Regulatory T Cells: Their Role, Mechanism of Action, and Impact on Cancer

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Anthony R. Cillo and Dario A.A. Vignali

Contents

A.R. Cillo • D.A.A. Vignali (✉)
Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

Tumor Microenvironment Center, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15232, USA
e-mail: dvignali@pitt.edu

6.1 Introduction

Generating antitumor immunity by using therapeutic monoclonal antibodies to block immune checkpoint receptors expressed on the surface of T cells has led to a revolution in the treatment of several solid tumors and hematologic malignancies [1]. T cells upregulate expression of immune checkpoint receptors following prolonged antigen stimulation, and expression of these receptors is associated with T cell dysfunction. Monoclonal antibodies targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), or programmed death-ligand 1 (PD-L1) have been successful in the clinic. These advances in immuno-oncology have led to prolonged survival in some patients with aggressive cancers, such as metastatic melanoma and non-small cell lung carcinoma [2]. Despite the success of immuno-oncology, there are still many patients that do not derive benefits from

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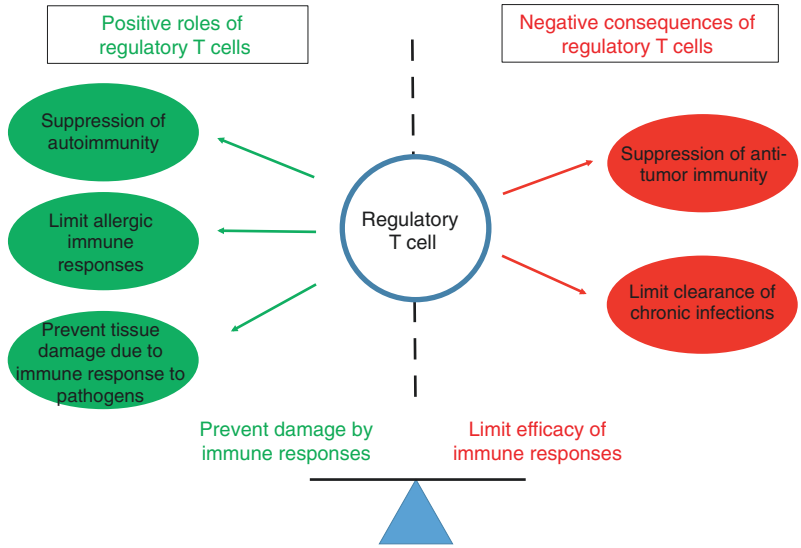

Fig. 6.1 Regulatory T cells (T_{regs}) limit self-directed immune responses but also suppress antitumor immunity and clearance of chronic viral infections. T_{regs} are essential for the maintenance of peripheral tolerance and control immune homeostasis through suppression of autoimmunity, limiting allergic immune responses and preventing damaging immune responses to pathogens. However, because of these normal immunosuppressive functions, T_{regs} also have negative consequences in that they can prevent effective antitumor immunity and limit clearance of chronic viral infections

blockade of inhibitory receptors, suggesting that additional immune mechanisms may need to be targeted to elicit an effective antitumor response. Regulatory T cells (T_{regs}) maintain peripheral tolerance by limiting inflammation and autoimmunity. However, T_{regs} also inadvertently limit the clearance of chronic viral infections and lead to tumor tolerance because of their homeostatic role in limiting tissue damage (Fig. 6.1). In this chapter, we discuss T_{regs} in the context of immuno-oncology, beginning with the discovery of T_{regs} and T_{reg}-specific cell markers, describing the immunosuppressive mechanisms of T_{regs}, and presenting evidence for the roles T_{regs} play in limiting antitumor immunity.

6.2 Discovery of a T Cell Population that Regulates Autoimmunity

T_{regs} were first discovered as a subpopulation of $CD4^+$ T cells that were responsible for preventing autoimmunity. Subsequent work over several decades has elucidated molecular pathways and surface receptors associated with T_{regs} and has led to an appreciation for the central role they play in maintaining peripheral tolerance *in vivo*. As such, research into the role of T_{regs} in cancer continues to expand, especially in the context of immunotherapeutic targeting. An initial discussion of the discovery and elucidation of surface markers for the identification of this T cell population is warranted to establish a basic understanding of the role of T_{regs} in immune homeostasis.

6.2.1 Suppression of Autoimmunity by $CD4^+$ T Cells

The importance of T_{regs} was first described in studies of autoimmunity in animal model systems dating back to the mid-1960s. These early studies showed that removal of the thymus from neonatal mice led to severe autoimmunity in many organs, including hematological disorders, endocrinopathies, gastritis, and oophoritis/orchitis [3]. These studies demonstrated an important role for T cells derived from the thymus in suppressing immune responses in a variety of tissues.

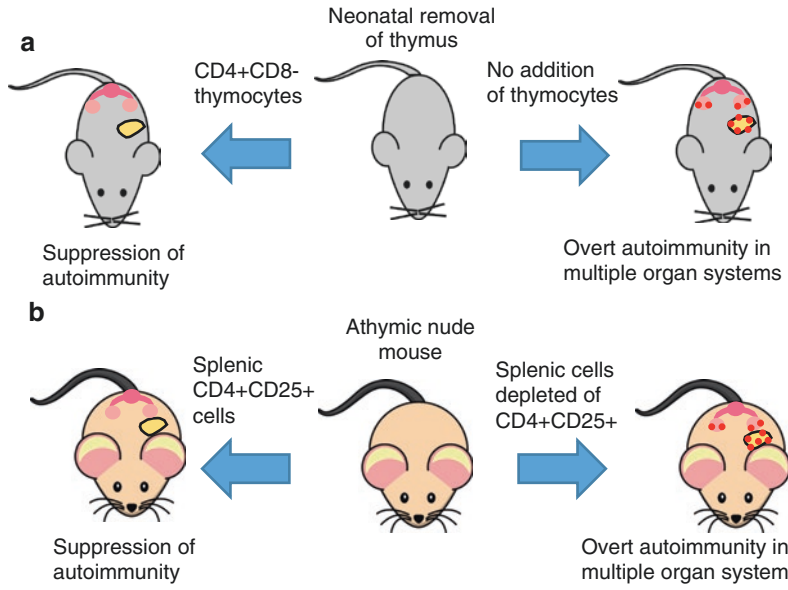

Fig. 6.2 Discovery of a T cell population that suppresses autoimmunity. (**a**) When the thymus is removed from neonatal mice within 3 days of birth, overt autoimmunity occurs in diverse organ systems such as the ovaries and pancreas. However, this autoimmunity can be prevented when $CD4^+CD8^-$ thymocytes are used to reconstitute these athymic mice. (**b**) Subsequent work showed that autoimmunity occurred in multiple organ systems when naturally athymic nude mice were reconstituted with splenic cells depleted of $CD4^+CD25^+$ T cells. Conversely, reconstitution of nude mice with splenic $CD4^+CD25^+$ T cells led to the suppression of autoimmunity. Collectively, these experiments demonstrated that thymus-derived $CD4^+CD25^+$ T cells that develop soon after birth are responsible for the suppression of autoimmunity

Importantly, these early studies also demonstrated that a T cell subpopulation was required to prevent autoimmunity as the transfer of $CD4^+CD8^-$, but not $CD4^-CD8^+$, thymocytes was sufficient to abrogate autoimmunity (Fig. 6.2a).

6.2.2 Markers of Regulatory T Cells

Subsequent studies sought to define cell-intrinsic markers of this suppressive cell population. Studies to identify markers of T_{regs} first focused on the observation that T_{regs} appeared to be an activated T cell population. A cell surface marker that is associated with T cell activation and function is the high-affinity IL-2 receptor α-chain (IL2Rα or CD25). The importance of CD25 in T_{reg} biology was discovered by comparing reconstitution of athymic nude mice with $CD4^+CD25^+$ versus $CD4^+CD25^-$ splenocytes. Reconstitution of nude mice with the $CD4^+CD25^+$ subpopulation led to suppression of autoimmunity, while the $CD4^+CD25^-$ T cell-reconstituted mice succumbed to autoimmunity (Fig. 6.2b) [4]. This study was the first to highlight the importance of CD25 as a marker of T_{regs}.

Advancing these observations demonstrated that both CD25 and IL-2 are essential for T_{reg} development and survival [5]. Although IL-2 contributes to survival and function of all T cells via CD25, it is critically important for T_{regs}, so much so that $CD4^+CD25^+$ T cells are highly enriched for T_{regs}. Importantly, T_{regs} do not secrete IL-2 themselves and therefore require paracrine production of IL-2 by other cell types to exert their effector function. Following the identification of $CD4^+CD25^+$ T cells as a suppressive cell population, additional studies sought to further define markers of T_{regs}.

The discovery of a genetic mutation that led to a severe autoimmune disease in mice (known as scurfy), and a similar disease in humans (known as immunodysregulation polyendocrinopathy enteropathy X-linked syndrome or IPEX), led

investigators to consider the role of this gene in T_{regs}. This single X-linked gene (now known as *Foxp3*) was found to encode a key transcription factor (FOXP3) that directs the formation and function of T_{regs} [6]. FOXP3 has been shown to be critical in driving the development and suppressive function of T_{regs} by controlling the transcription of key genes required for the maintenance and function of T_{regs}. FOXP3 drives or enhances the transcription of genes associated with suppression, such as CD25 and CTLA-4, while simultaneously suppressing the transcription of inflammatory genes, such as IFN-γ and IL-2. Expression of FOXP3 itself is driven by the epigenetic hypomethylation of the *Foxp3* promoter, which is considered a hallmark of T_{regs} [7]. While FOXP3 is a specific marker for T_{regs}, it should be noted that FOXP3 can also be transiently expressed in activated human $CD4^+$ T cells [8]. This transient expression of FOXP3 following activation can cause some effector T cells to appear to be $FOXP3^+$ T_{regs}, and therefore phenotypic identification of human T_{regs} should rely on a combination of markers (i.e., coexpression of CD25 and FOXP3 or the absence of CD127, as described below). Additionally, FOXP3 cannot be used to sort T_{regs} from unmanipulated samples from mouse or human donors, as it is a transcription factor that is expressed in the nucleus. Consequently, murine studies in which $FOXP3^+$ cells are purified routinely rely on the use of genetic reporters.

More recently, the absence of the interleukin-7 (IL-7) receptor (CD127) on $CD4^+CD25^+$ T cells has been described as a population that is enriched for $FOXP3^+$ T_{regs}. Conventional memory T cells require signals from IL-7 for their maintenance and as such express high levels of CD127 [9]. Conversely, T_{regs} do not express CD127 because FOXP3 suppresses transcription of the *Il7r* gene, leading to the absence of CD127 on cells that express FOXP3 [10]. In addition to being enriched for expression of FOXP3, cells that are $CD4^+CD25^+CD127^-$ are highly suppressive *in vitro*, demonstrating that this population is functionally T_{regs}. Collectively, these markers have facilitated the purification and analysis of T_{regs}. However, identification of additional markers would be beneficial.

6.2.3 Regulatory T Cell Origins: Thymus Versus Periphery

The role of T_{regs} in preventing autoimmunity was described using thymus-derived T_{regs} (tT_{regs}). However, populations of suppressive $CD4^+FOXP3^+$ T cells can also be generated outside of the thymus [11]. T_{regs} that develop *in vivo* outside of the thymus are known as peripheral T_{regs} (pT_{regs}). pT_{regs} differentiate from naïve $CD4^+$ T cells in the periphery following activation of naïve CD4+ T cells with suboptimal doses of antigen in the presence of transforming growth factor β (TGF-β). Activation of naïve CD4+ T cells under these conditions leads to the induction of FOXP3, the inability to secrete the effector cytokines IFN-γ and IL-2, and the ability to suppress proliferation of effector T cells *in vitro*. While tT_{regs} clearly limit autoimmunity *in vivo*, the role that pT_{regs} play is less clear [12]. One proposed function of pT_{regs} is to suppress immune responses to potentially damaging antigens, such as the gut microbiota, that are not recognized by the self-directed T cell receptor repertoire of tT_{regs}. Alternatively, pT_{regs} may be important for controlling immune responses in specific situations, such as in response to mucosal inflammation [13] or in controlling fetal-maternal tolerance [14]. It is clear that pT_{regs} can be induced in specific situations or to specific antigens *in vivo*, but their contribution relative to tT_{regs} in controlling autoimmunity and ultimately their role in cancer immunology requires further investigation. Taken together, tT_{regs} are indispensable for limiting autoimmunity *in vivo*, while pT_{regs} most likely play a role in controlling immune activation in specific scenarios where exogenous antigen-specific T_{regs} are required.

The identification of a $CD4^+$ T cell population that is essential to the prevention of autoimmunity has led to an entire branch of immunology dedicated to their study. Progress over several decades has led to substantial insight into the role of T_{regs} in suppressing autoimmunity, the origin and development of T_{regs}, and the appreciation for the essential role that FOXP3 plays in driving T_{reg} development and function. Identification of cell surface markers for T_{regs} has also accelerated their analysis *in vitro* and *in vivo*.

6.3 Regulatory T Cell Suppressive Mechanisms

Considerable attention has been devoted to understanding the mechanisms by which T_{regs} suppress immune responses. Broadly speaking, this can be broken down into two classes: mechanisms that are contact dependent and mechanisms that are mediated by soluble factors (Fig. 6.3) [15]. Contact-dependent mechanisms rely on direct interaction of T_{regs} with the cell types that are being actively suppressed. Soluble suppression mechanisms depend on either T_{reg} secretion of cytokines or metabolic inhibition of effector cells by T_{regs}. Both types of suppressive mechanisms can also be modulated and potentiated by the local microenvironment and cell-extrinsic pathways, as discussed below.

6.3.1 Contact-Dependent Suppression of Immune Responses

Early studies suggested that T_{regs} required direct contact with effector T cells or antigen-presenting cells to mediate suppression [16, 17]. Through physical interaction with either conventional CD4+ CD25- T cells or antigen-presenting cells, T_{regs} limited the production of IL-2 from effector T cells and prevented co-stimulation of effector T cells by antigen-presenting cells. T_{reg}-mediated suppression was lost following the addition of IL-2 or anti-CD28, underscoring that suppression by T_{regs} relied on deprivation of IL-2 and co-stimulation. These initial studies laid the framework for more in-depth analysis of the contact-dependent mechanisms used by T_{regs} to suppress immune responses.

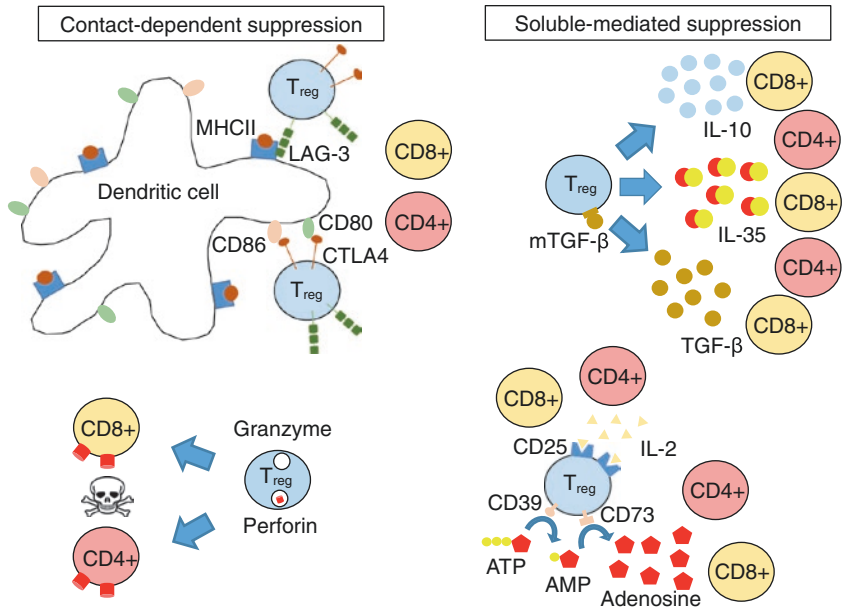

Fig. 6.3 Immunosuppressive mechanisms used by T_{regs}. T_{regs} suppress immune responses through either contact-dependent mechanisms or soluble mediators. Contact-dependent inhibition is achieved through interaction of CTLA-4 on T_{regs} with CD80/CD86 on dendritic cells or through interaction of LAG-3 on T_{regs} with major histocompatibility complex II on dendritic cells. The interaction of CTLA-4 with CD80/CD86 on dendritic cells prevents co-stimulation of CD28 on effector T cells with CD80/CD86, while LAG-3 prevents TCR/CD3-mediated activation of effector T cells. Expression of granzyme B and perforin in T_{regs} can lead to the suppression of immune responses through contact-dependent direct cytolysis of effector T cells. T_{regs} also can suppress effector T cells through soluble cytokines, such as IL-10, IL-35, and TGF-β. Alternatively, T_{reg} suppression with soluble mediators also occurs through metabolic disruption of effector T cells. This occurs through preferential uptake of IL-2 by T_{regs} due to high expression levels of CD25 (IL2Rα). The presence of the ectoenzymes CD39 and CD73 on the surface of T_{regs} can catalyze the breakdown of ATP into adenosine, which then can suppress effector T cells or dendritic cells

One surface molecule expressed by T_{regs} that mediates contact-dependent suppression is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). CTLA-4 is required to prevent systemic autoimmunity, and knockout of this gene in mice leads to fatal autoimmune-mediated destruction of multiple tissues [18]. CTLA-4 competes with CD28 for binding to the dendritic cell-expressed co-stimulatory molecules CD80 and CD86. Compared with CD28, CTLA-4 binds to CD80/CD86 with a higher affinity, effectively depriving conventional T cells of co-stimulation. Importantly, CTLA-4 is constituteively expressed by T_{regs} and mediates one form of contact-dependent suppression. T_{regs} can also further deprive effector T cells of co-stimulation through trans-endocytosis and subsequent degradation of CD80/CD86 from antigen-presenting cells [19]. In head and neck cancer patients, treatment with targeted chemotherapy led to an increase in intratumoral CTLA-4$^+$ T_{regs}, which was associated with poor clinical outcome [20]. Contact-dependent immunosuppression by CTLA-4$^+$ T_{regs} is necessary to maintain immune homeostasis, and CTLA-4$^+$ T_{regs} are likely to play a role in suppressing antitumor immunity.

Another molecule expressed on T_{regs} that contributes to contact-dependent suppression is lymphocyte-activation gene 3 (LAG-3) [21]. In addition to expression on T_{regs}, LAG-3 is upregulated on the surface of conventional T cells following activation. LAG-3 is associated with the T cell receptor (TCR) on the surface of T cells and binds major histocompatibility complex class II (MHC-II) molecules. This interaction between LAG-3 and MHC-II leads to inhibition of TCR/CD3-mediated T cell activation. LAG-3 can also directly modulate dendritic cell function by interacting with MHC-II and preventing dendritic cell maturation by depriving them of activating signals from conventional CD4$^+$ T cells. Consistent with a role for LAG-3 in contact-dependent suppression by T_{regs}, *in vitro* or *in vivo* blockade of LAG-3 reduces suppression by T_{regs} [22]. Further, genetic deletion of *Lag3* in mice also led to reduced suppressive activity [23]. In melanoma and colorectal cancer patients, LAG-3$^+$ T_{regs} are expanded in peripheral blood compared with healthy donors and are present at a higher frequency within lymph nodes containing tumor metastases compared with normal lymph nodes [24]. Furthermore, FOXP3$^+$LAG-3$^+$ cells were found to secrete IL-10 and TGF-β and to potently suppress proliferation in a contact-dependent manner *in vitro*.

A separate contact-dependent mechanism by which T_{regs} can exert effector function is through the release of cytolytic granules containing granzyme and perforin. Although this is a feature that is normally restricted to CD8$^+$ T cells, T_{regs} can express granzyme and perforin and can eliminate autologous cells through a perforin-dependent pathway [25]. T_{regs} expressing granzyme and perforin are therefore able to suppress immune responses through the direct elimination of effector T cells. Finally, one study demonstrated the importance of granzyme and perforin in the suppression of antitumor immunity in a murine cancer model, underscoring the importance of this contact-dependent mechanism in promoting tumor growth [26].

6.3.2 Suppression of Immune Responses via Soluble Factors

The second general mechanism by which T_{regs} can exert their suppressive function is either by secretion, uptake, or generation of soluble molecules. As discussed earlier, T_{regs} are characterized by constitutive expression of the IL-2 receptor CD25. This high expression of CD25 causes T_{regs} to preferentially bind IL-2, depriving conventional T cells of this important stimulatory cytokine. Effector T cell deprivation of IL-2 at inflammatory sites subsequently leads to loss of their effector function and apoptosis [27].

A second soluble mechanism used by T_{regs} to suppress immune responses is the secretion of cytokines, such as interleukin-10 (IL-10) [28]. In particular, IL-10 production by T_{regs} plays a role in controlling inflammation at mucosal sites, and mice that lack the *Il10* gene in T_{regs} develop spontaneous colitis and inflammation of the skin and lungs [29]. IL-10 can directly inhibit effector T cells through interaction with the

hetero-tetrameric IL-10 receptor complex, leading to activation of STAT3 and transcription of anti-inflammatory genes [30]. In addition to direct suppression of effector T cells, IL-10 can also suppress immune responses by limiting the ability of macrophages to produce inflammatory cytokines [31]. Similarly, IL-10 also prevents the maturation of dendritic cells and inhibits their expression of co-stimulatory molecules [32]. While T_{regs} are noted for their ability to secrete IL-10, other cell types, such as macrophages under certain conditions, also secrete IL-10 [28]. Although IL-10 is a highly pleiotropic cytokine, it has a clear role in suppression of immune responses by T_{regs}.

Another important cytokine produced by T_{regs} that has been shown to play a broad and important role in the immune system is TGF-β [33]. Unlike other cytokines, TGF-β is initially translated as an inactive protein that requires proteolysis for activation. Inactive TGF-β is non-covalently bound to latency-associated peptide (LAP) through an association with GARP on the surface of T_{regs} [34]. This membrane-bound form of TGF-β is then activated through several possible proteolytic pathways, allowing the activated form of TGF-β to perform its immunosuppressive function [35]. One of the first descriptions of a connection between TGF-β and T_{regs} was in a model of experimental autoimmune encephalitis (EAE) in mice, where oral tolerance was induced by feeding mice myelin basic protein [36]. Analysis of the $CD4^+$ T cells that infiltrated the nervous system to facilitate tolerance revealed that these cells produced TGF-β and prevented EAE. As with other T_{reg} molecules, knockout of TGF-β from murine T_{regs} leads to induction of autoimmune disease, underscoring the importance of TGF-β in immune homeostasis [37]. TGF-β suppresses effector T cell responses in several ways, including inhibiting IL-2 production and IFN-γ and perforin production in CD8+ T cells [38]. In head and neck cancer patients, an important role of TGF-β secreting T_{regs} has been described [39]. Taken together, secretion of TGF-β by T_{regs} plays an important role in maintaining immune homeostasis and can inhibit antitumor immunity.

Another important cytokine produced by T_{regs} to facilitate immunosuppression in murine models is interleukin-35 (IL-35) [40]. IL-35 is a member of the IL-12 family of heterodimeric cytokines and consists of one IL-12α subunit and one IL-27β/Ebi3 (Epstein-Barr virus-induced gene 3) subunit [41]. These cytokine genes are constitutively expressed in a subpopulation of murine T_{regs}, but not conventional T cells, and are upregulated following T_{reg} activation. IL-35 confers suppressive activity on naïve CD4+ T cells and directly suppresses division of conventional cells. Like other inhibitory cytokines, IL-35 can also drive the development of an induced T_{reg} population, called iTr35, that can suppress effector T cells via IL-35 [42]. IL-35 mediates signaling via a unique IL12rβ2:gp130 receptor heterodimer and a STAT1:STAT4 heterodimer [43]. In murine cancer models, IL-35 has recently been shown to play an important role in promoting tumor growth by contributing to T cell exhaustion in the tumor microenvironment [44]. Consistent with increased IL-35 production from highly activated T_{regs}, an IL-35 reporter mouse revealed enrichment of $IL\text{-}35^+$ T_{regs} in the tumor microenvironment, and neutralization of IL-35 or T_{reg}-specific genetic deletion of *Ebi3* led to enhanced antitumor immunity, which was mediated via enhanced cell proliferation and effector function and improved memory cell generation of effector T cells. T_{reg}-restricted deletion of *Ebi3* also led to reduced expression of the inhibitory receptors PD-1, LAG-3, and TIM-3, suggesting that IL-35 may promote exhaustion through upregulation of multiple inhibitory receptors [44].

Finally, T_{regs} can also mediate immunosuppression via the generation of adenosine, a labile, highly suppressive molecule [45]. Extracellular adenosine accumulates at sites of ischemia and inflammation *in vivo*. In the extracellular space, adenosine is generated by T_{regs} via breakdown of ATP. The extracellular ectoenzymes CD39 and CD73 on T_{regs}, or cells in close proximity, in tandem catalyze the breakdown of ATP to adenosine. While CD73 is broadly expressed on activated T cells and other cell types, CD39 expression is largely restricted to T_{regs}. Increased levels of adenosine at sites of inflammation

inhibit immune responses through interaction with either the adenosine A_{2A} receptor on effector T cells or the adenosine A_{2B} receptor on antigen-presenting cells. The interaction of extracellular adenosine with either receptor leads to increased intracellular levels of cAMP and limits the release of inflammatory cytokines from both effector T cells and antigen-presenting cells. T_{regs} can therefore limit the production of inflammatory cytokines locally by breaking down ATP into adenosine through extracellular ectoenzymes.

As described in this section, T_{regs} use multiple contact-dependent and contact-independent/contact-soluble mechanisms to suppress effector T cell responses and antigen-presenting cell development and function. Given the detrimental effects of autoimmunity and excessive immune responses and the diversity of cell populations and effector mechanisms they need to control, T_{regs} likely have evolved multiple immunosuppressive mechanisms to adequately control autoimmunity and inflammation in a variety of settings. An important question is whether certain mechanisms are more dominantly or preferentially utilized by T_{regs} in tumors and thus may be targeted therapeutically without substantially impacting the ability of T_{regs} to maintain immune homeostasis and peripheral tolerance.

6.3.3 Potentiation of Suppression and Survival

T_{regs} function in diverse environments and suppress a variety of cell types. Consequently, their function and survival are likely modulated or potentiated by a variety of environmental cues, many of which are likely poorly understood or have yet to be defined. Early studies suggested that T_{reg} suppression was contact dependent [16, 17]. However, this notion was inconsistent with the growing appreciation of the importance of cytokines in mediating T_{reg}-dependent suppression. This conundrum was resolved when a more recent study showed that it was not suppression by T_{regs} per se that was exclusively contact dependent but rather the boosting/potentiation of their suppressive activity that was contact dependent [46]. This study found that co-culture of T_{regs} with fixed or live conventional $CD4^+$ T cells or antigen-presenting cells was sufficient to boost the capacity of T_{regs} to suppress effector T cells across a permeable transwell membrane via IL-10 and IL-35.

The potentiation of T_{reg} function and survival was found to be mediated by neuropilin-1 (Nrp1) on the surface of T_{regs} via interaction with Sema4a [47]. Nrp1 is involved in normal neural and vascular development and also plays a role in tumor angiogenesis [48]. Signaling through the Nrp1/Sema4a interaction is necessary for T_{reg} suppression by soluble cytokines *in vitro* [47]. Nrp1 on T_{regs} limits Akt (protein kinase B [PKB]) activity via phosphatase and tensin homolog (PTEN), which in turn stabilizes the T_{reg} phenotype and enhances their survival and function. Consistent with a requirement for Nrp1 to mediate T_{reg} potentiation, genetic ablation of *Nrp1* in murine T_{regs} led to a significant enhancement of antitumor immunity *in vivo* but did not lead to overt autoimmunity or peripheral inflammation [47, 49]. These observations highlighted the unique role of Nrp1 in stabilizing and potentiating the survival and suppressive function of T_{regs} in the tumor microenvironment. However, it remains to be determined if the Nrp1 pathway is only utilized in the tumor microenvironment and if so why and if there are other mechanisms that regulate T_{reg} fate and function.

6.4 Relationship Between Regulatory T Cells and Cancer

T_{regs} are indispensable *in vivo* for their control of immune homeostasis through suppression of autoreactive T cells. Tumor tissue originates from healthy tissue, and as such T_{reg} suppression of autoreactive immune responses likely limits antitumor immune responses because of their normal role in protecting tissue from damage caused by overt inflammation. Experimental evidence from murine models has highlighted the importance of T_{regs} in suppressing antitumor immunity, while the presence of T_{regs} in human tumors correlates with poor prognosis.

6.4.1 Role of Regulatory T Cells in Suppression of Antitumor Immunity

The role that T_{regs} play in the suppression of antitumor immunity has been demonstrated in several mouse models. Mutation or elimination of the *Foxp3* gene in mice and humans leads to fatal autoimmunity, so germline deletion of *Foxp3* in mice cannot be used to study antitumor immunity. While T_{regs} can be limited or depleted in adult mice with antibodies targeting CD4 or CD25 or drugs such as cyclophosphamide, these treatments also impact activated effector T cells, confounding experimental interpretation [50]. Instead, inducible T_{reg}-targeting genetic systems have been used to transiently deplete T_{regs} in adult mice to study their role in suppressing antitumor immunity. One model that has been utilized in mice is insertion of the human diphtheria toxin receptor (DTR) under control of the *Foxp3* locus (*Foxp3*^{DTR}) [51, 52]. Following the administration of diphtheria toxin, all cells expressing FOXP3 are depleted, allowing for a direct assessment of the role of T_{regs} in suppressing antitumor immunity.

Using the *Foxp3*^{DTR} mice, depletion of FOXP3$^+$ T_{regs} has been performed in mice with a variety of implanted tumors. Following depletion of T_{regs}, mice had reduced tumor growth and prolonged survival compared with littermates that did not have their T_{regs} depleted [47, 53]. These mechanistic studies in mice have demonstrated that T_{regs} play an important role in suppressing antitumor immunity and that specific depletion of T_{regs} is sufficient to prevent tumor growth and prolong survival in mice. However, depletion of T_{regs} following administration of diphtheria toxin in the *Foxp3*^{DTR} mice is not specific for T_{regs} in the tumor microenvironment, and these mice quickly succumb to autoimmune disease despite their antitumor immune responses. This once again highlights the importance of T_{regs} in maintaining peripheral tolerance throughout life and suggests that systemic depletion of T_{regs} may not be a viable treatment option for cancer patients.

6.4.2 Local Expansion of Regulatory T Cells in Tumors

T_{regs} are present in many healthy tissues, and as tumors grow, T_{regs} can possibly expand locally through increased antigenic stimulation and subsequent proliferation (Fig. 6.4a). In the context of a tumor, tissue-resident T_{regs} specific for self-peptides presented on MHC receive additional anti-

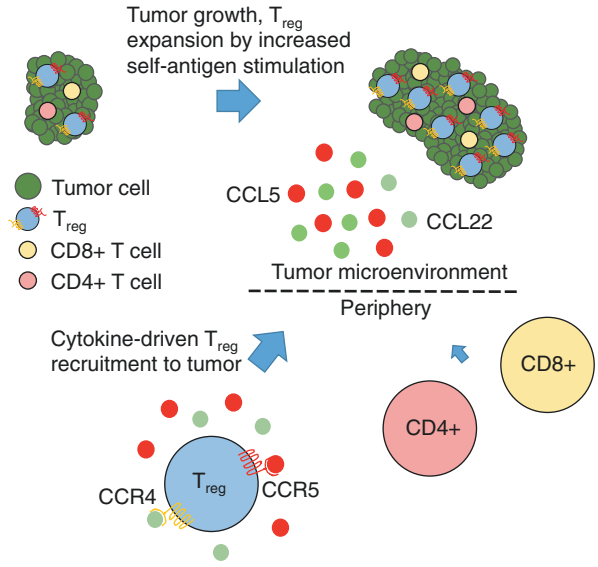

Fig. 6.4 Origins of T_{regs} in the tumor microenvironment. T_{regs} can increase in frequency within the tumor microenvironment as the tumor grows by increased self-antigen presentation and subsequent proliferation of T_{regs}. Enhanced recruitment of T_{regs} to the tumor microenvironment from the periphery can also occur through interaction of chemokine receptors on T_{regs} with chemokines produced by tumor cells, tumor-associated macrophages, or CD8$^+$ effector T cells within the tumor microenvironment. This chemokine signaling leads to the preferential accumulation of T_{regs} within the tumor

genic stimulation as the tumor grows, leading to expansion of local T_{regs}. In support of this concept, studies have found that T_{regs} within tumors have a distinct T cell receptor repertoire from conventional $CD4^+$ T cells [54] and that the T cell receptor repertoire of T_{regs} is largely skewed toward a few clonally expanded populations [55]. Also, T_{regs} are often the most proliferative immune cell type in tumors [47]. Expansion of T_{regs} specific for antigens present in normal tissue may partially explain the increased presence of T_{regs} within tumors, although this mechanism could exist in conjunction with enhanced trafficking of T_{regs} to tumors.

6.4.3 Regulatory T Cell Trafficking to Tumor Tissues

To perform their effector function, activated T_{regs} need to traffic to sites of inflammation within tissues. Trafficking of leukocytes is generally controlled via chemotactic cytokines known as chemokines. These chemokines interact with a specific array of cell surface transmembrane G protein-coupled receptors. For example, under normal physiologic conditions in the lymph node, expression of the chemokine receptor CCR7 on T_{regs} leads to their recruitment to T cell zones, where they have access to abundant IL-2 [56]. The recruitment of T_{regs} to T cell zones within lymph nodes highlights the function of chemokine receptors and ligands to target trafficking to specific anatomical locations. Similarly, secretion of specific chemokines by tumors or other immune cells within the tumor microenvironment can actively recruit T_{regs} through interaction with specific homing receptors on T_{regs}.

In cancer, specific chemokine/receptor interactions that recruit T_{regs} to the tumor are dependent on the tissue origin of the tumor and the cytokine milieu produced in the tumor microenvironment. The most commonly reported mechanism by which T_{regs} are recruited to the tumor microenvironment is through interaction of the chemokine CCL22 with CCR4 expressed on T_{regs}. First described in breast cancer, this pathway has been found to play an important role in recruiting activated T_{regs} to other tumor types including ovarian cancer, colorectal cancer, and head and neck cancer [57]. While tumor cells may actively secrete chemokines, tumor-associated macrophages (TAMs) are another major source of chemokines. TAMs were shown to be the major source of chemokines responsible for the recruitment of T_{regs} in ovarian cancer [58]. CCL22 secretion by tumor-infiltrating $CD8^+$ T cells can also drive T_{reg} recruitment into the tumor [59].

In the normal setting of inflammation, T_{regs} are recruited to limit tissue damage from the ensuing immune response. This is analogous to recruitment of T_{regs} to tumors via local expansion or chemokine-mediated recruitment. This highlights the co-opting of a normal biological process to promote tumor-induced tolerance. Studies in a wide variety of murine tumor models have demonstrated that T_{regs} play a central role in preventing antitumor immunity.

6.4.4 Regulatory T Cells and Prognosis

Many studies have evaluated associations between the frequency of T_{regs} in the tumor microenvironment and clinical outcome, including in head and neck [60], ovarian [58, 62], breast [63], pancreatic [64, 65], gastric [66, 67], lung [68], renal [69, 70], and liver cancers [71] and melanoma [61]. Studies assessing the frequency of T_{regs} within tumors have generally used tissue sections and have looked for FOXP3+ cells in the presence or absence of additional markers. Other studies have looked at the frequency of T_{regs} within tumors as a ratio of $CD8^+$ T cells to T_{regs} [72–75]. A recent meta-analysis of 17 different types of cancer across more than 15,500 cancer cases found that a higher frequency of T_{regs} in tumors was associated with poorer overall survival when considering all cancer types [76] (Fig. 6.5). Many of these studies used histology of tissue sections and have relied on identifying T_{regs} using staining for FOXP3 alone or with one or two other markers.

However, studies looking at the frequency of T_{regs} and their association with outcome have occasionally yielded conflicting results, with

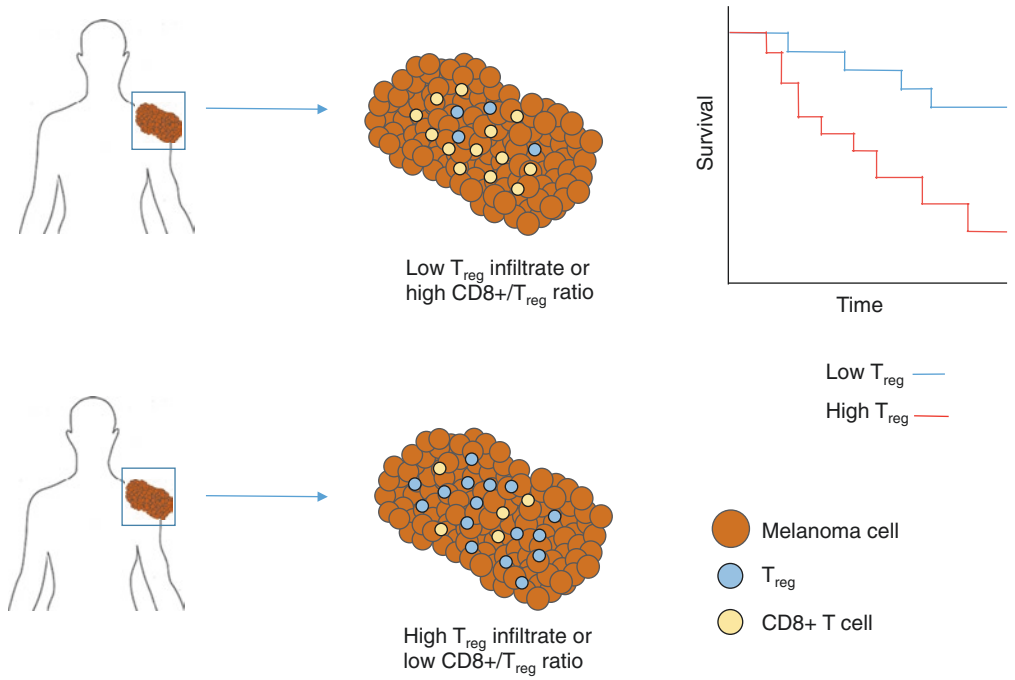

Fig. 6.5 Higher frequency of T_{regs} in tumors is associated with poorer prognosis. High absolute counts of FOXP3$^+$ T_{regs} within tumors or a low ratio of CD8$^+$ T cells to FOXP3$^+$ T_{regs} in tumors is associated with poorer outcomes (i.e., shorter overall survival) compared with low counts of FOXP3$^+$ T_{regs} or a high ratio of CD8$^+$ T cells to T_{regs}

some studies concluding that a higher frequency of T_{regs} is associated with poor clinical outcome and others showing that a higher frequency of T_{regs} is associated with better clinical outcome. These conflicting results have been found in studies looking at colorectal [77, 78], breast [79, 80], ovarian [81], and gastric [82] cancers.

Given the lack of clarity in the relationship between the frequency of T_{regs} in tumors and prognosis, recent work has attempted to further elucidate the role of FOXP3$^+$ T_{regs} in tumors. As discussed above, FOXP3 is predominantly expressed by T_{regs} but is also transiently expressed at lower levels in activated T cells. Given that human CD4$^+$FOXP3$^+$ T cells can contain both T_{regs} and activated effector T cells, studies that rely on FOXP3 histological analysis using no additional markers can have difficulty in accurately identifying T_{regs}. Erroneous identification of effector T cells as T_{regs} could underlie the variable conclusions reported. A recent study evaluating the role of CD4$^+$FOXP3$^+$ T_{regs} in colorectal cancer has sought to evaluate the role of FOXP3$^+$ T_{regs} versus FOXP3$^+$ effector T cells in controlling the prognosis in colorectal cancer [83]. These authors found that there were two distinct classes of colorectal cancer immune infiltrates: inflammatory and suppressive. Intriguingly, the authors found the role of FOXP3 expression was critically dependent on the class of immune infiltrate. In patients with suppressive tumors, a high frequency of FOXP3$^+$ T_{regs} was observed, and in this group, higher expression of FOXP3 was associated with poorer clinical outcome. In the second group of patients, with inflammatory tumors, a higher frequency of FOXP3$^+$ effector T cells was found. In this inflammatory group, higher expression levels of FOXP3 was associated with better overall survival. This important study highlights that not all human CD4$^+$ T cells that express FOXP3 are T_{regs} and that studies aimed at assessing the role of T_{regs} in tumors need to carefully classify FOXP3$^+$ cells as T_{regs} versus activated effector T cells. Additional studies in other cancer types are needed to fully understand the prognostic significance of T_{regs} in human cancer.

6.5 Immunotherapeutics and Regulatory T Cells

6.5.1 Altering the Balance Between Regulation and Inflammation

A hallmark of immunotherapy is the reinvigoration of the immune response against tumors. Exhausted $CD8^+$ T cells in the tumor microenvironment bear transcriptional hallmarks of dysfunction. $CD8^+$ T cells expressing inhibitory receptors exist on a spectrum of dysfunction, with higher levels of PD-1 expression, in conjunction with expression of additional inhibitory receptors such as LAG-3, TIM-3, and TIGIT, associated with the most dysfunctional cells [84]. Blockade of inhibitory receptors results in either partial or full functional reinvigoration of previously exhausted cells or a prevention of further exhaustion. However, not all patients respond to checkpoint inhibition. One potential explanation for the failure of patients who express PD-L1 within tumors to respond to PD-1/PD-L1 blockade is that their tumors may be enriched for T_{regs}. Increased frequencies of T_{regs} in $PD\text{-}L1^+$ tumors could lead to the failure of exhausted cells to be converted to effector cells due to the presence of suppressive cytokines, lack of co-stimulation, or inability to access presented antigens. Also, secretion of IL-35 by T_{regs} leads to expression of multiple inhibitory receptors on $CD8^+$ T cells, potentially rendering $CD8^+$ T cells unresponsive even in the presence of PD-1/PD-L1 blockade. Expression of multiple inhibitory receptors on $CD8^+$ T cells could significantly contribute to the lack of response following immunotherapy in patients, and many clinical trials are now currently investigating simultaneous blockade of multiple inhibitory receptors.

6.5.2 Potential Direct Effects of Therapeutics on Regulatory T Cells

Antitumor immunity is enhanced in some patients following blockade of CTLA-4 or PD-1/PD-L1. Much focus has been devoted to understanding the molecular dysfunction of effector $CD8^+$ T cells that express the inhibitory receptors CTLA-4 and PD-1 and the ways in which blockade of these inhibitory receptors improves the function of these effector cells. However, $CD8^+$ effector T cells are not the only cells that express these inhibitory receptors. Both effector $CD4^+$ T cells and T_{regs} can express inhibitory receptors, and their blockade, particularly on T_{regs}, may affect their frequency and function. While the most well-understood mechanisms governing immunotherapy are those that are controlled by effector $CD8^+$ T cells, potential effects of blockade of inhibitory receptors on T_{regs} are also an area of highly active research. PD-1 can be expressed on T_{regs}, but the effect of PD-1 signaling in T_{regs} is still unclear. CTLA-4 is also expressed on T_{regs}, and the role played by CTLA-4 in suppressing immune responses by T_{regs} is well described. However, the relative role that blockade of CTLA-4 on T_{regs} versus effector T cells has on antitumor immunity is an area of active research.

CTLA-4 is constitutively expressed by T_{regs} and is one of their key contact-dependent immunosuppressive mechanisms. Despite the appreciation of the role that $CTLA\text{-}4^+$ T_{regs} play in mediating immunosuppression, the enhancement of antitumor immunity has largely been attributed to the effects of blockade of CTLA-4 on conventional $CD8^+$ T cells [85]. The notion that the efficacy of CTLA-4 blockade is largely achieved through $CD8^+$ T cells is consistent with findings demonstrating that ligation of CTLA-4 limits activation of T cells in a cell-intrinsic manner. However, it is also possible that administration of anti-CTLA-4 may either deplete or alter the function $CD4^+FOXP3^+CTLA\text{-}4^+$ T_{regs}, leading to enhanced antitumor immunity.

Experimental evidence in support of the depletion of T_{regs} by CTLA-4 blockade comes from a mouse model in which the Fc receptor (FcR) portion of the antibody (the portion of the antibody that mediates antibody-dependent cellular cytotoxicity) of the CTLA-4 blocking antibody was mutated. In the absence of FcR binding, the effect of CTLA-4 blockade on tumor growth was largely lost, suggesting that antibody-dependent cellular cytotoxic elimination of T_{regs} may contribute to

the efficacy of CTLA-4 therapy [86, 87]. Secondly, emerging studies in humans have suggested that the efficacy of CTLA-4 therapy is dependent on the elimination of T_{regs} within the tumor microenvironment [88]. These studies in mice and humans demonstrate that depletion of CTLA-4$^+$ T_{regs} may play a role in the response following blockade of CTLA-4, but it is also possible that blocking this ligand on T_{regs} may affect their function. For example, CTLA-4 on T_{regs} interacts with CD80/CD86 on dendritic cells, leading to expression of indoleamine 2,3-dioxygenase (IDO). IDO-expressing DCs potently suppress T cell activation [89]. However, if CTLA-4 is blocked or CTLA-4+ T_{regs} are depleted, this may prevent upregulation of IDO on DCs, leading to enhanced antitumor immunity. Overall, the mechanisms of action by which CTLA-4 and PD-1/PD-L1 blockade enhanced antitumor immunity are areas of active investigation. It will be important to fully assess the impact of checkpoint inhibition of T_{reg} frequency and function.

6.6 Perspectives on the Importance of Regulatory T Cells in Immuno-Oncology

T_{regs} are an essential CD4$^+$ T cell subpopulation that control peripheral tolerance and immune homeostasis. However, T_{regs} can also limit antitumor immunity as demonstrated in a wide variety of mouse cancer models. There is also growing support for the importance of T_{regs} in limiting antitumor immunity in a broad range of human cancers. Consequently, effective therapeutic targeting of T_{regs} in cancer will likely be restricted to mechanisms that are selectively or preferentially utilized by intratumoral T_{regs}, without inducing detrimental autoimmune or inflammatory consequences. While new potential therapeutic targets for the selective modulation of intratumoral T_{regs} have recently been described, further T_{reg}-focused discovery efforts are clearly warranted. Whether T_{regs} limit the efficacy of checkpoint blockade (i.e., PD-1/PD-L1 or CTLA-4 blockade) is another important question that remains unresolved. The central role that T_{regs} play in normal physiology and cancer immunology suggests that future immunotherapeutics must carefully consider their impact on T_{reg} function in tumors and in the periphery.

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