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REVIEW ARTICLE

Targeting regulatory T cells in tumors

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Regulatory T (T_{reg}) cells play a crucial role in maintaining peripheral tolerance and preventing autoimmunity. However, they also represent a major barrier to effective antitumor immunity and immunotherapy. Consequently, there has been considerable interest in developing approaches that can selectively or preferentially target T_{reg} cells in tumors, while not impacting their capacity to maintain peripheral immune homeostasis. In this review, we describe our current understanding of the mechanisms underlying the recruitment, expansion, and suppressive activity of tumor-associated T_{reg} cells, and discuss the approaches used and the challenges encountered in the immunotherapeutic targeting of T_{reg} cells. In addition, we summarize the primary clinical targets and some emerging data on exciting new potential T_{reg} cell-restricted targets. We propose that discovering and understanding mechanisms that are preferentially used by T_{reg} cells within the tumor microenvironment will lead to strategies that selectively target T_{reg} cell-mediated suppression of antitumor immunity while maintaining peripheral immune tolerance.

Introduction

The past two decades have witnessed a paradigm shift in tumor biology, from the reductionist dogma that tumors are masses of malignant cells that acquire certain cell-autonomous properties, to the evolving view that tumors are aberrant organs in which transformed cells along with other recruited normal cell types conspire to foster tumor growth and metastasis [1]. This has led to the concept of a tumor microenvironment composed of cells from the immune system, the tumor vasculature and lymphatics, as well as fibroblasts, pericytes, and the occasional adipocyte [2,3]. The immune compartment of the tumor microenvironment, sometimes referred to as the 'immune contexture', has attracted extensive interest due to its importance both in the understanding of basic tumor biology and the implications for clinical applications [4]. The cellular composition of the tumor microenvironment is highly heterogeneous and includes almost all immune cell types, including $CD8^+$ T cells, $CD4^+$ T cells, regulatory T (T_{reg}) cells, macrophages, dendritic cells (DCs), natural killer (NK) cells, B cells, mast cells, and other cell types, with the relative percentages and phenotypes

Abbreviations
A2AR, adenosine receptor 2A; ADCC, antibody-dependent cell-mediated cytotoxicity; AML, acute myeloid leukemia; APCs, antigen-presenting cells; CTX, cyclophosphamide; DCs, dendritic cells; Foxp3, forkhead box protein 3; GITR, glucocorticoid-induced TNF receptor family-related protein; IDO, indoleamine 2,3-dioxygenase; IPEX, immune dysregulation, polyendorinopathy, enteropathy, X-linked; IS, immunoscore; mAbs, monoclonal antibodies; MDSCs, myeloid-derived suppressor cells; NK, natural killer; Nrp1, neuropilin 1; pDCs, plasmacytoid DCs; pT_{reg}, peripherally derived T_{reg}; RANKL, receptor activator of nuclear receptor kappa-B ligand; TAAs, tumor-associated antigens; TAMs, tumor-associated macrophages; T_{eff}, T effector; TNF, tumor necrosis factor; T_{reg}, regulatory T cell; tT_{reg}, thymus-derived $CD4^+Foxp3^+T_{reg}$; VEGF, vascular endothelial growth factor.

varying considerably between tumor types and even between patients with the same tumor type. This complexity provides a 'fingerprint' for the tumor microenvironment and can be highly correlative with clinical outcome. This has led to the development of Immunoscore (IS), an approach used to quantify $CD45RO^+$ memory cells and cytotoxic memory ($CD8^+$) T cells in the core and invasive margin of tumors as a possible prognostic indicator [5]. The IS assesses the immune infiltrate, and has been shown to provide high prognostic value for certain cancer types. For example, when compared to other clinical criterion, IS was shown to be a better predictor of disease recurrence in colorectal cancer patients following surgery [5]. From a functional perspective, one of the key features of the tumor immune contexture is its immunosuppressive environment, which underlies the basis for tumor escape from host immune destruction. While both tumor-intrinsic and -extrinsic mechanisms have been explored, it is generally accepted that T_{reg} cells, a small subpopulation of $CD4^+$ T cells endowed with potent suppressive capacity, play a pivotal role in inducing tumor-specific immune tolerance and will be the focus in this review.

T_{reg} cells, characterized by the expression of the transcription factor Forkhead box protein 3 (FoxP3) [6,7], maintain immune homeostasis, and prevent autoimmune and chronic inflammatory diseases [8]. Mutations in the *FOXP3* locus results in an early onset, fatal autoimmune, lymphoproliferative disease known as IPEX (Immune dysregulation, polyendorinopathy, enteropathy, X-linked) syndrome [9]. An analogous mutation in the murine *Foxp3* locus identified in the Scurfy mouse phenocopies the human disease [9]. There are two primary T_{reg} populations defined by the anatomical location of their development: thymus-derived $CD4^+Foxp3^+$ T_{reg} (tT_{reg}) cells and peripherally derived T_{reg} (pT_{regs}) cells. The different anatomical origins impact the functional specificity of these two T_{reg}-cell subsets, with tT_{reg} cells responsible for the maintenance of peripheral tolerance thereby limiting autoimmunity and tissue pathology [6,10], whereas pT_{reg} cells are thought to limit local immune pathologies at environmental boundaries (e.g. mucosal or fetus-maternal interfaces [11,12]). While the presence of pT_{reg} cells in the tumor microenvironment and their functional impact is an important topic (as reviewed in [13,14]), for this review we will not distinguish between pT_{reg} and tT_{reg} cells and instead will discuss the impact of T_{reg} cells on tumors in general.

T_{reg} cells have been shown to infiltrate human tumors and are thought to limit antitumor immunity. Indeed, initial observations from human ovarian cancer patients revealed that elevated frequencies of T_{reg} cells at the tumor site correlated with a poor clinical outcome [15]. However, later data gathered from a broader range of cancer types led to divergent correlations between intratumoral T_{reg}-cell number and disease outcome. Such discrepancies may be the result of three possibilities. First, an inability to clearly distinguish between T_{reg}-cells and activated T cells, which can express Foxp3. Second, T_{reg} cells may either promote tumor development by limiting antitumor immunity, or limit tumor development by limiting the stromal environment required for its growth and metastases [16]. Third, T_{reg} cells found in tumors may be heterogeneous in terms of their functional state and/or stability, which may in turn affect whether they have a positive or negative effect on tumor progression. Although the above issues warrant further study, there is a general agreement that T_{reg} cells impact the tumor microenvironment and that targeting them therapeutically could be beneficial. However, substantial depletion of T_{reg}-cell numbers and/or function could lead to deleterious autoimmune and inflammatory consequences. Thus, a key goal is to identify mechanisms or pathways that are selectively used and required for T_{reg} function in the tumor microenvironment but dispensable for peripheral immune control [17].

In this review, we will focus on the tumor-associated T_{reg} cells from the perspective of the mechanisms underlying their enrichment in tumors and promotion of their function, stability, and survival and how they are specifically or nonspecifically targeted by current cancer therapeutics. We will also discuss current therapeutic approaches that may impact T_{reg} function, and the challenges of developing novel targeting strategies that selectively target intratumoral T_{reg} cells while not impacting their maintenance of peripheral immune tolerance.

Phenotype of tumor-associated T_{reg} cells

Compared with conventional T helper cells, T_{reg} cells exhibit a distinct transcription signature that underlies their immune suppressive activity, yet they are known to be heterogeneous both in phenotype and function (as reviewed in [18]). It has been suggested that human T_{reg} cells, in particular, have a higher degree of heterogeneity although this may be due in part to the difficulty in distinguishing them from activated conventional T cells that transiently upregulate Foxp3 and CD25, emphasizing the need for additional markers or approaches to identify human T_{reg} cells. For

example, $CD4^+Foxp3^+$ cells with a low level of CD127 (IL-7 receptor α-chain) expression were defined as a highly purified T_{reg}-cell population with strong suppressive activity [19]. In another study, human $Foxp3^+CD4^+$ T cells were divided into three subfractions based upon the expression level of Foxp3 and the cell surface molecules CD25 (IL-2 receptor α-chain) and CD45RA. The $Foxp3^{hi}CD45RA^-CD25^{hi}$ and $Foxp3^{lo}CD45RA^+CD25^{lo}$ phenotypes corresponded to suppressive T_{reg} cells, whereas the $Foxp3^{lo}CD45RA^-CD25^{lo}$ fraction marks activated T effector (T_{eff}) cells without suppressive activity [20].

Intratumoral T_{reg} cells appear to have features that distinguish them from peripheral T_{reg} cells. They often acquire an activated-memory phenotype as marked by the upregulation of CD44 (marker of activated and memory T cells) and downregulation of CD62L and CCR7 (markers of naïve or lymphoid tissue-homing T cells) [21]. In multiple murine models, it was shown that tumor-infiltrating T_{reg} cells express high levels of Helios and Neuropilin 1 (Nrp1) [14]. They were also found to express high levels of molecules known to correlate with better suppressive function such as ICOS [22], CD39 [23], and Tim-3 [24]. In addition, T_{reg} cells from cancer patients, as compared to those in healthy subjects, are usually characterized by a distinct expression profile of chemokine receptors, such as CCR4 [15], CXCR4 [21], and CCR5 [25], which facilitates their migration into tumors in response to the corresponding chemokine ligands derived from tumor microenvironment. Recently, receptor activator of nuclear receptor kappa-B ligand (RANKL), a tumor necrosis factor (TNF) superfamily member, has been shown to mark a subset of T_{reg} cells that promotes the pulmonary metastasis of mammary carcinoma cells [26], but it is not yet clear if this is seen in multiple tumor types. While it has been suggested that the distinct phenotypic markers expressed by tumor-associated T_{reg} cells facilitate their entry and function as a consequence of tumor-derived environmental cues, further characterization of the phenotype of tumor-associated T_{regs} is required and may provide additional therapeutic targets.

Impact of T_{reg} cells on the tumor microenvironment

T_{reg} cells have a profound effect on the tumor microenvironment. Many studies have now shown that T_{reg}-cell ablation results in a dramatic reduction in tumor growth, often leading to complete tumor clearance [27–29]. As expected there are also profound cellular changes within the tumor microenvironment. In addition to suppressing the development and function of a variety of cell types, T_{regs} may also alter the function of certain cell populations. Initially, it was thought that T_{reg} cells primarily suppress antitumor effector cells, such as NKs, $CD8^+$, or $CD4^+$ T_{eff} cells, as their numbers are substantially increased in response to T_{reg}-cell ablation [28–31]. However, this may be too simplistic. First, although deletion of these effector cell types, especially $CD8^+$ T cells, negates the impact of T_{reg}-cell depletion and restores tumor growth, this does not reveal whether the impact of T_{reg} cells is direct or indirect, or which effector cell population is predominantly impacted [29]. Second, the innate immune components within tumors, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and other myeloid cells, are known to play unique or overlapping roles regulating the tumor microenvironment [32]. However, their interactions with T_{reg} cells are poorly understood [33]. While TAMs and MDSCs can facilitate the recruitment of T_{reg} cells into tumors and promote their local expansion, it is not clear whether and how T_{reg} cells impact MDSCs and TAMs. However, in a mouse model of oncogene-driven spontaneous melanoma (MT/Ret mice), T_{reg} cells control the number of $Ly6C^{hi}$ inflammatory monocytes, but not $CD8^+$ T cells or NKs, which are the critical mediators of the early antitumor response [34]. Another study using the same model showed that T_{reg} cells stimulate B7-H1 expression in MDSCs [35]. It has also been suggested that human T_{reg} cells are able to induce monocyte differentiation toward the tumor-promoting M2 subtype of TAMs [36]. Collectively, these results suggest that there is a collaborative network incorporating multiple immune suppressive cell types in the tumor microenvironment, which may not exist in most inflammatory situations. This may provide a unique opportunity to identify tumor-specific mechanisms within this T_{reg} orchestrated suppressive intratumoral cellular network that may be therapeutically targetable.

In addition to inducing immunosuppression, recent findings suggest that T_{reg} cells also modulate other physiological processes such as tumor angiogenesis and wound healing. A positive correlation between intratumoral T_{reg}-cell accumulation and markers of angiogenesis was observed in several cancer types including endometrial and breast cancer [37,38]. It was suggested that T_{reg} cells promote tumor angiogenesis both directly and indirectly. For example, T_{reg} cell-mediated suppression of tumor-specific T cells reduced their secretion of angiostatic cytokines TNFα and

interferon-γ (IFNγ) in the tumor, tipping the balance toward a proangiogenic environment [39–41]. T_{reg} cells can also contribute via production of the angiogenetic factor VEGFA, which is enhanced by tumor-associated hypoxia. T_{reg} cells also support other VEGFA-producing cell types including MDSCs and tumor cells, as eliminating T_{reg} cells causes reduction of the overall intratumoral VEGFA levels. Furthermore, transient T_{reg}-cell depletion leads to tumor rejection accompanied by vasculature normalization, suggesting that T_{reg} cells can promote neovascularization [30,42]. Thus, a better understanding of the interplay between T_{reg} cells and the tumor microenvironment may provide a molecular basis for designing novel therapeutic strategies.

Mechanisms of T_{reg}-cell enrichment within the tumor microenvironment

As a predominant immune cell type found in the tumor immune contexture, T_{reg} cells are usually present as a higher ratio of T_{reg} cells to T_{eff} cells within the tumor site compared to the circulation or secondary lymphoid tissues, suggesting a tumor-directed compartmentalized redistribution, retention, and/or expansion [43]. Such enrichment could be explained by several mechanisms, including preferential tumor-trafficking and retention, and the enhanced potential for T_{reg}-cell proliferation within tumor-draining lymph nodes and tumor sites.

Preferential recruitment and retention of T_{reg} cells to tumors

It has been hypothesized that activated T_{reg} cells preferentially migrate to sites of inflammation via a chemotactic network, such as the chemokines/chemokine receptor system. Consistent with this notion, T_{reg}-cell trafficking to tumors is triggered by a cohort of tumor-associated chemokines or hypoxia-induced factors, including CCL22, CCL17, CXCL12, CCL28, and vascular endothelial growth factor (VEGF) [44]. The CCL22/CCL17–CCR4 axis appears to be the most dominant mechanism responsible for T_{reg}-cell recruitment to the tumor, first highlighted in human ovarian cancer [15] and subsequently reported in other cancer types including breast [22], prostate [45], gastric cancer [46], and Hodgkin lymphoma [47]. Tumor cells as well as myeloid cells are the primary sources of CCL22 and CCL17, both of which bind to the chemokine receptor CCR4 expressed by T_{reg} cells. CCR4 blockade in mouse models using monoclonal antibodies (mAbs) or antagonists reduced intratumoral T_{reg}-cell numbers and enhanced antitumor immunity [47–49]. In addition, it has been shown that the CCL5/CCR5 pathway is involved in the recruitment of T_{reg} cells in a mouse model of pancreatic cancer [25]. Hypoxia-induced chemokine CXCL12 has also been reported to preferentially recruit T_{reg} cells, but not $CD8^+$ or $CD4^+$ T_{eff} cells, to human breast cancer, lung adenocarcinoma, and advanced cervical cancers [50–52]. Consistent with this, the CXCR4 antagonist AMD3100 has been reported to promote antitumor immunity in a mouse model of ovarian cancer [53]. CCL28, a chemokine prominently induced under hypoxic conditions in human ovarian cancer cells, was able to recruit T_{reg} cells expressing CCR10, while overexpression of CCL28 induced accelerated orthotopic mouse ID8 ovarian tumor growth through preferential recruitment of $CCR10^+$ T_{reg} cells [54]. Surprisingly, it was also suggested that $CCR10^+$ T_{reg} cells, in addition to suppressing effector T cells, facilitated tumor vascularization via production of VEGFA, further contributing to their tumor-promoting activity. It should be noted that VEGFA also serves as a chemo-attractant exploited by tumor cells to induce T_{reg}-cell infiltration through the surface receptor Nrp1, which is expressed on the majority of T_{reg} but relatively few T_{eff} cells. Interestingly, T_{reg}-restricted deletion of Nrp1 reduced T_{reg}-cell trafficking to tumors, thereby promoting tumor immunity and limiting tumor growth [55]. Thus, multiple components of the chemotactic network conspire to entice T_{reg} cells into tumors and facilitate their retention.

Tumor-specific expansion of T_{reg} cells

In addition to the preferential recruitment of T_{reg} cells into the tumor site, increased proliferation (with limited cell death) results in increased T_{reg}-cell expansion facilitating their dominance within the tumor microenvironment. This appears to be driven by antigen-, cytokine-, and metabolism-dependent mechanisms. An increased percentage of proliferating ($Ki67^+$) T_{reg} cells has been observed in a wide variety of tumor types, such as breast cancer and acute myeloid leukemia (AML), suggesting that T_{reg} cells exhibit increased proliferative potential in response to tumor growth. Consistent with these observations, one study showed that T_{reg} cells enter cell cycle much faster than conventional T cells during initial tumor emergence, as shown by the adoptive transfer of T cells into tumor-bearing mice and tracking the kinetics of cell proliferation *in vivo* [56]. Tumor-associated antigens (TAAs), which could be in the form of upregulated self-antigens, altered-self antigen as a consequence of post-transla-

tional modifications, or neoantigens generated by mutagenic events in tumor cells, may drive the early and rapid expansion of T_{reg} cells. Indeed, this proliferation potential was restricted to the $CD44^{hi}$ activated/memory rather than naïve T_{reg} populations further supporting a role of tumor-derived antigens. Interestingly, a recent study suggested that Aire is responsible for the thymic development of prostate antigen-specific T_{reg} cells that were subsequently enriched in the tumor microenvironment of mice with oncogene-induced prostate cancer [57]. Lastly, antigen presentation is required for both self and nonself antigen-induced T_{reg}-cell expansion, as T_{reg} cells expand in an MHC class II-sufficient environment but not in MHC class II-deficient tumor-bearing mice [58].

Cytokines, such IL-2 and TGFβ, have been suggested to favor the expansion of intratumoral T_{reg} cells over T_{eff} cells within the tumor microenvironment. IL-2, a key cytokine that is required for T_{reg}-cell function and homeostasis, is predominantly produced by local T_{eff} cells within the tumor. IL-2 is used preferentially by T_{reg} cells to facilitate their proliferation, in part due to higher expression of the IL-2 receptor, CD25. It has also been suggested that increased CD25 expression may also allow T_{reg} cells to act as an IL-2 'sink' thereby limiting T_{eff} cell proliferation [59], although this has been questioned by some [60]. This mechanism may be more prominent during the early phase of tumor progression when T_{eff} cells have not yet lost their ability to produce IL-2. Of note, high dose IL-2 infusion has been used clinically for the treatment of patients with metastatic melanoma and renal cell carcinoma with the goal of boosting antitumor T_{eff} cells. Unfortunately, clinical efficacy was observed in only ~20% of patients, which may have been due to preferential expansion of T_{reg} cells [61,62], highlighting the challenges of using IL-2 in the clinic.

TGFβ is a cytokine known to induce *de novo* pT_{reg} conversion from conventional T cells and expand T_{reg} cells in the periphery. Multiple cell types in the tumor microenvironment can produce TGFβ, including MDSCs, immature DCs, and T_{reg} cells. Interestingly, tumor cells can also enhance TGFβ secretion by MDSCs [63] and immature DCs [64]. T_{reg} cells can also produce TGFβ to mediate the suppression of T_{eff} cells while promoting their self-expansion in an autocrine manner [65].

Indoleamine 2,3-dioxygenase (IDO), an immunomodulatory enzyme associated with tumor tolerance that converts tryptophan (an essential amino acid) into kynurenine (an immunosuppressive metabolite), is mainly produced by intratumoral antigen presenting cells (APCs), especially plasmacytoid DCs (pDCs), and is also produced by a wide variety of tumors [66]. It has also been shown that IDO promotes the activation and expansion of T_{reg} cells in both mouse and human tumors [67,68]. Interestingly, it was suggested that there is a positive feedback loop between T_{reg}-cell activity and IDO production, as T_{reg} cells express the inhibitory receptor CTLA-4, thereby facilitating IDO production by APCs by ligating CD80/CD86 molecules. While ongoing clinical trials using IDO inhibitors for the treatment of a variety of solid tumors shows promise [69], other factors that promote the expansion of intratumoral T_{reg} cells may also be potential targets for cancer immunotherapy.

Mechanisms of T_{reg}-cell suppression in tumors

The mechanisms by which T_{reg} cells exert their immune suppressive function have been extensively studied [8]. The basic modes of T_{reg} suppression include expression of soluble (cell–cell contact independent) or membrane-tethered (cell–cell contact dependent) inhibitory molecules, direct induction of cytolysis of target cells, metabolic disruption, and inhibition of DC maturation. Although the utilization of these mechanisms in the tumor microenvironment have been assessed to some extent, there are clear gaps in our understanding of which mechanisms are most important in different mouse and human tumors, and the extent to which they differ across different tumor types [70]. In addition, it has recently emerged that there are mechanisms, such as the Nrp1:Sema4a axis, that are critically important for the stability and survival of T_{reg} cells thereby governing their impact on the tumor microenvironment [27]. Importantly, T_{reg} cells can not only directly suppress cells within the tumor and draining lymph nodes but can also impact the tumor microenvironment in a noncell-autonomous manner by regulating the phenotype and function of other immune cell types.

Suppressive cytokines made by T_{reg} cells

Suppressive cytokines, such as IL10, IL35, and TGFβ, are secreted by T_{reg} cells and required for their maximal suppressive function [8]. Although the suppressive function of T_{reg} cells isolated from cancer patients can be blocked by addition of neutralizing mAbs against IL-10 or TGFβ [71], the relative importance of T_{reg}-derived IL-10 and TGFβ remains surprisingly obscure. IL-35, an Ebi3:p35 heterodimeric member of the IL-12 family, is an inhibitory cytokine produced by mouse and human T_{reg} cells [72]. IL-35 contributes to the optimal suppressive activity of T_{reg} cells and is

also capable of inducing an IL-35-producing T_{reg}-cell population from conventional T cells, called iTr35 [73]. Although it has been suggested that additional cell types and even some tumor types can produce IL-35 [74,75], T_{regs} still appear to be the predominant source of IL-35. The observations in our laboratory suggest that a higher proportion of tumor-associated T_{reg} cells recruited to tumors in the B16 mouse melanoma model express IL-35 when compared with their peripheral counterparts [76]. Interestingly, tumor growth was reduced in mice treated with an IL-35-neutralizing mAb or mice with a T_{reg} cell-restricted deletion of *Ebi3*, thereby preventing IL-35 production, suggesting that T_{reg}-derived IL-35 may be an important suppressive mechanism within the tumor microenvironment.

Direct cytolysis of target cells

T_{reg}-mediated cytolysis of NKs and $CD8^+$ T cells via Granzyme B and Perforin may also be a relevant mechanism within tumors. Mice deficient in Granzyme B clear both allogeneic and syngeneic tumor cell lines better than wild-type mice, which can be partially reversed by adoptive transfer of wild-type, but not Granzyme B- or Perforin-deficient T_{reg} cells [77]. Moreover, while neither Granzyme B nor Perforin were expressed by naïve T_{reg} cells, they were expressed in 5–30% of intratumoral T_{reg} cells, suggesting that cytolysis is utilized as an active suppressive mechanism by tumor-associated T_{reg} cells.

Cell surface molecules on T_{reg} cells

Intratumoral T_{reg} cells express CD73 and CD39 that function in tandem to convert ATP into adenosine, a labile, highly suppressive molecule that binds to the adenosine receptor 2A ($A_{2A}R$) expressed on effector T cells [23]. The tumor microenvironment is known to have a high rate of cell turnover and the dying cells provide a significant source of extracellular ATP, which highlights the potential impact of $A_{2A}R$-mediated T_{reg} suppression. However, its importance within the tumor microenvironment needs to be explored further.

Multiple inhibitory receptors, including CTLA-4, PD-1, and LAG-3, are known to be expressed on exhausted intratumoral $CD8^+$ and $CD4^+$ T_{eff} cells and contribute to their functional impairment. They are also highly expressed on intratumoral T_{reg} cells and in most cases potentiate their functions. For example, CTLA-4 [78,79] and LAG-3 [80] facilitate interactions between T_{reg} cells and DCs, leading to inhibition of DC maturation, which in turn limits their ability to present antigen and prime T cells. Indeed, T_{reg}-restricted deletion of CTLA4 was shown to limit their suppressive function and thus promote tumor immunity and clearance [78]. Despite these early studies, more recent work has suggested that coinhibitory receptors expressed by T_{reg} cells may also limit their inhibitory functions in certain settings [81,82]. Considering the importance of coinhibitory receptors as immunotherapeutic targets in cancer, a thorough understanding of their functional impact on T_{reg} cells will be crucial for rational drug design.

Pathways that regulate T_{reg}-cell stability and function

Nrp1 is selectively enriched on $Foxp3^+$ T_{reg} cells in comparison to naïve $Foxp3^-$ conventional T cells [14]. Recent studies have suggested that the maintenance of a suppressive microenvironment by T_{reg} cells is substantially dependent on their lineage stability and survival, which is maintained by a novel Nrp1–Sema4a axis [27]. Although mice with a T_{reg} cell-restricted deletion of Nrp1 do not exhibit any autoimmune or inflammatory symptoms throughout their lifespan, they are highly resistant to several transplantable tumors, including B16 melanoma and MC38 adenocarcinoma. Indeed, their resistance to tumor growth resembles that induced by T_{reg}-cell ablation. Similar observations were obtained by treating tumor-bearing mice with an Nrp1 neutralizing mAb *in vivo*. The Nrp1–Sema4a axis was shown to be required for the lineage stability of intratumoral T_{reg} cells, but not peripheral T_{reg} cells, by limiting the activation of the serine/threonine protein kinase Akt, which would otherwise negatively regulate the activity of a key T_{reg}-cell transcription factor, Foxo1. The absence of this pathway leads to reduction in the expression of certain hallmark T_{reg}-cell signature genes (e.g., Helios), limits their survival by reduced expression of Bcl2, and upregulates expression of lineage defining transcription factors (e.g., T-bet, IRF4) and inflammatory cytokines (e.g., IFNγ). In the same study, it was shown that many cell types in the tumor microenvironment express Sema4a, with over half being pDCs [83] along with a significant proportion of activated T_{eff} cells [84]. In addition to maintaining T_{reg}-cell lineage stability, a previous study has also shown that Nrp1 promotes the prolonged interaction between T_{reg} cells and dendritic cells under physiological conditions [85], which may also be mediated by Sema4a. Thus Nrp1 plays a critical role in maintaining intratumoral T_{reg}-cell stability, survival, and function.

Clinical relevance of tumor-associated T_{reg} cells

A growing number of studies have shown that $CD8^+$ T-cell infiltration into tumors is a positive prognostic indicator [86,87]. However, the impact of T_{reg} cells has been more controversial. While several studies have suggested that a high density of T_{reg} cells within the tumor correlates with a poor clinical prognosis, there have been a number of studies showing a positive clinical prognosis, highlighting the importance of deciphering the clinical significance of T_{reg} cells further. For instance, studies in renal cell carcinoma [62], hepatocellular [88], and lung carcinoma [89] clearly demonstrated a poor prognosis associated with increased intratumoral T_{reg}-cell density. In contrast, data from head and neck cancer [90] and colorectal carcinoma [91] showed that infiltration of T_{reg} cells into the tumor correlates with improved disease outcome. Interestingly, contradicting observations do not seem to segregate between specific cancer types as both positive and negative correlations have been observed in breast and ovarian cancer (Table 1). While technical inconsistencies among these studies have been attributed to the differences observed, this may not provide the primary explanation [92]. These apparently contradictory results may be attributed to several biological factors. First, the impact of T_{reg} cells on tumor growth is influenced by the etiology of each tumor type. In most cases intratumoral T_{reg}-cell accumulation is predictive of poor prognosis; however, in the cases where chronic inflammation drives tumor initiation, such as colorectal carcinoma, T_{reg}-cell suppression of inflammation correlates with a favorable prognosis [32]. Second, there may be a temporal aspect to the impact of T_{reg} cells on tumor growth in early versus more advanced lesions. Although T_{reg} cells may play a beneficial role in con-

Table 1. Clinical significance of intratumoral T_{reg} cells. This table provides a list of tumor types, studies that have evaluated the prognostic value of T_{reg} cells, chemokine/chemokine receptor pathways that have been suggested to mediate T_{reg} recruitment, and therapies that have been assessed for their capacity to impact T_{reg} cells in the tumor types listed. Abbreviations: CTX, cyclophosphamide; Daclizumab, anti-CD25 humanized monoclonal antibody (Zenapax); Ipilumimab, human anti-CTLA-4 antibody; MEDI6383, OX40 agonist (MedImmune/AstraZeneca); ONTAK, diphtheria toxin-interleukin-2 fusion protein (denileukin diftitox).

Tumor type	T_{reg} prognostic indication	Proposed route of T_{reg} recruitment	Potential T_{reg}-modulating therapeutics and clinical trials (possible mechanism)
Hepatocellular	Poor [96,124]	CCR6/CCL20 [125] CCR4/CCL22 [126]	Metronomic CTX [124] (selective T_{reg} elimination)
Renal cell carcinoma	Poor [62]	CCR4/CCL22 [127]	ONTAK [109] Ipilimumab [128] CTX [129] (T_{reg}-cell depletion)
Lung carcinoma	Poor [24,89]	CXCR4/CXCl12 [51]	None
Cervical cancer	Poor [52]	CXCR4/CXCl12 [52]	Ipilimumab [NCT01693783] MEDI6383 [NCT02221960] (T_{reg}-cell functional inhibition)
Prostate cancer	Poor [45]	CCR4/CCL22 [45]	Ipilimumab [NCT01804465] (T_{reg}-cell depletion)
Melanoma	Poor [130] None [131]	CCR4/CCL22 [112]	Ipilimumab [113] (T_{reg}-cell depletion)
Breast cancer	Poor [22,50] None [132] Good [133]	CCR4/CCL22 [22] CXCR4/CXCL12 [50]	Daclizumab [134] (T_{reg}-cell depletion)
Ovarian cancer	Poor [15,54] Good [135]	CCR4/CCL22 [15] CCR10/CCL28 [54] CXCR4/CXCL12 [136]	Ipilimumab [NCT01611558] (T_{reg}-cell depletion)
Head and neck cancer	Good [90] Poor [137]	CCR4/CCL22 [138]	Ipilimumab [NCT01860430], [NCT01935921] (T_{reg}-cell depletion)
Lymphoma	Good [16] Poor [139]	CCR4/CCL22 [47]	Ipilimumab [140] (T_{reg}-cell depletion)
Colorectal cancer	Good [91] Poor [141]	CCR5/CCL5 [141]	None

trolling inflammation that can promote early cancer establishment, they may become detrimental in more advanced lesions by suppressing effector T cells and other immune populations [93]. Third, intratumoral T_{reg} cells may not be phenotypically and functionally homogeneous, complicating correlative assessment. Indeed, a closer association with poor clinical prognosis was observed by restricting analysis to $CCR4^+$ intratumoral T_{reg} cells [94]. This again highlights the importance of determining additional markers to define the T_{reg} subsets that dictate prognosis. Fourth, given the interplay between T_{reg} cells and other tumor-infiltrating immune cell subsets, multivariate measurements, including the ratio between $CD8^+$ effector T cells and T_{reg} cells, usually provide a more robust prognostic indicator than the number of T_{reg} cells alone [95,96]. Thus, a critical and thorough understanding of the clinical impact of T_{reg} cells in various human cancers is overdue.

Current clinical approaches targeting intratumoral T_{reg} cells

Breaking down tumor-specific tolerance is a key step in the development of effective and durable cancer immunotherapy, with T_{reg} cells a key target. Although several traditional anticancer drugs and immunotherapies impact intratumoral T_{reg} cells, current and future efforts have focused on developing therapies which specifically modulate intratumoral T_{reg}-cell number, activity, or trafficking, with several approaches exhibiting clinical benefits in a variety of murine tumor models and in human clinical trials (Fig. 1).

Nonspecific modulation of T_{reg} cells by traditional anticancer drugs

Although traditional chemotherapeutic agents at their conventional, clinically effective dose are generally

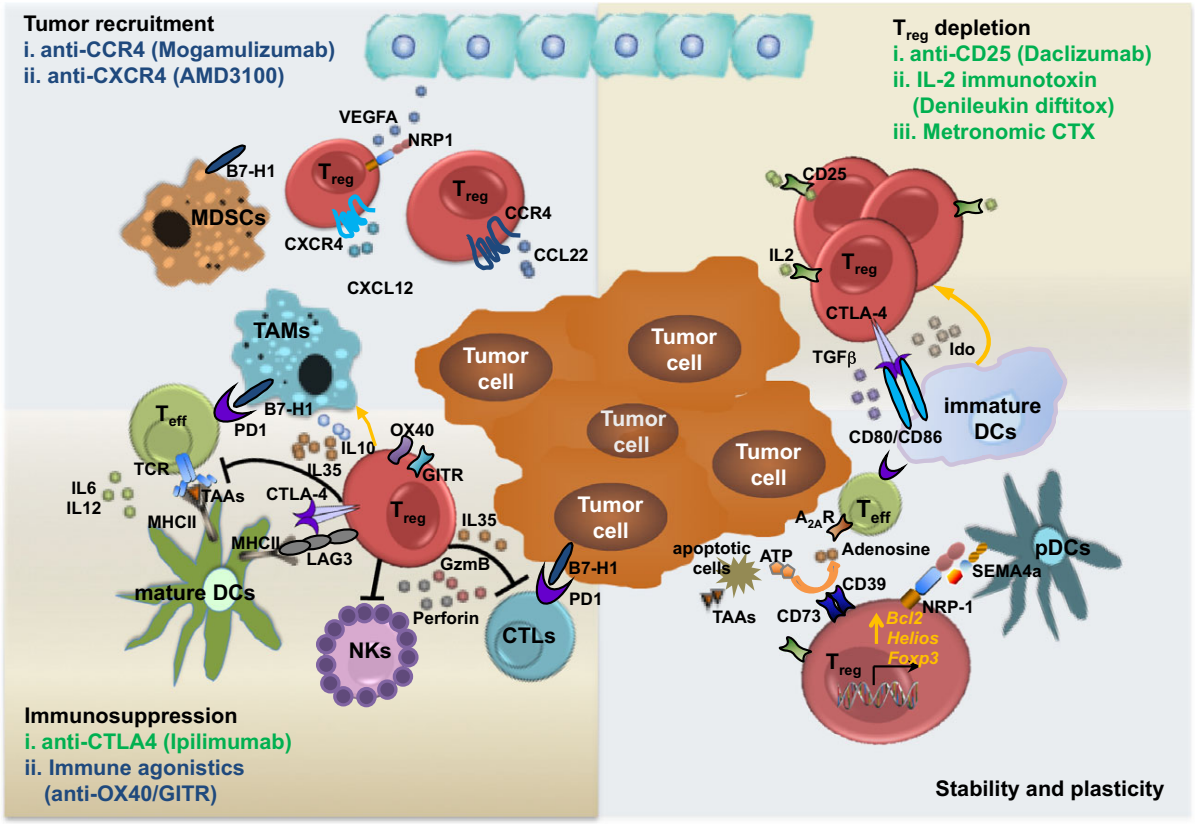

Fig. 1. Targeting T_{reg} cells in tumors. Several mechanisms that have been shown to mediate the recruitment, expansion, function, and stability of tumor-associated T_{reg} cells and are thus potential therapeutic targets as depicted (Green, FDA-approved; Blue, in clinical trials). Abbreviations: A2aR, adenosine receptor 2a; CTLs, cytotoxic T cells; DC, dendritic cells; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; NKs, natural killer cells; pDCs, plasmacytoid dendritic cells; TAA, tumor-associated antigens; Teff, effector $CD4^+$ T cells; T_{reg}, regulatory T cells; TCR, T-cell receptor.

immunosuppressive and eliminate highly proliferating cells, it has been shown that certain drugs when used at a lower dose, over a long period of time (termed as 'metronomic chemotherapy' [97]), can exhibit a broad spectrum of immunostimulatory effects. T_{reg} cells were shown to be preferentially affected, in part due to the fact that they exhibited a proliferative advantage over T_{eff} cells in most tumor microenvironments. One extensively studied example is cyclophosphamide (CTX), a frequently utilized traditional chemotherapeutic agent, which has been shown to reduce intratumoral T_{reg} cells and concomitantly reactivate intratumoral T_{eff} cells [98]. This effect was shown to be surprisingly durable despite a rapid rebound of peripheral T_{reg} cells [99], highlighting the restricted tumoral impact. Improved CTX selectivity and efficacy was observed in combination with an agonist antibody against the costimulatory receptor OX40 (CD134), as evidenced by the increased intratumoral T_{eff}/T_{reg} cell ratio and the regression of established B16 melanoma tumors [100]. However, the levels of circulating MDSCs in patients with early-stage breast cancer were increased [101], suggesting the clinical efficacy of a low-dose CTX regimen could be influenced by other factors, including cancer type and stage of disease, and the differential status of individual patients.

Some targeted therapies have been reported to downregulate the suppressive activity of T_{reg} cells. The therapeutic effects of the tyrosine kinase inhibitors sunitinib and sorafenib, which have been approved by the FDA for the treatment of renal cancer carcinoma, have been reported to inhibit intratumoral T_{reg} cells [102–104], while another tyrosine kinase inhibitor imatinib has been suggested to inhibit T_{reg}-cell expansion via inhibition of STAT3 and STAT5 signaling [105]. Although chemotherapeutic agents are able to limit T_{reg}-cell function and proliferation, they are not specific to tumor-associated T_{reg} cells and therefore are unlikely to be a long-term choice for T_{reg}-targeted approaches.

Direct intratumoral T_{reg}-cell depletion

Two agents for T_{reg}-cell depletion in humans have been developed; Daclizumab (Zenapax; PDL BioPharma, Incline Village, NV, USA), a monoclonal antibody against CD25, and Denileukin Diftitox (Ontak; Eisai, Inc. Woodcliff Lake, NJ, USA), an IL-2:diphtheria toxin fusion protein. Clinical trials with Daclizumab, when combined with dendritic cell vaccinations, have reported beneficial effects in glioblastoma [106] and breast cancer patients [107], but marginal efficacy in metastatic melanoma patients [108]. Similarly, Denileukin Diftitox, initially approved by the FDA for the treatment of T-cell lymphoma, was also effective in renal cell carcinoma patients [109], but had a negative outcome in clinical trials with metastatic melanoma patients [110]. Such conflicting results may be due to several reasons. First, the *in vivo* efficiency of T_{reg}-cell elimination was not uniform among different tumor types, partially due to variable CD25 expression [111]. Second, the timing of treatment may be a key factor impacting clinical outcome, as this may influence the extent to which beneficial effector responses, induced by concomitant vaccination, are blocked [108]. Although T_{reg}-cell depletion could induce severe systemic inflammation, this was not observed in most studies. Instead, one recent study revealed that anti-CD25 blockade preferentially inhibited the suppressive activity and stability of T_{reg} cells instead of inducing their elimination [107]. These findings suggested the T_{reg}-cell depletion regimens, upon further optimization, may prove to be effective. Alternatively, it is possible that optimal intratumoral T_{reg}-cell depletion using these approaches will result in systemic complications. Lastly, even if these approaches work, efficacy between different patients may be highly variable and difficult to predict.

Blocking T_{reg}-cell trafficking into tumors

Tumor-associated T_{reg} cells acquire a distinct pattern of chemokine receptor expression in comparison to peripheral T_{reg} cells and conventional T cells, which may provide another layer of selectivity for targeting T_{reg} cells in tumors. The CCR4–CCL22 pathway represents the dominant mechanism for intratumoral T_{reg}-cell recruitment into multiple tumor types, thereby providing a rationale for the development of CCR4 antagonists. An anti-CCR4 antibody, which was defucosylated to enhance antibody-dependent cell-mediated cytotoxicity (ADCC), to treat T-cell leukemia–lymphoma recently completed a Phase II clinical trial with promising results [49]. Initially, there were concerns that CCR4 targeting would negatively impact T_{eff} cells as a proportion of them also express CCR4. However, several studies have suggested that anti-CCR4 treatment consistently induces strong antigen-specific intratumoral $CD8^+$ T-cell accumulation and function rather than impacting their frequency [112]. It is possible that the positive benefits of limiting T_{reg} cell-mediated inhibition simply override the negative impact of partial T_{eff}-cell depletion within the tumor microenvironment. Thus, targeting differentially expressed chemokine receptors may prove efficacious.

Checkpoint blockade and immune agonists: preferential inhibition of T_{reg}-cell function?

Therapeutic targeting of inhibitory receptors, often referred to as checkpoint blockade, has garnered significant attention and is rapidly becoming standard of care for several tumor types. In just the past 4 years, four human mAbs have been approved by the FDA that target the inhibitory receptors CTLA-4 (Ipilimumab and Tremelimumab) and PD-1 (Nivolumab and Pembrolizumab), respectively. It was initially thought that the clinical efficacy of anti-CTLA4 was mainly through the reactivation of exhausted intratumoral $CD4^+$ and $CD8^+$ T_{eff} cells. It was subsequently suggested that CTLA-4 blockade may have a dual effect on both T_{eff} and T_{reg} cells, tipping the balance toward an effector response and triggering the tumor rejection. The clinical impact of anti-CTLA4 is supported by several observations. First, CTLA-4 is highly expressed by T_{reg} cells and appears crucial for their suppressive function, as demonstrated by a study in which mice with a T_{reg} cell-restricted deletion of *Ctla4* displayed a fatal autoimmune pathology similar to total T_{reg}-cell depletion [78]. Second, CTLA-4 mAbs that possess some Fcγ receptor ADCC activity were shown to limit intratumoral CTLA-4^+ T_{reg} cells [113]. In support of this notion, a positive correlation between the efficacy of Ipilimumab (which has a human IgG1 isotype and thus possesses some ADCC capacity) and reduction of T_{reg}-cell numbers has been reported in patients [114]. Furthermore, in a recent study examining the antitumor efficacy of different anti-CTLA-4 isotype variants that differ in their affinity for Fcγ receptors, tumor clearance in the MC38 and CT26 mouse models was partially or completely abrogated by mutating the Fc portion thereby limiting ADCC [115]. However, the other anti-CTLA4 mAb in the clinic, Tremelimumab, has a human IgG2 isotype negating any ADCC capacity and yet exhibits comparable clinical efficacy. Thus it remains unclear to what extent T_{reg} depletion plays a role in the mechanism of action of Ipilimumab. Taken together, these data suggest three possible interpretations. First, Ipilimumab has no T_{reg}-depleting activity and its efficacy, like Tremelimumab, is mediated by CTLA-4 blockade that limits T_{reg} function and facilitates CD8 and CD4 T_{eff}-cell expansion. In this instance, the apparent loss of T_{regs} may be a consequence of effector T-cell expansion which in turn limits T_{reg} proliferation. Second, Ipilimumab does have some T_{reg}-depleting activity but its ability to limit CTLA-4 activity on T_{reg} and effector T cells is less than Tremelimumab such that their mean clinical efficacy is the same. Third, Ipilimumab does have some T_{reg}-depleting activity, however this is incomplete and thus has no meaningful effect on T_{reg} activity at the population level. Thus the mean clinical efficacy of Ipilimumab and Tremelimumab is mediated by their capacity to limit T_{reg} function and facilitate CD8 and CD4 T_{eff}-cell expansion. Clearly, additional studies are required to resolve this uncertainty.

Several other molecules that are preferentially expressed by T_{reg} cells can also be targeted for functional modulation. GITR (glucocorticoid-induced TNF receptor family-related protein) is a costimulatory molecule that is constitutively expressed on T_{reg} cells and induced in conventional T cells upon activation. In contrast to inhibitory receptors, the engagement of GITR by its ligands boosts the effector function of $CD25^-$ conventional T cells while abrogating the suppressive activity of T_{reg} cells, a mechanism that may be exploited to restore protective immunity against tumors [116]. Indeed, it was shown in mouse models that the administration of agonistic anti-GITR mAb provoked potent antitumor responses manifested by increased IFNγ-producing $CD8^+$ and $CD4^+$ T_{eff}-cell infiltration, as well as enhanced T_{reg}-cell resistance [117]. Consistent with this, a combination of an agonist GITR mAb and a anti-CTLA-4 mAb exhibited enhanced efficacy against more advanced tumors, due to the distinct quantitative (increased number) and qualitative (enhanced activity) changes in T_{eff} cells induced by modulating CTLA-4 and GITR signaling, respectively [118]. However, it remains unclear what the effect of this combinational treatment has on T_{reg} number and function, warranting further study. Interestingly, another TNF receptor superfamily member, OX40, was found to 'mirror' GITR substantially in terms of expressional and functional impact on T cells, highlighting its potential as a target for immunotherapeutic development. OX40 is expressed at high levels in resting and activated T_{reg} cells, whereas it is only induced in a transient manner on effector T cells. Similar to GITR, it acts as a costimulatory receptor for T cells, whose engagement leads to enhanced effector responses but impaired T_{reg} cell-mediated immune suppression *in vivo*, as evidenced in both autoimmune and tumor settings [119,120]. Such T_{reg} inhibition is likely to be achieved by enhancing T_{reg}-cell death selectively at their site of activation (intratumoral but not systemically), a process that can be sensitized by chemotherapy-induced tumor antigen release [100]. In light of these promising results from animal models, human antibodies against GITR and OX40 are currently in clinical trials [121].

Concluding remarks and future directions

The immune suppressive activity of intratumoral T_{reg} cells represents a major hurdle for effective antitumor immunity, highlighting their potential as an immunotherapeutic target. Moreover, there is considerable interest in the possible synergistic opportunities of combining T_{reg} cell-targeted therapies with other modalities, such as immune checkpoint blockade, immune agonists, tumor-specific vaccines, radiotherapy, and chemotherapy. However, there remain several significant gaps in our knowledge and many challenges ahead.

First, a more in-depth understanding of basic T_{reg}-cell biology within the tumor microenvironment is warranted. T_{reg} cells differentially impact tumors of different etiologies, leading to complicated or even opposing clinical relevance across various cancer types. This highlights the importance of more in-depth mechanistic studies in a wide variety of human tumors. In addition, relatively little is known about the temporal requirement of T_{reg} cells at different stages of tumor progression and metastases, and whether this differs between tumor types. This analysis would not only identify additional potential therapeutic targets but would also likely inform clinical trial design and highlight additional biomarkers that could be used to assess T_{reg}-cell function and fate following therapeutic intervention.

Second, the techniques used to enumerate and phenotypically and functionally characterize human T_{reg} cells will critically influence clinical trial design and monitoring. While Foxp3 is a reliable and authentic marker of T_{reg} cells in mice, it can be limiting in certain human settings. Thus, the development of additional markers and/or approaches that can reliably identify all human T_{reg} cells would be highly desirable. Furthermore, it would also be desirable to define markers that identify more functionally suppressive T_{reg}-cell populations within the tumor microenvironment that could be selectively studied and targeted. For example, unlike its expression on murine T_{reg} cells, NRP1 is barely detected on naïve human peripheral T_{reg} cells but may be upregulated on T_{reg} cells infiltrating certain tumors [122,123]. This raises the possibility that $NRP1^+$ tumor-associated T_{reg} cells are a more suppressive population and have a closer correlation with clinical outcome.

Third, while potential therapeutic benefit may be obtained from current approaches designed to transiently deplete or limit T_{reg} cells, it is possible that this may lead to highly variable clinical observations. Thus, there is a clear need to develop more selective approaches to limit intratumoral T_{reg}-cell function without impacting peripheral immune homeostasis. Moreover, intratumoral T_{regs} may be a functionally heterogeneous population and thus single cell analyses, such as single cell RNA-seq, may facilitate further characterization. Going forward, it will be important to determine if there are defined subpopulations, with druggable targets, that are more critically involved in the promotion of tumor growth and/or blockade of antitumor immunity, and distinguishable for those that mediate peripheral immune homeostasis. Thus future studies aimed at developing therapies that surgically target certain tumor-promoting T_{reg} subpopulations may be important. Based on recent observations from our laboratory, we propose that this goal is achievable and may be efficacious, with IL-35 and the Nrp1:Sema4a axis as potential options.

Fourth, the role of costimulatory and coinhibitory molecules in modulating T_{reg} function and survival remains unclear and warrants further investigation and clarification. This is particularly important as we attempt to interpret clinical observations with checkpoint inhibitors and agonist antibodies that are in clinical trials and likely impact multiple T-cell populations.

Finally, the points raised above have focused on T_{reg} cell-intrinsic considerations. However, tumor tolerance is established via a complex network involving multiple suppressor cell types, as well as feed forward mechanisms mediated by tumor cells to reinforce the immune suppressive tumor microenvironment. Thus, we need a greater understanding of T_{reg} cell-extrinsic parameters. For example, MDSC, TAMs, pDCs, and other populations support the local expansion and functional modulation of T_{reg} cells, while T_{reg} cells in turn have a global and multifactorial impact on the tumor microenvironment. Thus, the identification of molecules or pathways promoted by tumor cells and other populations within the tumor microenvironment that lead to the modulation of T_{reg}-cell survival and function may also provide novel immunotherapeutic targets.

Thus, while many gaps remain and the true efficacy of targeting T_{regs} in tumors has yet to be determined, observations from both mouse models and human clinical trials and studies are extremely encouraging.

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Author contributions

D.A.A.V. and C.L. conceived the theme and outlines. C.L. wrote the manuscript and created the figure illustrations. C.J.W. and D.A.A.V. made the crucial editing.

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