, which is displayed by hind leg dragging in affected mice). Immunodepletion of $CD4^+$ or $CD8^+$ T cells from the mice before treatment abolished the combinatorial antitumor effect, confirming the expectation that 1MT acted indirectly through activation of T cell-mediated antitumor immunity. We have observed that combinatorial efficacy in achieving tumor regressions can be replicated by oral dosing of 1MT at 400 mg/kg on a b.i.d. schedule, again in the absence of any detectable side effects [156]. These striking findings were one harbinger in emerging concepts of immunochemotherapy, which is the combination of conventional chemotherapy with modalities that interfere with tumoral immune escape as a strategy to improve therapeutic outcomes [157], [158]. Our results demonstrating the powerful combinatorial effects of 1MT with conventional chemotherapy helped propel this compound onto a select list of immunotherapeutic agents identified by a National Cancer Institute (NCI) workshop panel in 2008 as having high potential for use in cancer therapy [159]. First-in-man trials were initiated that year with the D isoform of 1MT, a racemic agent. Phase IA single-agent evaluation and phase IB combinatorial evaluations (with taxotere or an adenoviral p53 vaccine) are now reported to be complete (H. Soliman, N. Vahanian, pers. comm.). Some preclinical results obtained during these trials have encouraged IDO inhibitor applications in immunochemotherapy, for example, with illustrations that the powerful efficacy of the anticancer drug imatinib (Gleevec®) in the treatment of solid gastrointestinal stromal tumors relies on a blockade in the IDO expression driven by activated KIT oncogenes in this disease [160].

6.2 Mechanisms of Action of D-1MT (Indoximod): Relief of mTORC1 Suppression by IDO

Since 1MT is a racemic compound, it was necessary to choose a single molecular species for clinical testing. For a variety of reasons, D-1MT was selected instead of L-1MT, but this choice has drawn some controversy because the mechanism of action of these isoforms, particularly D-1MT, has been somewhat enigmatic. Using classical in vitro assays that employ recombinant IDO1 enzyme and the non-physiological reductant methylene blue, L-1MT acts as a weak catalytic inhibitor. In contrast, under the same conditions, D-1MT exerts little, if any, effect as a catalytic inhibitor [150]. Our laboratory has observed that in cell-based assays D-1MT can inhibit IDO2 activity [137]. Further, efficacious effects of D-1MT which have been observed in the K/BxN mouse model of rheumatoid arthritis indicate a genetic requirement for *Ido2* but not *Ido1* in the drug-targeting mechanism (L. Merlo, E. Pigott, J. DuHadaway, S. Grabler, R.M., G.C.P., and L. M-N, unpublished observations). However, in studies conducted in different cell systems, other groups have not extended the evidence that D-1MT can inhibit IDO2 activity [53], [161], [162], finding instead that D-1MT was inactive and that L-1MT was either effective [53] or ineffective [53] in blocking

IDO2 catalytic activity (including in a human T-cell assay where IDO2 function was biologically relevant [53]).

Overall, it is clear that L-1MT is more potent than D-1MT as a biochemical inhibitor of IDO, but under traditional non-physiological assay conditions that may impact catalysis by IDO and possibly even more by IDO2 (which, in such assays, displays weaker catalytic activity). As a further complexity, D-1MT can also upregulate IDO expression in cells [50] albeit only at relatively high concentrations that may be irrelevant in vivo. Factors in choosing to clinically translate D-1MT instead of L-1MT included evidence that D-1MT is more potent at relieving IDO-mediated suppression of T-cell proliferation in mixed lymphocyte reactions involving human IDO^+ plasmacytoid DCs; displays superior anticancer activity relative to L-1MT in preclincal models, both as a single agent and in combination with chemotherapy; and has been genetically validated in terms of IDO targeting based on the loss of anticancer activity in *Ido1*-nullizygous mice [150]. As alluded to above, questions concerning D-1MT as a direct inhibitor of IDO enzymes [163] must be tempered by concerns about the use of non-physiological reductants in enzyme assays. This issue is critical to appreciate, because of emerging evidence that these reductants can exert differential effects on inhibitor binding and activity when compared to physiological reductants used in the reactions (R.M., J.D., and G.P., unpublished observations).

Our recent work in identifying mTORC1 suppression as an effector mechanism in IDO-mediated tryptophan catabolism also revealed a likely mechanism of action for D-1MT (which having entered clinical trials is now also known as NLG8189 or indoximod). Specifically, we found unexpectedly that D-1MT acts as a high-potency tryptophan mimetic in reversing mTORC1 inhibition and autophagic induction by IDO, even though D-1MT is insufficient to charge tryptophan-tRNA and therefore to rescue protein translation or return GCN2 to a quiescent state. Strikingly, D-1MT relieved mTOR suppression by IDO at even higher potency than L-tryptophan itself (i.e., at lower concentrations), within a nanomolar range concentration consistent with clinical pharmacodynamics associated with patient responses in phase I trials (H. Soliman, pers. comm.). The implications of this discovery are discussed in more detail below and elsewhere [138], [164], but they provide timely insight into the unique mechanism of action of D-1MT relative to indisputable catalytic inhibitors of IDO.

The findings suggest why D-1MT is generally superior to L-1MT at breaking IDO-dependent immune tolerance in preclinical mouse models of cancer [150]. D-1MT did not affect GCN2 activation status in Trp-deprived cells, like Trp or L-1MT, arguing that D-1MT may act exclusively by restoring the mTOR pathway, unlike Trp or L-1MT which would also be expected to restore GCN2 quiescence and block kynurenine production. Mechanistically, preliminary work suggests that L-1MT but not D-1MT can inhibit WARS1A-mediated tryptophan-tRNA amino acylation (R.M., unpublished observations), thereby explaining why D-1MT could not alter levels of uncharged Trp-tRNA that would be needed to reverse activation of GCN2 triggered by IDO-mediated Trp deficiency. In contrast, an inhibition of WARS1A activity by L-1MT could explain why L-1MT is inferior to D-1MT as an anticancer compound, because WARS1A inhibition would counteract IDO inhibition

by blocking elevation of uncharged Trp-tRNA levels that are needed to activate GCN2. Moreover, given evidence [138] that L-1MT may serve as a weak substrate of IDO, unlike D-1MT, it is conceivable that catabolism of L-1MT leads to production of the product N-methyl-kynurenine, which by activating the AhR pathway like kynurenine [165] may actually limit the immunostimulatory effects of L-1MT as an IDO enzyme inhibitor. Developing these concepts may yield a more complete understanding of why L-1MT serves as a poor physiological inhibitor of IDO function compared to D-1MT and therefore a weaker candidate for clinical exploration.

The discovery that D-1MT can reverse the suppression of mTORC1 by IDO is important in translational terms, because it suggests that D-1MT may have broader clinical uses against cancers that overexpress any tryptophan catabolic enzyme (IDO, IDO2, or TDO), perhaps even being suitable to combine with specific biochemical inhibitors of these enzymes. The definition of mTORC1 and PKC-θ as candidate pharmacodynamic markers for D-1MT responses may be useful in studying the response of patients recruited to ongoing phase IB/II cancer trials, addressing a current clinical need. In this regard, we note that the concentrations at which D-1MT affects these key immunoregulatory molecules are consistent with the clinical pharmacokinetics documented in human trials [166].

6.3 Discovery and Development of Novel Enzymatic Inhibitors of IDO

IDO has a number of appealing features as a target for small-molecule drug development. First, IDO is a single-chain catalytic enzyme with a well-defined biochemistry. Unlike many proposed therapeutic targets in cancer, this means that IDO is very tractable for discovery and development of small-molecule inhibitors. Second, the other known tryptophan-catabolizing enzyme on the kynurenine pathway, TDO2, is structurally distinct from IDO and has a much more restricted pattern of expression and substrate specificity, which mitigates "off-target" issues usually posed by novel agents. Third, bioactive and orally bioavailable "lead" inhibitors exist which can serve as useful tools for preclinical validation studies. Fourth, the *Ido1*-deficient mouse that has been constructed is viable and healthy [167], and further analysis encourages the notion that IDO inhibitors will not produce unmanageable, mechanism-based toxicities [168]. Fifth, pharmacodynamic evaluation of IDO inhibitors can be performed by examining blood serum levels of tryptophan and kynurenine, the chief substrate and downstream product of the IDO reaction, respectively. Lastly, small-molecule inhibitors of IDO offer logistical and cost advantages compared to biological or cell-based therapeutic alternatives to modulating T-cell immunity.

While early-phase clinical trials with D-1MT are currently ongoing, concerns regarding its inhibitory effects on IDO catalytic activity prompt the development of pharmacologically superior IDO inhibitory compounds. The rational design and development of new inhibitory compounds requires understanding of the IDO active

site and catalytic mechanism. Proposed models for the processes at work in the active site have been developed based on mechanistic studies [169]. The publication of an X-ray crystal structure for IDO complexed with a simple inhibitor [31] has greatly facilitated this work. Alternately, screening for novel inhibitors is likely to identify novel structural series to evaluate. Through this route, our group initially identified the natural product brassinin as an IDO inhibitor and evaluated brassinin derivatives for in vitro potency and cell-based activity [170]. Brassinin is a phytoalexin-type compound found in cruciferous vegetables that has potent chemopreventive activity against breast and colon cancer in rodent models [171], [172]. In order to probe the relationship between inhibitors and the active site, that is, to perform a structure–activity relationship (SAR) analysis, we synthesized a series of derivatives from the core brassinin structure [170]. Among the conclusions drawn, we determined that the indole core is not essential for enzyme inhibitory activity, consistent with the known promiscuity of the active site in IDO [36], thus broadening the spectrum of potential inhibitory compounds. In addition, we found that the dithiocarbamate segment of brassinin is an optimized moiety for inhibition, probably on the basis of chelation of the heme iron at the active site. Of the large number of derivatives evaluated, the most potent were only $\sim 1\ \mu M$ suggesting that it may be difficult to achieve significant improvements in potency within this simple structural class.

High-throughput screening of comprehensive compound libraries remains the most effective way to identify new structural series. A unique yeast screen has been used to identify IDO inhibitory compounds representing diverse structural classes [173] including several complex natural products with potent IDO inhibitory activity [174], [175]. The insight that a naphthoquinone pharmacophore might be at the core of several of the most potent IDO inhibitory compounds led us to conduct an SAR-driven study that yielded a promising series of pyranonapthoquinone-based IDO inhibitory compounds, some with inhibition constants of less than 100 nM [176]. Similar studies based on the phenylamidazole pharmacophore have likewise yielded a series of IDO inhibitory compounds, though not achieving the degree of inhibitory potency seen with pyranonapthoquinones [177].

Recently, starting from the IDO inhibitory compound 4-phenyl-1,2,3-triazole [178], Rohrig et al. employed computational structure-based methods to design a set of more potent bioactive inhibitors that lacked cellular toxicity and exhibited high selectivity for IDO over TDO2. Explanative power in understanding the activities of this set of compounds was gained through a quantitative SAR based on electrostatic ligand–protein interactions in the docked binding modes and on quantum chemically derived charges of the triazole ring.

Starting from another structural class, a new IDO inhibitor termed INCB024360 entered phase I trials for advanced malignancies in 2010. INCB024360 is a hydroxyamidine that competitively blocks the degradation of tryptophan to kynurenine by IDO with an IC_{50} of approximately 72 nM [179]. Oral administration of this compound in mice and dogs reduced kynurenine levels in the plasma as well as in tumors and TDLNs [180]. Using several mouse models, INCB024360 delayed tumor growth in wild-type mice but not in nude mice or *Ido1*−/− mice indicating not only that this drug targets IDO1 but also that it mediates its antitumor effects through the immune

system [179], [180]. The in vivo data complement in vitro experiments showing that INCB024360 does not inhibit IDO2 or TDO2 activity [179]. An important mechanistic observation is the ability of INCB024360 to increase the survival and decrease the apoptosis of DCs suggesting that this drug may improve the number of functional DCs, thereby allowing T cells to be more effectively primed against tumor cell antigens [179].

6.4 Inhibitors of IDO Expression: Gleevec and Beyond

Possible alternative targeting strategies include inhibiting IDO expression (upstream) or inhibiting the signaling pathway through which IDO acts (downstream). As mentioned earlier, some non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to indirectly block IDO activity by inhibiting COX2 [80], and the anti-inflammatory compound, ethyl pyruvate, previously found to inhibit NF-κB activity, has been shown in mouse models to be an effective inhibitor of IDO expression and to produce robust antitumor responses that were both T cell and IDO dependent [69]. Inadvertent targeting of IDO expression may already be providing a clinical benefit, as recent investigations into the therapeutic role of Gleevec in treating gastrointestinal stromal tumor (GIST) found that the inhibition of oncogenic Kit signaling could potentiate antitumor T-cell responses by interfering with the induction of IDO [181]. Downstream of IDO, the integrated stress-response kinase GCN2 has been identified as responding to tryptophan depletion to limit T-cell responses [136] and thus might represent an alternative target. Recent attention has also focused on AhR in mediating the downstream response to tryptophan catabolites, including kynurenine and kynurenic acid [87], [88]. The liver enzyme TDO2 catalyzes the same reaction as IDO, and recent reports indicate that TDO2 elevation in some cancers may serve as an alternate mechanism for eliciting the same immune escape mechanism [54], [182]. Thus, while targeting the IDO pathway has clearly been established as an attractive approach for leveraging cancer treatment, it remains to be determined how this will be translated to provide the greatest benefit to patients.

6.5 Potential Safety Risks of IDO Inhibitors Suggested by Studies of Ido1-Deficient Mice

As summarized above, small-molecule inhibitors of IDO are being developed to treat cancer, chronic infections, and other diseases, so the systemic effects of IDO disruption on inflammatory phenomena may influence the design and conduct of early-phase clinical investigations of this new class of therapeutic agents. In assessing potential safety risks that might be monitored clinically in patients during trials of IDO inhibitors, phenotypes revealed in *Ido1*-deficient mice may be useful to consider. In a recent report, our group summarized a set of cardiac and gastrointestinal

phenotypes we have observed during several years of study of *Ido1*-deficient mice in various contexts that may warrant consideration in planned assessments of the safety risks of IDO inhibitors [183]. The most striking phenotype observed to date was calcification of the cardiac endometrium proximal to the right ventricle. This phenotype was 30 % penetrant, specific to *Ido1* deficiency on the BALB/c strain background and sexually dimorphic in nature [183]. Additionally, we observed that administration of complete Freund's adjuvant containing Toll-like receptor ligands known to induce IDO caused acute pancreatitis in *Ido1*-deficient mice [183], with implications for the design of planned combination studies of IDO inhibitors with cancer vaccines. Further, in an established model of hyperlipidemia, caused by homozygous deletion of the murine low-density lipoprotein (LDL) receptor, we found that IDO deficiency caused a dramatic elevation in levels of serum triglycerides [183]. This risk factor may be relevant in cancer patients receiving IDO inhibitors who may also have occult unstable cardiovascular plaques. Lastly, we observed an increased sensitivity of *Ido1*-deficient mice to induction of acute colitis, with a marked elevation in tumor incidence, multiplicity, and staging in animals subjected to regimens of inflammatory colon carcinogenesis [183]. These findings suggested risks of colitis in the short term and colon carcinoma in the longer term in patients who may receive IDO inhibitors as part of their therapy. Here, we note that administration of D-1MT has never been observed to produce any of these phenotypes, but the benign nature of this compound may relate to its limited activity in blocking the IDO pathway at the level of mTORC1 restoration (or other targets), relative to more potent biochemical inhibitors in development. Together, while the phenotypes in *Ido1*-deficient mice characterized to date have been observed only under certain stress conditions, they suggest potential cardiac and gastrointestinal risks of IDO inhibitors, in particular as they will be tested in combination with other therapeutic modalities, that should be monitored in patients as this class of drugs proceeds through clinical development.

7 Conclusions

In a relatively short period, IDO has become recognized as a major regulator of the immune system. Pathophysiologically, IDO has been strongly implicated in tumoral immune tolerance and immune escape and appears to be widely overexpressed in cancer at the level of tumor cells and/or tumor-associated immune regulatory cells. IDO has a variety of characteristics that make it an appealing target for cancer drug development. To date, preclinical validation of IDO inhibitors suggests they may offer the greatest promise in combination with classical cytotoxic drugs, but their potential to heighten the response to active immunotherapeutic agents such as TLR ligands or tumor vaccines is also important to consider. Given the provocative preclinical findings that have emerged from studies of agents targeting IDO and the IDO pathway, one would expect therapeutic interest in this pathway to continue to grow.

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Chapter 12
Defining the Fate and Function of Effector T cells Through Galectin-1–Galectin-1 Ligand-Binding Interactions: Implications in Tumor Immunity

Charles J. Dimitroff and Gabriel A. Rabinovich

Abstract Galectin-1 (Gal-1), a member of an ancient family of animal glycan-binding proteins, has been implicated in a variety of biological events. Interactions between Gal-1 and Gal-1 ligands on T cells are critically involved in regulating the nature and intensity of T-cell-mediated inflammation and antitumor immunity. Appropriately glycosylated T-cell-membrane glycoconjugates operationally defined as Gal-1 ligands bind Gal-1 and elicit downstream cellular activities that dampen effector T-cell function. Together, these biological constituents represent promising targets in the development of novel anti-inflammatory and antitumor immune therapies. Whether through characteristic elevations in tumor-derived Gal-1 or an imbalance in regulatory and Gal-1 ligand$^+$ effector T-cell subsets during inflammation, the Gal-1–Gal-1 ligand-binding axis offers numerous cellular/tissue contexts to strategically interfere with Gal-1 efficacy. In this chapter, we will examine recent assessments of (1) Gal-1 expression and function in controlling both adaptive and antitumor T-cell immunity, (2) identity and function of T-cell Gal-1 ligands, and (3) targeting of the Gal-1–Gal-1 ligand axis to regulate inflammation or boost antitumor immune responses. These research disciplines collectively highlight the importance of understanding the identity and functional nature of Gal-1 and its ligands to strategically and safely manipulate the immune system to control immunopathologic conditions.

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1 Overview of Galectin-1 and Galectin-1 Research

1.1 Expression and Tissue Localization of Galectin-1

Gal-1 is an S-type lectin that contains numerous cysteine residues, which regulate its structure, oligomerization, and the binding activity of its carbohydrate-recognition domain (CRD) [1]–[3]. Of the 15 evolutionarily conserved β-galactoside-binding galectins, Gal-1 is the prototype variant with a single CRD existing in nature as monomeric, dimeric and/or multimeric forms. It is secreted through a nonclassical, Golgi-independent pathway and is structurally stabilized when bound to native cellular and extracellular ligands [4]–[6]. Gal-1 exhibits the highest affinity for N-acetyllactosaminyl moieties commonly found in extended antennae on N-glycans and core 2 O-glycans displayed by select membrane glycoproteins [7]–[18]. Dimerization of Gal-1 through noncovalent bonds offers optimal binding capacity, though oxidation–reduction flux in tissue microenvironments can affect intra-/intermolecular sulfhydryl bonding to induce/prevent oligomerization and CRD function [15], [19], [20].

Gal-1 is found in a variety of cells and tissues; however, it is expressed at relatively high levels by endothelial cells (ECs), activated B and T cells, inflammatory macrophages, decidual natural killer (NK) cells, and $FoxP3^{+}$ regulatory T cells [6], [21]–[34]. Furthermore, Gal-1 is markedly elevated in a number of tumor types, notably melanomas, lymphomas, gliomas, Kaposi's sarcoma, and carcinomas of the prostate, colon, ovary, and breast, and at the fetal–maternal interface [12], [26]–[28], [35]–[53]. Gal-1 functions in both physiologic and pathological contexts as a homo-/heterotypic cancer cell adhesion molecule, a modulator of immune cell growth and survival, and/or a regulator of adaptive and innate immune responses. Although first characterized as an adhesive molecule in the extracellular matrix mediating binding to ovarian cancer cells [54]–[56], Gal-1 is now more commonly known for binding to dendritic cells (DCs) and effector T cells, causing T-cell apoptosis or tolerization/exhaustion to help prevent fetus/graft rejection and autoimmunity and to promote tumor progression [13], [24], [37], [53], [57]–[63]. What is becoming increasingly clear is that tumor-, T-cell-, and EC-derived Gal-1 forms are involved in Gal-1-dependent immune regulation, fetal tolerance, and tumor growth. In melanoma and lymphoma models, complementary expression of Gal-1 incrementally influences various aspects of tumor growth by regulating antitumor immunity, tumor cell migration, and/or tumor angiogenesis [22], [26], [33], [35], [37], [39]–[41], [64]. Gal-1 localization in immune tissues, stroma, and cancer microenvironments and its critical roles in inflammation and malignancy have, thus, raised the level of significance of Gal-1 research in academia and the pharmaceutical industry.

1.2 Studying Gal-1 Effects and Cellular Targets

Understanding how Gal-1 binds N-acetyllactosaminyl-bearing receptors and transmits downstream intracellular signals, transcriptional activities, and production of anti-inflammatory/pro-tumorigenic factors are paramount to the goals of Gal-1 research. Considering the growing appreciation for (1) Gal-1 effects on immune mechanisms and malignancy [24], [53], [57], [59], [65]–[68], (2) the temporal and spatial localization of Gal-1 in tissues [6], [12], [21]–[33], [35]–[52], (3) innovative printed glycan array technologies [16], [17], [69], [70], (4) effective neutralizing Gal-1 antibodies (Abs) [12], [26], [36], [39], [40], [71], (5) inhibitors of Gal-1 binding or glycan formation, (6) regulators of Gal-1 function [33], and (7) stable mimetics of native Gal-1, Gal-1 research has been reinvigorated. In truth, studies on identity of Gal-1 ligands translating to a functional property and cellular trait, distinct from apoptosis induction, are now readily appreciated and a major focus in Gal-1 studies [13], [22], [24], [35], [36], [39], [40], [51], [72]–[75].

To study the cellular effectors of Gal-1, innovative tools designed to stabilize Gal-1 structure and function have been developed for probing the Gal-1 ligand and its function. While using Gal-1-deficient mice and component immune cell isolates from these mice have proven invaluable for studying Gal-1's role in adaptive and innate immune responses, more recent efforts using stabilized dimeric Gal-1 forms have allowed for observations at a wider range of concentrations. Historically, research using recombinant Gal-1 (rGal-1) has been limited due to the abundance of sulfhydryl groups and confounding effects caused by stabilization chemicals added to rGal-1 preparations. As rGal-1 formulations undergo rapid oxidation, aberrant oligomerization and abnormal folding of the CRD result in limited function in bioassays restricted to < 10 h [76]. To circumvent oxidative inactivation, rGal-1 preparations are commonly supplemented with reducing agents, such as dithiothreitol (DTT), that enhance CRD stability though alter the monomer–dimer–oligomer equilibrium. As a result, rGal-1 concentrations ≥ 7 μM are necessary to help prevent dissociation into monomeric forms that have weaker binding capacity [76]. At this high concentration, however, rGal-1 favors pro-apoptotic effects on activated leukocytes and leukemic cell lines, thereby obscuring other immunomodulatory activities controlling T-cell differentiation and cytokine biosynthesis. Furthermore, recent evidence confirms that DTT itself sensitizes cells to undergo apoptosis, which may also undermine authentic Gal-1 biological activity on T cells [77].

To avoid oxidative inactivation and monomer–dimer equilibrium dynamics, recent efforts have centered on developing structural stabilizers, such as immunoglobulin (Ig) fusions [70], [72], alkylation [78], leucine zippers [75], and a lectin-linker domain [20], to help maintain dimerization. While high concentrations elicit characteristic pro-apoptotic responses, use of lower and perhaps more physiologic concentrations of these dimeric Gal-1 mimetics can now be used to study new, previously unknown, Gal-1 effects. This is particularly suitable in controlled in vitro assays, where cell-specific responses of primary cell isolates, cell lines, or co-cell

culture systems are assayed. So, in addition to defining mechanisms of death induction, examining Gal-1-induced cytokine secretion or other surface immunoregulatory molecule expression can now be investigated [15], [70], [72], [74], [75], [79]–[82]. A Gal-1–human Ig chimera (Gal-1hFc), for example, can stably form functional and stable dimers below the 7-μM threshold, while retaining canonical β-galactoside-binding activity in the absence of reducing agents. Importantly, Gal-1hFc shares similar glycan recognition patterns as native and rGal-1, but it can also be used in bioassays for periods up to 48 h without losing functionality [18], [35], [41], [70], [73]. By probing for the Ig domain, Gal-1hFc can be used to detect Gal-1 ligands in mouse and human specimens by Western blot, flow cytometry, immunohistochemistry, and immunofluorescence assays, which have proven too difficult with rGal-1. Importantly, in cell cultures, use of Gal-1hFc has facilitated observations on elevated production of interleukin-10 (IL-10), programmed death-1 (PD-1), and cytotoxic T lymphocyte antigen-4 (CTLA-4) expression and on lower interferon-γ (IFN-γ) or IL-17 levels in activated T helper (Th) or polarized $CD4^+$ Th1 or Th17-cell subsets, suggesting that Gal-1 may indeed trigger a type 1 regulatory ($FoxP3^-$) Tr1-like cell phenotype. Future use of these dimeric Gal-1 mimetics will not only unveil new Gal-1-mediated immune functions but may also help discern ill-defined malignancy-associated effects on cancer cells.

1.3 Indirect and Direct Galectin-1 Effects on Effector T-cell Function

Gal-1 is a key regulator of T-cell-mediated inflammation and antitumor T-cell responses. By its restricted recognition of Gal-1-binding glycans on distinct membrane proteins expressed by DCs and certain effector T-cell subsets, Gal-1 binding selectively tolerizes, eliminates, and/or alters the differentiation of Th1/Th17 cells/DCs. Distinct glycan profiles on Th-cell subsets in fact enable Gal-1 selectively in binding on proinflammatory Th1- and Th17-cell subsets and not on naïve, Th2, or regulatory $FoxP3^+$ T cells [13]. Through the expression of pro-binding complex N-glycans, core-2 O-glycans, and/or asialo core-1 O-glycans or of anti-binding N-acetylneuraminic acid α2,6 galactose-R terminal residues [13], Gal-1 action can shape T-cell subset levels and corresponding T-cell-dependent processes. These effects ultimately tilt the balance between effector–regulatory Th-cell subsets to help silence inflammatory reactions or neutralize antitumor T-cell responses. This selectivity of distinct T-cell subsets imparts Gal-1 immunomodulatory activity on T cells; however, recent evidence suggests that T-cell subset skewing can also be encouraged via Gal-1 binding to antigen-presenting cells (APCs).

There is strong evidence that Gal-1 can, in fact, both indirectly and directly induce T cells to express immunoregulatory molecules. Through dependency on APCs relaying tolerogenic signals to T cells during activation/priming or through direct T-cell binding, Gal-1 can trigger expression of the immunoregulator IL-10 among other immune-dampening molecules.

1.3.1 Galectin-1-Dependent Regulation of Effector T Cells via DC Instruction

A pioneering report in 2009 provides convincing evidence that Gal-1 binding to DCs induces T-cell tolerization in an IL-10-dependent manner [22]. Whether Gal-1 binds to immature DCs or to antigen (Ag)-experienced APCs, which is governed in part by glycans on CD43, this study demonstrates that Gal-1 can limit DC maturation and priming potential and can elicit a paracrine effect during T-cell priming and activation. The acquisition of DC tolerogenic function is characterized by STAT3-dependent IL-27 secretion, which upon binding to IL-27R$^+$ T cells, stimulates the generation of IL-10-producing Tr1 cells. This Gal-1-dependent DC–T-cell tolerizing mechanism coincides with the peak and resolution phases of autoimmune responses and enhances tumor growth rates by the immunosuppressive effects of IL-10 [22]. Importantly, this study establishes the ability of an exogenous preparation of Gal-1 to influence the differentiation of a pure immune cell population compared with prior efforts using unsorted α-CD3-stimulated peripheral blood mononuclear cells (PBMCs).

1.3.2 Galectin-1-Dependent Regulation of Effector T Cells Without DC Input

Using new stable dimeric Gal-1 mimetics and advanced methods to polarize and expand Th-cell subsets, subsequent studies have tested the hypothesis that Gal-1 can directly differentiate and alter the function of undifferentiated Th cells or fully committed proinflammatory Th1-/Th17-cell subsets [73]. At non-death-inducing concentrations, Gal-1hFc chimera binds a select glycoform of CD45 on activated Th cells and Th1-/Th17-cell subsets that results in increased STAT1/STAT3/STAT6 phosphorylation signals and in upregulated IL-21 expression via transcriptional activity of c-Maf and the aryl hydrocarbon receptor (AhR) [73]. As a result, Gal-1-treated T cells characteristically upregulate IL-10 expression and, in the case of committed Th-cell subsets, Gal-1 causes a reversal of the ability to produce IFN-γ or IL-17 [41], [73]. Interestingly, these Gal-1-induced IL-10$^+$ T cells, which are negative for FoxP3, also show increased expression of PD-1 and CTLA-4. These Tr1-like cells functionally suppress T-cell proliferation, ameliorate allergic dermatitis, and promote tumor immune escape in an IL-10-dependent manner.

Direct Gal-1-dependent effects have also been further validated in studies assessing the role of native tumor-derived Gal-1 on sorted benign T-cell populations without DCs in culture assays performed in vitro. As such, strong evidence implicates Reed–Sternberg cell-derived Gal-1 from patients with Hodgkin's lymphoma in causing Th2 skewing underscored by IL-4 production and in elevating FoxP3$^+$ regulatory T-cell levels [26]. Likewise, clonal malignant T-cell-derived Gal-1 in patients with leukemic-cutaneous T cell lymphoma (L-CTCL) can attenuate benign T-cell proliferation and diminish Th1 responses [35].

Whether through stable dimeric Gal-1 mimetics or native tumor-derived Gal-1 present in supernatants, Gal-1 effects can be demonstrated through direct interaction with Th cells without co-culturing DCs. Notably, concentrations of dimeric Gal-1

are significantly lower and perhaps more physiologic than those needed to maintain dimers in rGal-1 preparations, which, as a consequence, only result in apoptotic induction.

2 Galectin-1-Binding Membrane Proteins (Galectin-1 Ligands)

2.1 Identity and Expression of Galectin-1 Ligands on T Cells

The significance of Gal-1 in regulating T-cell responses has revolutionized the therapeutic prospect of treating T-cell-dependent immunopathologies [83], [84]. While neutralization of Gal-1-binding activity through Ab or competitive inhibitor approaches is a promising strategy, targeting the ligand arm of the Gal-1–Gal-1 ligand axis also represents a potentially effective approach. Because Gal-1 ligand expression is restricted to activated $CD4^+$ T cells and $CD8^+$ T cells and polarized Th1- and Th17-cell subsets, there may, in fact, be more cell-specific downstream effectors following Gal-1 binding that represent potential therapeutic targets. To this end, Gal-1 ligand identity and expression in conjunction with downstream cellular effectors are still ill-defined and underscore renewed efforts to study Gal-1 ligands and binding consequences in T cells.

Dimeric Gal-1 has a high binding affinity for canonical type 1 (galactose β1,3-N-acetylglucosamine) or type 2 (galactose β1,4-N-acetylglucosamine) N-acetyllactosaminyl moieties. These disaccharides help comprise extended core 1 and core 2 O-glycan antennae and are variably contained in bi-/tri- or tetra-N-glycan antennae. Conversely, Gal-1-binding activity is prevented when these antennae are terminated with α2,6-sialylated residues [7], [13], [15]. As these structures can theoretically be expressed and found on the surface of all mammalian cells, expression of these N-acetyllactosaminyl moieties is restricted and, in most cases, differentiation- and developmentally dependent on the synchronized activity of glycosyltransferases and glycosidases.

Whether due to high cell-surface expression, precise localization in the "landscape" of the glycocalyx, and/or abundance of Gal-1-binding (galactose β1,3/4-N-acetylglucosamine) moieties, Gal-1 appears to only bind a limited subset of T-cell membrane proteins. That is, there may be additional specificity conferred by the membrane protein scaffold. In consideration of these attributes and cell-/differentiation-specific nature of Gal-1 binding, a discrete membrane protein that bears Gal-1-binding moieties on its N- and/or O-glycans is operationally defined as a *Gal-1 ligand*.

Given the advent of more stable dimeric Gal-1 mimetics that can be used in laboratory bioassays, recent efforts in Gal-1 ligand research have cemented and, in some cases, advanced our understanding of the Gal-1-binding repertoire on T cells and expanded our knowledge of how Gal-1 ligand engagement controls T-cell function.

2.1.1 Galectin-1 Ligands on CD4$^+$ T Cells

Early studies have shown that rGal-1 can bind CD2, CD3, CD4, CD7, CD43, and CD45 on primary activated CD3$^+$ T cells or human leukemic T-cell lines [7], [10], [11], [85]–[91]. By controlling for expression or by analyzing segregation and formation of surface microdomains following rGal-1 incubations, these studies demonstrate that this select glycoprotein repertoire helps regulate T-cell apoptotic activity. Moreover, modifying N- and O-glycan chains on these glycoproteins, through overexpression of key glycosyltransferases necessary for core 2 antennae formation or metabolic inhibition of N-/O-glycan biosynthesis, impinges on subsequent Gal-1-dependent apoptotic activity, suggesting that apoptotic activity is dependent on glycosylation activity and formation of Gal-1-binding moieties.

Results from these studies have recently been strengthened with direct evidence of Gal-1 binding to component N- and/or O-glycans on T-cell glycoproteins and have been expanded to include Gal-1 ligand analysis on primary undifferentiated and fully committed effector Th-cell isolates [41], [70], [73]. The identity of Gal-1 ligands on activated Th0 cells and on Th1- and Th17-cell isolates has been examined using Gal-1hFc, which is capable of detecting canonical Gal-1-binding N-acetyllactosaminyl moieties on both native and denatured glycoproteins. Fluorescence-activated cell sorting (FACS), immunofluorescence, immunoprecipitation, and Western blotting analyses with Gal-1hFc together show that CD43 and CD45 are the major Gal-1-binding Th-cell glycoproteins [41], [70], [73]. These studies show that CD43 and CD45 are indeed key Gal-1 ligands on mouse and human Th cells. Binding of Gal-1 to CD45, in particular, has been shown to transmit pro-apoptotic activity or, at low concentrations, immunoregulatory activity defined by IL-10 expression [40], [68], [71]. These Gal-1-induced IL-10$^+$ (FoxP3$^-$) Th cells show elevated PD-1 and CTLA-4 levels, resembling Tr1 cells that can suppress T-cell proliferation and T-cell-dependent inflammation and promote tumor immune evasion in an IL-10-dependent manner [41], [70], [73]. What these studies reveal is that, unlike FoxP3$^+$ regulatory Th cells which are negative for Gal-1 ligand, Gal-1-induced IL-10$^+$ Tr1-like cells also express Gal-1 ligands.

The existence of Gal-1-induced Tr1-like cells challenges our current belief in which Gal-1 ligands are preferentially expressed on effector nonregulatory Th cells. These results compel reconsideration of the postulate that Gal-1 ligand$^+$ T cells in inflamed tissues or tumors represent effector proinflammatory or antitumor T cells. As such, in diagnosing melanomas and in prognosticating a personalized treatment plan, pathologists often annotate the level of tumor-infiltrating lymphocytes (TILs) as rationalized by their presumed antitumor activity. The possibility that Gal-1 ligand$^+$ TILs are IL-10 producers and are not representative of an antitumor TIL infiltrate potentially undermines TIL assessments as a measure of tumor progression. An imbalanced level of Gal-1 ligand$^+$ IL-10$^+$ Th cells in tumors may, in fact, implicate an aggressive tumor phenotype. To help distinguish these immune states relative to Gal-1 ligand expression, a dual-marker approach, such as Gal-1 ligand and IL-10 or IFN-γ and IL-17, will need to be employed in future analyses to accurately characterize tumor immune activity in vivo (Fig. 12.1)

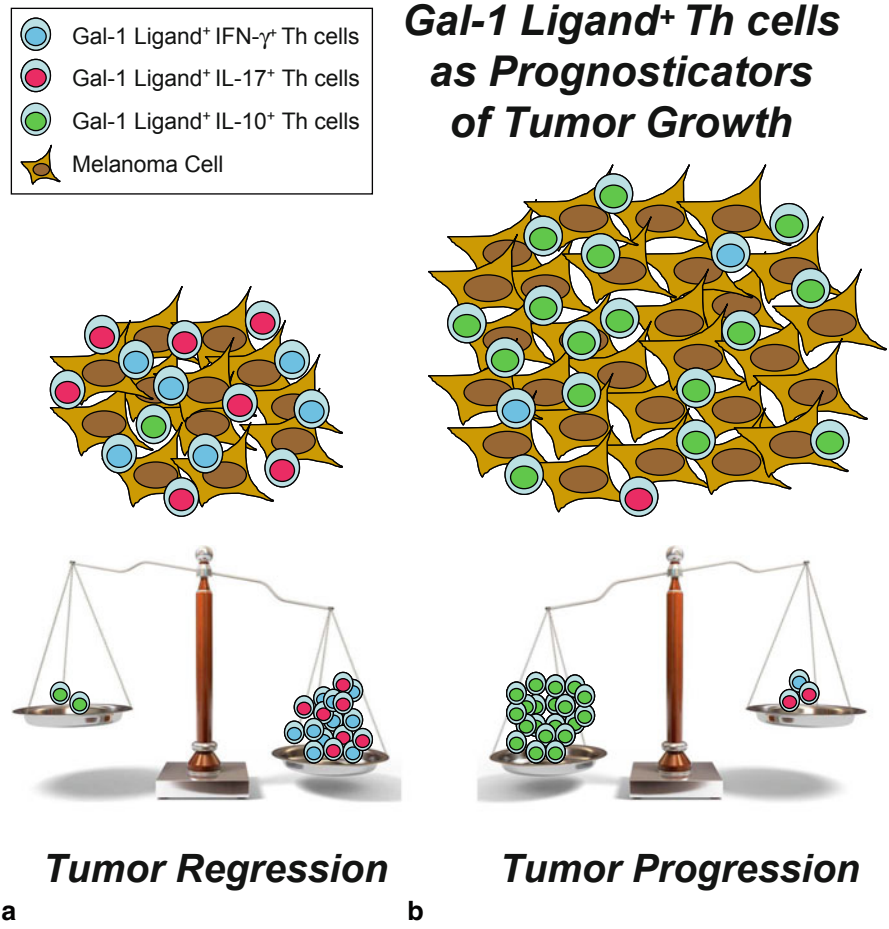

Fig. 12.1 Hypothetical distribution scenarios of Gal-1 ligand$^+$ Th cells in regressing or expanding tumors. Gal-1 ligands are expressed on effector Th1 cells and Th17 cells and on IL-10$^+$ Tr1-like cells [13], [41], [70], [73]. To examine the level and effector function of Th cells in tumors as a prognostic measure of tumor growth, Gal-1 ligand$^+$ Th cells could be distinguished as either immunoregulatory or antitumor. That is, dual analysis methods should be employed to help discriminate Gal-1 ligand$^+$ IL-10-producing cells from Gal-1 ligand$^+$ IFN-γ-/IL-17-producing cells. **a** In this scenario, a regressing melanoma may be inferred from the imbalance of Gal-1 ligand$^+$ IFN-γ$^+$ or IL-17$^+$ Th cells to Gal-1 ligand$^+$ IL-10$^+$ Tr1-like cells. **b** Alternatively, melanoma progression could be indicated by an imbalance of elevated Gal-1 ligand$^+$ IL-10$^+$ Th cell numbers to low Gal-1 ligand$^+$ IFN-γ$^+$ or IL-17$^+$ Th-cell levels

2.1.2 Galectin-1 Ligands on CD8$^+$ T Cells

Data obtained through incubations of CD8$^+$ T cells (cytotoxic T cell, CTLs) with dimeric Gal-1 or by examining immunity in Gal-1-deficient mice or in mice bearing

Gal-1-silenced tumors suggest that the fate and function of CTLs, including viral- and tumor-specific CTLs, are likely regulated by Gal-1. Evidence shows that Gal-1 can suppress proliferative activity, inhibit IFN-γ expression, enhance IL-10 expression, modulate TCR signaling, and/or promote apoptotic activity in the CTL subset [37], [40], [41], [63], [74], [92]–[96]. Given the functional consequences and alignment with Gal-1 ligand-associated data on Th cells, there is a paucity of data on the identity of Gal-1 ligand(s) on primary mouse and human CTLs. Furthermore, connections between a distinct Gal-1 ligand and a specific functional consequence on CTLs are lacking.

While the T-cell receptor (TCR) and CD43 and CD45 are candidate Gal-1 ligands on CTLs based on their abundance and influence on cell proliferation on T cells [97], direct evidence showing Gal-1-binding to these structural constituents on CTLs has still not been demonstrated. Moreover, the relative contribution of N- and/or O-glycans contributing to Gal-1-dependent apoptosis in CTLs remains inconclusive [98]. Recent data on N-glycans from activated CTLs compared with naïve CTLs, however, show that they contain more and longer multi-antennary structures consistent with an increase in Gal-1-binding N-acetyllactosaminyl moieties [99], [100]. Further studies are needed to help clarify the relative contribution of N- and/or O-glycans on distinct CTL membrane proteins from Ag-specific CTL isolates stimulated through authentic APC-T cell priming and co-expression of stimulatory/inhibitory activation molecules.

2.2 Glycosyltransferase Regulators of Galectin-1-Binding Activity

Synthesis of Gal-1-binding moieties on a T-cell membrane glycoprotein (i.e., formation of Gal-1 ligand activity) is governed by the activity of β1,4-galactosyltransferases and β1,3-N-acetylglucosaminyltransferases. The coordinated action of these enzymes lends to the construction of Gal-1-binding type 2 (galactose β1,4-N-acetylglucosamine) N-acetyllactosaminyl moieties in extended core 2 O-glycans or in complex bi-/tri-/tetra-antennary N-glycans. Moreover, Gal-1 preferentially recognizes linear repeats of N-acetyllactosamines referred to as poly-N-acetyllactosaminyl glycans ((galactose β1,4 N-acetylglucosamine β1,3)$_n$) [15], [101].

While there are striking differences in Gal-1-binding activity between naïve and activated/memory T cells, levels of β1,4-galactosyltransferases and β1,3-N-acetylglucosaminyltransferases are surprisingly relatively unchanged [18], [99]. The subset of enzymes that are in fact altered and help regulate Gal-1 ligand activity are core 2 β1,6-N-acetylglucosaminyltransferase-1 (C2GnT) [10], β1,6-N-acetylglucosaminyltransferase (Mgat5) [102] and α2,6-sialyltransferase (ST6Gal-1) [7], [13], [15], [99], [100]. Upon activation of a naïve T cell, a core 2 O-glycan is initiated by elevated levels and action of C2GnT [10]. Following β1,6-transfer of the N-acetylglucosamine to N-acetylgalactosamine in the core 1 backbone via C2GnT, Gal-1-binding galactose β1,4-N-acetylglucosamine moieties are sequentially added to the core 2 extension. T-cell activation is also regulated through the collaborative activity of N-glycan branching N-acetylglucosaminyltransferases

(Mgat1, Mgat2, Mgat4/b, Mgat5) that are essential for development of multi-antennary N-glycans, whose antennae can then serve as acceptors for synthesis of N-acetyllactosaminyl [14], [103], [104]. Mgat5, in particular, exhibits a β1,6-N-acetylglucosaminyltransferase activity that helps generate the tetra-antennary N-glycan variant, which is commonly used for poly-N-acetyllactosamine formation and enhancement of galectin-binding avidity [102]. The role and activity of ST6Gal-1, to the contrary, have been shown to be critical in preventing Gal-1 binding [7], [13], [15], [99], [100]. When galactosyl residues on N-glycan antennae are terminated with an α2,6-linked N-acetylneuraminic acid, Gal-1 is incapable of recognizing internal N-acetyllactosaminyl moieties, which is characterized on Th2 cells [13].

In all, C2GnT, Mgat5, and ST6Gal-1 have been shown to play key roles in regulating the Gal-1 ligand activity of activated Th cells and CTLs. Nevertheless, in that nonphysiologic anti-CD3 TCR stimulation is commonly used for generating "activated" T cells in Gal-1-binding and glycosyltransferase analyses, further studies using native memory Th- and CTL-cell isolates, including Ag-specific T cells created by native APC-mediated Ag priming, need to be conducted to validate and possibly refine our knowledge of the glycobiological regulators of Gal-1 ligand activity. Of note, it is apparent that terminating an N-acetyllactosaminyl moiety with an α1,3-fucose residue can inhibit Gal-1 binding [15], [70]. However, determining whether a distinct role for leukocytic α1,3-fucosyltransferase (FT) 4 and 7 activity in the negative regulation of T cell Gal-1 ligand activity has not been formally studied. The collaborative activity of FT4 and 7 in skin-homing Th cells, for example, is a fundamental requirement in generating E-selectin ligand activity and consequent skin-homing capabilities [105]–[110]. As such, upon entering and residing in the skin, one could postulate that expression α1,3-fucosyl moieties via T-cell FT4 and 7 activities may help lower susceptibility to Gal-1-mediated immunomodulation and maintain normal immunologic function in skin.

3 Targeting Galectin-1– Galectin-1 Ligand Interactions

The importance of Gal-1–Gal-1 ligand-binding interactions on T cells for controlling immunopathologies and tumor immune evasion has been cemented during the last decade. Due to the potential of suppressing inflammation and augmenting antitumor T-cell immunity, there is a growing translational interest for developing methods to regulate this binding axis. Considering our contemporary knowledge of temporal and spatial expression of Gal-1 and its ligands in the immune system, we are now poised to strategically target this binding axis to effectively enhance or limit Gal-1-mediated immunosuppression in vivo. Advances in neutralization approaches, including functional blocking anti-Gal-1 monoclonal Abs, structural mimetics of Gal-1-binding moieties, metabolic inhibitors of Gal-1-binding glycans, and regulators of Gal-1 expression, are now available for therapeutic assessment in research models of inflammation and cancer.

3.1 Competitive Inhibitors of Galectin-1-Binding

3.1.1 Neutralizing Anti-Galectin-1 Antibodies

Recent evidence shows that inhibiting Gal-1-binding activity with neutralizing anti-Gal-1 monoclonal Abs critically defines an immunomodulatory role for Gal-1 in T-cell culture systems [40], [71]. In one study, using neutralizing anti-human Gal-1 monoclonal Ab (8F4F8G7) effectively averts Gal-1-dependent viral-specific CTL apoptosis [40]. This monoclonal Ab has also been shown to be effective in blunting Gal-1-dependent angiogenic activity associated with tumor growth and progression [12], [36]. In other work, efforts to demonstrate Gal-1-dependent immunoregulation are validated by using blocking anti-mouse Gal-1 (clone 201002) and anti-human Gal-1 (clone 25C1) monoclonal Abs to block Gal-1-mediated immunosuppressive activity of both mouse and human FoxP3$^+$ regulatory T cells [71].

In all, data from these reports highlight the effectiveness of neutralizing monoclonal Ab approaches and confer the specificity needed to help differentiate Gal-1's role from other galectins, such Gal-3 and Gal-9, which may also have immunoregulatory activities [83]. Future efforts to humanize these neutralizing monoclonal Abs have now intensified given the prospect of antagonizing Gal-1 function to help boost antitumor immune responses in patients with cancer.

3.1.2 Lactosyl Mimetics

One of the most commonly used reagents for inhibiting the function of Gal-1 is lactose (galactose β1,4 glucose). Whether by pre-incubating Gal-1 preparations with lactose prior to Gal-1 ligand analyses or by adding lactose to T-cell cultures for assaying Gal-1 efficacy, low millimolar concentrations can effectively block Gal-1 binding. More recent efforts using more potent digalactosyl mimetics as Gal-1 competitive inhibitors have revealed therapeutic value in antagonizing Gal-1 activity to improve CTL expansion and function in vitro and tumor immunity in vivo. A thiodigalactoside (TDG) molecule has been shown to effectively inhibit galectin-1 function in tumor cell–CTL tumoricidal assays, resulting in enhanced CTL activation, expansion, and tumoricidal activity [111]. Moreover, tumor immune responses are boosted in tumor-bearing mice treated via in vivo administration of TDG [111]. These observations are reinforced in subsequent work evaluating TDG's in vivo efficacy, showing that TDG treatment can increase effector T-cell levels in the blood of tumor-bearing mice and augment the number of TILs and corresponding apoptotic$^+$ tumor cells in tumors [30], [96]. Results from these studies depict the promise of using small-molecule TDG or TDG-like structures to block Gal-1 immunosuppression. However, TDG and lactose have broad specificity for other members of the galectin family, which may not be involved in tumor immune escape, angiogenesis, or metastasis, thus precluding their direct use in the clinics. Moreover, understanding whether these lactosyl mimetics are well tolerated in humans and whether they possess sufficient half-life to elicit Gal-1 antagonism and immune boosting is vital to engender further optimism as a future cancer immunotherapeutic approach.

3.2 Metabolic Inhibitors of Galectin-1-Binding Carbohydrates

Analogs of glucosamine were originally designed to therapeutically antagonize the heightened production and utilization of nucleoside triphosphates in cancer cells. The intent was to interfere with glycoconjugate, DNA, and/or RNA synthesis via lowering of endogenous adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), thymidine triphosphate (TTP), and uridine triphosphate (UTP) levels [112], [113]. Subsequent analyses on tumor cells revealed a striking deglycosylation effect at non-growth-inhibitory concentrations [55], [56], [114]. In later work using a 4-fluorinated analog of N-acetylglucosamine (4-F-GlcNAc), results indicate that 4-F-GlcNAc can effectively limit the expression of N-acetyllactosamines necessary for E-selectin- and Gal-1 binding to T cells [18], [110], [115]–[117]. By lowering endogenous uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) levels [18], 4-F-GlcNAc metabolically antagonizes N-acetyllactosamine formation, which has been shown to be more effective at preventing Gal-1 binding than E-selectin binding [18]. Current studies have now focused on antagonizing Gal-1-mediated effects on proinflammatory Th1 and Th17 cells and CTLs in mouse models of cancer [41]. Data show that, compared with untreated controls in Gal-1^{+} tumor-bearing mice, 4-F-GlcNAc treatment limits the production of Gal-1-induced IL-10^{+} Th cells, while it increases Th1- and Th17-cell levels. The net immunologic effect results in attenuated tumor growth rates. The pharmacologic intent in this pathobiologic context is to capitalize on high glycometabolic activity in effector T cells and make them resistant to Gal-1 binding and related immune evasion of Gal-1^{hi} tumors, such as melanomas and lymphomas. Given the effectiveness of 4-F-GlcNAc-dependent antitumor T-cell immunity, an advantage is that it could deplete Gal-3- and Gal-9-binding N-acetyllactosaminyl moieties. Because these galectins can also impact immune mechanisms [65]–[67], [83], the potential of inhibiting binding moieties for all three galectins could indeed synergize the antitumor T-cell response, making N-acetyllactosamine lowering a very effective approach [118]. Moreover, tailoring 4-F-GlcNAc treatment during the in vitro expansion of tumor Ag-specific T cells for adoptive-transfer clinical protocols could potentially result in Gal-1-resistant antitumor T cells with improved longevity in vivo [118].

3.3 Regulators of Galectin-1 Binding

An interesting study by Thijssen et al. shows that a potent peptide inhibitor of angiogenesis and corresponding tumor growth [119]–[121], otherwise known as anginex, has a distinct affinity for Gal-1 [33]. This binding activity has been postulated to be a key mechanism by which anginex inhibits tumor angiogenesis via antagonism of Gal-1's pro-angiogenic effects on ECs [64]. By extension, it can be hypothesized that anginex could interfere with Gal-1's ability to recognize T-cell Gal-1 ligands and trigger downstream immunoregulatory activity. However, most recent data indicate that, when Gal-1 is bound by anginex, Gal-1's affinity for glycoproteins is

in fact markedly amplified [122]. So, even though anginex could potentiate Gal-1 binding to Gal-1 ligands on T cells and encourage tumor immune evasion, the anti-angiogenic activity of anginex appears to outweigh the pro-tumorigenic immune activity, resulting in limited tumor growth.

3.4 Gal-1 Mimetics as Anti-Inflammatory Therapeutics

Interference of Gal-1-binding activity or Gal-1 ligand formation implicates these methods for enhancing effector T-cell responses in the context of antitumor immunity. On the contrary, by exogenous administration of stable dimeric Gal-1 mimetics, such as Gal-1hFc, the pharmacologic rationale is to encourage immunoregulation and alleviate T-cell-mediated inflammation. This would increase the efficacy of Gal-1 treatment on experimental models of autoimmune disease such as that observed with recombinant Gal-1 [13], [34], [123]–[127]. As an example, Gal-1hFc, which contains a mutated hFc domain incapable of binding the Fc receptor, is designed to specifically bind Gal-1 ligands and elicit a Gal-1 ligand-specific response and not elicit non-specific antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity [35], [70], [73]. This Gal-1hFc mimetic has been shown to attenuate T-cell-dependent allergic dermatitis and kill granulocytes extracted from the synovium of inflamed joints in patients with rheumatoid arthritis [70]. These results implicate Gal-1hFc as a potential anti-inflammatory therapeutic. Interestingly, other strategies using human Fc fusions with inflammatory mediators, namely tumor necrosis factor (TNF) receptor and CTLA-4, have been effective in controlling rheumatoid arthritis in humans [128]–[130]. These Ig chimeric molecules have shown desirable biodistribution, pharmacokinetics, and inflammatory cell targeting characteristics and have now stimulated the development of other biologic–human Fc fusion constructs to blunt inflammation. Considering indiscriminate immunosuppression and increased susceptibility to tumorigenesis and opportune infections, Gal-1hFc can selectively target effector Th1/Th17 cells or inflammatory granulocytes, induce apoptosis, or convert Th1/Th17 cells to Tr-1-like cells and dampen inflammation. Future in vivo studies using additional models on inflammation need to be conducted to evaluate any potential immune toxicities as well as an anti-inflammatory efficacy of Gal-1hFc.

4 Conclusion

In this chapter, we review the most recent studies on how Gal-1 can impinge on effector T-cell function to modulate inflammatory and tumor immune responses. These studies collectively advance our understanding of which T-cell Gal-1 ligands bind Gal-1 and how these binding interactions are regulated and transmit Gal-1 efficacy. Furthermore, data from these studies justify further interrogation of Gal-1 ligand identity and Gal-1-binding effects on newly described T-cell subsets and on native

tissue-resident T cells to fully appreciate the therapeutic potential of targeting Gal-1–Gal-1 ligand-binding interactions. Indeed, firm evidence of Gal-1 ligand repertoire and corresponding Gal-1 effects on native CTLs is lacking, while both Gal-1 effects and glycome-related factors controlling Gal-1 ligand formation on Ag-specific T cells have not been fully characterized. What raises the significance of these uncertainties even further is the great pharmacologic promise of the biologics/drugs/Abs presented here that have been designed to block Gal-1–Gal-1 ligand interactions. As these approaches can effectively limit anti-inflammatory responses or improve antitumor T-cell responses, comprehensive characterization of their biologic activities and specificities in vivo is still needed to rationalize their safety in humans.

Conflict of Interest Authors do not report any conflicts of interest.

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Chapter 13
Arginine Metabolism, a Major Pathway for the Suppressive Function of Myeloid-Derived Suppressor Cells

Paulo C. Rodríguez and Augusto C. Ochoa

Abstract Various mutations in cancer create a microenvironment surrounding the tumor, characterized by the presence of a chronic inflammatory infiltrate which facilitates the growth of the tumor cells, enhances angiogenesis and more importantly, inhibits any protective immune response. One of the most prominent inflammatory cells are the so-called myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells that are potent inhibitors of T cell, NK cell, and dendritic cell functions. Recent findings in tumor-bearing mice and patients with cancer indicated that the increased metabolism of the nonessential amino acid L-Arginine by MDSC-producing Arginase I inhibits T-cell-lymphocyte responses. Here, we discuss some of the most recent concepts of how MDSC expressing Arginase I may regulate T-cell function in cancer and suggest possible therapeutic interventions to overcome this inhibitory effect. In addition, we discuss how metabolic limitation of L-Arginine can be used as a novel therapy to downmodulate T-cell responses in several diseases. Altogether, this chapter emphasizes the importance of the metabolism of the amino acid L-Arginine as a regulator of inflammation-linked diseases and also suggests the potential use of this pathway as a therapy to control unbalanced T-cell responses in autoimmunity and transplantations.

Keywords Arginine · Myeloid-derived suppressor cells · Immune response · L-Arg starvation · Arginase I expression · tumors-infiltrating MDSCs · Hematopoietic progenitors · Molecular mechanisms · MDSCs in vivo

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1 Introduction

Current concepts in cancer development and progression have convincingly demonstrated that malignant tumors create a chronic inflammatory microenvironment that promotes their growth and invasive properties. Until recently, the presence of this inflammatory response was poorly understood. The bands of inflammatory cells and fibrotic tissues seen surrounding many solid tumors were thought to be the remains of a failed attempt by the immune system to control the growth of the malignant cells. Similarly, the high numbers of granulocytes found in the peripheral blood of some cancer patients without an active infection were classified as leukemoid reactions and were primarily considered to be a nonspecific effect of the continued tumor growth. Research during the past two decades has instead shown that these inflammatory cells are induced by tumors and play an important role in supporting carcinogenesis and their growth, invasion, and metastatic spread. The heterogeneous population of cells that make up this chronic inflammatory microenvironment is composed primarily of $CD11b^{+}$ myeloid cells that are highly suppressive of antitumor T-cell responses. However, they also promote angiogenesis, induce regulatory T cells, and even protect tumor cells from the effects of chemotherapy and radiation therapy. The molecular mechanisms used by these cells to suppress T-cell function include depletion of amino acids arginine, tryptophan, and cysteine, the production of reactive nitrogen species such as nitric oxide (NO) and peroxynitrites (NOO^{-}), and the production of reactive oxygen species (ROS). Here, we discuss the most recent data on how myeloid-derived suppressor cells (MDSCs) metabolizing L-arginine (L-Arg) may regulate the production of reactive nitrogen species and ROS and suppress T-cell function in cancer and other diseases.

2 Alterations of the Immune Response in Cancer

A dysfunctional immune response in cancer patients manifested by the loss of delayed-type hypersensitivity was demonstrated several decades ago, but the underlying mechanisms were unknown [62], [63], [110], [109]. Initial hypotheses included the presence of "blocking antibodies," the production of suppressor factors by tumor cells, and the generation of suppressor macrophages [31], [32], [107]. Murine models also showed that tumor growth was associated with a progressive decrease in T-cell function that could be reestablished through the use of prostaglandin inhibitors or low-dose chemotherapy [28], [29], [56]. These concepts were incorporated early into the immunotherapy trials where low-dose cyclophosphamide or local radiation was used as a preconditioning regimen preceding adoptive cellular therapy. However, early immunotherapy trials in human patients failed to reproduce the therapeutic successes seen in murine models (with 3–5-day-old tumors) (reviewed in [26]). In fact, several vaccine trials demonstrated that tumors were able to progress even in the presence of a strong T-cell response [102].

In the early 1990s, the identification of several discrete but specific alterations in T cells from mice or patients with cancer, such as a decreased expression of the T-cell receptor ζ chain (CD3ζ), a diminished level of the tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, and the inability to upregulate Jak-3 and to translocate nuclear factor kappa B (NF-κB)–p65 [19], [45], [55], [62], [64], [114], [115], provided the first molecular mechanisms to explain the decreased T-cell response in cancer. Almost simultaneously, investigators developed cellular and molecular models that provided important insights into the multiple mechanisms by which cancer and chronic inflammatory diseases could selectively inhibit T-cell responses [41], [81], [101]. These models facilitated the discovery of immunoregulatory mechanisms such as the expression of checkpoint molecules on T cells and antigen-presenting cells (APCs) [18], [43], [48–50], the development of regulatory T cells [60], [61], and the accumulation of tumor-induced MDSCs [7], [20], [80], [93].

Using cocultures of activated murine peritoneal macrophages and T cells, Otsuji et al. [77] and Kono et al. [45], [46] first demonstrated that activated peritoneal macrophages cocultured with T cells induced the loss of the CD3ζ chain of the T-cell receptor and suppressed T-cell responses in vitro. This effect was blocked by the use of oxygen radical scavengers, suggesting that it was, in part, mediated by ROS [14]. Soon after, Schmielau et al. described the presence of an increased number of activated neutrophils in the peripheral blood of patients with advanced pancreatic and breast cancer who also showed a diminished expression of the CD3ζ chain [91]. Changes in the expression of the CD3ζ chain were also found in patients with metastatic renal cell carcinoma [117] where increased granulocyte counts were found to be associated with a poor outcome [82]. In addition, Zea et al. and Baniyash et al. [6], [115], [118] demonstrated that the changes in T cells were also found in infectious diseases such as tuberculosis and leprosy, suggesting that the chronic inflammatory microenvironment rather than the tumor cells were responsible for the induction of T-cell dysfunction.

Which tumor factors lead to the activation and/or accumulation of MDSCs? Gabrilovich et al. [113] and Bronte et al. [17] demonstrated that the vascular endothelial growth factor (VEGF), granulocytic colony-stimulating factor (G-CSF), and granulocytic–monocytic colony-stimulating factor (GM-CSF) produced by tumor cells arrested the differentiation of myeloid cells, resulting in the accumulation of immature myeloid cells (iMCs) that, in turn, suppressed T-cell function. These suppressive myeloid cells were found to be increased in patients with head and neck, breast, and lung cancer [2], [3] and were initially thought to block T-cell responses by producing interleukin-10 (IL-10), transforming growth factor beta (TGF-β), and prostaglandin E_2 (PGE_2). However, it soon became apparent that MDSCs had additional and more potent inhibitory mechanisms that had not previously been described.

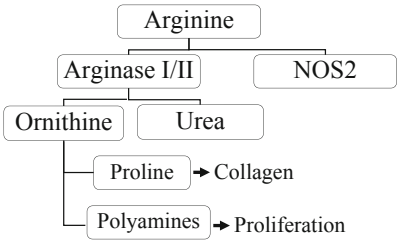

Fig. 13.1 L-Arginine metabolism in myeloid cells. L-Arginine is metabolized in myeloid cells through arginase I and II and nitric oxide synthase 2 (NOS2). L-Arginine hydrolysis through arginase I and II results in the production of urea and ornithine, the later being a major precursor for the synthesis of polyamines and collagen.

3 Metabolism of L-Arg by Myeloid Cells

In addition to the production of immunosuppressive cytokines, MDSCs were found to rapidly deplete the amino acid L-Arg from the microenvironment. L-Arg is the substrate for at least four enzymes that exist as multiple isoforms in MDSCs: nitric oxide synthases (NOS1, NOS2, and NOS3), arginases (arginase I and II), arginine glycine amidinotransferase (AGAT), and L-Arg decarboxylase (ADC) [66]. The normal serum concentration of L-Arg is maintained through a combination of dietary intake and de novo synthesis. Dietary L-Arg is taken up by intestinal epithelial cells and traverses the plasma membrane via the y+ system of cationic amino acid transporters (CATs) [13]. De novo synthesis of L-Arg occurs primarily in the kidney as a result of recycling of citrulline produced in the intestine [65]. Once L-Arg is transported into the cytoplasm, its metabolism depends on the type of cell. In myeloid cells, L-Arg is primarily metabolized by the inducible NOS (iNOS) or by arginase I or II (Figs. 13.1 and 13.2). iNOS metabolizes L-Arg to produce citrulline and nitric oxide, the latter of which plays an important role in cytotoxic mechanisms in myeloid cells and vasodilatation in endothelial cells [4], [34]. Alternatively, arginase I and arginase II metabolize L-Arg to L-ornithine and urea, the first being the precursor for the production of polyamines essential for cell proliferation and an important mechanism for detoxification of protein degradation in hepatocytes [65]. Two other enzymes, ADC and AGAT, convert L-Arg to agmatine, which, in turn, is converted to putrescine and urea by agmatinase [66]. Mammalian ADC is highly expressed in the brain [39], [120], while AGAT is expressed in the brain and heart [15], [37]. ADC and AGAT appear to be less important in the immune response.

The expression of arginase I and NOS2 in murine macrophages is differentially regulated by Th1 and Th2 cytokines [33], [68] with interferon gamma (IFN-γ) up-regulating NOS2 exclusively and IL-4, IL-10, and IL-13 inducing arginase I [67], [89]. The mitochondrial isoform arginase II is not significantly modulated by Th1 or Th2 cytokines [83]. In turn, arginase I and NOS2 appear to modulate each other's expression. The inhibition of arginase I leads to an increased NOS2 expression and, consequently, increases the production of NO [12]. Moreover, the upregulation of

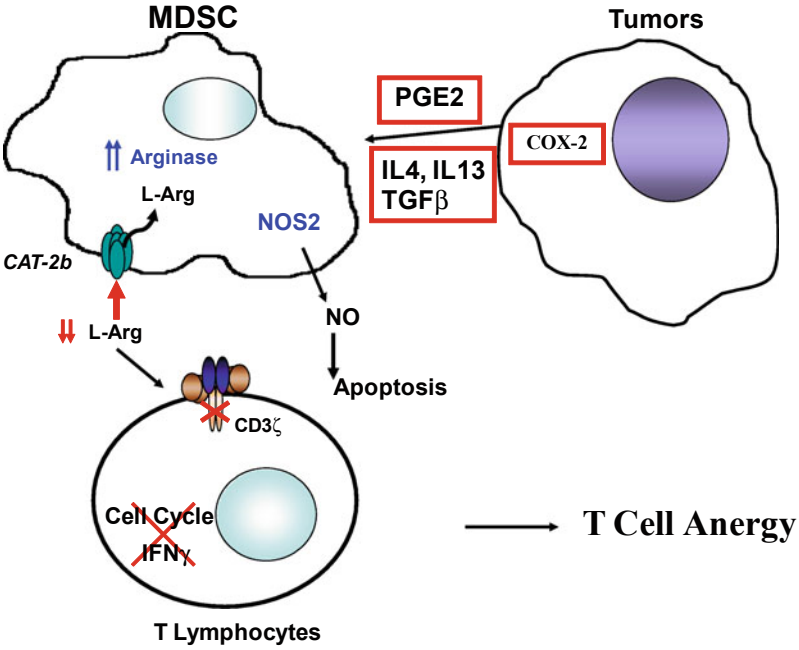

Fig. 13.2 T cell dysfunction induced by arginase I. Tumor cells expressing COX-2 and releasing PGE_2, and high levels of different mediators present in the tumor microenvironment including TGF-b, IL-4, and IL-13 induces the expression of arginase I and CAT-2B in MDSC. This leads to a reduction of extra cellular levels of L-Arginine, which finally activates GCN2 and blocks the expression of multiple genes including CD3 z, IFNg, cyclin D3 and cdk4. A similar reduction occurs in patients with cancer, but through the release of arginase into the extracellular environment. Furthermore, MDSC release NO, which is implicated in low recognition of antigens and direct induction of apoptosis in T cells.

arginase I inhibits NOS activity and contributes to the pathophysiology of several diseases including vascular dysfunction and asthma [119]. The mechanism of inhibition of NOS2 expression by arginase I appears to be mediated by L-Arg depletion, which blocks the translation of NOS2 [57]. In addition, low levels of nitric oxide induce nitrosylation of cysteine residues of arginase I which increases its biological activity, further reducing L-Arg [90].

The expression of arginase I or NOS2 also has effects on the extracellular levels of L-Arg. Peritoneal macrophages stimulated with IL-4 plus IL-13 increase the expression of arginase I and CAT-2B, which results in a rapid increase in the uptake of extracellular L-Arg with the consequent reduction of L-Arg in the microenvironment. In contrast, macrophages stimulated with IFN-γ that preferentially increase the expression of NOS2 do not increase CAT-2B expression and do not deplete L-Arg from the microenvironment [83]. Results from the arginase I and arginase II knockout mice confirm that only arginase I is able to deplete serum levels of L-Arg [16], [38]. Coculture experiments of macrophages producing arginase I and activated T cells

resulted in the loss of CD3ζ, an arrest in T-cell proliferation, and the inability of T cells to produce IFN-γ (but not IL-2). The addition of arginase inhibitors or exogenous L-Arg reversed the CD3ζ loss and reestablished T-cell proliferation [83]. These results were confirmed with macrophages from arginase I-conditional knockout mice (unpublished findings). In contrast, T cells cocultured with macrophages expressing NOS2 did not develop these alterations.

4 Effects of L-Arg Starvation on T Cells

The association between an increased expression of arginase I, a decrease in L-Arg levels, and changes in T-cell responses was first suggested by experiments showing that mice undergoing extensive surgery developed thymic involution and a decrease in splenic T cells. This effect was prevented by the injection of L-Arg [5]. Our initial experiments demonstrated that culturing T cells in a tissue culture medium with L-Arg levels $< 50\ \mu M$ resulted in a significant decrease in cell proliferation [104]. In addition, T cells activated in an L-Arg-free environment developed all the alterations previously described in tumor-bearing mice and cancer patients, i.e., the decreased expression of CD3ζ, an inability to upregulate Jak-3, a decreased translocation of NF-κB–p65, and the inability to produce IFN-γ [116]. More importantly, Rodriguez et al. also showed that the absence of L-Arg arrested T cells in the G_0–G_1 phase of the cell cycle, while T cells cultured with L-Arg progressed easily through the S and G_2–M phases [86]. This arrest in cell cycle progression was caused by a selective inability to upregulate cyclin D3 and cdk4, which did not affect other cyclin proteins [86]. In fact, silencing cyclin D3 in T cells resulted in a similar inhibition of proliferation as that caused by the absence of L-Arg. Additional research showed that L-Arg starvation impaired the expression of cyclin D3 and cdk4 in T cells through a decreased mRNA stability and diminished rate of translation [86], [88].

How does the depletion of one amino acid, L-Arg, leads to the specific molecular changes that result in T-cell anergy? Previous work had shown that amino acid starvation leads to the accumulation of empty aminoacyl-transfer RNAs (aminoacyl-tRNAs), which leads to the activation of general control nonrepressed 2 (GCN2) kinase that, in turn, phosphorylates the translation initiation factor eukaryotic initiation factor 2 alpha (eIF2α). The phosphorylated form of eIF2α binds with high affinity to eIF2β, blocking its ability to exchange guanosine diphosphate (GDP) with guanosine triphosphate (GTP), which inhibits the binding of the eIF2 complex to methionine-aminoacyl-tRNA. This results in a decreased initiation of global protein synthesis. Our results show that T cells cultured in a medium without L-Arg have high levels of phospho-eIF2α and a decreased global protein translation that preferentially impairs the synthesis and expression of the RNA-binding protein HuR, which confers stability to messenger RNA (mRNA) containing AUUA-rich elements such as cyclin D3 [86], [88]).

5 Arginase I Expression in Tumors-Infiltrating MDSCs

Some tumor cell lines including non-small lung carcinoma and breast carcinoma have been shown to express arginase [10], [95], [103]. This was thought to be a mechanism for the production of polyamines needed to sustain the rapid proliferation of tumor cells. Our results suggest instead that arginase I is primarily expressed in MDSCs infiltrating tumors, which inhibits T-cell function and represents a potent mechanism for stromal remodeling and for tumor evasion [84].

Two major subsets of MDSCs have been reported: granulocytic MDSCs (G-MDSCs) that are $CD11b^{+}$ $LY6G^{+}$ $LY6C^{low}$ and monocytic MDSCs (M-MDSCs) that are $CD11b^{+}$ $LY6G^{-}$ $LY6C^{high}$ [111]. However, several reports have also shown the presence of tumor-infiltrating MDSCs that express $CD11b^{+}$ $LY6G^{-}$ $LY6C^{-}$, a phenotype reminiscent of alternatively activated macrophages [17], [84]. In addition, granulocytic and monocytic subpopulations of iMCs can be found in the bone marrow of healthy mice, but these do not appear to suppress T-cell function [8], [53], [97]. These variations in MDSC phenotype appear to be the result of the different combinations of soluble factors produced by different tumor types. The balance between G-MDSCs and M-MDSCs, their biology, and the mechanisms that lead to their accumulation is still a matter of significant research. Recent data suggest that M-MDSCs may be precursors of G-MDSCs [112]. G-MDSCs are the major source of arginase I in tumor-bearing hosts and are significantly more potent inhibitors of T-cell function in vitro [84], whereas tumor-associated M-MDSCs primarily metabolize L-Arg through NOS2 [111]. In addition, recent publications demonstrate that MDSCs promote angiogenesis and create a "pre-metastatic niche" for circulating tumor cells [27].

In spite of the phenotypic differences, researchers have shown that the depletion of both G- and M-MDSCs using antibodies against the myeloid differentiation antigen GR-1 anti-GR-1) antibodies induces an antitumor effect mediated by $CD8^{+}$ T cells [35], [80], [93].

6 Molecular Mechanisms of Tolerance Induced by MDSCs

The mechanisms by which MDSCs induce T-cell tolerance include the production of arginase I, peroxynitrites, or ROS (H_2O_2). The effect of arginase I does not require cell-to-cell contact, while peroxynitrites and H_2O_2 require close proximity of MDSCs and T cells. Our data suggest that the depletion of extracellular L-Arg by arginase I represents one of the primary mechanisms for the induction of T-cell tolerance [83]–[85]. In fact, the depletion of L-Arg through these mechanisms is not limited to the tumor microenvironment but can also be measured in the depletion of L-Arg levels in the plasma of patients with renal cell carcinoma [87], [117]. Furthermore, the addition of arginase I inhibitors nor-N(omega)-hydroxy-L-arginine (nor-NOHA) or NOHA in vitro, or its injection in tumor-bearing mice, prevents the loss of T-cell function and results in an immune-mediated antitumor response which inhibits tumor growth in a dose-dependent manner [84].

Peroxynitrites and H_2O_2 also produced by MDSCs cause T-cell tolerance through cell-to-cell contact. This mechanism appears to require the coexpression of arginase I and NOS2 [7], as shown by the fact that the addition of NOS2 and arginase inhibitors to cocultures of MDSCs and activated T cells completely reestablishes T-cell function [9]. It is possible that this cell–cell suppression of T-cell function is primarily mediated by the production of peroxynitrites. Under limiting amounts of L-Arg, NOS2 preferentially produces peroxynitrites ($ONOO^-$) instead of nitric oxide (NO). $ONOO^-$ are highly reactive oxidizing agents that nitrate proteins and induce T-cell apoptosis [42]. Nytosylation also appears to affect the conformational flexibility of the T-cell antigen receptor and its interaction with the major histocompatibility complex (MHC) in $CD8^+$ cells. Therefore, MDSCs can directly disrupt the binding of specific peptides on MHC to $CD8^+$ T cells [58], [73]. MDSCs coexpressing arginase I and NOS2 can also impair $CD8^+$ T-cell function by blocking their ability to secrete IFN-γ when stimulated with specific antigens [23], [51], [53], [84], [97], [106]. This suppression requires the production of IL-13 and IFN-γ [25], [96], [97] and signaling through the STAT1 transcription factor [51]. In addition, MDSCs have been shown to induce regulatory T cells by producing high levels of stem cell factor (SCF) [36], [79].

7 MDSCs in Human Tumors

Human MDSC phenotypes vary significantly ranging from iMCs [94], [100] to activated granulocytes [87]. In cancer patients, M-MDSCs have been characterized as expressing either $CD14^+HLA\text{-}DR^{lo}$ or $CD11b^+CD14^-CD33^+CD15^-$, while G-MDSCs express $CD11b^+CD14^-CD33^+CD15^+CD66b^+$ [24], [30]. A study of 117 patients with metastatic renal cell carcinoma (RCC) demonstrated a six- to tenfold increase in arginase activity in the peripheral blood mononuclear cells (PBMCs), as compared to normal controls [117]. Separation of the different subpopulations of MDSCs demonstrated that the major source of arginase were G-MDSC which separated with the PBMCs when centrifuged over Ficoll–Hypaque [87]. These cells suppressed the ability of T cells to proliferate and produce IFN-γ in vitro.

Human MDSCs differ in several ways from murine MDSCs. Normal human granulocytes constitutively express arginase I as a potent antibacterial and antiviral mechanism. Arginase I expression in mature human granulocytes does not appear to be upregulated by cytokines such as IL-4, IL-13, or TGF-β. However, hematopoietic stem cells can be induced to express high levels of arginase I when cultured in a medium with GM-CSF, G-CSF, and IL-6 [59]. Human MDSCs also differ from murine MDSCs in their mechanism of arginine depletion. Human MDSCs do not uptake L-Arg (as murine MDSCs do). Instead, arginase I is stored in primary [69] or gelatinase granules [40] and is released into the microenvironment at the time of degranulation, depleting the local levels of L-Arg. T cells stimulated in this L-Arg-depleted microenvironment develop a loss of the CD3ζ chain expression and are unable to produce IFN-γ and to proliferate [47], [70], [117]. In fact, the high levels

of arginase I released in the sera of renal cell carcinoma patients result in a decrease of L-Arg to < 50 μM (normal control levels are 50–150 μM) and a significant increase in ornithine levels, a result of arginine metabolism by arginase I. Therefore, high arginase I levels have a systemic metabolic effect (L-Arg depletion) and block the protective T-cell responses [117].

8 Generation of MDSCs from Hematopoietic Progenitors

The process of myelopoiesis and commitment to a myeloid-cell lineage is tightly regulated. Accumulating evidence however indicates that tumor-derived factors alter this process and result in an increased number of iMCs, the majority of which are MDSCs [24]. Different cytokines including VEGF and GM-CSF participate in the recruitment of MDSCs from the bone marrow, [17]. In fact, reports by Ohm and Carbone show that serum levels of VEGF directly correlated with numbers of MDSCs in the blood and spleen and are associated with poor prognosis in cancer patients [74]–[76]. Tumor-derived VEGF has been previously associated with an arrest in dendritic cell maturation through the inhibition of NF-κB signaling [22], [78]. Treatment of MDSCs with all-*trans* retinoic acid appears to counter the inhibition of NF-κB signaling and promote MDSC differentiation into mature APCs [52]. Interestingly, however, treatment of patients with IL-2 and the VEGF-R inhibitor, becavizumab, resulted in an increase in MDSCs [87].

Increased levels of GM-CSF have also been associated with MDSC-dependent suppression which was reversed by the use of neutralizing antibodies to GM-CSF [8]. Similar effects on MDSCs have been suggested with other growth factors including Fms-like tyrosine kinase 3 (Flt3) ligand [99], stem cell factor (SCF) [79], and S100 calcium-binding protein A9 (S100A9) [11]. In a recent study, Youn et al. demonstrated in a series of elegant experiments that M-MDSCs from tumor-bearing mice were able to acquire a granulocytic morphology in the presence of tumor cell-conditioned medium in vitro or after the adoptive transfer to tumor-bearing recipients, effectively converting into G-MDSCs. This process appeared to be controlled by epigenetic silencing of the retinoblastoma gene through modifications mediated by histone deacetylase 2 (HDAC-2) [112]. Another study suggested instead that G-CSF, GM-CSF, and IL-6 are the central mediators of the maturation of hematopoietic progenitors into MDSCs [59]. A complete understanding of these pathways could identify new molecular targets aimed at blocking MDSC maturation.

9 Induction of Arginase and Other Suppressive Mechanisms in MDSCs

In vitro studies had shown that murine macrophages cultured with IL-4 + IL-13 (and TGF-β) increased the expression of arginase I and their ability to suppress T cells. We explored whether these factors were being produced by tumors and

whether they induced arginase I in MDSCs in vivo. Experiments using 3LL Lewis lung carcinoma and Colon carcinoma cell line MCA-38 however failed to show the production of these cytokines by these cell lines. Instead, what we found was the expression of high levels of cyclooxygenase-2 (COX-2) and the production of high quantities of prostanoids including PGE_2. COX-2 inhibitors or silencing of COX-2 in tumor cells completely blocked their ability to induce arginase I in MDSCs [85]. Consequently, treatment of tumor-bearing mice with COX-2 inhibitor sc-58125 decreased the expression of arginase I in MDSCs infiltrating the tumor and induced an immune-mediated antitumor effect [85]. Similar results have been reported in mice bearing the 4T1 breast carcinoma [98] and in mice with 1,2-dimethylhydrazine diHCl (1,2-DMH)-induced colon carcinoma [105]. Other factors may also play a role in the induction of arginase in MDSCs including hypoxia-inducible factor 1 (HIF-1) and HIF-2 (reviewed in [92]), IL-4, IL-13, and IFN-γ [25] in mice and IL-8 in human MDSCs (Rotondo et al). In addition, CCAAT enhancer-binding protein beta (C/EBPβ) [59] and STAT3 [108] have been proposed as molecular regulators of arginase in tumors.

10 Inhibition of MDSCs in vivo

Blocking the accumulation of MDSCs in tumor-bearing hosts has been achieved in animal models or patients with antibodies against Gr-1, CD11b, and CSF1, inhibitors of CSF1 receptor (CSFR1/c-fms), and the multi-targeted receptor tyrosine kinase inhibitor sunitinib. In addition, the use of the antimetabolites gemcitabine (GEM) and 5-fluorouracil (5-FU) has also shown the ability to deplete MDSCs and partially restore T-cell function in tumor-bearing hosts [21], [44], [54], [71], [72], [79]. However, the effects of these anti-MDSC approaches on specific MDSC subpopulations remain unclear. Preliminary data suggest that sunitinib may block the proliferation of M-MDSCs and impair the survival of G-MDSCs [44]. A goal of targeted depletion of selective MDSC subpopulations, or the silencing of specific suppressive mechanisms from MDSCs, may allow us to enhance the efficacy of immunotherapy and other forms of cancer treatment.

11 MDSCs: Lessons from Other Diseases and Future Applications

MDSCs are not unique to cancer. Trauma patients and patients with chronic infections including active pulmonary tuberculosis also have increased numbers of MDSCs expressing arginase I that inhibit T-cell function. These data suggest that MDSCs may represent a normal process triggered by tissue damage (danger signal) with the aim of protecting the integrity of the tissues and "healing" the initial injury. This mechanism was described in the late 1980s by Albina et al. studying the healing

of surgical wounds [1]. They described that the tissue surrounding a surgical wound was initially infiltrated by cells expressing iNOS which would most likely eliminate microbial agents contaminating the wound. This surge was followed by cells expressing arginase I which metabolize L-Arg to ornithine, which, in turn, would trigger the synthesis of collagen by fibroblasts, ultimately leading to the healing of the surgical wound. The local depletion of L-Arg would also prevent T cells from infiltrating a healing tissue and cause chronic inflammation at the site. In cancer or chronic infections, tissue damage would also trigger a similar response with the proliferation of fibroblasts producing collagen, aimed at isolating and healing the damaged tissue (i.e., malignant growth). As a matter of fact, many tumors are surrounded by dense fibrous tissue that makes its surgical excision difficult. The major difference between both disease processes (surgical wound vs malignant tumor) is that the surgical wounds heal, thus ending the role for arginase-producing MDSCs. In contrast, malignant tumors do not stop growing and destroying tissue (would not "heal") promoting instead a chronic inflammatory process mediated by MDSCs. The continuous production of arginase I would ultimately lead to the depletion of L-Arg from the microenvironment and the development of T-cell anergy. Therefore, our working hypothesis has been that tumors "hijack" a normal healing process by promoting the differentiation and activation of MDSCs expressing arginase I, which not only creates a nurturing stroma for the tumor cells but also inhibits any protective antitumor T-cell response. Although this is likely to be an oversimplified version of the complex mechanisms triggered in vivo, it provides a model to understand a complex event in the development of cancer, which could enable the design of new therapeutic approaches to interrupt this dysfunctional response.

In summary, the role of MDSCs in the development of malignant tumors has clearly been demonstrated over the past two decades. The mechanisms that induce these immunosuppressive cells are primarily produced in the tumor microenvironment and include cytokines such as IL-4, IL-13, or TGF-β, or prostaglandins (Fig. 13.2). The combination of factors is likely to vary between the different types of tumors. However, understanding how these factors stimulate the maturation of MDSCs and the molecular mechanisms that regulate their function should help develop new targeted therapies to inhibit MDSCs and enhance the efficacy of cancer therapies.

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Chapter 14
The Hypoxia-adenosinergic Immunosuppression and Redirection of Immune Response in Tumor Microenvironment

Akio Ohta and Michail Sitkovsky

Abstract In this chapter, we will focus on physiological regulators of activated immune cells in cancerous tissue microenvironments. This consideration started when we were contemplating the molecular mechanism that would be responsible for the so-called Hellstrom Paradox. Indeed, it was not explained why cancer patients often have tumor-recognizing effector T cells without having tumor rejection. The latest great advances in identification of various immunological negative regulators of immune response still left room for tumor defense by physiological inhibitors of antitumor T and natural killer (NK) cells. We started by assuming that cancerous tissues could be misguidedly protected by the same mechanism, which saves lives by protecting vital tissues from collateral damage by overactive immune cells during the antipathogen immune response. In our search for a mechanism that protects tissues from collateral damage, we first focused on intracellular cyclic adenosine monophosphate (cAMP) which was long known to be immunosuppressive. It was important to identify which of the many Gs protein-coupled receptors is actually physiologically responsible for inhibition of immune response in tumor microenvironment. Levels of extracellular adenosine are high in inflamed and cancerous tissues corresponding to local hypoxia. A2A and A2B subtypes of adenosine receptor, which are coupled to cAMP-elevating Gs protein, are predominantly expressed in immune cells. Indeed, extracellular adenosine endogenously generated by degradation of adenosine triphosphate (ATP) could suppress immune response and immunoregulation by adenosine was notable in tumor microenvironment. Blockade of the hypoxia-adenosinergic immunosuppression may be a promising approach to eradicate cancer, especially when it is combined with adoptive immunotherapy or cancer vaccine.

Keywords Tumor microenvironment · Hypoxia · Adenosine · A2A adenosine receptor · A2B adenosine receptor · T cell · Regulatory T cell · Myeloid-derived suppressor cells · Adoptive immunotherapy · Cancer vaccine

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1 Hypoxia

1.1 Immunosuppression in Hypoxic Tissue Microenvironment

Tissue oxygen levels are not uniformly distributed in the same organ. Levels of oxygenation depend on diffusion of oxygen from blood vessels, distal area being more hypoxic [1], [2]. Pathological conditions in inflamed tissue and tumor are related to the formation of even less oxygenated microenvironments [2], [3]. Tissue inflammation inflicting damage to blood vessels reduces oxygen supply [3]. Combined with increased oxygen demand by accumulated inflammatory cells, inflamed tissue becomes deeply hypoxic [4].

Tumors are also often hypoxic for reasons different from those causing hypoxia in inflamed tissues. Oxygen demand is high in tumors because of aggressive proliferation of tumor cells. In addition, disorganized blood vessel formation in tumors is making blood flow sluggish, and therefore oxygen supply is low [5], [6]. Interface between tumor cells is tightly packed, preventing oxygen diffusion to the inside of tumor tissue [6], [7]. Hypoxia in tumors correlates with poor prognosis because hypoxic tumors are refractory to radiotherapy and chemotherapy [5]–[7]. Moreover, hypoxia is conductive to the establishment of tumor microenvironment, which is potentially suppressive to antitumor immune activities [8]–[10]. Hypoxia has been shown to suppress immune functions of T cells, natural killer (NK) cells, and antigen-presenting cells (APCs).

1.2 Suppression of T-cell Immunity Under Hypoxia

In vitro T-cell activation under hypoxia impairs proliferation of activated T cells and their effector functions such as cytotoxicity and cytokine production [1], [11]–[13]. Hypoxia blocks Ca^{2+} increase after stimulation of T-cell receptor (TCR) [14]. Whole body exposure of mice to hypoxic atmosphere inhibited T-cell activation in vivo [15]. In that study, the extent of T-cell activation correlated with the levels of oxygenation in the spleen. Indeed, degrees of T-cell activation were attenuated in poorly oxygenated environment as detected by covalent binding of nitroimidazole compound, Hypoxyprobe-1 [15].

Exposure to hypoxia induces cellular stress response to adapt energy deprivation. One of the most important events is stabilization of hypoxia-inducible factor-1α (HIF-1α), which upregulates glycolytic enzymes, angiogenesis, and erythropoiesis [16], [17]. In T cells, however, HIF-1α was reported to diminish TCR signaling [14]. Higher interferon gamma (IFN-γ) production and stronger cytotoxicity in T cells lacking HIF-1α suggest a negative regulatory role of HIF-1α [18], [19].

2 Adenosine

2.1 Formation of Extracellular Adenosine in Tumor

Tumors have been found to contain high levels of extracellular adenosine [20], [21], one of the potential immunosuppressive molecules. Enzymatic degradation of extracellular adenosine triphosphate (ATP) leads to an increase of extracellular adenosine. 5'-Ecto-nucleotidases are responsible for this metabolism: CD39 converting ATP to adenosine diphosphate (ADP) and to adenosine monophosphate (AMP) and CD73 catalyzing adenosine formation from AMP. CD73-deficient mice maintain extracellular adenosine concentration at low levels physiologically and even after the induction of inflammation, suggesting that conversion of adenine nucleotides accounts for a large part of extracellular adenosine formation [22]–[24]. Extracellular adenosine may be removed by further metabolism to inosine by adenosine deaminase and by cellular uptake through nucleoside transporters. Adenosine kinase in the intracellular compartment metabolizes adenosine to AMP, making room for further adenosine uptake. Inhibitors of adenosine deaminase, nucleoside transporters, and adenosine kinase increase extracellular adenosine levels, indicating significance of these mechanisms in the regulation of extracellular adenosine [25]–[28].

Increase of extracellular adenosine levels has been observed during inflammation [29]–[32]. By causing tissue injury, inflammation is able to increase extracellular content of adenine nucleotides and facilitate metabolism to produce adenosine. Cellular damage is considered to cause leakage of adenine nucleotides to extracellular space [4]. Increased release of adenine nucleotides was reported in activated neutrophils and irritant-treated keratinocytes [33], [34].

Subsequent to extracellular increase of adenine nucleotides, tissue hypoxia facilitates conversion to adenosine through upregulation of CD39 and CD73 levels [35], [36]. In parallel, hypoxia inhibits adenosine kinase [37]–[39]. Thus, tissue hypoxia is conductive to extracellular accumulation of adenosine [40] by increasing adenosine formation and by suppressing its removal. Intratumoral hypoxia caused by poor oxygen supply in spite of increasing demand of oxygen favors adenosine accumulation in the tumor. Various tumor cells expressing CD73 also contribute to the production of extracellular adenosine [41]–[44]. These findings correspond to the increase of adenosine levels in tumor tissue (Fig. 14.1).

2.2 Extracellular Adenosine as an Immunoregulatory Molecule

Adenosine is abundant in cells for its use in energy and nucleic acid metabolism. But its presence in the extracellular compartment results in distinctive effects on the cardiovascular system, neuronal cells, kidney, fat tissue, platelets, and leukocytes. In the mid-70s, incubation with adenosine was known to induce cyclic adenosine monophosphate (cAMP) in various cell types including T cells. The increase of cAMP

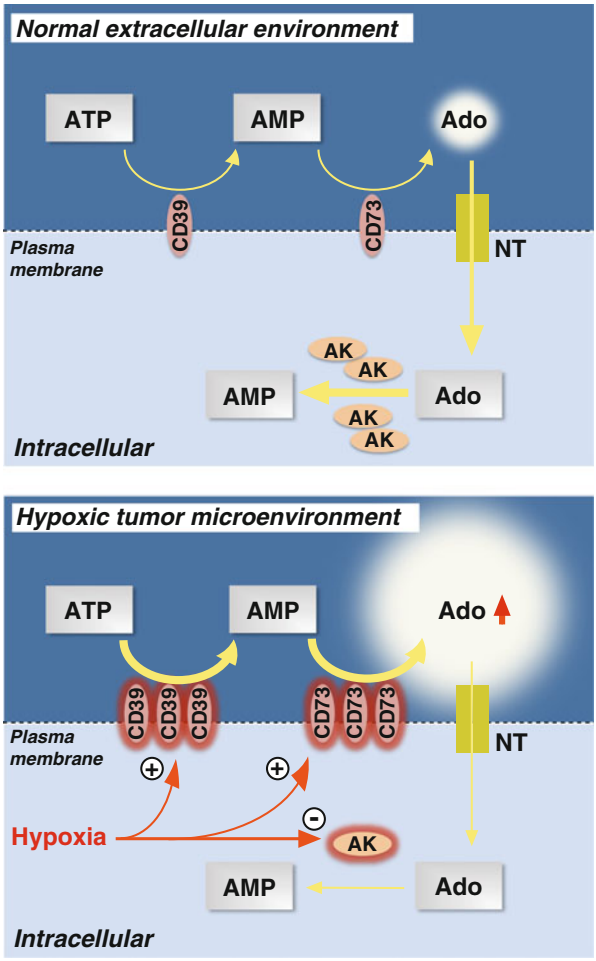

Fig. 14.1 The enhancement of extracellular adenosine generation in *hypoxic tumor microenvironment*. Adenine nucleosides (*ATP*, *ADP*, and *AMP*) in the extracellular compartment are catabolized to adenosine by the activities of *CD39* and *CD73* ecto-enzymes. In normoxic microenvironments (oxygen tension $> \sim 3\,\%$), the concentration of extracellular adenosine is kept low by, e.g., adenosine kinase and adenosine deaminase (not shown in this figure) and cellular uptake is regulated through nucleoside transporter (*NT*). However, hypoxia in tumor microenvironment can change the balance of extracellular adenosine formation and removal in favor of the accumulation of extracellular adenosine. Upregulation of *CD39* and *CD73* under hypoxia accelerates extracellular formation of adenosine. In addition, hypoxia down-regulates adenosine kinase (*AK*) and impairs removal of adenosine

in T cells led to speculation of receptor-mediated signaling, but at that time, the function of adenosine signaling was discussed in regard to energy production [45]–[47]. Effects of adenosine on T-cell function were reported in the 1980s, showing the inhibition of T-cell proliferation, interleukin-2 (IL-2) production, and B-cell helper function [48]–[50]. In parallel, the increase of cAMP was demonstrated to suppress IL-2 production, B-cell helper function, and cytotoxicity of T cells [51], [52]. These early studies implied that extracellular adenosine is inhibitory to T cells through the induction of cAMP.

Meanwhile, the presence of multiple adenosine receptor subtypes was speculated based on the different selectivity of synthetic adenosine derivatives [53], [54]. Since the first cloning of adenosine receptor in 1989, four different adenosine receptors have been identified to date. Among these, A2A and A2B adenosine receptors are cAMP-inducing receptors coupled to Gs protein, while A1 and A3 adenosine receptors are

coupled with Gi protein to reduce cAMP levels [55], [56]. Indeed, adenosine A2A receptor (A2AR) is the predominant subtype in T cells [57], [58].

Similar to the inhibitory effects on polymorphonuclear cells and macrophages, suppression of T-cell activation by A2AR agonist was shown in two papers published in 1997 [57], [59]. In these papers, treatment with A2AR agonist resulted in decreased T-cell proliferation, downregulation of activation marker CD25, and decreased cytotoxicity with a reduced level of Fas ligand expression.

2.3 Mechanism of A2AR-mediated T-cell Inhibition

Subsequent studies revealed more details of T-cell suppression due to A2AR signaling. A2AR stimulation at the time of T-cell activation significantly reduced proliferation of T cells and their effector functions including cytotoxicity and production of cytokines such as IL-2, IFN-γ, and TNF-α [60]–[62]. Both $CD4^+$ and $CD8^+$ T cells are susceptible to this mechanism [62]. A2AR is also expressed in human T cells, and A2AR agonist was shown to be suppressive to effector functions of human T cells such as cytokine production and cytotoxicity [63], [64]. Inhibition of T-cell activation correlates well with the interruption of TCR signaling by A2AR stimulation [61], [65], [66]. A2AR agonist diminished phosphorylation of ZAP70 after TCR stimulation together with downstream phosphorylation of ERK. Inhibition of Akt phosphorylation by A2AR agonist also suggests interruption of the phosphatidylinositol-3-kinase pathway. Since A2AR stimulation induces cAMP, protein kinase A-dependent phosphorylation of COOH-terminal Src kinase may inhibit Lck activation in the early stage of TCR signaling [67].

The helper function of $CD4^+$ T cells is important in activating both cellular immunity and humoral immunity depending on functional differentiation of $CD4^+$ T cells into T helper 1 (Th1) and Th2 cells. Although A2AR agonist can inhibit development of both Th1 and Th2 cells [68], large declines in IFN-γ and IL-2 production by exposure to A2AR agonist indicated a strong suppression of Th1-type cellular immune responses [60]–[62]. Inhibition of Th1 cell development is consistent with changes in cytokine production from APCs in which A2AR agonist diminishes IL-12, but augments IL-10 [69], [70].

A2AR stimulation not only blocks activation of T cells immediately, but also elicits sustained inhibition of T-cell activities by inducing activated T cells with impaired effector functions. As mentioned above, T-cell activation in the presence of A2AR agonist reduced IFN-γ production from activated cells. However, when these cells were restimulated after the removal of A2AR agonist, IFN-γ-producing activity was still less than normal activated T cells [61], [62]. The induction of such anergic T cells suggests that the T-cell inhibitory effect of adenosine may be persistent even after clearance of adenosine (Fig. 14.2a). This property of A2AR signaling may be relevant to the memory of exposure to extracellular adenosine, where persistent elevation of cAMP was observed after transient exposure to adenosine [57].

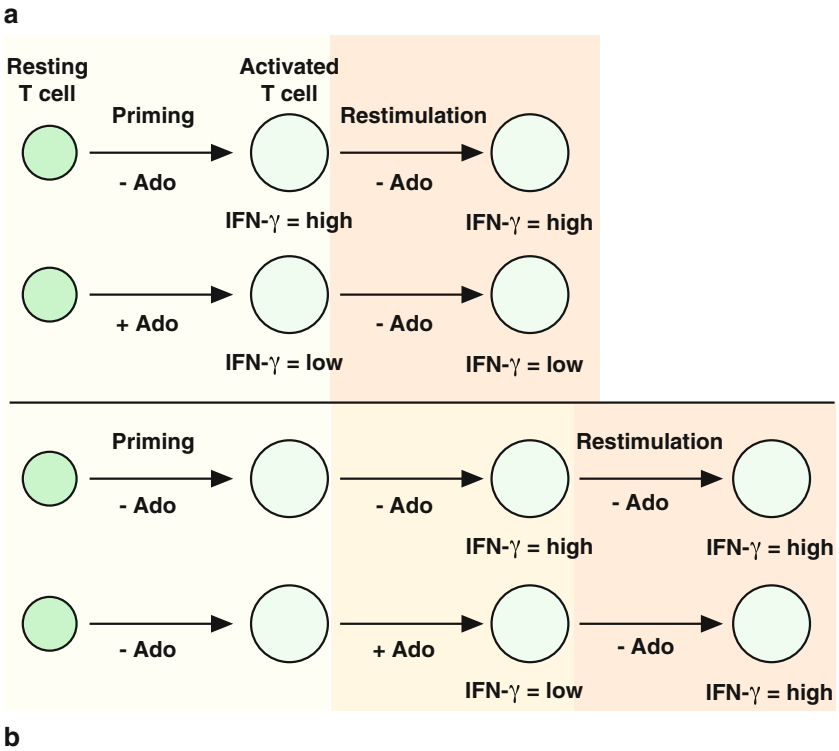

Fig. 14.2 Early stages of priming and activation of *resting T cells* are highly susceptible to the A2AR-mediated immunosuppression. **a** When *resting T* cells are stimulated in the presence of A2AR agonist, the *activated T cells* produce very low levels of *IFN-γ*. The impairment of IFN-γ-producing activity persists even after removal of A2AR agonist. **b** A2AR agonist can inhibit *IFN-γ* production from already *activated T cells*, but only in the very presence of A2AR agonist. After the removal of A2AR agonist, *IFN-γ* production from T cells returned to normal levels

Comparison between resting and activated T cells showed that already activated T cells are relatively resistant to A2AR-mediated inhibition (Fig. 14.2b). When activated T cells were restimulated, A2AR agonist still inhibited T-cell proliferation and IFN-γ production. After removal of the A2AR agonist, however, the effector function of these T cells came back to the same levels as in activated T cells that were never cultured with A2AR agonist [71], [72]. Therefore, although A2AR agonists can inhibit activities of the already activated T cells, the inhibitory effect did not persist after its removal. This result indicates that A2AR stimulation does not switch fully functional effector T cells to the anergic phenotype. The inhibition of activated effector cells by extracellular adenosine is highly territorial: only in extracellular adenosine-rich tissue microenvironment, but not in the neighboring adenosine-low microenvironment.

2.4 *Adenosine Promotes Immunosuppressive Activity of Regulatory T Cells*

Regulatory T cells (Treg) were initially identified as $CD4^+$ T cells constitutively expressing CD25 at high levels. Activation of Treg follows the normal scheme of T-cell activation, but activated Treg spontaneously inhibit activation of other effector T cells. Since the lack of immunoregulation by Treg causes severe autoimmune diseases, Treg are indispensable for the control of immune activation against self-antigens in peripheral tissues [73], [74].

It was suggested that Treg development and effector functions are under control of the hypoxia-adenosinergic pathway, and the model was proposed to potentially unify the diverse functions of Treg [75]. A large body of published data are consistent with the model where Treg development and their immunoregulatory activity are mediated by the interplay of the cAMP-elevating adenosine receptors, HIF-1α, and subsequent cAMP response element (CRE)- and hypoxia response element (HRE)-mediated transcription in Treg and effector cells. Accordingly, HRE- and CRE-driven activities of Treg may be required to achieve a maximal level of immune suppression.

As a subset of T cells, Treg express functional A2AR as well [76]. In contrast to negative effects on activities of most T cells, A2AR stimulation rather promotes immunoregulatory activity of Treg [76]. In isolated spleen cells, containing both effector T cells and Treg at physiological ratio, T-cell stimulation in the presence of A2AR agonist inhibited activation of effector T cells but increased Treg population. A2AR stimulation not only increased the number of Treg but also augmented the T-cell inhibitory activity of Treg. Corresponding to the enhanced immunoregulatory activity, A2AR agonist upregulated cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression in these Treg. The importance of CTLA-4 in the immunosuppressive activity of Treg was demonstrated by systemic lymphoproliferation and autoimmune disease in mice with Treg-specific deletion of CTLA-4 [77].

Adoptive transfer of Treg reduced ischemia-reperfusion injury in vivo, but pre-treatment of Treg with A2AR agonist before transfer augmented the efficacy of this treatment [78]. Moreover, A2AR-deficient Treg were less effective compared to wild-type Treg, suggesting the in vivo significance of A2AR signaling in regulating the immunosuppressive activity of Treg.

Treg may develop either during T-cell maturation in the thymus (natural Treg) or in the peripherals by functional differentiation of mature T cells (inducible Treg). Analysis of A2AR-dependent Treg expansion showed the involvement of natural Treg proliferation and induction of new Treg [76]. The promotion of inducible Treg has been speculated from an upregulation of FoxP3 mRNA in T-cell culture treated with A2AR agonist [61]. FoxP3 is a transcription factor involved in the regulation of immunosuppressive activity. It was further confirmed that A2AR agonist expanded transforming growth factor beta (TGF-β)-inducible Treg both in vitro and in vivo [79]. Besides A2AR, adenosine 2B receptor (A2BR) may be also involved in the increase of inducible Treg [80].

Upstream adenosine receptor signaling, hypoxia may be also involved in the regulation of Treg. Indeed, FoxP3 is inducible by hypoxia in T cells, and HIF-1α

mediates hypoxic induction of FoxP3 [81], [82]. However, subsequent studies provided evidence for complicated role of HIF-1α in the regulation of Treg. Initially, studies using mice with HIF-1α-deficient T cells demonstrated HIF-1α-mediated downregulation of Treg and reciprocal increase of Th17 [83], [84]. In contrast, more recent papers showed that hypoxia induces FoxP3 and increases Treg abundance [82]. In this setting, HIF-1α is necessary for optimal immunosuppressive activity of Treg. Nonetheless, local oxygen levels may be an important regulator of Treg. Hypoxia in tumors may be relevant to increase of Treg population in tumor microenvironment.

While adenosine can control the immunoregulatory activity of Treg, Treg may utilize adenosine in their mechanism of immunosuppression. Treg express CD39 and CD73, extracellular nucleotidases that catalyze degradation of ATP to adenosine and increase extracellular adenosine concentration [85]–[87]. The produced adenosine, in turn, interacts with A2AR and blocks activation of T cells. This mechanism may explain why A2AR-deficient effector T cells were resistant to immunoregulatory cells [88] and why CD73-deficient Treg were less effective in inhibiting ischemia-reperfusion injury [78]. Furthermore, adenosine produced from Treg may autonomously target Treg to enhance their activity.

Thus, A2AR-mediated signaling promotes immunoregulation by Treg both quantitatively and qualitatively. The outcome of this effect is consistent with the direct inhibition of effector T-cell activation by A2AR-mediated signaling. In addition to the direct inhibition of T-cell activation, A2AR agonist also provides longer lasting T-cell inhibition by at least two different mechanisms. When present at the time of T-cell priming, A2AR agonist induces longer lasting inhibition of antigen-specific T-cell response by developing anergic effector T cells. In addition, when enforced in the presence of A2AR agonist, Treg may provide long-lasting suppression of antigen-specific T-cell response ([76], [78]; Fig. 14.3).

2.5 Myeloid-derived Suppressor Cells

Together with Treg, myeloid-derived suppressor cells (MDSCs) represent major immunoregulatory cells contributing to immunosuppressive environment in tumors [89]. Adenosine promotes expansion of MDSCs in A2BR-dependent manner. Indeed, the number of tumor-infiltrated MDSCs is low in A2BR-deficient mice [90]. Hypoxia also promotes differentiation and function of MDSCs [91], suggesting significance of the hypoxia-adenosine pathway in regulating MDSCs in tumors.

2.6 Antigen-presenting Cells

Adenosine receptor stimulation of APCs inhibits T-cell stimulating activity. A2AR agonists inhibit IL-12 production but induce IL-10 from dendritic cells [69], [70]. This change in cytokine milieu is suppressive to the induction of Th1 cells and

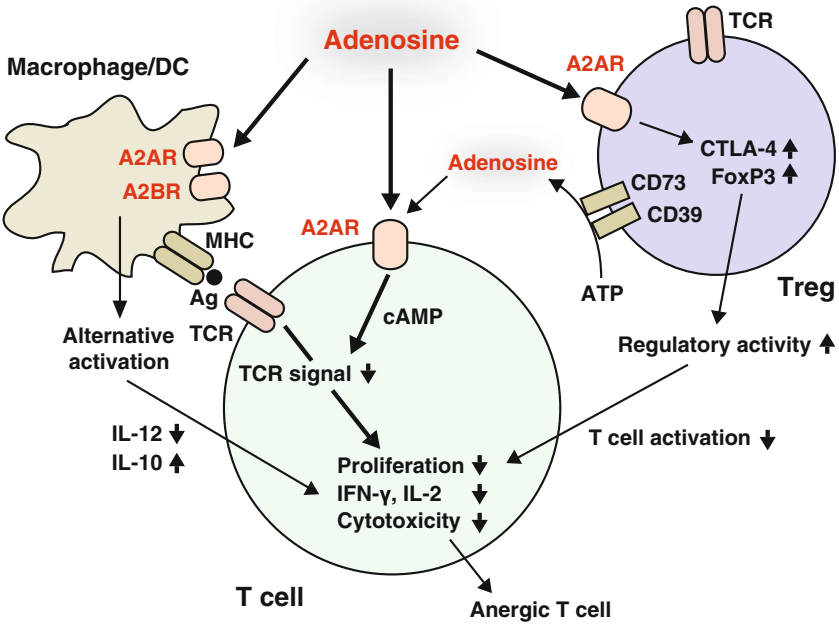

Fig. 14.3 Regulation of T cells' effector functions by extracellular adenosine. Signals from *A2AR* on *T cell*'s surface directly inhibit the TCR-mediated activation. As a result, *A2AR* stimulation diminishes various T-cell functions including proliferation, cytokine production, and cytotoxicity. The impairment of effector functions in activated T cells can persist even after removal of agonist, suggesting the development of anergic T cells. Adenosine also indirectly influences T-cell activation by inducing alternative activation of antigen (*Ag*)-presenting cells. Macrophages and dendritic cells (*DC*s) activated in the presence of adenosine produce less *IL-12* and more *IL-10*, changing cytokine milieu for functional differentiation of T cells. Moreover, A2AR stimulation promotes Treg expansion and their immunosuppressive function. Thus, adenosine signaling suppresses T-cell activation both directly and indirectly. Therefore, T-cell inhibitory effect of A2AR/A2BR-mediated immunosuppressive signaling is both immediate (i.e., by directly inhibiting the T-cell activation signal) and long-lasting (anergic T cells and Treg)

therefore inhibitory to cellular immune responses. While A2AR stimulation suppresses activation of APCs to proinflammatory phenotype, adenosine induces alternative activation of APCs via A2BR [69], [70], [92], [93]. Alternative activation induces arginase, indoleamine-2,3-dioxygenase (IDO), TGF-β, and COX-2 in APCs, and such APCs inhibit optimal activation of T cells [94]. A2AR agonist also induces VEGF from macrophages [95], [96], suggesting adenosine switches APCs to tolerogenic and angiogenic phenotype.

Dendritic cells exposed to hypoxia express lesser levels of major histocompatibility complex (MHC) and co-stimulatory molecules [97], [98]. Hypoxia inhibits phagocytosis by dendritic cells, decreasing capture of antigen [99]. Therefore, those dendritic cells under hypoxia have impaired T-cell stimulatory capacity as APCs.

2.7 NK Cells

Adenosine suppresses NK cell activities. A2AR agonists are suppressive to IFN-γ production and cytotoxicity of lymphokine-activated killer (LAK) cells and NK cells from mice and humans [100]–[102].

Hypoxia also suppresses activity of NK cells [103]. Closely relevant to the inhibition of NK cell-dependent cytotoxicity, hypoxia downregulates NKG2D ligands including MHC class I chain-related molecules on tumor cells [104], [105]. Since NKG2D is an activating receptor of NK cells, hypoxic tumor cells are induced to be resistant to NK cell-dependent cytotoxicity. These observations suggest biological significance of oxygen tension in the regulation of antitumor immune responses.

3 Endogenous Adenosine as a Physiological Regulator of Immune Response

3.1 A2AR

A2AR stimulation suppresses immune responses through Gs protein-mediated cAMP increase. However, there are many other cAMP-elevating receptors on the surface of immune cells that can transduce immunosuppressive signals when activated pharmacologically. A brief inventory of such molecules includes prostaglandin E_2, adrenaline, histamine, and small peptides such as vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP). Adenosine has been regarded as just one of such anti-inflammatory small molecules, but in recent years, recognition of adenosine became prominent because of its nonredundancy as an endogenously produced immunoregulator [106]–[108].

One of the most important features of A2AR is its critical role in physiological regulation of immune responses. Acute hepatitis induction in A2AR-deficient mice resulted in remarkable exaggeration of liver damage and proinflammatory cytokine levels [109], [110]. The result demonstrated that (1) endogenously produced adenosine can control the intensity of immune response through A2AR and (2) A2AR signaling is critical to stop inflammation because other immunoregulatory mechanisms could not compensate for the lack of A2AR-mediated immunosuppression.

Adenosine-dependent immunoregulation may represent the tissue's negative feedback response to overwhelming inflammation [106], [110]. Tissue damage inflicted by proinflammatory activities triggers an accumulation of extracellular adenosine. Indeed, an increase of adenosine levels was observed during inflammation [29]–[32]. Tissue hypoxia and nucleotidase activities of CD39 and CD73 are responsible, at least in part, for the increase in extracellular adenosine [22]–[24]. The increased adenosine transmits a signal to immune cells through A2AR to stop proinflammatory activities and prevent further tissue damage. Interruption of this sequence, e.g., A2AR-deficiency and A2AR antagonism, means loss of a brake on inflammation.

Exaggerated inflammation in A2AR-deficient mice was demonstrated in various tissues and in various causes of inflammation [32], [111]–[114], suggesting that the adenosine–A2AR system is a universal mechanism in the vital body to prevent excessive tissue damage.

This discovery offered a solution to a clinically important issue that, as opposed to recruitment of anti-inflammatory effects by targeting A2AR with agonists, it is possible to enhance inflammation by blocking the action of endogenous adenosine by A2AR antagonist. Intake of A2AR antagonists may be detrimental to inflammatory disorders; however, we may take advantage of this mechanism in the treatment of cancer.

3.2 A2BR

Another Gs protein-coupled adenosine receptor is adenosine A2B receptor (A2BR). Affinity of adenosine to A2BR is lower than A2AR; however, local adenosine levels in hypoxic tissue can be high enough to stimulate A2BR [115], [116]. A2BR is expressed on macrophages, dendritic cells, endothelial cells, epithelial cells, mast cells, and fibroblasts, and it has distinctive effects on inflammatory responses [115], [116]. A2BR agonist was shown to block inflammatory tissue injury in experimental models. Exacerbation of colitis, lung inflammation, and ischemia-reperfusion injury in A2BR-deficient mice suggests pathophysiological significance of endogenous adenosine signaling through A2BR [117]–[121]. Since A2BR stimulation changes the functions of macrophages and dendritic cells as APCs, T cells may receive indirect immunoregulatory effects from A2BR [92], [93], [115], [116]. Thus, increase of extracellular adenosine triggers anti-inflammatory negative feedback responses via A2AR and A2BR. The adenosine–A2AR/A2BR pathway may be vital as an immunoregulatory mechanism in tumor.

4 Cancer

Tremendous efforts by tumor immunologists have significantly advanced the understanding of tumor-associated antigens and improved induction of effector T cells recognizing tumor cells as foreign [122], [123]. It also became clear that the immunosuppressive environment in tumors is a potential problem in tumor eradication by immune cells. Tumors often have infiltration of T cells that can be reactive against the tumor cells, but the tumor-infiltrated T cells are inactive in attacking the tumor in vivo. In mice manipulated to express the same antigen in both normal and tumor tissues, the same effector T cells were disabled only in tumor [124]–[126]. Such studies provide a direct evidence for the existence of potentially immunosuppressive tumor microenvironment. Tumors may employ various mechanisms to evade immune response, e.g., Treg, MDSCs, anti-inflammatory cytokines, and IDO [9], [127].

Advances in T-cell technology developed methods of inducing antitumor effector T cells. However, efficacy of these antitumor T cells may be limited if they are sensitive to immunosuppression in tumor microenvironment. Disengagement of antitumor effectors from immunosuppressive mechanism in tumor will significantly improve the outcome of tumor immunotherapy.

Hypoxia, which is frequently observed in tumors, may play a role in the establishment of immunosuppressive environment. Hypoxia is conductive to the increase of extracellular adenosine levels, and indeed high levels of extracellular adenosine were observed in tumors [20], [21]. Various effects of hypoxia in vivo and in vitro are mediated by the interaction of extracellular adenosine with A2AR [32], [40], [128]–[132]. There is a similarity between tumor-infiltrated T cells and T cells activated in the presence of adenosine in terms of preferential suppression of effector functions [62]. Thus, adenosine may represent one of the potentially immunosuppressive mechanisms in tumors. This concept was established in a tumor inoculation study in which A2AR-deficient mice, but not wild-type mice, demonstrated regression in growing tumors [21]. Improvement of T cell-mediated tumor eradication upon inactivation of A2AR suggests nonredundance of the adenosine–A2AR pathway in the immunosuppressive tumor microenvironment. Tumors protect themselves utilizing the body's common rule: You shall not take vengeance when you see adenosine.

The enhanced tumor regression in A2AR-deficient mice suggested that A2AR-antagonists might be useful to break immunosuppression in tumors and improve tumor immunotherapy. Indeed, A2AR antagonists such as caffeine, ZM241385, and SCH58261 blocked tumor growth by promoting antitumor immune responses [21], [42], [133]. Significant reduction of intratumoral blood vessels in A2AR antagonist-treated mice suggests that the treatment not only enhances antitumor immune response but also blocks adenosine-induced angiogenesis in tumors [21]. The countermeasure to immunosuppression in tumors in conjunction with successful induction of antitumor effector T cells may significantly improve the outcome of tumor immunotherapy.

In addition to A2AR, A2BR also participates in the protective mechanism of tumor against immune response. Retardation of tumor growth was observed in A2BR-deficient mice [134]. Treatment with A2BR antagonist is also inhibitory to tumor growth in wild-type mice, but not in T cell-deficient mice [135]. Enhanced T-cell infiltration into the tumor by A2BR antagonist suggests that extracellular adenosine in tumor discourages antitumor immune response through both A2AR and A2BR.

The critical role of adenosine-dependent immunosuppression in tumors was also demonstrated by the promotion of antitumor immunity in the absence of CD73 [136], [137]. The lack of CD73, ecto-nucleotidase, sharply decreases extracellular adenosine formation and promotes proinflammatory responses [22]–[24]. Neutralization of CD73 by the injection of antibody inhibited tumor growth and promoted antitumor immune response [42], [138]. CD73 expression on tumor cells plays an important role in immunosuppression and tumor metastasis. Indeed, tumor cells lacking CD73 are susceptible to antitumor immunity [42], [43]. Not only CD73 expression on tumor cells but also CD73 expression on normal cells plays a significant role. Inoculated tumors grow slower in CD73-deficient mice because of stronger antitumor

T-cell response [139]. Moreover, CD73 deficiency resulted in the inhibition of carcinogenesis thanks to T cell- and NK cell-dependent immune response [140]. These studies suggest that, besides blockade of adenosine signaling by adenosine receptor antagonists, prevention of extracellular adenosine formation by targeting CD73 may be a promising countermeasure to immunosuppressive tumor microenvironment.

5 Natural A2AR Antagonists: Caffeine and Theophylline

Caffeine and theophylline are representative nature-derived methylxanthines, and they are the most widely consumed A2AR antagonists in the form of beverage, food, and medication. It is known that the psychostimulatory effect of caffeine is attributable to antagonism of the adenosine–A2AR interaction [141], [142]. Indeed, caffeine exacerbated inflammatory tissue damage in experimental acute hepatitis by blocking A2AR [133], [143]. While caffeine and theophylline block A2AR-mediated cAMP increase, a high concentration of these compounds actually increase cAMP levels by inhibiting cAMP phosphodiesterase. Therefore, while low doses of caffeine exacerbate inflammatory tissue damage, caffeine can be anti-inflammatory at high doses [133], [143]. Normal caffeine consumption in humans raises caffeine concentration enough to antagonize A2AR [141], [144], [145]. Since anti-inflammatory high dose may not be reproduced by normal caffeine consumption in humans, the immune-enhancing effect will be clinically more relevant.

In tumor immunotherapy, proinflammatory action of natural adenosine receptor antagonists may be beneficial in promoting antitumor immune response. Co-treatment with caffeine significantly improved tumor eradication by endogenously developed and adoptively transferred antitumor T cells [21]. The enhancement of antitumor activity by caffeine may be relevant to some epidemiological studies that have suggested inverse association between cancer incidence and coffee consumption. The statistics suggest that coffee consumption dose-dependently decreased incidence of breast, liver, colon, lung, skin, and endometrial cancer [146]–[154].

6 Conclusion

Hypoxia in tumors may be implicated to the establishment of immunosuppressive environment. Hypoxia inhibits diverse antitumor immune responses at least in part by upregulation of extracellular adenosine. Adenosine stops antitumor immune response through A2AR and A2BR on immune effector cells. This direct action of adenosine can immediately suppress immune responses in tumor microenvironment. Adenosine evokes longer lasting immunoregulation, which persists in immune cells even after the disappearance of adenosine. Cell activation in the presence of adenosine induces anergic T cells and alternative activation of APCs.

Furthermore, adenosine promotes cellular immunosuppressive activities. Adenosine promotes expansion of Treg and their immunoregulatory activity. MDSCs were

also shown to increase in response to adenosine and hypoxia. The increase of professional immunoregulatory cells may be an important component of tumor microenvironment, which is harsh to immune effectors. Thus, the hypoxia-adenosine pathway involves direct inhibition of antitumor effector cells and long-term effect by developing tumor microenvironment favoring immunosuppression.

Treatment with adenosine receptor antagonist and CD73 inhibitor may be a promising approach to improve antitumor immunity. Since this treatment is compensatory to the current approach that focuses on the numerical increase of antitumor T cells, it will be more efficacious when combined with cancer vaccines and adoptive immunotherapy [21], [123], [155]. In cancer adoptive immunotherapy, downregulation of A2AR on the antitumor T cells is expected to promote their efficacy in vivo. In addition to A2AR antagonist treatment after cell transfer, transfer of cells that were created to be insensitive to adenosine may also be worth exploring [21], [72].

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Chapter 15
Molecular Pathways in Antigen-Presenting Cells Involved in the Induction of Antigen-specific T-cell Tolerance

David M. Woods, Andressa Laino, Alejandro Villagra and Eduardo M. Sotomayor

Abstract There is now an undisputed understanding that tolerance to tumor antigens imposes a significant barrier to cancer immunotherapy. Bone marrow (BM)-derived antigen-presenting cells (APCs) play a central role in the induction of tolerance in a wide variety of malignancies. Here, we discuss receptor–ligands, intracellular signaling pathways and epigenetic mechanisms that, given their role in regulating the inflammatory properties of APCs, influence the functional outcome (i.e., priming versus tolerance) of antigen-specific T cells. The identification of these mechanisms and pathways has provided novel molecular targets to potentially revert mechanisms of T-cell unresponsiveness in cancer.

Keywords Antigen-presenting cells · Tolerance · Signaling pathways

1 Introduction

In the new century, our view of immune system activation has changed dramatically given the identification of inhibitory signaling pathways in immune cells that, by counteracting positive/activating pathways, greatly influence the initiation, magnitude, and duration of immune responses. These findings have led immunologists to redefine the concept of immune activation as the net outcome of "turning on" activating genes and "downregulating" genes with inhibitory function [1]. By

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extension, it was proposed that these inhibitory regulatory pathways also play a role in the induction and maintenance of peripheral tolerance to self-antigens. Experimental evidence supporting the role of negative regulatory pathways in immune tolerance has been provided by studies in mice with targeted disruption of specific inhibitory molecules in which unchecked inflammatory responses and autoimmunity were commonly observed [2], [3].

Bone marrow (BM)-derived antigen-presenting cells (APCs), in particular dendritic cells (DCs), play a central role in the generation of productive antigen-specific T-cell responses [4]. However, these same cells are also required for the induction of T-cell tolerance [5]. This dual function of APCs was attributed initially to the existence of specific APC subpopulations: those that preferentially induce T-cell priming and those involved in the induction of T-cell anergy [6]–[8]. The demonstration, however, that a single APC subpopulation can induce both T-cell outcomes [9], led to the alternative explanation that perhaps the functional status of the APC at the time of antigen presentation, rather than a static phenotype of the APC, could be the central determinant of T-cell activation versus T-cell tolerance. Indeed, it has become increasingly clear now that antigen encounter by BM-derived APCs in the presence of inflammatory mediators and/or microbial-derived molecules, such as Toll-like receptor (TLR) ligands, triggers their maturation to a functional status capable of generating strong T-cell responses, while antigen capture by these same APCs in the absence of inflammatory signals, or in the presence of inhibitory mediators, leads instead to the development of antigen specific T-cell tolerance [10].

Given this plasticity of a defined APC population to induce divergent T-cell outcomes, it was subsequently proposed that a delicate balance between activating and inhibitory pathways in the APC might play a role in influencing whether T cells would be activated or rendered tolerant following antigen recognition. As such, significant effort has been devoted in recent years to uncover those signaling pathways in APCs that, by regulating the inflammatory properties of these cells, might be central in the decision leading to T-cell activation versus T-cell unresponsiveness. In this chapter, studies that provided some of the answers to these important questions are reviewed. Receptor–ligand interactions, novel intracellular signaling pathways, and epigenetic mechanisms that, by limiting the ability of the APC to stimulate antigen-specific T cells, are important in preserving tolerance to self-antigens are discussed. Although these negative regulatory pathways in APC impose a significant barrier to our efforts to overcome immune tolerance to tumor antigens, their identification has provided novel molecular targets to potentially revert mechanisms of T-cell unresponsiveness in cancer.

2 APCs and Tolerance to Tumor Antigens

An unexpected finding in the field of tumor immunology was the discovery that most of the antigens expressed by tumor cells were not necessarily neo-antigens uniquely present in cancer cells, but rather tissue differentiation antigens shared between the

tumor and normal tissues [11], [12]. These surprising findings prompted some investigators to hypothesize that perhaps the greatest obstacle for harnessing the immune system against tumors is the immune system itself and, more specifically, its complex mechanisms for establishing T-cell tolerance against self and, by extension, to tumor antigens, most of them also "self" [13]. In the mid-1990's, the demonstration by the Bogen's and Levitsky's groups that antigen-specific $CD4^+$ T cells were rendered tolerant during tumor growth *in vivo* provided the first experimental evidence supporting the immune tolerance hypothesis [14], [15]. Since then, several studies have confirmed that this state of T-cell unresponsiveness occurs during the progression of both hematologic and solid tumors expressing model or true tumor antigens [16]–[18] and that this unresponsiveness also affects the $CD8^+$ T-cell compartment [17], [18], [19], [20]. Furthermore, the demonstration that T-cell tolerance is seen during the progression of spontaneously arising tumors [21] and more importantly during the growth of human malignancies [22], [23] led to the undisputed realization that tolerance to tumor antigens, through mechanisms akin to those that regulate responses to self-antigens, represents an important immunosuppressive strategy by which tumor cells might escape T-cell mediated antitumor responses.

This, at the time, alternative view of tumor immunity has intimately linked the cancer immunology and autoimmunity fields. For instance, several principles learned from the better understanding of the cellular and molecular mechanisms by which tolerance to self is maintained in normal conditions, or broken in autoimmune diseases, have been applied to identify tolerogenic mechanisms in cancer patients [24]. One such mechanism was provided by the identification of the central role that BM-derived APCs play in the induction of tolerance to self-antigens [25], [26], a concept that was then extended to the field of tumor immunology with the unambiguous demonstration by us and others that BM-derived APCs are also required for the induction of tolerance to antigens expressed by tumor cells [27], [16]. These studies also provided evidence that the intrinsic antigen-presenting capacity of tumor cells has little influence over T-cell priming versus tolerance, a critical decision that is regulated at the level of the APC.

DCs, macrophages, and B cells are all BM-derived cells that express major histocompatibility complex (MHC) as well as co-stimulatory molecules and, as such, can potentially present tumor antigen to antigen-specific T cells. Although it is plausible that under particular conditions, each subpopulation might induce T-cell tolerance [28]–[32], several lines of evidence have pointed to DCs as playing a central role in influencing the delicate balance between immunity and tolerance *in vivo* [33], [34]. In support of this statement, it has been shown that in the steady state, DCs continually migrate between lymphoid and nonlymphoid tissues capturing self- and harmless environmental proteins through endocytic receptors, such as DEC 205 [5]. Several studies tracking the fate of cellular antigens, particulate antigens, and antigen-pulsed DCs at the site of injection and in draining lymphoid organs [35], [36], [26] have clearly established that antigen presentation by DCs in the steady state, which is characterized by the absence of inflammation, induced a modest T-cell proliferation but not polarization into T helper 1 (Th1) or Th2 subsets. Instead, after several rounds of cell division, almost all the antigen specific T cells are deleted and those that remain

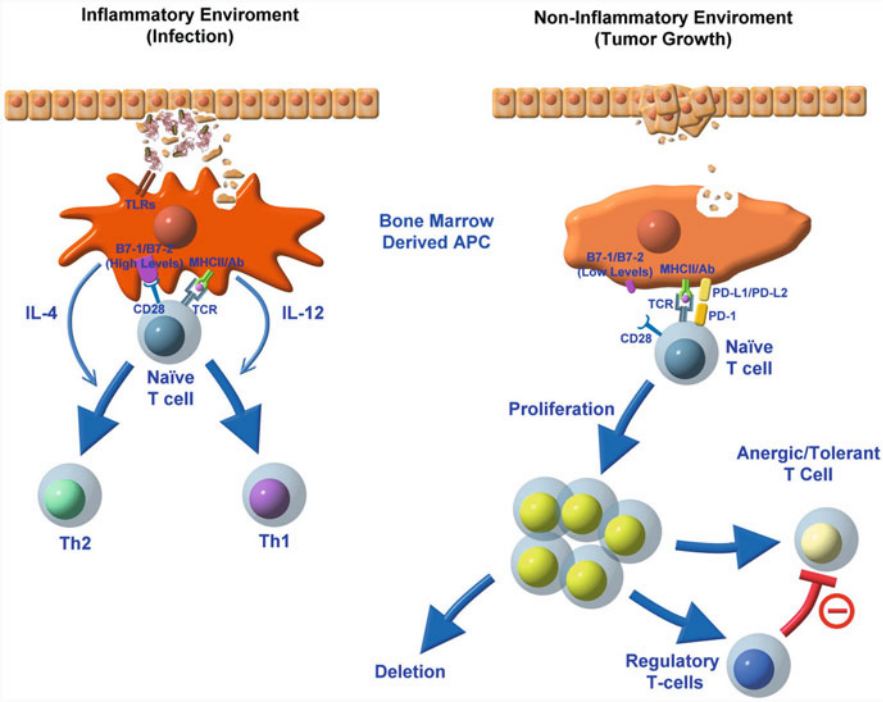

Fig. 15.1 Comparison of antigen presentation in inflammatory and noninflammatory environments. In the presence of TLR agonists (e.g., lipopolysaccharide), APCs present MHC-bound antigen while expressing high levels of the co-stimulatory molecules B7-1 and B7-2 and polarizing cytokines resulting in activation of T cells. In contrast, antigen presentation by APCs in the absence of inflammation results in lack of co-stimulation by CD28 ligands or co-stimulation of T cells by negative regulatory molecules such as PD-L1. Ultimately, this results in deletion or anergy formation of reactive T cells

are functionally anergic even to cognate antigen administered with strong immune adjuvant, such as complete Freund's adjuvant (CFA) [37], [38].

The above scenario also typifies how DCs would normally encounter tumor antigens *in vivo* and has been proposed as an explanation for how tolerance to tumor antigens is induced by these cells (Fig. 15.1). But unlike the steady state, in which the lack of inflammatory stimuli during antigen encounter by DCs is considered to be the major determinant of tolerance induction, in the tumor-bearing host, the encounter of tumor antigens by DCs not only occurs in the absence of inflammatory signals needed for efficient maturation/activation of these cells, but also in the presence of inhibitory factors such as vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), macrophage colony-stimulating factor (M-CSF), transforming growth factor-β (TGF-β), IL-10, prostaglandin E2 (PGE2), and gangliosides that further suppress DC maturation [39]. In this adverse environment, DCs likely acquire tolerogenic properties that would in turn lead to the induction and maintenance of T-cell tolerance to tumor antigens [40]. A better understanding of those ligand–receptors

and/or intracellular pathways involved in the generation of tolerogenic APCs will provide novel molecular targets for therapeutic approaches that, by converting APCs from "tolerizing" into "activating" in tumor-bearing hosts, might ultimately result in breaking of the remarkable barrier that tolerance to tumor antigens has imposed to cancer immunotherapy.

3 Signaling Pathways in APCs Influencing Antigen-specific T-cell Activation Versus Tolerance

3.1 Tyrosine Kinase Receptors

A wide spectrum of cellular functions, such as cell proliferation, differentiation, survival, and metabolism, are regulated through tyrosine kinase receptors (TKRs). TKRs are characterized by their intrinsic tyrosine kinase activity. Ligand recognition by the TKR extracellular domain causes the receptor to dimerize or oligomerize, which in turn activates its tyrosine kinase activity and initiates a specific signaling transduction cascade. Three TKR families of interest have been identified in macrophages and other monocyte-derived cells: the receptor for macrophage colony-stimulating factor (M-CSF) involved in the survival of circulating monocytes and tissue macrophages and the closely related Tyro3 kinase and STK (mouse)/RON (human) receptors [41]. A number of studies have demonstrated the important role of TKRs, especially those belonging to the Tyro3 kinase receptor family in limiting macrophage and DC activation [42].

The Tyro3 family of TKRs is composed of three members named Tyro3, Axl, and Mer (TAM), all having low basal activity levels. TAM TKRs are activated by the binding of growth arrest specific gene 6 (GAS6) or Protein S, proteins that bind to phosphatidylserine on the extracellular surface of apoptotic cells [43]. These TKRs are well established as important in the phagocytosis of apoptotic cells in immune, nervous, and reproductive tissues.

The TAM protein family was first identified in cells of the rat nervous system by using a homology-based cloning. This approach identifies novel receptor tyrosine kinase (RTK) members because of the high similarity that exists on the tyrosine kinase (TK) domain of different RTKs [44]. The central role of this receptor family in immune regulation was first highlighted by studies in triple mutant mice lacking Tyro3, Axl, as well as Mer ($TAM^{-/-}$) [45]. Four-week-old $TAM^{-/-}$ mice were found to have a progressive enlargement of the spleen and lymph nodes that was caused by aberrant T-cell and B-cell proliferation. Lymphocytes from these mice also showed evidence of being activated, as demonstrated by their increased expression of CD44 as well as by production of interferon-γ (IFN-γ). Eventually, these animals developed autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematous. Given that Tyro3 receptors are expressed in monocytes, macrophages and DCs, but not in B cells or T cells, it was concluded that the constitutive immune activation observed in these mutant mice was not due to an intrinsic defect in the lymphocyte

compartment, but the result of lack of TKR in nonlymphocytic cells. Studies of APCs from TAM$^{-/-}$ mice revealed that these cells are indeed functionally hyperactive and display higher levels of MHC class II and B7.2 co-stimulatory molecules relative to wild-type cells before and after activation with lipopolysaccharide (LPS). In addition, higher levels of TNF-α and IL-12 cytokines were produced by TAM$^{-/-}$ macrophages in response to LPS-stimulation. These results were reminiscent of previous studies in mice in which Mer kinase activity is suppressed (mer kinase-deficient mice or mer^{kd}) [46]. LPS-injected mer^{kd} mice showed an increase in tumor necrosis factor α (TNFα) production *in vivo* that correlated with an enhanced susceptibility to endotoxic shock, which could be reverted when animals were treated with TNFα-blocking antibodies. Furthermore, macrophages from mer^{kd} mice displayed increased nuclear factor kappa B (NFκB) translocation to the nucleus and TNFα hyper-production when stimulated with LPS *in vitro*. Finally, sera from mer^{kd} mice showed an increase in anti-DNA antibodies as compared to wild-type mice, and later in life, they develop autoimmunity features, such as development of systemic lupus erythematous [47].

Among its known roles in regulation of APC function, the Mer receptor is required for efficient phagocytoses of apoptotic bodies by macrophages and DCs [48]. Indeed, while Mer, Axl, and Tyro3 combinations are expressed differentially in differing cell types, Mer expression is seen among all phagocytic cells. GAS6 is the common ligand for all the members of the Tyro3 kinase family [10]. The demonstration that GAS6 protein mediates the binding of phosphatidylserine displayed by cells that have initiated apoptosis suggested a potential role of the Tyro3 kinase family in the clearance of apoptotic cells. Indeed, injection of labeled apoptotic cells in animals with deficient mer receptor function resulted in excessive accumulation of apoptotic bodies [48]. Furthermore, *in vitro* studies showed that macrophages isolated from mer kinase-deficient mice displayed a marked decrease in their ability to phagocytose apoptotic cells, but retained the ability to phagocytose bacteria, beads, and opsonized cells. Additionally, the second known ligand of TAMs, Protein S, has also been shown to activate Mer on macrophages. When Mer is blocked through neutralizing antibodies or when Protein S oligomerization with phosphatidylserine is prevented, phagocytosis of apoptotic cells is significantly reduced [49]. These data demonstrate mer RTK as an important scavenger receptor in macrophages.

Deficient removal of cellular debris in animals with deficient mer receptor function resulted in persistence of self-antigens that could explain why these animals are prone to develop autoimmune diseases [50]–[53]. Nonetheless, it does not explain why mer^{kd} macrophages displayed overproduction of pro-inflammatory cytokines and co-stimulatory molecules following LPS stimulation. It has been proposed that phagocytosis of apoptotic bodies might inhibit the production of inflammatory cytokine in APCs through an enhancement in the production of anti-inflammatory mediators, such as IL-10 and TGF-β [54], [55]. In addition, it has been shown that uptake of apoptotic bodies by DCs prevents translocation of NF-κB into the nucleus which leads to diminished production of pro-inflammatory mediators in response to LPS stimulation. Decreased NF-κB nuclear translocation induced by apoptosis seems to be dependent on the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT

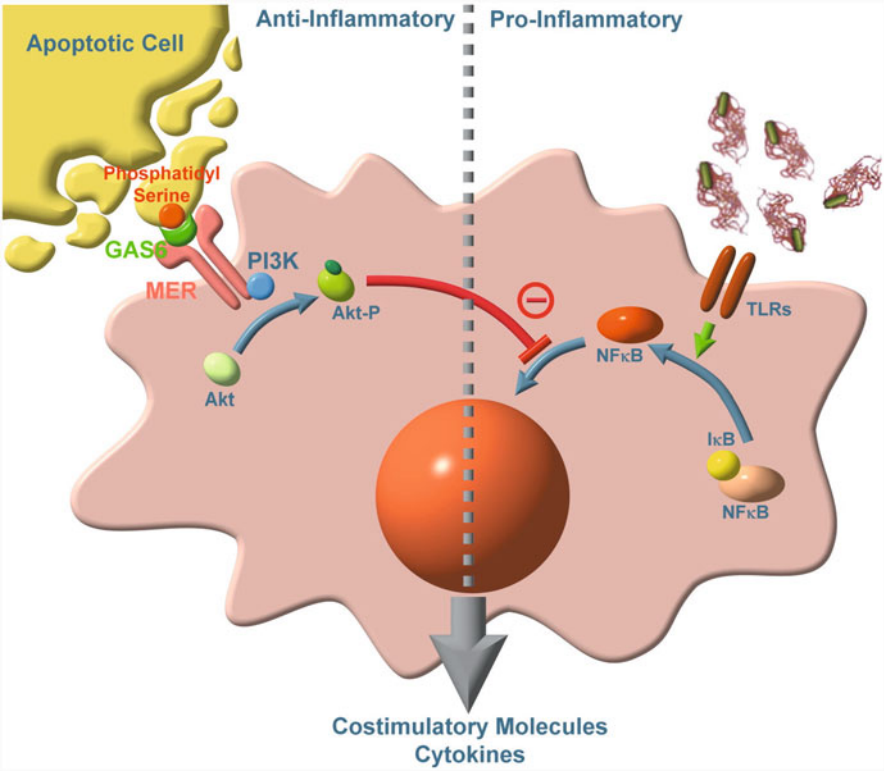

Fig. 15.2 Phagocytosis of apoptotic cells inhibits APC pro-inflammatory function by inhibiting NFκB translocation to the nucleus. In apoptotic cells, extracellular translocation of phosphatidylserine results in binding of the soluble protein GAS6. The Tyro3 kinase Mer recognizes GAS6, allowing the phagocytosis of apoptotic bodies. This further results the phosphorylation of AKT and subsequently the inhibition of NFκB translocation to the nucleus, thereby blocking proinflammatory function

pathway since it was prevented by PI3K inhibitors [56]. Given that mer-deficient macrophages have impaired phagocytosis of apoptotic bodies [48], the absence of this negative regulatory mechanism will be associated with increased NFκB translocation and enhanced pro-inflammatory response to LPS. Therefore, in mice in which signaling through the tyro3 kinase receptors and, in particular, mer has been abrogated, the accumulation of self-antigen due to deficient phagocytosis combined with the presence of APCs displaying enhanced pro-inflammatory features might result in aberrant activation of the lymphocytic compartment, breaking of tolerance to self, and the subsequent development of autoimmunity (Fig. 15.2). In these mice, however, several questions remain unanswered, such as the potential contribution of the microbial flora. Important information will be obtained by crossing TAM mice or mer^{kd} mice with myeloid differentiation primary response gene ($MyD88^{-/-}$) or mice lacking specific TLRs, specially $TLR4^{-/-}$ mice, given the increase sensitivity of TAM and mer^{kd} mice to LPS, the ligand for TLR4.

3.2 Signal Transducers and Activators of Transcription

There are seven mammalian signal transducers and activators of transcription (STATs): STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. These proteins are highly homologous in several regions, including a N-terminal domain (NTD), a coiled-coil domain (CC), a b-barrel DNA binding domain (DBD), a linker domain (LD), a SRC homology 2 (SH2) domain, and a C-terminal transactivation domain (TAD), which is located at the carboxyl terminus [57], [58]. However, the amino acid sequence diversity and their tissue-specific distributions account for the diverse roles of STATs in response to extracellular cytokines.

STAT3 modulates the expression of important genes involved in the regulation of a variety of physiological and nonphysiological cellular functions, including innate and acquired immune responses [59], [60]. The Stat3 pathway is activated in response to a wide variety of cytokines, such as the IL-6 and IL-10 family of cytokines, granulocyte colony-stimulating factor (GCSF), leptin, IL-21, and IL-23. The genes regulated by STAT3 are equally diverse and include IL-17, IL-23, B-cell lymphoma-extra large (Bcl-xL), Bcl-2, myeloid cell leukemia-1 (MCL1), CCDN1, VEGF, c-Myc, p53, in addition to others [61], [62]. Generalized, STAT3 has a dual effect in tumor inflammation and immunity by enhancing pro-oncogenic inflammatory pathways, including NF-κB and IL-6-GP130-Janus kinase (JAK) pathways and by adding a brake to the STAT1 antitumor immune responses, mainly mediated by T cells.

Given the central role of STAT3 in regulating inflammatory responses, it should be of no surprise that disruption of this signaling pathway in APCs influences the inflammatory status and the functional outcome of antigen-specific $CD4^+$ T-cells. Indeed, one study revealed that inhibition of the STAT3 signaling pathway in macrophages and DCs using the tyrosine kinase inhibitor, tyrphostin AG490, resulted in enhanced priming of naïve antigen-specific T-cells and the restoration of responsiveness of anergic $CD4^+$ T-cells *in vitro*. Importantly, the ability of AG490-treated APCs to break T-cell tolerance correlated with a complete inhibition of Stat3 DNA-binding activity in these APCs [63]. Studies in macrophages isolated from Stat3-deficient mice revealed that LPS stimulation rendered these APCs capable of effectively priming naïve antigen-specific T cells and able to overcome the state of unresponsiveness of tolerized T cells *in vitro*. Additionally, in mice lacking functional STAT3 in macrophages and neutrophils, the *in vivo* response to a tolerogenic stimuli is T-cell priming rather than T-cell tolerance, uncovering a previously unknown role for STAT3 in the induction of immune tolerance [63].

Phenotypic and functional analyses of macrophages isolated from Stat3-deficient mice provided important insights into the potential mechanism(s) by which these APCs can restore the responsiveness of tolerized T cells. Freshly isolated peritoneal exudate macrophages (PEMs) from Stat3−/− mice displayed an increased expression of MHC class II molecules as well as B7.1 and B7.2 co-stimulatory molecules relative to nonstimulated PEMs from control mice. LPS-stimulation of PEMs from Stat3−/− mice resulted in significantly higher mRNA levels of the chemokines RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-2, IP-10,

and the cytokine IL-6 as compared to LPS-stimulated PEMs from control mice. In addition, IL-12 production was detected in LPS-stimulated Stat3−/− PEMs while no IL-12 could be detected in PEMs from control mice. Importantly, no IL-10 was detected in the supernatants of LPS-stimulated Stat3−/− PEMs, cytokine that was present at significant levels in the supernatants of Stat3+/+ PEMs controls. Further studies demonstrated that supernatants from LPS-stimulated STAT3−/− macrophages were sufficient to effectively break antigen-specific T-cell tolerance and that this ability was dependent on the combined effect of IL-12 and RANTES [63].

It is important to note that macrophages devoid of Stat3 share phenotypic and functional characteristics displayed by macrophages lacking the aforementioned tyro3 family of receptor tyrosine kinase TAM mutant mice. Freshly isolated macrophages from these mice have increased expression of MHC class II molecules, produce elevated amounts of IL-12 in response to LPS, and induce strong lymphocyte activation. These findings are quite similar to those observed in STAT3−/− macrophages. It is noteworthy, however, that while genetic disruption of all three inhibitory Tyro3 receptors (triple mutant mice) is required to generate "inflammatory" macrophages, a similar outcome can be achieved by disruption of the STAT3 signaling pathway in these cells. The common findings in macrophages from STAT3−/− mice and in TAM triple mutant mice raise the interesting possibility that STAT3 may represent a common signaling pathway linking different inhibitory receptors with their downstream intracellular targets. It is plausible therefore, that the activated phenotype of STAT3−/− PEMs could be related to an enhanced activity of different pro-inflammatory pathways that are tightly regulated by an intact STAT3 signaling in these APCs.

Recently, STAT3 was also discovered to regulate expression of the programmed death ligand 1 (PD-L1) on APCs [64]. PD-L1 expression was induced in $CD14^+$ monocytes stimulated with both LPS and R848, a TLR7 agonist, while blocking STAT-3 activation in these cells prevented PD-L1 expression. Crucially, chromatin immunoprecipitation of STAT3, but not STAT1, showed binding to the PD-L1 gene promoter. As PD-L1 expression by APCs is well characterized in its ability to induce tolerance, this newly discovered role of STAT3 as a positive regulator of PD-L1 expression expands STAT3's known roles as a negative regulator of inflammation.

The molecular mechanism(s) through which STAT3 signaling pathway exerts its inhibitory effects in APCs are not fully elucidated. It is likely that a greater amount of indirect mechanisms also govern STAT3's ability to influence the function of APCs since global chromatin-binding assays have shown that STAT3 binds approximately 3,000 gene promoters [65]. Among the indirect mechanisms of action, one that has gained particular attention relates to the intimate link between STAT3 signaling and IL-10, a cytokine with well-known anti-inflammatory properties. It has been shown that binding of activated STAT3 to the IL-10 promoter is required for efficient expression of the IL-10 gene and protein production [66]. In turn, IL-10 can enhance STAT3 activation in those cells expressing IL-10 receptor, forming a positive feedback mechanism to amplify and maintain production of this cytokine. Supporting these findings, studies have demonstrated that IL-10 mediated anti-inflammatory effects, which depend on STAT3 signaling, require synthesis of *de novo* proteins [67].

Intriguingly, when macrophages are treated with IFN-γ or IFN-α prior to IL-10 stimulation, STAT1 is activated over STAT3, resulting in increased STAT1:STAT3 heterodimer formation at the expense of STAT3 homodimers [68], [69].

In addition to IL-10, other cytokines, such as IL-6, can induce high levels of STAT3 phosphorylation in APCs. However, the anti-inflammatory effect associated with STAT3 activation is observed only in cells stimulated with IL-10 but not in response to IL-6. For instance, IL-10 but not IL-6 can significantly reduce the ability of APCs to produce IL-12 and TNFα in response to LPS [70]. In explanation of the generation of opposing effects by IL-6 and IL-10 signaling through STAT3, recent experiments suggest that STAT3 activation by IL-6 is transient, while IL-10 activation is long lasting. Using time course microarrays of human DCs, a nearly identical transcriptional response to IL-6 and IL-10 was seen 45 minutes post stimulation. However, the gene profile began to diverge after 2 hours post stimulation. A temporally related decrease in STAT3 phosphorylation via IL-6 signaling was also seen. When STAT3 activation was truncated by IL-10-blocking antibodies, gene profile and STAT3 dephosphorylation profile were similar to IL-6 signaling. A possible explanation is altered suppressor of cytokine signaling 3 (SOCS3) expression kinetics in IL-6 stimulated cells compared to IL-10, with IL-10 stimulation favoring higher expression [71]. In agreement, studies have also implicated SOCS3 to be responsible for the different inflammatory responses to IL-10 and IL-6 stimulation. SOCS3 exerts its regulatory role via binding to the phosphorylated subunit glycoprotein 130 (gp130) that is present in the IL-6 receptor but not in the IL-10 receptor [70]. In animals with an intact SOCS3, IL-6 induction of STAT3 phosphorylation decreases faster, relative to the levels of phosphorylated STAT3 induced by IL-10. In SOCS3−/− animals, however, the differences in the kinetics of STAT3 phosphorylation in response to IL-10 and IL-6 are no longer observed [[72], [70]]. Furthermore, unlike wild-type mice, treatment of SOCS3−/− deficient animals with IL-6 reproduced the anti-inflammatory effect associated with IL-10 treatment [70]. These data suggest that persistent STAT3 signaling is required for this pathway to exert its anti-inflammatory effect.

The anti-inflammatory role of STAT3 in APCs cannot be solely explained by its effect upon IL-10. Although APCs devoid of STAT3 share phenotypic characteristics with APCs from IL-10−/− mice, important differences among these cells still remain. Similar to our findings in PEMs lacking STAT3, alveolar macrophages from IL-10−/− mutant mice display increased expression of B7.1 and B7.2 costimulatory molecules [73]. However, while no changes in the expression of MHC class II molecules were observed in IL-10-deficient macrophages, a significant increase in the expression of MHC class II molecules is a characteristic of macrophages lacking STAT3. The ability of STAT3 to regulate MHC class II expression has been attributed at least in part to the regulatory role of STAT3 upon cathepsin S, a protease involved in cleavage of the invariant chain (Ii). Studies using the STAT3 inhibitor cucurbitacin I (JSI-124) have also pointed to an increase in translocation from intracellular compartments to the cell surface as an explanation for the increased expression of in MHC class II molecules in APCs [74]. Finally, studies have found

that pathways other than IL-10 signaling, such as NF-κB activation by TLRs, M-CSF signaling and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function, which are negatively regulated by STAT3, might also play a role in the enhanced innate immunity observed in mice with disruption of this signaling pathway in APCs [75], [76].

3.3 Bruton's Tyrosine Kinase

Bruton's tyrosine kinase (BTK) is well described to play crucial roles in B-cell differentiation, activation, and survival [77]. More recently, BTK has also been shown to negatively regulate the T-cell stimulatory function and maturation of DCs [78]. Initially, Kawakami et al. made the observation that BM-derived DCs from BTK-deficient mice had increased expression of MHC class II after LPS stimulation. Under the hypothesis that BTK has a negative regulatory role in DC function, the authors next demonstrated that BTK-deficient DCs were better able to activate T cells, evidenced by increased cytokine production and proliferation, in both *in vitro* and *in vivo*. To explain this increased T-cell activation, it was further observed that BTK-deficient DCs produce less IL-10 after LPS stimulation. As STAT3 regulates the expression of IL-10 [66], its role in the observed BTK-deficient DC phenotype was investigated. To this end, it was shown that BTK-deficient mice, post-LPS stimulation, had decreased phosphorylation of tyrosine 705 (Tyr-705) and serine 727 (Ser-726) residues of STAT3, markers of STAT3 activation. Importantly, similar reduced Tyr-705 and Ser-727 phosphorylation was seen when cells were treated with a BTK inhibitor. Additionally, decreased STAT3 DNA binding was shown. Finally, it was shown that blocking of IL-10 ameliorated Tyr-705 phosphorylation. From these observations, it was concluded that BTK negatively regulates DC function through autocrine secretion of IL-10 and STAT3 activation. Independent studies have further provided support of the interaction of BTK in the STAT3 pathway [79].

3.4 Suppressors of Cytokine Signaling

Communication among immune cells is fundamental in order to elicit a coordinated immune response. Cytokines released by immune cells orchestrate such a response by binding to specific cell surface receptors and activating proteins that will carry on the signal from the cell surface to the nucleus. Members of the JAK protein family bind constitutively in a specific manner to the cytoplasmic domains of cytokine receptor chains. After ligand engagement, dimerization or higher order oligomerization of receptor complexes occurs, allowing JAK phosphorylation. Activated JAK proteins will then recruit and phosphorylate specific STAT proteins. Activated STATs dimerize, dissociate from the receptor, and translocate to the nucleus where they will induce gene expression. Among the STAT-activated genes there will be those that

mediate the cytokine biological effect but also genes that are involved in turning-off cytokine signal when their production is no longer needed. The SOCSs are a protein family, consisting of eight members, the cytokine-inducible SH2 domain-containing protein (CIS) and SOCS1 through SOCS7 each of which has a central SH2 domain and a C-terminal 40 amino acid sequence known as the SOCS box. While the SH2 domain binds phosphorylated tyrosine residues present in activated members of the cytokine signaling pathway, the SOCS box targets the complex for ubiquitination, proteosome degradation, and, as such, termination of cytokine signaling and its biological effect. Given that overexpression of certain SOCS inhibits signaling by a variety of cytokines through the JAK/STAT pathway, it has been proposed that SOCS proteins are critical in providing a negative feedback loop for cytokine production.

SOCS1 has been shown to play a central role in regulation of autoimmunity and in tumor rejection. This protein was first discovered using two-yeast hybrid assay to identify molecules that interact with Jak2 [80]. In addition to the SH2 and SOCS box domain, another region denominated by kinase inhibitory region (KIR) might play a role in SOCS1 mediated inhibition of Jak2. KIR might increase the binding strength of SOCS1 to Jak2 and block the access of substrates and/or ATP to the kinase catalytic pocket [70]. The importance of SOCS1 in controlling autoimmunity was unveiled in mice lacking functional SOCS1 (SOCS1$^{-/-}$ mice) [81]. These animals die within 2–3 weeks after birth because of a complex organ pathology that includes peripheral T-cell activation and massive infiltration of macrophages in the liver, spleen, lung and heart. These pathologic findings seem to be related to aberrant IFN-γ responses, since treatment with IFN-γ-blocking antibodies or by crossing the SOCS1$^{-/-}$ strain with IFN-γ$^{-/-}$ animals prevented development of disease.

SOCS1 deficiency in the hematopoietic compartment is thought to be sufficient to cause disease since transfer of SOCS1$^{-/-}$ BM into irradiated Jak3$^{-/-}$ recipients resulted in premature lethality [82]. In addition, SOCS1$^{-/-}$ Rag2$^{-/-}$ mice do not develop pathologic abnormalities suggesting that lymphocyte subsets contribute to the SOCS1$^{-/-}$ pathology [82]. However, SOCS1 deficiency in T/natural killer T (NKT) cells alone is not sufficient to cause inflammatory pathology. Indeed, experimental studies in conditional knockout (KO) mice in which SOCS1 was deleted in CD4^{+} T cells, CD8^{+} T cells, and NKT cells but not in NK cells, B cells, monocytes, or granulocytes (flox–SOCS1 mice crossed with mice expressing the cre-recombinase protein under the control of the Lck promoter) did not show any abnormal activation and/or inflammatory changes [83]. Conversely, in mice in which SOCS1 expression was specifically restituted in T and B cells (SOCS1$^{-/-}$ Tg) autoimmunity developed [84]. These animals die within 6 months when kept in pathogen-free conditions and within 3 months when kept in regular conditions. Splenomegaly and lymphadenopathy were observed as early as 10 weeks after birth and these findings coincided with the accumulation in the spleen of phenotypically mature DCs displaying high levels of co-stimulatory molecules. Studies of BM-derived DCs from SOCS1$^{-/-}$ Tg animals showed that these cells display enhanced responses to IL-4 and IFN-γ stimulation. Finally, autoimmune disease in SOCS1$^{-/-}$ Tg animals resembles systemic lupus erythematous, and the skin lesions, glomerulonephritis, hypergammaglobulinemia, and

autoantibody production seem to be the result of aberrant B-cell activation in these mice. Of note, SOCS1$^{-/-}$ DCs constitutively produce TNF-family B-cell growth factors and induce strong B-cell proliferation and antibody production [84].

The studies above point toward the APC as being the critical cell in which disruption of the negative regulatory effect of SOCS1 results in dramatic pro-inflammatory changes and development of autoimmunity *in vivo*. A more recent study supports the ability of SOCS-1-deficient DCs to disrupt T-cell tolerance [85]. In this study, the authors used lentiviral transfection to introduce SOCS1 siRNA into BM-derived DCs. SOCS1-deficient DCs were matured *ex-vivo* with LPS and loaded with a self-antigen (TRP2) expressed by melanocytes. Adoptive transfer of LPS matured SOCS1$^{-/-}$-deficient DCs, but not regular DCs, resulted in the development of autoimmune disease as evidenced by skin depigmentation or vitiligo. In addition, SOCS1-deficient cells induced strong antitumor responses against B16 melanoma tumors expressing the TRP2 antigen. Although, the mechanism(s) involved in the generation of antitumor responses are not fully elucidated, IL-12 signaling seems to play an important role. Indeed, disruption of IL-12 signaling by using IL-12 receptor KO SOCS1-deficient DCs suppresses their ability to induce autoimmunity or antitumor immune responses. Furthermore, IL-12 signaling in the absence of SOCS1 results in persistent STAT4 activation and increased production of IL-12. Interestingly, SOCS1-deficient DCs are characterized by an increased life span since they were detected up to 4 days following their adoptive transfer into recipient animals. In sharp contrast, wild-type DCs are nearly undetectable 48 hours after adoptive transfer. How much the immune response induced by SOCS1$^{-/-}$ DCs depends on their increased survival remains to be elucidated.

In summary, SOCS1 has been unveiled as a master regulator of innate immune responses (Fig. 15.3). SOCS1-deficient DCs and macrophages stimulated with microbial products or cytokines are hyper-activated and as such prone to initiate pathological immune responses that might lead to autoimmunity. A better understanding of the mechanisms by which SOCS1 regulates inflammation would have a significant impact not only in the autoimmunity field but also will provide novel molecular tools to overcome immune tolerance to tumor antigens.

4 Epigenetic Regulation of Immune Responses

It has become increasingly evident in recent years that epigenetic mechanisms are intimately involved in the regulation of the immune response. Epigenetics, generally defined as changes in gene expression independent of changes in the DNA sequence, encompasses a variety of mechanisms including microRNAs, DNA methylation, and chromatin/histone modifications. Among these mechanisms, histone acetylation/deacetylation has garnered particular attention due to observations, mainly through the use of histone deacetylase inhibitors, of their importance in regulating the inflammatory response of immune cells (Fig. 15.4).

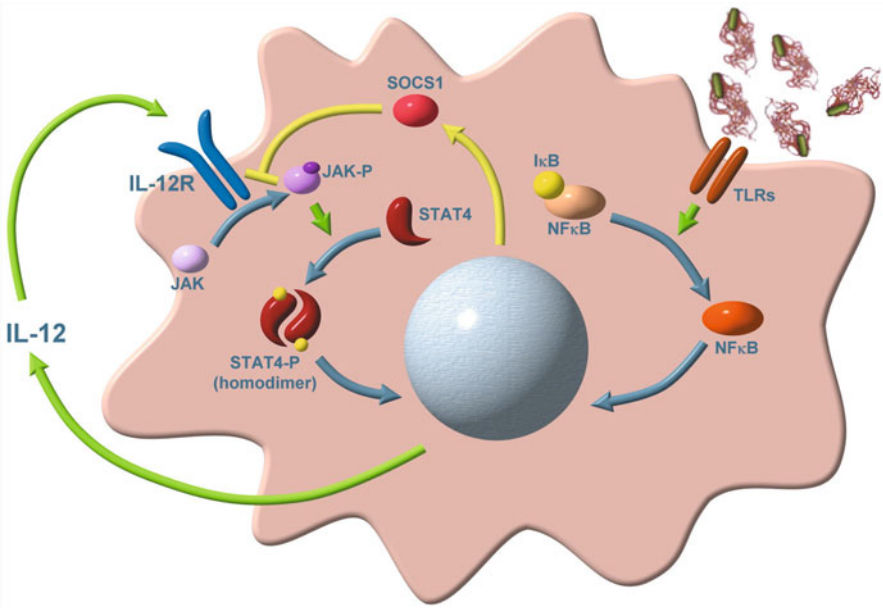

Fig. 15.3 Regulation of innate immune responses by SOCS1. IL-12 production is induced in macrophages and DCs by TLR ligands via NFκB activation. Through autocrine/paracrine mechanisms, IL-12 signaling leads to the production of negative regulators needed to limit the cytokine's effects. Cells lacking SOCS1 are hyperactivated in response to microbial products and/or cytokines resulting in unrestrained inflammation and the development of autoimmune disease

4.1 Histone Deacetylases

One of the most studied posttranslational protein modifications is the acetylation of lysine amino acids. Initially, these modifications were found at the N-terminal end of histones as a transcriptional regulatory mechanism. Generally, in a nonmodified steady state, the highly positive N-terminal ends of histones wrap around histones, generating an obstacle for the binding of transcription factors and the recruitment of other proteins that need to read the "writing pattern" on nucleosomes to exert their transcriptional functions. In this context, acetylation of histones neutralizes these positive charges promoting a relaxed nucleosome conformation and allows the binding of transcription factors and "writer" proteins. Acetyl modifications are introduced by a heterogeneous group of proteins named histone acetyltransferases (HATs), most of them forming multi-protein complexes that can be selectively recruited to DNA sequences upon exogenous or endogenous cellular stimuli [86]. In opposition, these acetyl modifications can be removed by another group of proteins, histone deacetylases (HDACs). While originally described as histone modifiers, HDACs have more recently been demonstrated to modify a variety of other proteins involved in diverse

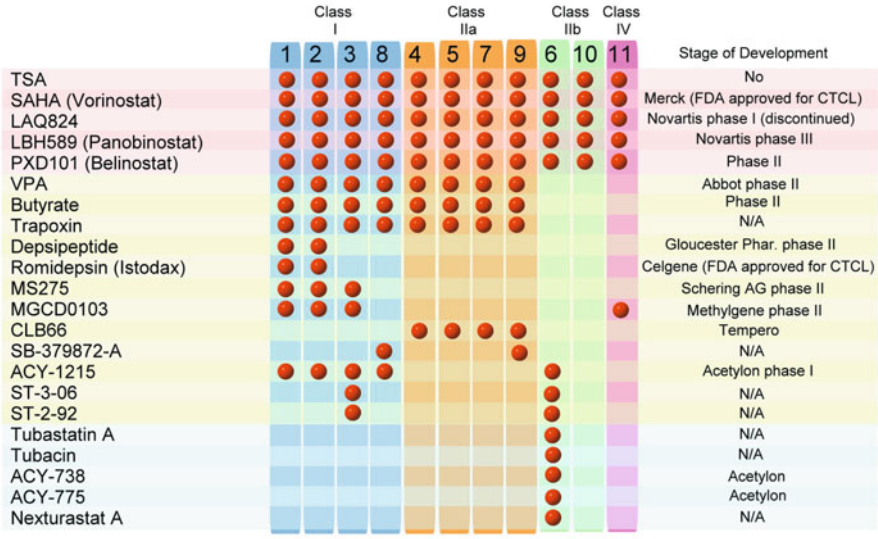

Fig. 15.4 List of histone deacetylase (HDAC) inhibitors and their specificity

cellular processes nonrelated to the chromatin environment. This includes deacetylation of multiple nonhistone targets, such as proteins involved in cell cycle/apoptosis and immune regulation [87], [88].

The 18 HDACs identified in humans are subdivided in two families: the classical HDAC family of zinc-dependent metalloproteins, composed by classes I, II, and IV and the class III nicotinamide adenine dinucleotide (NAD+)-dependent belonging to the sirtuin family of HDACs. The class I HDACs (HDAC1, 2, 3, and 8) are most closely related to the yeast deacetylase RPD3, and the class II HDACs are subdivided into class IIa (HDAC4, 5, 7, and 9) and Class IIb (HDAC6 and 10), both subclasses sharing homology with the yeast deacetylase HDA1 [89]. Finally, the newest HDAC discovered, HDAC11, comprises its own class IV and does not share homology with either RPD3 or HDA1 yeast deacetylases. HDAC KO mice have severe malfunctions at multiple cellular processes and are in some cases embryonically lethal (HDAC1, 3, and 7) or lethal at the perinatal stage (HDAC2, 4 and 8).

Cytokine production in APCs is known to be regulated by changes in the acetylation status of gene promoter region histones [90], and recently the roles of HDACs in the regulation of not only cytokine production but also co-stimulatory and antigen presentation have been discovered. Given the clinical use of HDAC inhibitors (HDACi) in the treatment of cancer and its continued exploration in the treatment of inflammatory conditions, an expanded understanding of their effect on APCs is crucial. Currently, the exact roles of HDACs in the regulation of APC function remain less than well characterized; however, the knowledge acquired thus far demonstrates that HDACs play key roles in regulating the immune response. Briefly discussed below is an overview of the current knowledge of the role of HDACs in regulating

APC function. For a more in-depth review of the subject, readers are referred to a more in depth review on the subject [87].

Several studies have shown the ability of various HDACi to modulate the expression of immunologically relevant cell surface markers. This modulation includes the upregulation of MHC I, MHC II, and CD40 [91], as well as the co-stimulatory molecules CD80 and CD86 [92]. Illustrative of the duality of HDACi with regard to their influence on tolerance vs. inflammation, some reports have demonstrated downregulation of these same molecules resulting from HDACi treatment [93], [94]. For example, treatment with the HDACi sodium butyrate results in downregulation of MHC I expression [95]. With regard to regulation by specific HDACs, the mechanisms behind these disparate findings remain largely unknown. However, experiments have shown that the class I HDACs: HDAC1, HDAC2, and HDAC8 interact with the promoter region of MHC class I resulting in deacetylation of the region [96].

In much the same fashion as cell surface markers, there are numerous reports on the effects of HDACi on cytokine production by APCs. Even more so than with cell surface markers, reports of HDACi influence on the cytokine production by APCs are convoluted. Indeed, dependent on cell type, HDACi, and disease context, inhibition of HDACs can lead to inhibition or enhanced expression of inflammatory cytokines and/or suppressive cytokines. In one study, treatment of DCs with the HDACi trichostatin A (TSA) reduced expression of the pro-inflammatory cytokines IL-12, TNF and IL-6 [94]. In a separate study, TSA was shown to also increase expression of the anti-inflammatory cytokine IL-10 [97]. Conversely, yet another study showed that treatment with LAQ824 results in an increased expression of IL-12 and a reduction in IL-10 [92]. Ultimately, these differences resulting from HDACi treatment significantly alter the ability of APCs to activate T cells [98], [99]. While these results and others paint a clear picture that modulation of HDACs heavily influences the cytokine production by APCs, the specific HDACs regulating these responses are less well understood. With regard to current knowledge, it is known that expression of IL-10 is positively and negatively regulated by two HDACs, HDAC6 and HDAC11, respectively [100]. Furthering the idea of counter regulation by different HDACs, IFN-β is inhibited by HDAC1 and HDAC8 and enhanced by HDAC6 [101]. Additionally, both HDAC2 and HDAC3 have known roles in regulating the anti-inflammatory cytokine IL-4 [102]. Finally, as mentioned previously, both STAT1 and STAT3 are known to be regulated by acetylation. Indeed, HDAC1 [103], HDAC2, and HDAC3 [104] have been shown to have roles in the regulation of STAT3, and HDAC3 also having a role in STAT1 regulation [105]. While several studies have shown that HDACs directly promote or inhibit cytokine production, it is highly probably that many of the effects seen by HDACi treatment result from modulation of STAT1 and STAT3 acetylation. Given the disparate nature of these STAT molecules, this may also account for many of the disparate results seen in cytokine production resulting from HDACi treatment.

5 Concluding Remarks

Since the initial description, well over a decade ago by the Bogen's and Levitsky's group of the phenomenon of tumor-induced antigen-specific T-cell tolerance, significant advances have been made in the understanding of the cellular and molecular mechanisms underlying this phenomenon. BM-derived APCs cells and specifically DCs, through mechanisms akin to those that regulate responses to self-antigens, have been shown to be central in the induction of this state of T-cell unresponsiveness. More recently, studies of receptor–ligands and intracellular signaling pathways in APCs have unveiled a complex network in which a delicate balance among stimulatory and inhibitory pathways critically influences the inflammatory status of these cells and as such their ability to induce priming versus tolerance of antigen-specific T-cells. These studies have also shown the dominant role of inhibitory pathways in preserving tolerance towards self-, since their genetic and/or pharmacologic disruption in APCs was associated with over-activation of the immune system and development of autoimmunity. Inhibitory signaling pathways, like those described here, under normal conditions, act as stringent safeguard mechanisms, preventing the development of autoimmunity. However, these same pathways also impose a significant barrier to efforts to overcome tolerance to tumor antigens and effectively harness the immune system against malignant cells. Therapeutic strategies targeting these molecular pathways in APCs hold the hope of directing the immune response away from tolerance toward a robust antitumor effect. Future studies not only will provide answers to several questions that remain in our understanding of inhibitory signaling pathways in APCs, but they will likely provide novel targets to augment antitumor immune responses while minimizing "collateral damage" to normal tissues.

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Chapter 16
Overcoming Immune Suppression: Therapeutic Strategies Targeting T-Cell Function in Cancer

Jeffrey S. Weber

Abstract The first clinical success in overcoming immune suppression in cancer was shown by the development of human antibodies with antitumor activity against the checkpoint proteins cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1). In 2011, the Food and Drug Administration (FDA) approved the anti-CTLA-4 antibody ipilimumab for metastatic melanoma. Patients with objective responses to ipilimumab can have remissions lasting more than 5 years, although significant immune-related toxicity can occur in 5–15 % of patients. More recently, PD-1 and PD-L1 antibodies have been tested in the clinic, with PD-1 antibodies nivolumab and MK-3475 showing impressive response rates of 20–40 % in previously treated patients. These antibodies are being tested in randomized phase II and III registration studies in metastatic melanoma and non-small cell lung cancer.

Denileukin difitox, or ONTAK, a fusion of interleukin-2 (IL-2) with diphtheria toxin, and daclizumab, an IgG2 humanized antibody against CD25, have been used to deplete CD25-expressing T regulatory cells (Tregs) in cancer patients, but these strategies have resulted in transient depletion only.

Nonmyeloablative (NMA) chemotherapy using fludarabine and cyclophosphamide have been employed to transiently deplete Tregs while mintaining overall lymphodepletion in the setting of autologous stem cell transplantation or adoptive cell therapy. This approach cannot provide long-term Treg inhibition, but has been successful in promoting long-term response to tumor-infiltrating lymphocyte (TIL) therapy.

Inhibitors of indoleamine-2,3-dioxygenase, or IDO that is secreted by dendritic cells (DCs) and depletes tryptophan needed for the growth and viability of activated T cells are being tested in the clinic. Myeloid-derived suppressor cells (MDSCs) derived from either a monocytic or a granulocytic lineage can suppress T-cell responses by both direct contact-dependent and indirect mechanisms via nitric oxide and other oxidative stresses. Their inhibition by a variety of agents including gemcitabine and all *trans*-retinoic acid (ATRA) has been evaluated in the clinic to indirectly increase T-cell reactivity.

Keywords CTLA-4, PD-1, and PD-L1 antibodies · Adoptive cell therapy · Myeloid-derived suppressor cells · T regulatory cells

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1 Introduction

A variety of agonistic and antagonistic factors influence immune homeostasis and are responsible for the induction of T-cell tolerance. The programmed death-1 (PD-1, CD279) [38], [45], [99], [134] and cytotoxic T-lymphocyte antigen-4 (CTLA-4, CD152) receptors [22], [35] provide critical signals that modulate adaptive immune responses in humans, but the kinetics and levels of their expression are often altered in malignancy. Both molecules are found on T regulatory cells (Tregs), on which CTLA-4 is highly expressed. In both naïve and memory T cells and Tregs, activation depends on the binding of CD28 to the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) on antigen-presenting cells (APCs), while CTLA-4, a member of the CD28 family, has a counter-regulatory effect since it can down-modulate T-cell activity when it preferentially binds to B7-1 and B7-2. Inhibition of CTLA-4 might thus represent a strategy to overcome T-cell suppression and reverse its impact on Tregs. PD-1 is another member of the CD28 family that is upregulated on activated T cells, B cells, dendritic cells (DCs), and macrophages. Engagement of PD-1 transduces a variety of signals that inhibit the function of those immune cells. PD-1 has two known ligands, PD-L1 (CD274, B7- H1) and PD-L2 (CD273, B7-DC). While PD-L1 expression is induced on a variety of lymphoid and peripheral tissues, including tumor cells, PD-L2 is more restricted to myeloid cells, including DCs [134]. CTLA-4 is expressed on activated T cells, Tregs, and rarely on tumor cells, and its binding to B7-1 and B7-2 down-modulates T-cell responses, decreases expression of interleukin-2 (IL-2), and diminishes proliferation of T cells [35]. These regulatory molecules can protect against autoimmunity by maintaining peripheral tolerance of self-antigens. Expression of PD-1 and CTLA-4 on T cells and binding to their ligands have been shown to diminish tumor immunity and facilitate chronic infection and tumor progression. High levels of PD-1 have been found on "exhausted" T cells, specifically circulating tumor antigen-specific T cells, and on tumor-infiltrating lymphocytes (TILs) [1], [20], [37], [72], [154]. PD-1 blockade on those cells with an antibody augments their antitumor activity, secretion of interferon-gamma (INF-γ) and other cytokines, and promotes their proliferation to reverse the exhausted phenotype [155], [165]. PD-1 blockade is another means of overcoming T-cell checkpoint inhibition and may alter the influence of that molecule on Tregs.

When the binding of PD-1 and CTLA-4 to their ligands is inhibited, antitumor immune effects are seen in a variety of mouse models [10], [58], [59], [103]. Human antibodies against CTLA-4 and PD-1 have been shown to have antitumor activity in the clinic, and in 2011 the Food and Drug Administration (FDA) approved the anti-CTLA-4 antibody ipilimumab for metastatic melanoma [12], [31], [55], [126], [158], [163]. The two receptors are both expressed on activated T cells with different kinetics, but their ligands are differentially expressed both temporally and spatially. In the clinic, the anti-CTLA-4 antibody ipilimumab has shown a 10 % response rate by standard Response Evaluation Criteria In Solid Tumors (RECIST) criteria, with another 10 % of patients having clinical benefit shown by long-term tumor stability without progression or progression followed by regression [55], [103], [126], [158],

[163]. Patients with complete responses can have remissions lasting more than 5 years [31], [118]. The drug has significant immune-related toxicity, with grade 3 colitis occurring in 5–15 % of patients depending on the dose. Ipilimumab was approved in March 2011 based on the results of two phase III randomized trials showing prolonged survival for patients receiving the drug compared to control-treated patients [55], [123]. More recently, a variety of PD-1 and PD-L1 antibodies are being tested in the clinic, but the PD-1 antibody BMS 936558, now known as nivolumab, has shown impressive response rates of 20–30 % (20–40 above) in previously treated patients [145]. This antibody is being tested in randomized phase III registration studies in metastatic melanoma and non-small cell lung cancer.

A number of agents have been tested for their ability to directly inhibit the activity of Tregs. Denileukin difitox, or ONTAK, is a fusion of IL-2 with the diphtheria toxin, which upon internalization into an IL-2 receptor bearing T cell is capable of inducing cell death by inhibition of protein synthesis. That agent has been approved by the FDA for treatment of cutaneous T-cell lymphoma, but has been employed in a number of trials as a strategy for Treg depletion and inhibition, since Tregs are characterized, among other features, by high levels of expression of CD25, the low-affinity IL-2 receptor alpha (IL-2Rα). In a similar strategy, daclizumab, an immunoglobulin G2 (IgG2) humanized antibody against CD25, has been used to deplete CD25-expressing Tregs. A significant issue in the field of Treg depletion is the fact that to date no marker absolutely specific for those cells has been defined that might constitute a proper target for depletion, and most strategies have resulted in transient depletion only. Cross-reactivity of anti-CD25 antibodies with activated effector T cells is also a concern.

Nonspecific means like NMA chemotherapy using agents like fludarabine and cyclophosphamide have been employed to transiently deplete Tregs while maintaining overall lymphodepletion in the setting of autologous stem cell transplantation or adoptive cell therapy. It has been employed in an adoptive transfer strategy pioneered at the National Cancer Institute (NCI), but also tested at several other centers in the USA and abroad. In this approach, fludarabine specifically acts to deplete lymphocytes, resulting in a transient depletion of CD4+/CD25hi/Foxp3 positive Tregs over a period of several weeks, during which antitumor effector cells are adoptively transferred and high-dose IL-2 is added. This approach cannot provide long-term Treg inhibition.

Finally, indirect means of suppressing Treg activity has been employed using inhibitors of indoleamine-2,3-dioxygenase, or IDO, which is secreted by antigen-presenting cells (APCs) like DCs and acts to deplete tryptophan, an amino acid needed for the growth and viability of activated T cells. Myeloid-derived suppressor cells (MDSCs), a group of cells derived from either a monocytic or granulocytic lineage, can suppress T-cell responses by both direct contact-dependent and indirect mechanisms that act via nitric oxide and other oxidative stresses [96]. They have been shown to augment the activity of Tregs, and their inhibition by a variety of agents including gemcitabine and all *trans*-retinoic acid (ATRA) has been evaluated

in the clinic to indirectly increase T-cell reactivity. In the ongoing sections [92], we will summarize and review the important clinical details of attempts to alter immune suppression in patients with cancer, focusing on melanoma, an immunogenic tumor in which many immune reagents have been tested.

2 CTLA-4 as a Target: Preclinical Data

CTLA-4 is a checkpoint regulatory protein predominantly expressed within T cells. The molecule was cloned in 1988 [3] and was found to be critical for the maintenance of T-cell homeostasis and tolerance. When a naïve T cell is stimulated via the T-cell receptor (TCR), CTLA-4 is delivered to the cell surface and localizes at the immunologic synapse [81]. CTLA-4 then preferentially binds to co-stimulatory molecules CD80/CD86 on APCs in competition with CD28 on T cells [16], [23], [24], resulting in decreased TCR signaling via the *Akt* pathway [147]. Although CTLA-4 delivery to the immune synapse depends on events that are downstream of TCR signaling [128], it is unclear how CTLA-4 engagement results in a decreased TCR signal after it binds CD80/CD86. Knockout mice deficient in CTLA-4 developed lymphoid proliferation and diffuse lymphadenopathy and died early during development of heart failure secondary to lymphocytic myocarditis [144], [151], [157]. The immune-related pathologic changes observed in mice genetically deficient of CTLA-4 depended on CD28 interacting with its ligands B7.1/B7.2. This has been shown by the lack of disease in mice deficient in CTLA-4, B7.1, and B7.2, and the protection against development of autoimmunity afforded by the use of CTLA-4 Ig in mice genetically deficient of CTLA-4.

Work from a number of laboratories demonstrated that both CD4+ and CD8+ T cells lacking CTLA-4 in vitro and in vivo showed high rates of proliferation and an activated phenotype, consistent with its checkpoint inhibitory function [17], [19], [44], [46], [90]. Absence of CTLA-4 impacted on the proliferation of CD4+ T cells in animal models, resulting in a shifted CD4/CD8 ratio. Depletion experiments showed that CD4+ T cells were required for the immune tissue infiltration seen in the knockout mice and that CD8+ T-cell activation was CD4+ T-cell dependent [111]. Data from experiments with CTLA-4-blocking antibodies showed that CTLA-4 binding induced peripheral CD4+ T-cell tolerance [41] and modulated cell cycle progression by T helper cells [46], [90]. CTLA-4 engagement also played an indirect role in CD8+ T-cell responses since in experiments with pmel-1 TCR transgenic mice that were also CTLA-4−/− deficient, autoimmune vitiligo developed in a CD4+ T cell-dependent manner [161]. In mice that were both transgenic for a different TCR and also CTLA-4 deficient, recall CD8+ T-cell responses significantly increased, suggesting that CTLA-4 may regulate CD8+ memory responses but not their induction [132]. Antibody-mediated blockade of CTLA-4 augmented T-cell immune responses [75], [77], and a blocking antibody against CTLA-4 combined with a cellular-based tumor vaccine induced regression of established poorly immunogenic tumors in several models including the well-characterized poorly

immunogenic B16 melanoma [148], [149], [169]. Poorly immunogenic tumors did not exhibit regression with anti-CTLA-4 alone, but did so only when combined with a granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting tumor cell vaccine in association with autoimmune vitiligo [58], [59]. Tumor regression was T-cell dependent since the benefit of CTLA-4 blockade was eliminated in mice genetically deficient for TCR-expressing cells. CTLA-4 was detected on the surface of CD4+CD25hiFOXp3+ natural Tregs found in high numbers in the circulation of and within tumors of cancer-bearing murine and human hosts. Was the therapeutic activity of CTLA-4- blocking antibodies in murine models explained by elimination of Treg activity? This was not substantiated in animals or in patients receiving a human anti-CTLA-4 antibody, as indicated below. Recent work has indicated that it is the ratio of T effector to Tregs within the tumor microenvironment that is critically altered by anti-CTLA-4 antibodies in rodents and humans, and that the local ratio of those cells is associated with the clinical benefit of CTLA-4 abrogation [26].

3 CTLA-4 as a Target: Clinical Data

Preliminary clinical studies with the CTLA-4- blocking human IgG1 antibody ipilimumab included single-dose pilot studies in small numbers of patients followed by repeat-dosing phase II trials which included a peptide vaccine. In the first published experience from Hodi et al. [56] six patients were treated with a single dose of 3 mg/kg, with minimal evidence of side effects. Clinical benefit was observed in three patients who had previously been treated with a GM-CSF-transduced cell-based vaccine. No patient had tumor regression, but three had pathologic evidence of necrosis of bulky tumors that were subsequently resected. This may have been beneficial since two of the patients had long-term freedom from progression after resection of their large, necrotic tumors.

In a follow-up trial, the investigators then chose a dose of 3 mg/kg for further testing, since it was previously believed to be safe, and calculated that it would achieve serum antibody levels of 10 μg/mL believed to be active. The antibody was administered with a multi-peptide vaccine derived from melanoma antigen gp100 and emulsified with the oil-in-water-based adjuvant Montanide ISA 51. In an initial pilot trial, they treated 14 patients and found a unique spectrum of dose-limiting and severe colitis, rash, and hypophysitis that suggested ipilimumab had achieved a diffuse level of activation of T cells [112]. They subsequently treated 56 patients who had failed prior IL-2 as well as other therapy with ipilimumab at doses between 1 and 3 mg/kg, and described seven responders (two complete, five partial) for an overall response rate of 13 % [7]. Five of the seven responses were sustained for more than 2 years, and 5 of 14 patients who had grade III or higher immune-related adverse events (irAEs) clinically responded versus 2 of 42 without immune-related side effects ($p = 0.008$) [87]. These irAEs consisted of colitis, diarrhea, hypophysitis, hepatitis, nephritis with azotemia, rash, and vitiligo. They were inflammatory in nature and appeared to be consistent with reversal of immune suppression and tolerance induced by Tregs.

A dose-ranging phase I trial of single administration of two different preparations of ipilimumab was then carried out at doses from 2.8 to 20 mg/kg. This was followed by a small phase II extension of the trial in which 23 patients with stage IV melanoma received 10 mg/kg of ipilimumab four times every 3 weeks [159]. Two patients in the phase II extension of that trial had an objective response, and an additional seven patients had stable disease for a disease control rate (DCR) of 39 % and a median overall survival of 13.5 months for the extension cohort; both of the responders and three patients with stable disease had not progressed at 24 months.

In a small randomized phase II trial, 73 patients with metastatic melanoma who were previously untreated received ipilimumab alone at 3 mg/kg four times at a 4-week interval or combined with dacarbazine given over 5 days every 4 weeks [50]. There was a 17 % response rate with a 14.8-month median survival achieved in the combination arm, compared with 9 % and 11.2 months for the monotherapy arm. The favorable results of that small, randomized phase II trial supported a registration trial of ipilimumab plus dacarbazine versus dacarbazine alone in 502 front-line melanoma patients in whom there was a clear benefit for the ipilimumab-containing arm in terms of response rate, progression-free survival (PFS), and overall surivival [126]. With more than 30 months of follow-up for all patients, median survival was improved for ipilimumab with dacarbazine compared to dacarbazine alone to 11.2 months from 9.4 months, and overall survival was superior in the combination arm, $p = 0.001$, supporting the subsequent FDA registration of ipilimumab for melanoma.

At the NCI, 139 patients were treated with multiple doses of ipilimumab ranging from 3 to 9 mg/kg, with some intra-patient dose escalation, with or without a peptide vaccine [31]. In that trial, a majority of the patients received the vaccine, and a 17 % objective response rate with a 15.7-month median survival was noted among patients who had predominantly failed IL-2 or chemotherapy. Based on those and other data, 676 second-line melanoma patients were randomized to receive ipilimumab at 3 mg/kg, a multi-peptide vaccine, or the combination of ipilimumab and vaccine in a registration trial [55]. Response rate and overall survival were superior in either ipilimumab-containing arms compared to the vaccine alone control arm. Median survival was improved from 6.4 months in the vaccine-alone control arm to 10.0 and to 10.4 months in the two ipilimumab arms with $p = 0.004$. These data directly led to the registration of ipilimumab in the USA.

In order to address the question of the optimal dose of ipilimumab, a three-arm randomized phase II trial of 216 previously treated melanoma patients was conducted [163]. Patients received ipilimumab at a dose of 0.3, 3, or 10 mg/kg four times administered every 3 weeks. Patients with the best response of stable disease or who had any regression were able to have further "maintenance" therapy with ipilimumab every 3 months until dose-limiting toxicity, progression, or refusal. A clear dose response for objective response rate and for the frequency of irAEs was observed in that trial. These data supported the choice of the 10 mg/kg dose for subsequent registration trials in metastatic and adjuvant settings and provoked an ongoing randomized phase III trial of ipilimumab at 3 mg/kg compared to 10 mg/kg in stage IV melanoma patients. In order to avoid some of the gastrointestinal side effects of ipilimumab, a randomized phase II trial was conducted in which budesonide, an

oral non-absorbed steroid, was used prophylactically in 115 first-line and second-line melanoma patients who received ipilimumab at 10 mg/kg every 3 weeks and were randomly allocated to receive either budesonide or placebo from the start of treatment [158]. The primary endpoint of the trial was the rate of grade 2 or more diarrhea. There was no impact of budesonide upon diarrhea or any irAE, but response rates were 12.1 and 15.0 % in the budesonide and placebo arms, respectively. Median survivals were excellent at 15.3 and 17.1 months, respectively. Rates of grade 3–4 irAEs were about 40 % in either arm. Those favorable phase II data taken together supported two registrational trials with ipilimumab: a front-line trial of dacarbazine (DTIC) plus ipilimumab versus DTIC alone in 502 patients, and a second-line trial of 676 patients who were human leukocyte antigen (HLA) A*0201 positive and who were randomly allocated 3:1:1 to receive either ipilimumab at 3 mg/kg with a peptide vaccine, ipilimumab at 3 mg/kg alone, or the peptide vaccine alone. The results of the trial have been summarized above [55], [126]. Recent follow-up data from the second-line and front-line randomized trials suggest that at 3, 4, and 5 years, there is a plateau of overall survival with ipilimumab, often described as the tail on the curve, raising the possibility that some patients may be cured of melanoma. In a long-term follow-up of 177 patients treated with ipilimumab on four different trials at the NCI over the past 8 years, 17 patients or 9 % sustained a complete response with ipilimumab or with additional surgery [118]. All of those patients except one were alive without progression at a median of 5 years after treatment, again suggesting that they may be cured of melanoma. Ipilimumab has been shown to have activity in patients with brain metastases, with a 30 % DCR in the central nervous system (CNS) in one study [88]. Unusual kinetics of response have been seen with ipilimumab, with slow regression, long times to complete response, prolonged durations of stable disease, and even progression followed by regression in patients [162]. Another unique aspect of ipilimumab treatment is the so-called abscopal effect in which tumor destruction induced locally in a progressing patient on ipilimumab leads to subsequent tumor shrinkage at distant sites [116]. Patients who fail one immunotherapy, such as high-dose IL-2, can subsequently respond to treatment with ipilimumab, suggesting that there may be different factors that are associated with the likelihood of responding to different immune modulators [65]. Ipilimumab has also been tested, and has been shown to have preliminary evidence of activity, in combination with other agents in small cell and non-small cell lung cancer, and in hormone-resistant prostate cancer [85], [86], [122].

The important questions of whether ipilimumab affected Treg numbers or function, or what other correlates of response with ipilimumab exist, have been addressed in a number of clinical studies. In one study, the phenotype of Tregs was assessed before and after ipilimumab with vaccine at doses ranging from 1 to 3 mg/kg. No differences in phenotype or numbers of CD4/CD25/FOXP3 positive cells were observed after ipilimumab [87]. In a second study of Treg numbers and function in patients with resected stage IIIC and IV melanoma who received ipilimumab at 10 mg/kg, no differences in phenotype, numbers or function of Tregs were observed 24 weeks after initiation of ipilimumab [131]. In contrast, murine studies have demonstrated that not only does anti-CTLA-4 antibody impact on Treg numbers and function in

the peripheral blood but the ratio between T effector cells and Tregs infiltrating the tumor may also be associated with a favorable outcome [26]. Ipilimumab has been associated with increased numbers of activated HLA DR+ T cells, an increased ratio of activated inducible costimulator (ICOS)+/CD4+ T cells to Tregs as well as levels of NY-ESO antibodies, although this has been controversial [43], [69], [80], [171], [172]. Administration of ipilimumab has also been found to be associated with an influx of CD8 effector cells infiltrating tumors in a neo-adjuvant study in patients with stage III melanoma who subsequently had their tumors resected. In a small study of nine patients with melanoma or prostate cancer who developed colitis after ipilimumab therapy, an immunohistochemical analysis of colon biopsies was performd to determine if the colitis correlated with depletion of intramucosal FOXP3(+)Tregs, which normally express CTLA-4 [84]. No evidence of FOXP3(+) T-cell depletion was found in any of the nine patients who developed colitis, suggesting that numbers of Tregs in the gut were not impacted by ipilimumab therapy. Three patients with colon cancer, four with non-Hodgkin's lymphoma, and four patients with prostate cancer who had received a cell vaccine were treated with ipilimumab. Tregs as detected by expression of CD4+CD25+FOXP3+ CD62L+ cells declined within a week after ipilimumab but rebounded to levels at or above baseline values at the time of the next infusion 3 weeks later, suggesting that any alterations in numbers of Tregs induced by ipilimumab were transient. In melanoma patients treated with ipilimumab after a cell vaccine strategy, the extent of treatment-induced tumor necrosis was linearly related to the natural logarithm of the ratio of intratumoral CD8(+) effector T cells to FOXP3(+)Tregs in posttreatment biopsies [54]. Taken together, these findings support the idea that while ipilimumab may alter the relationship of effectors to Tregs in the tumor microenvironment, the available data do not support the idea that it functions by Treg depletion or functional inhibition within the peripheral T-cell compartment or the tumor microenvironment. The data on induction of NY-ESO-specific T cells or antibodies and their association with response are intriguing, but there are contradictory data derived from different groups, and those ideas need to be validated using specimens from large multicenter studies.

Tremelimumab is another human antibody directed against CTLA-4. In its initial phase I studies, four patients of 29 treated with escalating doses had responses with durations longer than 24 months, with an additional five patients without disease progression at 24 months [123]. Based on these promising initial data, phase II second-line and phase III front-line trials of the drug as a single agent were performed. Tremelimumab was not shown to significantly prolong survival or result in a high response rate when administered at a dose of 15 mg/kg every 90 days in metastatic melanoma [68], [125]. Nonetheless, a series of correlative studies were conducted with this drug in order to determine what factors in the tumor microenvironment, and within the peripheral blood compartment, might be associated with its clinical benefit. In an initial study of 12 patients whose peripheral blood was characterized, there was no significant change observed in the proportion of antigen-specific T cells by tetramer assays. Additionally, there was no post-dose alteration in other antigen-specific CD8+ cell populations, FoxP3 transcripts, changes in surface expression of T-cell activation or memory markers, or phenotype of Tregs [25]. In contrast, in another small study of 10 patients, tremelimumab rapidly restored

the effector and memory CD4+ and CD8+ T-cell pool and TCR-dependent T-cell proliferation that became entirely resistant to Treg-mediated suppression. PFS and overall survival were associated with the resistance of peripheral blood lymphocytes to Treg-mediated inhibitory effects, but not to the number of Tregs [91]. In a study of the tumor microenvironment on patients receiving tremelimumab, regressing lesions had diffuse intratumoral infiltrates of CD8(+) T cells that were markedly increased compared to a baseline biopsy. Non-regressing lesions had sparse, patchy CD8(+) intratumoral infiltrates. Patients with regressing lesions had an increased frequency of CD8(+) T cells with or without a concomitant increase in CD4(+) T cells. Two of three responding patients showed a slight increase in the number of FoxP3(+) T cells in the posttreatment biopsies [124]. In a study of tremelimumab with exemestane in 26 patients with breast cancer, increased peripheral blood CD4+ and CD8+ T cells expressing ICOS were observed, and a marked increase was seen in the ratio of ICOS+ T cells to FoxP3+ Tregs, again suggesting that it was the relationship of effector cells to Tregs and not a specific impact on Tregs that was associated with CTLA-4 blockade [150]. This was confirmed by in vitro experiments suggesting that depletion of Tregs was required to observe a maximal augmentation of immune reactivity when tremelimumab was added to purified peripheral blood effector cells [138], and by experiments from patients treated with tremelimumab which demonstrated that both numbers and function of Tregs were maintained after tremelimumab treatment, similar to the situation with ipilimumab [67]. In another study of the tumor microenvironment, there was a significant increase in infiltrating CD8(+) T cells in biopsy samples taken after tremelimumab treatment. There was no difference between the absolute number, location, or cell density of infiltrating cells between clinical responders and patients with nonresponding lesions who showed acquired intratumoral infiltrates. There were no differences in indicators of cell replication (Ki67) or the Treg marker FOXP3, suggesting that numbers of tumor-infiltrating Tregs were not impacted by tremelimumab [57]. Finally, in a recent study of circulating cell phenotypes in patients receiving both tremelimumab and INF-α, the treatment induced clinically significant antitumor responses by inhibiting CTLA-4 suppressive effects on T effectors as shown by augmented circulating activated T cells, and less so by affecting T-regulatory phenotypes [140]. Taken together, in vitro and in vivo data using both ipilimumab and tremelimumab, both human blocking antibodies to CTLA-4, make it appear unlikely that its activity is due to abrogation of Treg numbers or function, and that it may primarily affect the relative balance in the tumor microenvironment between the activity of effector cells compared to regulatory cells.

4 PD-1/PD-L1 as a Target: Preclinical Studies

PD-1 is expressed on activated T and B cells and monocytes, and acts as an immune checkpoint regulatory protein. It regulates the balance between immune activation and T-cell tolerance [39], [61]. The induction and maintenance of T-cell tolerance

requires the presence of PD-1, and the expression of the PD-L1 ligand on non-hematopoietic cells can limit effector T-cell responses and protect against immune-mediated tissue damage. The PD-L1 and PD-L2 ligands, otherwise known as B7-H1 and B7-H2, are found on tumor cells, APCs, as well as placental tissue and non-hematopoetic cells that infiltrate tumors [52]. PD-1 is located on T and B cells, natural killer (NK)-T cells, activated monocytes, and DCs. It can also be expressed on Tregs, as can PD-L1 [8], [107], [139], [152].CD80 or B7-1 can bind PD-L1, and an inhibitory bidirectional interaction may occur between PD-L1 and B7-1, revealing that the B7:CD28 family can regulate T-cell interactions and tolerance [15].

PD-1 is a transmembrane protein that is encoded by the *Pdcd1* gene located on chromosome 2 in humans [66]. PD-1 functions by binding several phosphatases that can inhibit TCR signaling through dephosphorylation of TCR signaling intermediates. The src homology-2 (SH2)-domain containing tyrosine phosphatases SHP-1 and SHP-2 bind to PD-1 [135]. The inhibitory function of PD-1 for those phosphatases is lost when a tyrosine-based inhibitory motif is mutated, suggesting that it mediates functional PD-1 induced T-cell inhibition [104], [135]. PD-1 ligation inhibits phophoinositol-3 kinase activity and downstream activation of Akt and several known components of the TCR complex [21]. Soluble PD-1 inhibited DC function and increased IL-10 production without affecting DC IDO. These effects were reversed by a blocking anti-PD-1 antibody, suggesting that generation of a suppressive DC phenotype is associated with PD-1 and PD-L1 and/or PD-L2 interaction [108].

Older mice that are genetically deficient at the PD-1 locus can develop autoimmune glomerulonephritis and other immune conditions [74]. *Pdcd1−/−* mice generated in Balb/c mice develop a dilated cardiomyopathy associated with an autoantibody against cardiac troponin [100], [101]. This strain-specific autoimmunity occurs sooner in development when PD-1 deficiency is bred into mice with a genetic background rendering them susceptible to autoimmune diseases. These data all taken together support a role for PD-1 in the induction and/or maintenance of tolerance and natural suppression of autoimmunity.

PD-L1, the principal ligand of PD-1, is found on murine and human T and B cells, DCs, macrophages, mesenchymal stem cells, and bone marrow-derived mast cells. PD-L1 is highly expressed on tumors and on tumor cell lines in mice and humans, which may promote tumor-induced immune suppression by generating an inhibitory PD-1 signal. PD-L1 expression has been found by immunohistochemical analysis on a wide variety of solid tumors of epithelial, mesenchymal, and neuro-ectodermal origin including breast, lung, colon, ovarian, melanoma, bladder, liver, salivary, stomach, gliomas, thyroid, thymic epithelial, head, and neck cancer [30], [49], [60], [71], [98], [102], [105], [136], [143].

Expression of PD-L1 in tumors is augmented in the setting of genetic loss of phosphatase and tensin homolog (PTEN) which leads to activation of Akt, a common event in melanoma [166]. Expression of PD-L1 on tumors was found to be directly correlated with numbers of TILs and with poor clinical outcome for kidney, ovarian, bladder, breast, gastric, and pancreatic cancer but not small cell lung cancer [25], [43], [54], [68], [69],[84], [123], [125], [171]. Blockade of the PD-1–PD-L1 interaction might directly diminish tumor-induced immune suppression and reverse

the inhibition observed in "exhausted" PD-1-expressing T cells. Treatment with an anti-PD-L1 antibody or the injection of tumor cells into *Pdcd1−/−* mice resulted in increased antitumor responses [53], [62], [109]. Treatment with anti-PD-L1 antibody in vivo delayed the growth of tumors induced by PD-L1-expressing mouse myeloma cell lines [53]. PD-L1 expression on the immunogenic tumor P815 was associated with resistance to immunotherapy, but anti-PD-L1 blockade using a monoclonal antibody (mAb) restored the immune response to anti-CD137 therapeutic mAb [109]. Treatment with anti-PD-L1 antibody can augment the therapeutic effect of adoptive T-cell immunotherapy, and administration of anti-PD-L1 with activated T cells augmented rejection of a PD-L1-expressing squamous cell carcinoma [63].

PD-1 has been found to be highly expressed on lymphocytes that infiltrate human tumors. It can be found on circulating endogenous or vaccine induced melanoma antigen-specific T cells, and is also overexpressed on TILs from melanomas [36], [89], [137], [164], suggesting that those T cells exhibit an "exhausted" phenotype. Blockade of PD-1 with a mAb increased the proliferation of melanoma antigen-specific CTL, augmented their resistance to inhibition by Tregs and decreased the inhibitory function of Tregs. PD-1 blockade also reversed the increased levels of PD-1 and PD-L1 induced on melanoma antigen-specific CTL by Tregs and reversed the inhibition of cytokine expression by tumor antigen-specific CTL which was mediated by by Tregs. PD-1 blockade also resulted in decreased intracellular FoxP3 expression by Tregs [2].

5 PD-1/PD-L1 as a Target: Clinical Studies

Preclinical data obtained with human specimens suggested that PD-L1 tumor expression was a negative prognostic factor for clinical outcome [156], [160], [173], but that PD-L1 expression was also associated with IFN-γ-expressing T cells infiltrating tumors [51]. These data, along with the compelling preclinical data cited above, strongly supported the testing of anti-PD-1 and anti-PD-L1 antibodies in the clinic. A total of three PD-1 antibodies, two PD-L1 antibodies, and a PD-1 fusion protein are currently being tested in the clinic in cancer patients. The nivolumab and Merck MK-3475 anti-PD-1 molecules and the BMS 936559 anti-PD-L1 antibody have been tested extensively in several hundred patients each with metastatic cancer. Nivolumab is a human IgG4 antibody that was evaluated in a phase I single-adminstration dose escalation study in 39 patients with various solid tumors, including melanoma, renal cell cancer, lung cancer, and prostate and colorectal cancer [141]. Several responses in melanoma, colorectal cancer, and renal cell cancer were observed with single dosing from 0.3 to 10 mg/kg that was associated with modest toxicity and a serum half-life of 20 days. The spectrum of toxicities of all three antibodies was somewhat reminiscent of the irAEs observed with ipilimumab, but the overall incidence of grade 3 side effects was less than that seen with ipilimumab, but hyper- or hypothyroidism, as well as pneumonitis were observed more frequently. Multi-dosing of nivolumab every 2 weeks resulted in a response rate of 28 % as second-line therapy

in ipilimumab-naïve patients with metastatic melanoma, and 20 % in patients with non-small cell lung cancer, at doses ranging from 0.1 to 10 mg/kg [145]. Responses were durable, with many responses at 24 months without progression. The kinetics of response with this antibody were similar to those seen with ipilimumab, and progression followed by subsequent regression after rechallenge with nivolumab has been observed. When this antibody was combined with a peptide vaccine as second-line therapy for metastatic melanoma, a 25 % response rate was seen. Progression of disease in that trial was associated with elevation in numbers of CD4/CD25/FOXPp3 positive CD127 negative Tregs, and with increase in CTLA-4 on those cells, and on both CD4 and CD8 T cells overall [11]. These data suggested that after treatment with PD-1 antibody, ipilimumab might be an effective treatment which impacts on Tregs in patients that progressed after anti-PD-1. That idea is being tested in two trials, one of simultaneous PD-1 antibody and ipilimumab and the other of reciprocal sequential treatment with both antibodies.

The chimeric anti-PD-1 antibody CT-011 has been tested primarily in hematologic malignancies, with occasional responses in acute myeloid leukemia (AML), indolent lymphomas, and chronic lymphocytic leukemia [73]. It has been evaluated in melanoma, and clinical reports are pending. The MK-3475 anti-PD-1 antibody from Merck has been tested in melanoma at doses from 1 to 10 mg/kg [9]. It is a high-affinity IgG4 humanized antibody, with a half-life of 13.6 days. In its initial phase I dose escalation testing, responses were seen in melanoma patients at all doses. Only grade 1 and 2 toxicities were initially observed. In expansion cohorts in which 132 ipilimumab-experienced and ipilimumab-naïve melanoma patients were treated, the rate of grades 3–4 immune-related side effects was 5 %. The response rates by immune-related response criteria (irRC) were 55 % in 57 ipilimumab naïve melanoma patients, and 41 % in 27 melanoma patients who were ipilimumab experienced. These encouraging data have supported a randomized phase II study of MK-3475 at either 10 or 2 mg/kg compared to chemotherapy with PFS as the primary endpoint with a crossover design. BMS 936559, a fully human IgG_4 antibody against PD-L1, one of the ligands of the PD-1 receptor, has been tested in patients with various solid tumors [110]. A total of 207 patients received anti-PD-L1 antibody including 75 with non–small cell lung cancer, 55 with melanoma, 18 with colorectal cancer, 17 with renal cell cancer, 17 with ovarian cancer, 14 with pancreatic cancer, 7 with gastric cancer, and 4 with breast cancer. The median duration of therapy was 12 weeks or one and a half treatment cycles (range, 2–111 weeks). Treatment-related grade 3 or 4 toxic effects occurred in 9 % of patients. A complete or partial response was observed in 9 of 52 patients with melanoma, 2 of 17 with renal cell cancer, 5 of 49 with non-small cell lung cancer, and 1 of 17 with ovarian cancer. Responses lasted for 1 year or more in 8 of 16 patients with at least 1 year of follow-up. This human antibody was tested at doses of 0.1, 1, 3, and 10 mg/kg in patients with melanoma, with an overall response rate of 17 %. Interestingly, both anti-PD-1 and anti-PD-L1 antibodies have demonstrated the same slow kinetics and sensitivity to retreatment after progression seen with ipilimumab [13].

Adoption of Lymphoid-Depletion Protocols Prior to TIL Transfer The realization that homeostatic lymphoid proliferation that occurred after lymphoid depletion

in rodent models resulted in increased immune "space" that favored the outgrowth and proliferation of adoptively transferred effector cells with increased antitumor activity led to the adoption of a lymphoid depletion protocol for preparation prior to TIL transfer [40], [82], [153]. Animal model data suggested that lymphoid depletion allowed for a diminution or Tregs and lowered "sink" for cytokines like IL-7 and IL-15 and that greater lymphoid depletion was more effective than less [119], [170]. An initial small pilot trial tested the idea that lymphoid depletion with chemotherapy prior to adoptive cell transfer yielded a significant proportion of long-term responders [95]. Following that trial, 43 patients with stage IV melanoma who received NMA chemotherapy prior to TIL and one cycle of high-dose IL-2 were found to have an overall response rate of 49 % [32]. Five of those patients (12 %) had a complete response and were alive and progression free at times ranging from 6.9 to 8.6 years. The 16 partial responders all had progressive disease with 15 of the 16 progressing 2–36 months after treatment, and one who did not progress until 7 years after treatment. The patients in this NMA-only TIL trial showed no association between clinical response and sex, age, HLA type, metastatic stage, or numbers of TIL cells administered. Clinical responders did differ from nonresponders, tolerating fewer doses of post-infusion IL-2. This regimen in various forms has been repeated at several other institutions recently, including Moffitt Cancer Center in Tampa and MD Anderson Cancer Center in Houston. Investigators at MD Anderson have treated 38 patients with metastatic melanoma [33], differing from the NCI strategy in that they employed no selection steps prior to the rapid expansion (REP) phase. Patients were administered two cycles of high-dose IL-2, one immediately after TIL infusion and a second one 21 days later. The MD Anderson investigators achieved an overall response rate of 47 % with a complete response rate of 5 %, with the longest follow-up being 36 months. At Moffitt Cancer Center, 13 patients were treated with TIL selected for antigen reactivity similar to the NCI protocols, to which were added the fastest growing cultures expanded in 24 well plates in spite of selection [120]. In that trial, 31 % of patients have had a partial response, and 15 % have had a complete response; these results are similar to the NCI data of 37 % partial and 12 % complete responses. The longest duration of follow-up of the Moffitt patients is 15 months. In those patients, clear elimination of Tregs at the time of lymphoid depletion with chemotherapy was documented, albeit with a relatively rapid recovery of CD4+CD25highFOXP3+CD127low Tregs to baseline or above after 2 weeks. While no functional studies have been done, it appears that NMA mediates a transient Treg depletion only. Further trials using NMA chemotherapy and total body irradiation (TBI) show even better response rates, with a higher proportion of long-term survivors in complete remission [34], [113].

Lymphoid Depletion Prior to TIL Transfer to Deplete Tregs Work with murine models of adoptive cell therapy detailed above suggested that the addition of radiation and other means of inducing lymphodepletion augmented the therapeutic effect of adoptively transferred T cells [12], [126]. To evaluate this phenomenon in patients, TBI was added to NMA chemotherapy with 2 days of cytoxan and 5 days of fludarabine. When 2 or 12 Gy of TBI was added to TIL plus IL-2, the response rates

were 52 % and 72 % respectively, compared to 49 % with NMA alone. Responses were seen in all sites of tumor including brain. There was one treatment-related death in the 93 patients described in one report from the NCI. Host lymphodepletion was associated with increased serum levels of the lymphocyte homeostatic cytokines IL-7 and IL-15. When Tregs were assessed in the periphery of patients undergoing lymphoid depletion, their numbers dropped precipitously after lymphodepeletion but returned toward baseline after initiation of high-dose IL-2, suggesting that the use of lymphodepleting chemotherapy provided a transient diminution of Tregs, but which may have been enough to promote the activity of adoptively transferred T cells to achieve regression of disease.

6 T Regulatory Cells: Targeting the IL-2 Receptor with ONTAK

ONTAK, or denileukin diftitox, a fusion protein of IL-2 and diphtheria toxin, has been evaluated for its ability to enhance antitumor immunity by the selective elimination of Tregs displaying the high-affinity IL-2 receptor (the α–β–γ trimer) [168]. The idea that ONTAK could deplete Tregs and thereby induce regression of cancer was tested in a phase II trial in melanoma. After ONTAK administration in 60 patients with metastatic disease, there was a partial response rate of 16.7 % noted with 5 % stable disease and 15 % mixed responses [142]. Many of the responses were assessed by positron emission tomography (PET) scan alone, which rendered the data difficult to interpret. The 1-year survival rate was 40 %, not appreciably different from that observed with the use of chemotherapy in melanoma. This agent indeed can transiently diminish numbers of circulating Tregs in primates and in humans, but it can also exert a potent cytotoxic effect on NK cells, a phenomenon that paradoxically could impair its antitumor properties [5]. Coadministration of IL-15 with ONTAK alleviated this problem by selectively protecting potentially NK cells, while allowing the depletion of immunosuppressive Tregs in cancer patients. A sensitive FOXP3 methylation assay was used to assess peripheral blood Treg depletion with ONTAK and other strategies. In nine control patients, blood T regulatory frequencies varied over time; there was a 46 % reduction in one patient [6]. In treated patients, a more than twofold decrease in Tregs was observed in only one out of 11 patients receiving cyclophosphamide and in four out of 13 receiving daclizumab, but there was no decrease in Tregs in any of the six patients who received denileukin diftitox. As a positive control, a more than twofold increase in peripheral blood Tregs was detected in four out of nine patients who were treated with IL-2. Thus, none of the Treg-depleting strategies that were tested led, in the majority of patients, to a conservative 50 % reduction in blood Tregs. In contrast, when tested with a number of cancer vaccine strategies, ONTAK was found to alter T cell-mediated immune responses [83]. Tregs in spleen, peripheral blood, and bone marrow of normal C57BL/6 mice were significantly reduced after a single intraperitoneal injection of denileukin diftitox; the reduction was evident within 24 h and lasted for approximately 10 days [28]. Injection of denileukin diftitox 1 day before vaccination enhanced antigen-specific

T-cell responses above the levels induced by vaccination alone. In one pilot study of 15 patients with gastrointestinal cancer, ONTAK given as a single dose or in repeated doses was followed by immunization with DCs modified with a fowlpox vector encoding the carcinoembryonic antigen, called rF-CEA(6D)-TRICOM [83]. Circulating CD4(+)CD25(high)FOXP3(+) Tregs detected by flow cytometry were depleted by a factor of two to three after multiple doses of denileukin diftitox. Earlier induction of, and overall greater exposure to, an anticarcinoembryonic antigen (CEA) T-cell immune response was observed in the multiple-dosing group, but not in the single-dose ONTAK group. These data suggested that combining Treg depletion using ONTAK with a vaccine to enhance tumor antigen-specific immune responses might increase antitumor immune responses, but the clinical manifestations of this approach were unclear. ONTAK-mediated T regulatory depletion resulted in enhanced stimulation of proliferative and cytotoxic T-cell responses in vitro but only when it was omitted early during T-cell priming. The drug also had no efficacy in previously treated non-small cell lung cancer patients [94]. ONTAK significantly reduced the number of Tregs present in the peripheral blood of metastatic renal cell carcinoma patients and blocked Treg-mediated immunosuppression in vivo. ONTAK -mediated elimination of Tregs followed by vaccination with an RNA-transfected DC strategy significantly increased tumor-specific T-cell responses in renal cell cancer patients when compared with a DC vaccination alone [42]. In contrast, a study in melanoma that used a different schedule of ONTAK failed to show any evidence of Treg depletion [5]. Thirteen patients (12 with metastatic melanoma and one patient with metastatic renal cell cancer) were treated with one of the two FDA-approved doses of ONTAK (seven patients at a dose of 9 μg/kg and six patients at 18 μg/kg). No patient was shown to have an objective clinical response. FOXP3 expression did not decrease significantly by flow cytometry in the overall group although it did decrease modestly among patients receiving the 18 μg/kg dose with $p = 0.031$. ONTAK did not alter the suppressive activity of CD4/CD25/FOXP3 positive cells measured using an in vitro coculture assay that included an allogeneic mixed lymphocyte reaction. Increased or rebounding numbers of lymphocytes in patients that usually occurred after treatment with IL-2 did not occur when ONTAK was added to IL-2. ONTAK has also been used to treat patients with diffuse B-cell lymphoma that did not express the low-affinity IL-2Rα. Of 25 patients treated with a combination of rituximab and ONTAK, 13 patients (57 %) experienced grade ≥ 3 adverse events and one patient (4 %) died [29]. In correlative studies, soluble CD25 and the number of CD25+ T cells decreased after treatment; however, there was a compensatory increase in levels of circulating IL-15 and interferon gamma-induced protein 10 (IP-10). Thus, although the addition of denileukin diftitox to rituximab decreased the number of CD25+ T cells in that trial, denileukin diftitox contributed to the toxicity of the combination without an improvement in response rate or time to progression. Where does that leave us with the idea that ONTAK can be used to effectively deplete Tregs in cancer patients? The answer is that it can indeed reduce the numbers of Tregs in cancer, but the effect of ONTAK is transient and unlikely to have a major impact on immunity. Concerns remain about the effects of the drug on priming of an immune response and on effector T-cell activity. Measurement of antigen-specific immunity impacted by ONTAK did not correlate with clinical benefit from the drug.

7 T Regulatory Cells: Targeting the IL-2 Receptor with Daclizumab

An alternative approach to depletion of CD25-expressing Tregs with an IL-2-toxin fusion molecule is the use of an antibody directed specifically against the CD25 IL-2 low-affinity receptor α. Daclizumab is a chimeric anti-CD25 humanized antibody that can deplete CD25-expressing Tregs [4]. Metastatic melanoma patients that were HLA A*0201 positive were vaccinated with cytokine-matured DCs that were pulsed with a mixture of class I restricted tumor peptides and the T-cell helper protein keyhole limpet hemocyanin (KLH). Half of the patients received daclizumab, given either 4 or 8 days before DC vaccinations [79]. It was noted that daclizumab-mediated inhibition of the low-affinity IL-2Rα-expressing cells during vaccine-based immunotherapy in mice resulted in depletion of Tregs (73 % reduction; $p = 0.0154$) but also unfortunately abolished vaccine-induced immune responses [64]. However, during lymphodepletion induced with chemotherapy, IL-2Rα blockade with daclizumab decreased Tregs (93 % reduction; $p = 0.0001$) without impairing effector T-cell responses, resulting in significantly augmented antitumor efficacy. In vitro, daclizumab does not mediate antibody-dependent or complement-mediated cytotoxicity but can downregulate FOXP3 expression selectively within the CD25(high) CD45RA(neg) T regulatory population. Daclizumab-treated CD45RA negative Tregs lost their ability to suppress T-cell activity and regained their ability to produce INF-γ, which is consistent with those cells becoming reprogrammed to be effector cells. Daclizumab administered during a different DC vaccination strategy prevented the induction of specific antibodies in vivo but not the presence of antigen-specific T cells. Daclizumab, however, did prevent CD25(+) T cells from acquiring effector function. Fewer patients pretreated with daclizumab developed functional, vaccine-specific effector T cells and antibodies compared with controls. Daclizumab pretreatment had no significant effect on PFS compared with the control group in that study, again emphasizing that targeting the CD25 low-affinity IL-2Rα to deplete Tregs was practical, but transient, and while associated with detectable augmentation in antigen specific T-cell responses, was not clearly associated with clinical benefit. A study tested the ability of daclizumab to safely and specifically deplete Tregs in patients with glioblastoma (GBM) who were treated with temozolomide (TMZ) at doses that became lymphodepleting [93]. Daclizumab was given at the time of epidermal growth factor receptor variant III (EGFRvIII)-targeted peptide vaccination to amplify the anti-EGFRvIII immune response. Daclizumab was well tolerated, with no symptoms of autoimmune side effects and resulted in a significant reduction in the frequency of circulating CD4+Foxp3+ Tregs detected by flow cytometry compared to a control population. There was a significant ($p < 0.0001$) negative correlation between the frequency of Tregs as a proportion of total T cells and the level of EGFRvIII-specific humoral responses which suggested that the depletion of Tregs was associated with an increased vaccine-induced humoral immune response. In order to test its impact on Tregs in vivo, daclizumab was combined with a glycoside cancer vaccine in previously treated patients with metastatic breast cancer [130].

Daclizumab administration led to a significant and prolonged decrease in numbers of Tregs. High levels of CD8 and CD4 T-cell priming and boosting to a number of vaccine antigens were observed in the absence of autoimmune side effects. Therefore, daclizumab-induced CD25 blockade depleted and selectively "reprogrammed" patient Tregs when administered with a vaccine therapy in metastatic breast cancer.

Daclizumab administration during recovery from a lymphodepleting regimen of TMZ in patients with GBM multiforme reduced the frequency of Tregs (48 % reduction; $p = 0.0061$) while permitting vaccine-stimulated antitumor effector cell expansion to occur [93]. Daclizumab efficiently depleted all CD25 expressing cells, including CD4(+)FoxP3(+)CD25(high) Tregs from the peripheral blood within 4 days of administration. Thirty days after administration, daclizumab was no longer detectable in the circulation and all CD25(+) cells had reappeared, suggesting that the depletion of Tregs was transient. The challenge with daclizumab was to deplete the Tregs while sparing activated T effector cells.

8 Targeting Myeloid Derived Suppressor Cells (MDSCs) with Retinoids and Chemotherapy

Local retinoid production has been shown to increase within the tumor microenvironment up to fivefold compared with normal surrounding tissue, with a commensurate increase in retinoid signaling to tumor-infiltrating antigen-reactive T cells [121]. Conditional disruption of retinoid signaling in CD8(+) T cells using a dominant negative retinoic acid (RA) receptor α construct established that RA signaling was required for tumor-specific CD8(+) T-cell expansion and accumulation and for protective antitumor immunity [48]. An in vivo analysis of antigen-specific CD8(+) T-cell responses revealed that early T-cell expansion was retinoid-independent; however, later T-cell expansion and accumulation was diminished in the absence of retinoid signaling. These data suggested that retinoid function was essential for the survival of tumor-reactive CD8(+) T cells within the tumor microenvironment. The use of 13-*cis*-retinoic acid resulted in an increased percentage of peripheral blood lymphoid cells expressing surface markers for T-helper cells with only minimal effect on NK cell marker expression. In contrast, beta-carotene caused an increase in the proportion of cells expressing NK cell markers with lesser impact on T-helper cell phenotypic markers [115]. Modest increases in the percentage of cells expressing Ia antigen, transferrin, and IL-2 receptors were produced by both drugs. These data indicate that both retinoids and carotenoids can induce major changes in immune phenotypes in vivo in humans at doses relevant to their potential clinical use. Retinoids like ATRA, a stimulating agent for MDSC differentiation, did not induce them to convert to immunogenic APCs, but NKT ligand-loaded, ATRA-treated MDSCs could be converted into immunogenic APCs to augment T-cell-mediated immune responses and reverse MDSC-induced immune suppression. These effects were mediated by NKT cells secreting IFN-γ and ATRA-mediated increases in glutathione (GSH) levels. Combined treatment with differentiating and activating agents was required for the conversion of

MDSCs into immunogenic APCs that stimulated T cells [117]. Administration of the chemotherapy agent paclitaxel significantly decreased accumulation and immunosuppression mediated by tumor-infiltrating MDSCs without impacting on normal hematopoiesis [78]. This was accompanied by inhibition of p38 mitogen-activated protein kinase (MAPK) activity, tumor necrosis factor alpha (TNF-α) production, and S100A9 expression in MDSCs. The production of mediators of chronic inflammation that could be immunosuppressive in the tumor milieu also was decreased. Reduction in tumor burden and prolonged survival after administration of paclitaxel was associated with, and depended on the restoration of CD8 T-cell effector function, implicating adaptive immunity as playing a role in tumor regression mediated by cytotoxic drugs. The ability of paclitaxel to block the immunosuppressive activity of MDSCs in vivo and, thereby, augment T-cell activity was associated with a decrease in immunosuppression and chronic inflammation in the tumor microenvironment which might enhance subsequent immunotherapy or targeted therapy. Docetaxel, another taxane, significantly inhibited tumor growth in 4T1-Neu breast cancer-bearing mice and decreased the proportion of MDSC among splenocytes [76], [133]. This treatment also increased antigen-specific CTL responses. Docetaxel-pretreated MDSCs cocultured with OT-II clonal CD4 splenocytes in the presence of its antigen ovalbumin (OVA) (323–339) induced antigen-specific CD4 activation and expansion in vitro. In characterizing the phenotype of MDSCs for activating type 1 M1 (C-C chemokine receptor type 7, CCR7) and suppressive type 2 M2 (mannose receptor (CD206)) markers, MDSCs from untreated tumor-bearing mice were primarily of the CD206 (+) M2 type with few CCR7(+) M1-like cells. Docetaxel skewed the MDSCs toward an M1-like phenotype, resulting in 40 % of MDSCs expressing CCR7 in vivo and in vitro, and a number of activating macrophage differentiation markers such as major histocompatibility complex (MHC) class II, CD11c, and CD86 were upregulated. Docetaxel also induced cell death selectively in CD206 (+) M2-like MDSCs while sparing the M1-like MDSCs, amplifying its immune stimulating effects. Inhibition of the signal transducer and activator of transcription 3 (Stat3) nuclear regulatory factor was found to be, in part, responsible for the observed results.

Sunitinib, an FDA-approved pan-tyrosine kinase signal inhibitor with clinical activity in renal cell cancer also inhibited Stat3 in renal cell cancer-associated MDSC, downregulated expression of genes associated with angiogenesis, and caused a decrease in numbers of MDSCs and Tregs that infiltrated tumors [70], [106]. These results suggest that Stat3 activity was important for antitumor activity of sunitinib in renal cell cancer, and Stat3 inhibition promoted both a proapoptotic effect of sunitinib on tumor cells and had important effects on the tumor immunologic microenvironment. A significantly higher percentage and infiltration of CD8 and CD4 cells was found in tumors of sunitinib-treated mice compared with control-treated mice. The expression of regulatory checkpoint molecules CTLA-4 and PD-1 on both CD4 and CD8 T cells, and PD-L1 expression on MDSC and plasmacytoid DCs was also significantly decreased after sunitinib treatment. Treatment of tumor-bearing mice with methyl-2-cyano-3, 12-dioxo-oleana-1,9(11)-dien-28-oleic acid, methyl ester (CDDO) eliminated the suppressive activity of intratumoral MDSCs but did

not affect their overall numbers independent of its antitumor activity [167]. Methyl-CDDO treatment decreased tumor size in mice. Experiments with severe combined immunodeficient-beige mice indicated that this antitumor effect was largely mediated by T cells. Methyl-CDDO significantly enhanced the antitumor effect of a cancer vaccine. Treatment of pancreatic cancer patients with methyl-CDDO did not impact the number of MDSCs detected in the peripheral blood but significantly improved the immune response to vaccine. In contrast, gemcitabine (gemzar) and 5-fluorouracil (5-FU), activated the nonobese diabetic (NOD)-like receptor family, pyrin domain containing-3 protein (Nlrp3)-dependent caspase-1 activation complex (called the "inflammasome") in MDSCs, inducing production of IL-1β, which decreased antitumor immunity [97]. Chemotherapy-triggered IL-1β secretion relied on lysosomal permeabilization and the release of cathepsin B, which bound to Nlrp3 and induced caspase-1 activation. MDSC-derived IL-1β induced secretion of IL-17 by CD4 (+) T helper cells, which decreased the anticancer effects of the chemotherapy. Gemzar and 5FU exerted greater antitumor effects when tumors were established in Nlrp3(-/-) or Casp1(-/-) mice or wild-type mice treated with interleukin-1 receptor antagonist (IL-1Ra). These results suggest that activation of the Nlrp3 inflammasome in MDSCs by 5FU and gemzar paradoxically limited the antitumor effects of these chemotherapeutic agents and suggest that the use of an IL-1Ra might unmask the beneficial MDSC-depleting activity of gemzar.

9 Conclusions

A number of therapeutic strategies to overcome immune suppression in cancer patients have been developed that fall into three categories: the use of antibody-mediated blockade of T-cell regulatory checkpoint proteins like CTLA-4 and PD-1; direct abrogation of the function of Tregs using the CD25 IL-2 Rα as a target; and inhibition of the function of MDSCs as a means of indirectly impacting on T-cell activation and proliferation. Herein, we have reviewed the different strategies to promote immunity that has concentrated on melanoma, and conclude that while significant success has been seen with the first strategy of checkpoint blockade inhibition, direct and indirect abrogation of Treg function has had a more checkered track record. The inability to define a specific marker that identifies Tregs or MDSCs and allows for their targeting without compromising effector T cell and antigen-presenting function is a profound limitation of all attempts to functionally inhibit those important cells. In contrast, our knowledge of new regulatory molecules on effector T cells that limit their function has expanded in the past few years to encompass lymphocyte activation gene-3 (LAG-3), Tim-3, ICOS, B and T lymphocyte attenuator (BTLA), and others, all of which theroretically can be targeted with human antibodies that abrogate their inhibitory functions or promote activating functions. While there is little evidence even in murine models that checkpoint protein inhibition mediates clinical benefit by exclusively blocking those proteins on Tregs, it may be that it is the dynamic relationship between relieving checkpoint inhibition of effector cells and regulatory

cells that is most important. While the search for the holy grail of targeting Tregs and MDSCs with exquisite specificity continues, new antibodies with anticancer activity not just in melanoma but also in other solid tumors singly and in combination are likely to have great promise therapeutically for years to come.

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