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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

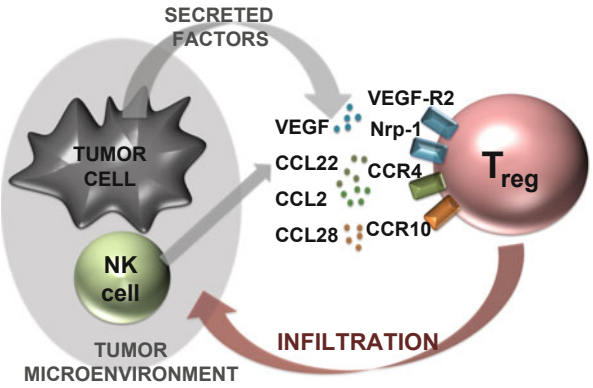

Fig. 1.1 Mechanisms of T_{reg} recruitment to the tumor microenvironment. Tumor cells secrete VEGF, which binds to T_{reg}-expressed VEGF-R2 and Nrp-1 and mediates recruitment into tumors. Tumor cells also secrete chemokines CCL22, CCL2, and CCL28, which recruit T_{reg} through the cognate receptors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

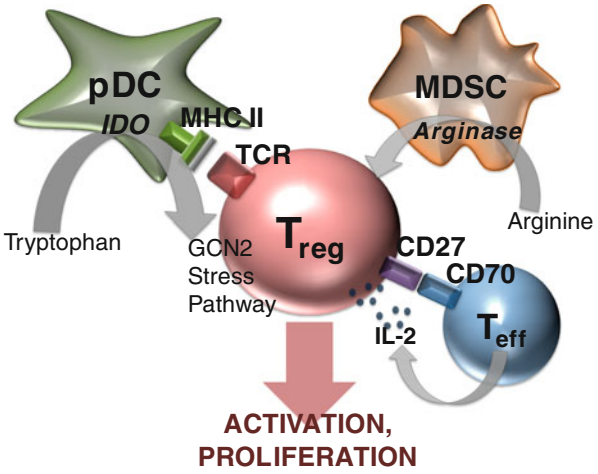

Fig. 1.2 Mechanisms of T_{reg} activation within tumors and draining lymph nodes. Foxp3$^+$ T_{reg} are activated by plasmacytoid dendritic cells (pDCs) expressing IDO in tumor-draining lymph nodes. This activation requires MHC expression by antigen presenting cells, and activation of the GCN2 stress 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,

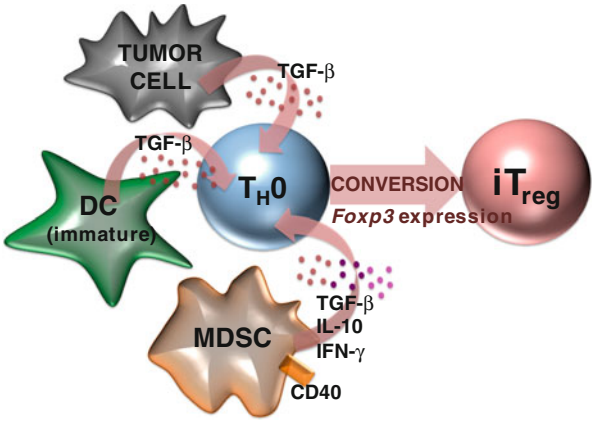

Fig. 1.3 Mechanisms of iT_{reg} conversion by tumor cells and associated antigen presenting cells. Tumor cells and immature DCs produce TGF-β, which results in the conversion of CD4 T cells to Foxp3-expressing iT_{reg} within tumors. MDSCs can also produce TGF-β, and have been shown to drive iT_{reg}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

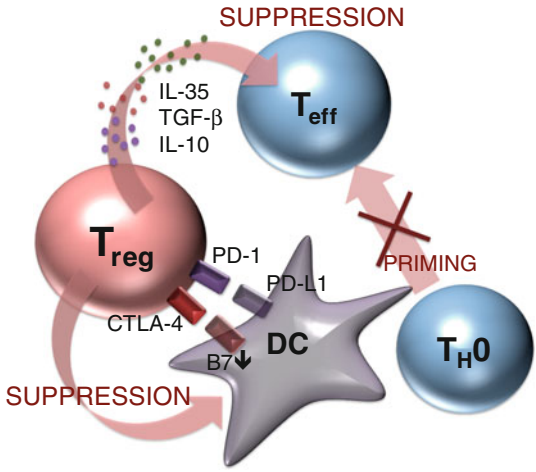

Fig. 1.4 Mechanisms of T_{reg} suppression of antitumor effector T cells. T_{reg} production of IL-35, TGF-β, IL-10, has been implicated in the direct suppression of effector T-cell responses. T_{reg} also impair DC function through CTLA-4 and PD-1. CTLA-4 expressed by T_{reg} has been shown to downregulate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