Published in final edited form as:
Cell Mol Life Sci. 2009 August ; 66(16): 2603–2622. doi:10.1007/s00018-009-0026-2.

The Development and Function of Regulatory T Cells

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Abstract

Regulatory T cells [Tregs] are a critical subset of T cells that mediate peripheral tolerance. There are two types of Tregs: natural Tregs, which develop in the thymus, and induced Tregs, which are derived from naïve $CD4^+$ T cells in the periphery. Tregs utilize a variety of mechanisms to suppress the immune response. While Tregs are critical for the peripheral maintenance of potential autoreactive T cells, they can also be detrimental by preventing effective anti-tumor responses and sterilizing immunity against pathogens. In this review, we will discuss the development of natural and induced Tregs as well as the role of Tregs in a variety of disease settings and the mechanisms they utilize for suppression.

Keywords

Regulatory T cells; Foxp3; peripheral tolerance; inflammatory bowel disease; allergy; type 1 diabetes; multiple sclerosis; tumors

Introduction

The immune system uses many mechanisms to maintain immunologic self-tolerance and protect the host against exacerbated responses to foreign antigens. The existence of regulatory T cells [Tregs] that actively suppress the function of conventional T cells is a key mechanism by which the immune system limits inappropriate or excessive responses. The concept of 'suppressor' T cells has been around since the early 1970's [1,2] but because of the difficulty in proving their existence, due to the absence of the molecular and cellular tools we enjoy today, this concept was largely forgotten and relegated to phenomenology. In 1995, a landmark study by Sakaguchi and colleagues described a unique $CD4^+CD25^+$ T population that had potent regulatory activity [3]. In order to avoid the lingering skepticism that still surrounded 'suppressor' T cells, this sub-population was referred to as regulatory T cells [Tregs]. This discovery reawakened interest in the concept that there were specific subpopulations that could inhibit and regulate the immune response. In 2003, the Treg field made another significant leap forward with the discovery of *Foxp3*, a forkhead family transcription factor, as a critical regulator of Treg development, function and homeostasis [4–6]. Genetic mutations in the gene encoding *Foxp3* have been identified in both mice and humans [6,7]. The importance of Tregs first became apparent in patients with mutations in *Foxp3* who develop a severe, fatal systemic autoimmune disorder called Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, [8]. IPEX patients present at an early age in males and causes severe enlargement of the secondary lymphoid organs, insulin-dependent diabetes, eczema, food allergies and concomitant infections. Currently the only cure is bone marrow transplantation.

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A spontaneous *Foxp3* mutation in mice, known as "scurfy", results in symptoms that that are very similar to that seen in IPEX patients. It quickly became apparent that Tregs were very important regulators of peripheral tolerance and immune responses.

There are two types of $CD4^+$ Tregs, 'natural' Tregs (nTregs) and induced Tregs (iTregs), which are primarily defined by where they develop. nTregs develop in the thymus during the course of positive and negative selection, while iTregs develop in the periphery from conventional $CD4^+$ T cells following antigenic stimulation under a variety of conditions. Both nTregs and iTregs must achieve a fine balance between maintaining peripheral tolerance by suppressing potential autoimmune responses, while also controlling responses to infections. Often achieving this balance can be contradictory with Tregs overperforming or underperforming. For instance, Tregs have been shown to dampen local anti-tumor responses and prevent sterilizing immunity against certain chronic infectious agents. On the other hand, Tregs can occasionally be ineffective in mediating peripheral tolerance leading to an exacerbated inflammatory/allergic reaction or autoimmunity. Given this paradigm, therapeutic targeting of Tregs will have to be carefully controlled to ensure that cancer, for instance, is not replaced with rampant autoimmunity.

In this review, we will cover the development and function of both nTregs and iTregs, providing a brief overview of the various mechanisms used by Tregs to mediated suppression. We will illustrate the role of both Treg populations, as well as the mechanisms they utilize, in several contrasting disease settings. While most of the focus of this review is on murine Tregs, studies with human Tregs have been highlighted throughout.

Development of nTregs

nTregs, like all T cells, arise from progenitor cells in the bone marrow and undergo their lineage commitment and maturation in the thymus (Figure 1). nTregs comprise a small population, only ~5–10% of peripheral $CD4^+$ T cells [9], however their existence is vital. nTregs migrate from the thymus into the periphery after day 3 of life, and thymectomy of mice at day 3 results in lethal autoimmunity due to the lack of peripheral Tregs [10].

While there is no cell surface marker that uniquely identifies nTregs, there are a number cell surface proteins that are preferentially expressed on nTregs (Figure 1). The first identified was the high affinity IL-2 receptor α chain, CD25 [3]. This seminal discovery suggested that CD25 could be used as a marker for Tregs. However, CD25 is not unique to nTregs, as conventional T cells express CD25 when activated by T cell receptor (TCR) ligation. Since this original publication, numerous studies have been dedicated to the identification of specific cell surface markers expressed by nTregs. Purification of human nTregs, but not murine nTregs, can be enhanced by excluding T cells that express CD127. It has recently been shown that CD127 expression is inversely correlated with Foxp3 expression and suppressive capacity of nTregs [11]. It is well known that activation of a conventional T cell requires both TCR ligation as well as signaling through co-stimulatory molecules. Therefore, it is not surprising that nTregs express both effector molecules, such as CTLA-4, LAG-3, CD39 and CD73, and co-stimulatory molecules, such as CD28, CD80/86 (B7), CD40, OX40 and 4-1BB [12–14]. However, none described to date are restricted to Tregs [14]. Other molecules that may be useful surface markers of nTregs, but whose functions are not clearly elucidated yet, include neuropilin-1 and folate-receptor 4 (FR4). The most specific marker of Tregs is *Foxp3*, which is expressed exclusively in thymus-derived nTregs and certain peripheral iTreg populations that have suppressive capabilities. However due to its nuclear expression, *Foxp3* cannot be used to purify or mark nTregs [5,6]. In contrast to murine T cells, human $CD4^+CD25^-$ T cells that are activated can upregulate expression of Foxp3 without acquiring suppressive capacity [15]. However, genetic mutations in the gene encoding *Foxp3* are fatal for both mice and

humans [6,7]. These findings suggest that although not all Foxp3$^+$ human T cells are suppressive, *Foxp3* still appears to be the "master regulator" of nTreg development and function. In accordance with its important role in nTreg function, it was also demonstrated that conventional T cells forced to express *Foxp3* via retroviral transduction acquired regulatory capacity both *in vitro* and *in vivo* [4,5].

Signals that influence the development of nTregs *in vivo* are not entirely clear. However, it is known that nTreg development is influenced by co-stimulatory molecules and cytokines, TCR and antigen affinity, and the location and context within the thymus where antigen is encountered.

Co-stimulatory Molecules and Cytokines

Like conventional T cells, Tregs are selected by peptides presented by antigen presenting cells (APCs) in the thymus. However, co-stimulatory molecules such as CD28 [16], CD80/86 (B7), CD40 [12] and IL2rβ [17] appear to be especially critical for the development of nTregs. Mice deficient in these molecules have reduced numbers of nTregs with impaired suppressive capacity. Also critical to the development of nTregs are IL-2 and, to a lesser degree, TGFβ. Although nTregs express the high-affinity IL-2 receptor CD25, they do not make IL-2 themselves due to chromatin inaccessibility of the IL-2 locus [18]. Consequently, nTregs are absolutely dependent upon paracrine IL-2 for survival and growth. In addition to IL-2, TGFβ also appears to play a role in the development of nTregs. It was originally thought that nTreg development was independent of TGFβ since Treg development is unaltered in TGFβ receptor dominant negative mice [19]. However, it was recently shown that sustained TGFβ is required to maintain Foxp3 expression and suppressor function of peripheral nTregs both *in vitro* and *in vivo* [20]. Clearly, further studies will need to be performed to determine whether TGFβ has an analogous role in the thymus.

TCR Ligation, Strength of Signal and Self-Peptides

Conventional T cells undergo selection in the thymus based on the strength of signal they receive from thymic APCs [DCs, medullary (mTEC) or cortical epithelial cells (cTEC)] presenting self peptides. Strong signals due to high affinity or avidity interactions between the TCR and MHC:peptide complexes results in negative selection. No or very low signal leads to thymocyte death by neglect. Positive selection of thymocytes that will survive and populate the periphery occurs when intermediate to weak signals are delivered via engaged TCR (reviewed in [21]). However, it has been suggested that nTregs differ in the selection process in that they are positively selected on a TCR affinity/signal strength that is between that required for the positive and negative selection for conventional T cells [22]. Like conventional T cells, nTregs have a polyclonal TCR repertoire and are selected on self-peptides. However, unlike conventional T cells, Tregs appear to have more exacting requirements regarding the composition of self and the amount of signal required for their development as they usually do not develop in mice that express a single TCR [23]. However, Treg development is restored if a significant amount of the cognate antigen for which the TCR is specific is expressed in the thymus. To further study the affinity of nTregs for self-peptides, TCR genes from a large cohort of conventional T cells and nTregs were cloned and sequenced [24]. Like TCRs from conventional T cells, nTreg TCRs were extremely diverse, but the overlap between TCR sequences of conventional T cells and nTregs was minimal, approximately 15–20%. However, unlike conventional T cells, nTregs were capable of recognizing and signaling in response to self-peptides. More recently, hundreds of conventional T cell and nTreg TCRs were sequenced and quite different conclusions drawn [24,25]. In these studies, the data suggest that nTregs do not preferably recognize self-antigens, but instead express a polyclonal TCR repertoire that is comparable to conventional T cells. Collectively, these contrasting conclusions suggest that significantly more analysis will be required before firm conclusions can be made.

While some of these data suggest that nTregs have "self-reactive" TCRs, an alternative theory is that nTregs simply have a lower activation threshold than that of conventional T cells. It is important to note that in essence all selected TCRs are self reactive. If they were not, they would not have driven positive selection. So the more important criterion might be the combination of TCR affinity for self, coupled with the differential ability of conventional versus regulatory T cells to propagate a signal that mediates a functional outcome. Indeed, it has been shown that human Tregs are responsive to TCR stimulation at 10- to 100-fold lower antigen concentrations than that required to activate a human conventional T cell [26]. This sensitivity may, in part, be the result of their expression of additional or enhanced levels of certain co-stimulatory and/or accessory molecules. Alternatively, there may be differences in the strength or efficiency of the TCR:CD3 signaling cascade. While it has been previously demonstrated that nTregs require activation to achieve optimal suppression regardless of the antigen specificity of the target cell [27,28], recent evidence suggests that nTreg activation may not be required to achieve some level of suppression [29]. This suggests that the requirements needed to initiate trademark responses, proliferation or suppression, are different between conventional T cells and nTregs, respectively. Moreover, when bone marrow cells were transduced to express a nTreg-derived TCR and adoptively transferred into irradiated mice, the ratio of nTreg:conventional T cell was dramatically increased [30]. However, such analysis has so far only been conducted on a very limited number of Treg-derived TCRs and thus additional studies are required before a definitive conclusion can be drawn.

It has also been suggested that the self-peptides that mediate selection of nTregs in the thymus may also promote their expansion and the conversion of conventional Tregs into "induced" Tregs (iTreg) in the periphery [31]. However, as highlighted above more recent studies have suggested that there is nothing 'unique' about the TCR repertoire expressed by nTreg and that they do not preferentially have a higher affinity for self-antigens [24,25]. Moreover, nTregs recognize foreign antigens with the same frequency as conventional T cells, leading some to suggest that thymic peptides that participate in selection may be different to peptides that drive proliferation and function of peripheral nTregs [25]. Therefore, it has recently been proposed that the stage at which T cells are committed to the Treg lineage occurs prior to TCR-mediated selection and that even weak TCR:self peptide-MHC interactions are sufficient to allow survival in the thymus [32–34].

Site of nTreg Development

In addition to TCR signal strength and affinity for peptide, the context within the thymus where antigen is encountered may have an impact on whether a thymocyte is selected, deleted or becomes a Treg. Using an inducible promoter system to quantitatively alter the degree of thymic expression of an MCC-derived T cell epitope, it was shown that the absolute number of nTregs remained constant despite different degrees of expression [34]. The authors found that the number of conventional T cells that were deleted increased with increasing thymic antigen, however the number of nTregs was unchanged, owing to resistance to deletion. This suggests that it is not the affinity for self-peptide, but rather, the context or niche in which the T cells encounter antigen that is most important in determining whether the cell is selected or deleted [34].

Control of the peptide repertoire that is expressed in the thymus undoubtedly affects the T cells that develop. The autoimmune regulator gene (*Aire*) promotes the expression of tissue-specific self-antigens by thymic medullary epithelial cells [35]. Aire expression is critical for the establishment of central tolerance toward certain self-peptides, allowing thymocytes that are strongly self-reactive to be deleted [21,36]. *Aire*-deficient mice develop multi-organ autoimmunity due to compromised negative selection. However, it has also been suggested that other forms of tolerance, such as impaired development of nTregs, may play a role in this

autoimmunity. A number of reports suggest that in addition to promoting deletion of self-reactive conventional T cells, *Aire*-expressing stromal cells may also enhance *Foxp3* upregulation in $CD4^+$ thymocytes and nTreg development in response to self-peptides [37–39]. However, other studies have shown that in the absence of *Aire*, antigen specificity of the nTregs is modestly altered, but the number, frequency and function of nTregs remain intact [21,35,40,41]. Therefore, while it seems possible that *Aire*-dependent antigens may play a role in the thymic development of nTregs, this issue is controversial and still remains to be resolved.

Another currently debated issue is that of the timing and location of nTreg development. Using $Foxp3^{gfp}$ reporter mice it was shown that $Foxp3^+$ cells are found only in the thymic medulla suggesting that nTreg development occurred relatively late in thymic development [42]. In addition, a group of epithelial cells expressed in the thymic medulla called the Hassall's corpuscles were suggested to play a role in the differentiation and development of human nTregs. Human Hassall's corpuscles express thymic stromal lymphopoietin (TSLP) which conditions thymic DCs to express CD80 and CD86. Ligation of MHC and CD80/86 by $CD4^+$ thymocytes induced differentiation $CD4^+$ thymocytes into $Foxp3^+$ nTregs [43]. However, recent studies have challenged the idea that nTreg differentiation occurs this late in thymic development. Although *Foxp3* is not detected in the thymic cortex, some studies suggest that commitment to the nTreg lineage might occur in the cortex, at an earlier point in thymic development [25,40,44,45]. Indeed, by limiting the expression of MHC to mTEC, cTEC and/or DCs it has been shown that multiple accessory cell types in both the thymic cortex and medulla are capable of mediating Treg development [45]. In further support of this hypothesis, researchers have shown that limiting the capacity of $CD4^+CD8^+$ (DP) thymocytes to influence early γδ T cell or αβ T cell progenitors leads to preferential differentiation of T cells into $Foxp3^+$ nTregs [33]. Moreover, this appears to occur prior to, and independent of, agonist selection. Another study elegantly showed that within the thymic $CD4^+$ population, there exists a $CD25^{high}$ population that represents the immediate precursors to nTregs which are primed and ready to express *Foxp3* following stimulation with IL-2 or IL-15 [46]. Not only does this suggest that a precursor population exists that are poised to become nTregs, but it also suggests that in addition to TCR engagement, IL-2 and the IL-2R play a pivotal role in the development of nTregs.

Maintenance of nTreg Homeostasis

Signals that are important for nTreg development also appear to be important for their maintenance in the periphery (Figure 1). By generating mice with a stop codon in the *Foxp3* gene, investigators were able to monitor the transcription of the Foxp3 allele in the absence of Foxp3 protein synthesis [38]. In comparing these Tregs to nTregs, which transcribe and translate Foxp3 protein, and to conventional T cells, the authors could decipher Foxp3 protein-dependent features from those associated with Foxp3 expression. Their results indicated that Foxp3 plays a critical role in the peripheral maintenance of nTreg phenotype stability, including anergy and dependence on IL-2. Therefore, it appears that in addition to its role in nTregs development, Foxp3 helps to maintain the stability nTreg features in order to preserve the nTreg lineage. TGFβ signaling in peripheral nTregs is critical to their ability to suppress a variety of cell types including Th1 cells, $CD8^+$ cytotoxic T cells and NK cells [47]. In addition to this role in the function of nTregs, TGFβ is also important in maintaining Foxp3 expression, nTreg homeostasis and maintenance in the periphery [20].

Perhaps most critical to the maintenance of nTregs are CD28 and IL-2. Owing to their dependence on paracrine IL-2 for survival [18], nTregs are beholden to conventional T cells for peripheral maintenance. *Cd28*-deficient mice have reduced numbers of nTregs in the spleen and lymph nodes and the resulting nTregs have reduced suppressive capacity when compared to wild-type nTregs [48]. CD28 appears to support the survival of nTregs by enhancing IL-2

production by conventional T cells in addition to maintaining CD25 expression on nTregs [42,49]. IL-2 signaling seems to be critical, not just for survival of nTregs, but also for their generation and function in the thymus and the periphery. Utilizing mice that were deficient in IL-2, IL-2Rα and IL-2Rβ, studies show that while IL-2 and IL-2Rα were dispensable for nTreg thymic development, IL-2Rβ was absolutely required [42]. Genetic deficiency of CD25 in humans results in similar clinical manifestations as IPEX, underscoring the importance of IL-2 in nTreg function and maintenance [50]. In addition, in both murine and human studies, neutralizing anti-IL-2 induces multi-organ autoimmunity and significantly reduces the number of nTregs in the thymus and periphery [51,52]. This suggests that both thymic development and peripheral expansion of nTregs absolutely require IL-2.

Development of iTregs

While thymically-derived nTregs play a critical role in immune homeostasis, it is clear that regulatory T cells can be induced from conventional, naïve $CD4^+$ T cells both *in vitro* and *in vivo*. One of the challenges in the field of regulatory T cells is determining the relative contributions of nTregs versus iTregs *in vivo*. Indeed, it appears that the main difference between the two subsets is their origin of development (thymus versus periphery) (Figure 1). However, it has been proposed that nTregs and iTregs differ not primarily in their origin, but rather as a consequence of differentiation through antigen exposure and specific factors that are highly expressed in distinct settings [53]. The importance and contribution of each particular iTreg subset is most likely dictated by the context of the antigen and environment. Two main subsets of iTregs that are generated in the periphery have been described, based upon the cytokines that cause their induction: type 1 regulatory T cells (Tr1), which are induced by IL-10 [54,55], and T helper 3 (Th3), which are induced by TGFβ [56]. While both subsets are generated in the presence of different cytokines, they exert their suppressive activity through secretion of the same cytokines that are responsible for their induction, IL-10 and/or TGFβ, respectively. While TGFβ and IL-10 are the primary cytokines involved in iTreg formation, it has also been demonstrated that IL-4 and IL-13 can induce the development of $Foxp3^+$ Tregs from $Foxp3^-$ naïve T cells independently of TGFβ and IL-10 [57]. Both IL-4 and Il-13 signal through the IL-4Rα chain, suggesting an essential role for this receptor in the generation of Tregs in the periphery.

There has been considerable interest in determining the role of each Tregs subset in immune function, primarily because iTregs represent a powerful therapeutic tool for autoimmune disease, inflammation and anti-tumor treatment. While they are distinct, both subsets have similar phenotypes and utilize overlapping mechanisms of suppression. Both nTregs and iTregs share cell surface markers characteristic of an activated T cell, such as CD25, CTLA-4, GITR, CD62L, $CD45RB^{lo}$ (reviewed in [58]). The transcription factor Foxp3 is expressed by Th3 cells following induction. However, Tr1 cells do not express Foxp3, either constitutively or following activation either *in vitro* and *in vivo* [55]. Transfer of either iTregs or nTregs has been shown to prevent the development of autoimmune disease in several models as well as help promote transplantation tolerance [3,59–62]. While their phenotype and modes of suppression may be similar, nTregs and iTregs in mice appear to have different requirements for their development. While nTregs may develop in response to self antigen, or at least strongly ligating peptides in the thymus, iTregs develop in response to weaker, sub-optimal TCR stimulation and exogenous antigens in the periphery [63,64]. Although it remains possible that the TCR repertoire of iTregs includes high affinity for self antigens, these cells are primarily generated in inflammatory settings in the presence of anti-inflammatory cytokines. Co-stimulation through CD28 is also required for the generation of nTregs while iTregs are able to develop in the periphery in its absence [64,65]. Furthermore, *in vitro* conversion of iTregs can occur in the absence of CD28 stimulation and result in cells that remain functional following *in vivo* transfer [66]. In fact, recent studies have indicated that co-stimulation can

actually hinder iTreg development *in vitro*, which may explain the reduced stability of Foxp3 expression and limited lifespan of iTregs stimulated in the presence of anti-CD28 antibodies [67,68]. Because of the potential for therapy, there has been a great deal of interest in generating and expanding human iTregs *in vitro*. Human iTregs have been generated *in vitro* using in a variety of conditions, including allogeneic DCs [69,70], anti-CD3 and 4C8 administration [71] and SEB exposure [72]. Furthermore, human iTregs could be generated *in vitro* in the absence of TGFβ [73,74]. Finally, one study has shown that human Tr1 cells can be generated from $CD4^+$ T cells in the presence of IL-2 through the engagement of CD3 and the complement regulator CD46 [75].

In addition to strength of signal, many other factors can contribute to the generation of iTregs, including antigen and route of exposure, cytokines, tissue specific factors and APCs. Intranasal or oral exposure to antigen tends to selectively induce the generation of iTregs [76,77]. APCs play an important role in iTreg generation. Monocyte-derived DCs, including plasmacytoid dendritic cells (pDCs) can induce Treg formation [70,78,79]. DCs in the gut associated lymphoid tissue (GALT) are particularly efficient at inducing Treg formation [76,77,80] as well as DCs present in tumor microenvironments [81]. It has been shown that Tregs can induce DCs to become tolerogenic [82], which in the GALT provides a positive feedback loop, in the presence of TGFβ, that leads to further induction of iTreg formation [80]. In addition, it has been shown that a population of macrophages ($CD11b^+F4/80^+CD11c^-$) in the lamina propria (LP) could induce $Foxp3^+$ Tregs in a retinoic acid (RA)-, TGFβ- and IL-10-dependent manner [83]. Finally, studies have shown that NKT cells can play a role in iTreg generation by induction of tolerogenic APCs in response to oral antigen [84] as well as in EAE [85]. Tregs have been shown to induce suppressive properties in other T cell populations, a process often referred to as ‘infectious tolerance’. Early studies showed that T cells from tolerized animals could be transferred and retain their tolerant state [86]. It was later shown that the maintenance of this transplantation tolerance was due to Tregs inducing the conversion of the suppressed T cells into iTregs [87]. Others have shown that co-culture of nTregs with naïve $CD4^+$ T cells leads to iTreg formation whose suppressive ability was dependent on the cytokines IL-10 or TGFβ [88,89].

Tregs also exhibit considerable plasticity in the periphery. There have been a number of studies that have demonstrated a reciprocal relationship between Th17 cells and Tregs (reviewed in [90]). While TGFβ induces the conversion of naïve $CD4^+$ T cells into iTregs, the addition of IL-6 and IL-21 inhibits this process and promotes Th17 conversion [91]. Furthermore, activated Tregs, which produce high levels of TGFβ differentiate into Th17 cells in the presence of IL-6 [92]. In contrast, RA drives the induction of Tregs and inhibits Th17 differentiation, presumably by enhancing TGFβ signaling and inhibiting IL-6 signaling [76].

Type 1 Regulatory T Cells

Tr1 cells are defined by their requirement for IL-10 for their induction and their ability to produce high levels IL-10 and TGFβ to mediate suppression (Figure 1) [54,93]. Co-culture of both mouse and human $CD4^+$ T cells with Tr1 cells in transwell assays reduced the proliferative response of the $CD4^+$ T cells. The addition of neutralizing antibodies to IL-10 and TGFβ reversed the action of Tr1 suppression, indicating an essential role for these cytokines in Tr1 mediated suppression [54]. While the true hallmark of Tr1 cells is high level IL-10 production, additional cytokines such as IL-5 and IFNγ are also secreted by Tr1 cells depending upon the experimental conditions [93]. Thus far, no specific markers have been identified for Tr1 cells. While their proliferative capacity is low, they can expand in the presence of IL-2 and IL-15, due to the high levels of receptors for these cytokines expressed following activation [94]. However, *Foxp3* is not expressed by Tr1 cells [55] and, like TGFβ-dependent converted Tregs,

Tr1 cells can be generated in the absence of nTregs [95], suggesting Tr1 cells may also be developmentally distinct [55].

The induction of Tr1 cells is primarily mediated by IL-10 producing APCs which occur in a variety of immunological settings. Studies have shown that production of IL-10 by immature DCs (iDC), tolergenic myeloid DCs and pDCs induces the generation of Tr1 cells in transplant settings and in response to allergens, pathogens and tumor antigens in mice and humans [93] [89]. The secretion of IL-10 has been shown to be important in mediating suppression of murine and human T cells *in vivo*, such as those responsible for the development of inflammation in the gut [54,96]. Mice deficient in *Il10* succumb to inflammatory bowel disease (IBD), which can be prevented in young mice by the addition of exogenous IL-10. As Tr1 cells produce high levels of IL-10, transfer of these cells can prevent induction of IBD by $CD4^+$ effector T cells [96]. In addition to IBD and mucosal immunity, Tr1 cells have been shown to play a key role in regulating allergic immune responses in a wide range experimental conditions (reviewed in [93]).

Th3 Cells and the Role of TGFβ in iTreg Development

Perhaps the most prominent factor in the conversion of naïve $CD4^+$ T cells to iTregs is the cytokine TGFβ. These iTregs, defined by some as Th3 cells, develop both *in vitro* and *in vivo* in the presence of TGFβ. It is well established that antigen stimulation of naïve murine $CD4^+$ T cells *in vitro* in the presence of TGFβ leads to the induction of Foxp3 expression and regulatory activity [59,63,64]. Furthermore, a substantial number of studies have demonstrated that TGFβ induces conversion of $CD4^+$ T cells *in vivo* in both mice and humans. These iTregs are particularly important in a variety of disease settings and in maintaining tolerance to antigens expressed in the intestinal tissue, which has been described as a “privileged site” for iTreg differentiation [59,63,97–100]. Interestingly, the TGFβ-dependent generation of iTregs can occur in mice that completely lack nTregs [59,64,87], providing support that iTregs are developmentally distinct from nTregs.

TGFβ-deficient mice succumb early on to spontaneous autoimmune disease [101]. Similarly, mice expressing a dominant-negative form of the TGFβRII manifest a similar, systemic disease, characterized by spontaneous T cell activation and infiltration [19]. The importance of Tregs in mice lacking TGFβ was not fully appreciated prior to the discovery of TGFβ-induced Treg formation. nTregs develop normally in TGFβ-deficient mice, indicating production of TGFβ by nTregs is not required for suppression of inflammation [102]. Their maintenance and function in the periphery, however, is adversely affected in the absence of TGFβ [20]. The importance of TGFβ-induced iTreg generation in controlling disease is evident from studies showing that $CD4^+$ and $CD8^+$ T cells from mice resistant to TGFβ signaling are unable to be controlled by Tregs upon transfer, resulting in IBD [103] and reduced tumor rejection [104], respectively. These data indicate that TGFβ plays pivotal roles in regulating tolerance via the maintenance and function of nTregs and by directly regulating conventional T cells.

As mentioned, a great deal of research in the iTreg field has focused on the mucosal tissues of the small intestine, as it has many features that make it a highly tolerogenic, and thus suitable, environment for iTreg formation. In particular, high concentrations of anti-inflammatory cytokines such as TGFβ, IL-4 and IL-10, which increases the generation of both TGFβ-induced Th3 and IL-10-induced Tr1 cells [54]. Recently, however, exciting new research had shed additional light on the mechanism of iTreg formation in the GALT, including factors that specifically promote the immune integrity of this highly antigenic environment. It has been known for some time that T cells express homing receptors that mediate migration into the gut, namely the integrin α4β7 and the chemokine receptor CCR9 [105,106]. Several groups have shown that these gut homing receptors are induced on T cells by a key metabolite of vitamin

A, retinoic acid (RA), that is generated by DCs in the GALT [106]. Reduced numbers of T cells were found to home to the gut tissues in mice that were either deficient in vitamin A or retinoic acid receptor (RAR) signaling [106]. TGFβ produced by a variety of cells in the intestine also induces the up regulation of the integrin αEβ7 (CD103) on DCs, a population of cells that are particularly capable of producing RA [76,80]. Work by several groups has now shown that RA enhances the TGFβ-dependent conversion of T cells into iTregs [67,77,80].

Analysis of the cell types involved in iTreg conversion revealed that DCs derived from the spleen were unable to induce Foxp3 expression in naive T cells. However, DCs from the peyer's patches (PP), MLN and lamina propria (LP) of the gut could induce the generation of iTregs [77,80]. This induction correlated with CD103 expression, as $CD103^+$ DCs, but not $CD103^-$ DCs, were able to induce conversion, and required TGFβ, as neutralizing anti-TGFβ antibodies blocked this process [80]. While RA alone was not able to induce conversion, inhibitors of retinol dehydrogenases greatly diminished the conversion process [77,80]. In addition, RA enhanced upregulation of α4β7 on the converted iTregs allowing them to home to the GALT more efficiently [67,80,107]. Lastly, RA was able to enhance the TGFβ-dependent conversion of iTregs *in vitro*, which upon adoptive transfer in an IBD model, were able to suppress the induction of colitis more efficiently than TGFβ alone treated cells [76]. These data indicate that, at least in some disease models, the mechanisms by which Tregs develop and function may be a possible way to target Tregs for therapeutic benefit. While there is considerable overlap with regards to cytokine usage, perhaps additional tissue specific and/or environmental factors may also play a role in maintaining tolerance, similar to those found in the GALT.

Role and Mechanisms of Treg Suppression in Disease

How Tregs suppress has generated a tremendous amount of research effort since Tregs were identified [3]. There have been numerous manuscripts and reviews discussing and dissecting the mechanisms of suppression used by Tregs (see our previous reviews for a more in depth discussion of basic Treg mechanism: [14,108]). From these studies, it has become clear that Tregs do not rely on a single mechanism of suppression but rather have an arsenal of regulatory mechanisms at their disposal. These can be divided into four basic modes of action as depicted in Figure 2: inhibitory cytokines, cytolysis, metabolic disruption and modulation of APC function [14,109,110]. For the purpose of this review, we will provided a brief summary of the mechanisms of suppression utilized by Tregs followed by a more in depth analyses of studies that have contributed to our current knowledge of both the role and mechanisms of Treg-mediated suppression in various key disease settings. The six types of disease states that we will focus on are inflammatory bowel disease (IBD), allergy and asthma, type I diabetes (TID), multiple sclerosis (MS), tumors and infections.

Mechanisms of Treg Suppression

The inhibitory cytokines IL-10, TGFβ and the recently described IL-35 areexpressed by Tregs and are considered a major mechanism of suppression utilized by Tregs. Interestingly, the concept of a soluble factor mediating Treg suppression is still controversial, considering the cell to cell contact dependence that was thought to be required to mediate suppression. However there is a growing list of *in vivo* studies describing the importance of Treg-derived IL-10, TGFβ and IL-35 in the suppression of various immune responses [14,109,110].

Cytolysis by Tregs is mediated by granzyme A in humans and granzyme B in mice. While the dependence of perforin remains in question, it is clear that in both *in vitro* and *in vivo* models Tregs appear capable of killing target cells in a granzyme-dependent manner [111,112].

Recently, there have been several studies describing novel mechanisms of Treg suppression mediated through metabolic disruption. In these studies, the conventional T cells were

suppressed by: (1) IL-2-deprivation mediated apoptosis, (2) the generation of pericellular adenosine by CD39 and CD73 and the subsequent activation of the adenosine receptor 2A on conventional T cells, and (3) the transfer of the inhibitory second messenger cyclic AMP (cAMP) into conventional T cells via gap junctions [14,109,110].

Finally, Tregs can suppress target cells by augmenting APC function. This is primarily mediated through the interaction of cell surface molecules on Tregs such as CTLA-4 and the lymphocyte activation gene 3 (LAG-3) and their interaction with CD80/CD86 and MHC class II, respectively, on APCs [14,109,110]. This interaction results in the reduced ability of the APCs to activate conventional T cells. Additionally, there is evidence to suggest that Tregs can mediate the production of the immunoregulatory tryptophan–degrading enzyme, indoleamine 2,3-dioxygenase (IDO) by DCs [113]. Taken together, these mechanisms provide a potent arsenal for Tregs to utilize in maintaining peripheral tolerance and this is perhaps a reflection of the fact that Tregs need to suppress multiple cell types in a variety of distinct anatomical and disease settings.

Inflammatory bowel disease

IBD encompasses Crohn's disease and ulcerative colitis which manifests as chronic inflammation of the gastrointestinal tract. Disease susceptibility is governed by genetic and environmental factors. Individuals with IBD exhibit aberrant inflammation towards the normal bacterial flora in the gut. Several murine models, including DSS, TNBS and T cell-induced colitis, have been utilized to understanding the etiology and mechanisms underlying IBD. Among these murine models, the T cell transfer model is a well established system in which the role of Tregs in protection against IBD has been dissected. Therefore, for the purpose of this review we have mainly focused on the studies pertaining to the function of Tregs in mediating protection from IBD in this model. In the murine T cell transfer model, a small number of naïve $CD4^+CD45RB^{hi}$ T cells are transferred into immunodeficient *Rag*$^{-/-}$ or scid mice which leads to Th1-mediated colitis in approximately 4–6 weeks. This condition can be prevented or reversed by co transfer of Tregs or by adoptive transfer of Tregs once the pathology is established [61,114]. The majority of studies point to the cytokines IL-10 and TGFβ as key regulators used by Tregs to control IBD. In addition, $CCR4^+$ and $CCR7^+$ Tregs are found to be important in mediating the activity of Tregs at sites of inflammation in an IBD setting. Recently our lab has demonstrated a critical role for IL-35 in regulating IBD [115].

The importance of IL-10 in immunoregulation became evident upon analysis of *Il10*$^{-/-}$ mice, which develop enterocolitis [116]. However, under germ-free or Helicobacter-free conditions this does not occur, suggesting that IL-10 is important in controlling the inflammatory responses against certain commensal bacteria in the normal flora. Indeed, administration of rIL-10 can protect scid mice from colitis following transfer of $CD4^+CD45RB^{hi}$ T cells in the absence of Tregs [117]. While these studies indicate a role for IL-10 in maintaining intestinal homeostasis, a direct link between IL-10 and Treg function was not provided until it was demonstrated that $CD4^+CD45RB^{lo}$ T cells isolated from *Il10*$^{-/-}$ mice failed to prevent IBD when co-transferred with $CD4^+CD45RB^{hi}$ cells [118]. Moreover, the $CD4^+CD45RB^{lo}$ T cells from *Il10*$^{-/-}$ mice induced inflammation in *Rag*$^{-/-}$ mice when administered alone. Consistent with these reports, treatment with anti-IL-10R antibody abolishes the protection mediated by Tregs in a T cell transfer model as well as in *H.hepaticus*-induced intestinal inflammation, suggesting a critical role for Treg-derived IL-10 in regulating intestinal inflammation. IL-10 produced by Tr1 cells does not appear to play a role in controlling inflammation as wild-type $CD4^+CD45RB^{lo}$ T cells could protect against colitis induced by *Il10*$^{-/-}$ $CD4^+$ effector T cells [119]. This suggested that differentiation of IL-10-induced Tr1 cells from $CD4^+$ $CD45RB^{lo}$ effector T cells is not critical for the control of inflammation. This, however, does not exclude the possibility that IL-10 producing cells develop from the $CD4^+CD45RB^{lo}$ population.

Although these studies provided evidence for a major role of IL-10 in the function of Tregs in IBD, treatment with anti-IL-10R antibody did not completely abolish the protection from colitis in CB-17-scid mice. In addition, *Il10*$^{-/-}$ CD4^{+}CD25^{-} T cells were also protective [119], thus providing contradictory evidence for the role of Treg-derived IL-10 in the regulation of inflammation. However, deletion of IL-10 specifically in Tregs shed insight into these contradictory data. Conditional IL-10 deletion in Tregs resulted in colonic inflammation. However, inflammation, onset and incidence of disease were less severe than that seen in the *Il10*$^{-/-}$ mice [120]. These studies point to the fact that even though IL-10 derived from other cell types partially alleviate the symptoms, IL-10 derived from Tregs plays an important role in suppressing the local inflammation. This is consistent with evidence that in IBD, IL-10-producing Tregs are selectively enriched in the colonic LP and secondary lymphoid organs [121]. Similarly in humans, Crohn's disease patients have intestinal CD4^{+} T cells that are defective in producing IL-10 [122].

IL-10, however, is not the sole player in the control of intestinal homeostasis. Other factors such as TGFβ and IL-35 also play a role. TGFβ deficient mice develop early multifocal inflammatory disease which leads to their death within 3–5 weeks of age [123]. In the absence of T cell specific TGFβ, mice develop colitis [101]. Tregs from these mice potentiated disease rather than cause protection in an IBD model [124]. Furthermore, anti-TGFβ neutralizing antibodies abrogate the ability of wild-type Tregs to mediate protection [125]. While it is clear that Treg-derived TGFβ is important in controlling colitis, there is evidence that TGFβ derived from other cells contributes to this protection. CD4^{+}CD25^{-} T cells that express latency associated peptide (LAP) on the surface were found to suppress colitis induced by naïve T cells [126]. In addition, co-administration of *Tgfb*$^{-/-}$ Tregs with anti-TGFβ neutralizing antibody could not protect from naïve T cell induced colitis [103]. Finally, anti-TGFβ antibody was also shown to enhance colitis induced by CD4^{+}CD25^{-} T cells [127]. Taken together, these reports suggest that Treg-derived TGFβ, along with iTreg-derived TGFβ and additional gut-specific factors, play a critical role in IBD [128].

Recent work from our laboratory described a novel inhibitory cytokine, IL-35, that is preferentially expressed by Tregs and important for maximal Treg function [115,129]. IL-35 is a heterodimeric cytokine composed of Ebi3 and p35 chains and a new addition to the IL-12 family of cytokines. Tregs from either *Ebi3*$^{-/-}$ or *Il12a*$^{-/-}$ mice failed to cure IBD in *Rag*$^{-/-}$ mice suggesting an important role for IL-35 in Treg-mediated protection from IBD [115]. Interestingly, initial studies with human Tregs suggest that EBI3 and hence IL-35 is not constitutively expressed in human Tregs [130]. However, further analysis is required to determine whether IL-35 is up regulated in human Tregs during an inflammatory response.

Taken together, it is apparent that IL-10, TGFβ and IL-35 are important mediators of suppression in Treg-mediated protection in IBD. It will be important to determine the relative contribution of each these cytokines in IBD in relation to one another. Furthermore, it remains unclear whether other Treg mechanisms play a role in Treg-mediated protection against IBD. In addition, they may ultimately form the basis of a future therapeutic modality.

Allergy and Asthma

The importance of Tregs in an airway hypersensitivity reaction (AHR) was clearly demonstrated in a study in which depletion of Tregs in allergen-sensitized mice resulted in increased levels of Th2 cytokines, IgE, and AHR [131]. In humans, Tregs from allergic donors have been found to be functionally defective in their ability to suppress the proliferation and IL-5 secretion of allergen-stimulated CD4^{+} T cells compared to Tregs obtained from non-allergic donors [132]. In patients with atopic dermatitis, the functional defect does not reflect the reduction in numbers of Tregs [133]. However, children with asthma have fewer Tregs than the non-asthmatic patients [134]. Both nTregs and antigen-induced Tr1 cells have been

implicated in controlling the allergen-induced Th2 response in mice and humans. The importance of IL-10 in protection against allergy became evident from human studies in which non-allergic individuals have a higher numbers of IL-10-producing activated T cells in response to IL-10 [135]. A role for Treg-derived IL-10 was clear when transfer of antigen-specific Tregs prior to allergen challenge inhibited allergic symptoms and Th2 cytokine production in an IL-10-dependent manner [136].

A role for TGFβ has also been described in allergic immune responses. Membrane bound TGFβ was found to be expressed in tolerized $CD4^+$ T cells in response to respiratory antigens [137]. Consistent with the protection mediated by nTregs, iTregs inhibit the development of allergen-induced AHR [138]. Ovalbumin-specific T cells producing IL-10 [139] and TGFβ [140] could mediate protection against Th2-mediated AHR. In an antigen-dependent murine asthma model, adoptive transfer of Tregs over expressing TGFβ effectively suppressed AHR [141]. However, an indispensable role for Treg-derived IL-10 was evident as cells over expressing TGFβ but lacking IL-10 could not confer complete protection against AHR [141]. Similarly, from studies in humans it was noted that individuals with high doses of allergen exposure demonstrate a preferential switch towards IL-10-secreting Tr1 cells [142]. Another mechanism by which nTregs and Tr1 cells might be indirectly contributing to suppression of allergy is by inducing IgG4 and suppressing IgE production [143].

Due to importance of Treg-derived IL-10 and TGFβ in curtailing allergic conditions, Tregs represent an attractive cell type for therapeutic manipulation in these inflammatory diseases. Recent studies suggest that recombinant IL-2 in combination with anti-IL2 mAb reduces the severity of allergen-induced inflammation in the lung following expansion of Tregs *ex vivo* [144]. This outcome can be correlated to the increase in the Treg numbers seen following this treatment [145]. Collectively, these studies suggest that Tregs are important in regulating inflammatory conditions and that IL-10 and TGFβ produced by Tregs represent attractive candidates for therapeutic approaches. It should be noted that while IL-10 and TGFβ appear to be important contributors to Treg-mediated control of allergy and asthma, it remains unclear whether other Treg mechanisms can also control these inflammatory diseases.

Type I Diabetes

Accumulating evidence suggests that failure of suppression of autoreactive T cells by Tregs can result in autoimmune disorders such as type 1 diabetes (T1D) [146,147]. The Non Obese Diabetic (NOD) mouse is one of the best and most extensively studied spontaneous models of an autoimmune disease. Type 1 diabetes appears in female mice by 12–16 weeks of age and is preceded by a long phase of asymptomatic pre-diabetes characterized by progressive insulitis starting at 3 weeks of age. A protective role for Tregs in preventing spontaneous diabetes in NOD mice as well as diabetes induced by diabetogenic T cells has been established [148, 149]. First, the occurrence of diabetes in NOD mice is correlated with the reduced functional capacity of Tregs over time [146,150]. Additionally, the reduced susceptibility of conventional T cells to Treg-mediated suppression has been reported in diabetic humans [151] as well as in NOD mice [147]. Second, induction of disease by diabetogenic T cells in NOD.scid mice is prevented by co-injection of Tregs from young, pre-diabetic mice or islet-specific iTregs [152]. Lastly, depletion of Tregs in young NOD mice with anti-CD25 mAb also accelerates disease onset and increases incidence in both male and female mice [153]. The protection mediated by Tregs can be antigen-specific or non-specific. However, the suppression by islet antigen-specific Tregs, such as those derived from BDC.2.5 transgenic mouse, is more potent than that by the polyclonal Tregs as fewer are required to suppress the disease [154].

From NOD mouse models there is evidence that Tregs mediate their function in T1D through the action of CTLA-4 and TGFβ. The loss of activity of CTLA-4 and TGFβ adversely affects the incidence and acceleration of T1D [147]. Additionally, a cocktail treatment of anti-CTLA-4

and anti-TGFβ (but not anti-TNFα) antibodies results in poly-autoimmune syndrome characterized by colitis, sialitis and gastritis [147]. Furthermore, *Cd28*$^{-/-}$ NOD mice, which lack Tregs, exhibit an increased rate of T1D onset when treated with anti-CTLA-4 and anti-TGFβ [155]. It is unclear, however, if TGFβ from other cellular sources may be playing a role.

Therapies utilizing Tregs for treatment of T1D is an area of active investigation. Two different approaches have yielded promising results: induction of Tregs *in vivo* and adoptive transfer of *in vitro* cultured Tregs. Administration of non-mitogenic, CD3-specific antibodies in NOD mice induces a population of Foxp3^{+} Tregs that suppress T1D in a TGFβ-dependent manner [152]. This has proved to be successful in clinical trials in humans where administration of hOKT3γ1(Ala-Ala) (a humanized Fc mutated anti-CD3 monoclonal antibody), halted disease progression for more than one year [156,157]. Recently, treatment with rapamycin, which promotes the development of Tr1 cells and Foxp3^{+} Tregs, successfully prevented diabetes in mice [158]. Finally, *ex vivo*-expanded antigen-specific Tregs can suppress ongoing diabetes, providing a viable strategy for therapy [159].

Multiple Sclerosis

Multiple Sclerosis (MS), results from autoimmune destruction of the myelin sheath and inflammation of the brain and spinal cord [160]. The importance of Tregs in this disease has been suggested by studies in humans documenting the functional decline of Treg activity in MS patients [161]. The frequency or number of Tregs present in the periphery appears to be normal in MS patients [161–163] but the cells are not functional *in vitro* [162,164]. This reduced functional capacity is correlated with reduced Foxp3 protein levels [164]. Consistent with these reports, the presence of Tregs in the CNS is associated with recovery of MS while depletion of Foxp3^{+} Tregs exacerbates the disease [165]. The mouse model of MS, experimental autoimmune encephalomyelitis (EAE), shares many features with the human disease. Both show involvement of encephalitogenic myelin-specific T cells in the resulting pathology which consists of perivenular lesions in the CNS and extensive demyelination and axonal damage. In this model, adoptive transfer of polyclonal or antigen-specific Foxp3^{+} Tregs can prevent disease progression.

At present, IL-10 and TGFβ appear to be the primary mediators of Treg function in the cure of, or protection against, EAE. In an induced model of EAE, it was demonstrated that anti-CD3-stimulated cells from naïve SJL mice secreted IL-10 and treatment with a soluble IL-10R antibody partially reversed the suppressive capacity of Tregs [166]. The primary evidence for a role for IL-10 in *in vivo* protection against EAE has been derived from adoptive transfer studies where wild type but not *Il10*$^{-/-}$ Tregs from unimmunized SJL/J donors partially protected recipient mice from $PLP_{139–151}$-induced EAE [166]. In support of this, disruption of the *Il10* or *Il10r* gene in mice resulted in failure to inhibit MOG peptide-induced EAE suggesting that IL-10 is important in mediating protection against EAE [167]. Additionally, it has been demonstrated that there is a significant accumulation of IL-10-producing Tregs in the CNS which correlated with the recovery phase. These cells expressed Foxp3 and could mediate suppressive activity *in vitro* [165]. Histological and flow cytometric analysis by several groups have also revealed that the Tregs localize to the CNS during inflammation [168,169].

The importance of TGFβ in recovery from EAE was demonstrated in two independent studies. First, it was demonstrated that the percentage of CD4^{+} cells expressing TGFβ LAP on the cell surface increased significantly in blood and spleen of EAE-recovered mice as compared with the naïve mice [170]. Second, *in vivo* neutralization of TGFβ prevented recovery from disease [170]. Recently it was found that CD4^{+}CD25^{+}LAP^{+} cells suppress MOG-specific immune responses in a TGFβ-dependent manner by inducing Foxp3 and by inhibiting IL-17 production [171]. Therapeutic approaches focusing on enhancing Treg number or function for protection from EAE may be of great benefit to patients suffering from MS. Recently, genetically modified

polyclonal Tregs expressing a chimeric receptor consisting of an MBP epitope bound to the extracellular and transmembrane domain of the CD3ζ chain results in functional Treg activation upon encounter with the MBP specific autoreactive T cells. These receptor-modified Tregs inhibited the onset as well as the progression of EAE in an antigen-specific manner [172].

Tumors

The primary objective of Tregs is to maintain peripheral tolerance which involves policing and preventing anti-self reactivity. However, tumors are seen as self and thus Tregs try to prevent anti-tumor specific T cells from clearing the tumor, making Tregs a significant barrier for effective immunotherapy. Both the adaptive and innate immune responses are important in tumor clearance. If Tregs are capable of suppressing the beneficial anti-tumor response, they should be present in the tumor environment. Indeed, there are numerous publications that report an increase in the number of Tregs in the local tumor environment of humans suffering from melanoma [173], lymphoma [174], and ovarian [175–177], pancreatic [178], breast [178], gastric [179,180], and lung [175,181] cancers. However, there are conflicting reports as to whether the presence of Tregs in the local tumor environment is indicative of a poor prognosis [176,177] or a positive prognosis [182–184]. Therefore, further analysis is required to correlate prognosis with Treg frequencies in humans. While it is difficult to discern these discrepancies, it is worth noting that in these studies the presence of Tregs was determined by staining for Foxp3, which does not necessarily correlate with human Tregs as it as been shown that human conventional T cells can express Foxp3 following activation [185–187]. A recent paper clearly demonstrates that the $Foxp3^+$ tumor infiltrating T cells were Tregs by both staining and functional analysis [176]. Importantly, they found that the presence of Tregs correlated with a poor prognosis. In murine tumor models, there is strong evidence to suggest that in the absence of Tregs a proper antitumor response can be mounted resulting in the clearance of the tumor. The original studies to demonstrate this were done by depleting the Tregs *in vivo* with anti-CD25 depleting antibodies [188,189]. Together, the data generated with human cells and murine models strongly suggests a role for Tregs in suppressing the anti-tumor response.

There are a number of studies that have attempted determine what mechanism Tregs yse to suppress the anti-tumor response, with the majority focusing on the inhibitory cytokines TGFβ and IL-10. It was initially demonstrated in both humans and mice that Tregs are capable of suppressing both adaptive and innate aspects of the anti-tumor immune response in a TGFβ-dependent manner by suppressing $CD8^+$ T cells and NK cells, two of the immune system's primary weapons against tumors [104,190]. More recently in a murine tumor model, an effective anti-tumor response could be generated by blocking Treg function with a novel peptide inhibitor that bound TGFβ on the cell surface of Tregs suggesting a direct role of TGFβ in the anti-tumor response [191]. Together with TGFβ, it was demonstrated that the potent inhibitor cytokine IL-10, was important in the Treg suppressive response in a UV-induced tumor model using IL-10 deficient mice [192]. The reliance of Tregs on IL-10 to suppress an anti-tumor response in humans was also observed in an early study involving Tregs isolated from head and neck squamous cell carcinoma [193]. Although the data was generated *in vitro*, it was clear that in humans, Tregs are capable of suppressing the anti-tumor response in an IL-10/TGFβ-dependent manner. Taken together there is convincing evidence to suggest that the suppressive cytokines IL-10 and TGFβ are important mechanisms by which Treg mediate suppression within the tumor microenvironment of patients and murine tumor models.

Interestingly, Tregs may also augment APC function in a suppressive cytokine-dependent manner. Studies with human Tregs, describe a novel interaction between IL-10-producing Tregs and APCs in which IL-10 causes the up-regulation of B7H4 on APCs rendering them immunosuppressive [194]. This was followed by a subsequent paper that showed that B7-

$H4^+$ tumor infiltrating macrophages were immunosuppressive in a human ovarian carcinoma [195]. These papers provide evidence for a cytokine-driven, APC altering mechanism by which IL-10 produced by Tregs renders APCs immunosuppressive in the tumor environment.

Another mechanism in the Treg arsenal is the suppression of APC function via direct contact within the local tumor microenvironment resulting in the reduction of APC-mediated activation events. In a recent paper, CTLA-4 on Tregs mediated the downregulation of CD80 (B7.1) and CD86 (B7.2) on APCs thereby affecting the ability of the APCs to activate other T cells by reducing CD28 co-stimulation. This Treg-mediated CTLA-4/APC interaction was important in suppressing the anti-tumor response, as a robust anti-tumor response was mounted in the presence of CTLA-4 deficient Tregs compared to wild type Tregs [196]. Additionally, there is evidence to suggest that Tregs interact with APCs through CTLA4 and cause the APCs to up regulate production of the tryptophan–degrading enzyme, indoleamine 2,3-dioxygenase (IDO) which is a potent immunoregulatory enzyme [197]. Interestingly, IDO-producing pDCs isolated from tumor draining lymph nodes activate Tregs which cause the upregulation of the programmed cell death ligand 1 (PD-L1) and PD-L2 expression on target DCs resulting in the suppression of the effector T cell response [198]. Additionally, this could also lead to a feedback mechanism in which Tregs, through CTLA-4 interaction with DCs, cause an increase in IDO production [113,199] and subsequently an increase in the number of IDO^+ pDCs which could in turn activate Tregs.

The primary method of tumor clearance is granzyme B-dependent cytolysis mediated by $CD8^+$ T cells and NK cells. It was initially demonstrated that Tregs can also kill conventional T cells in a granzyme B-dependent, perforin-independent pathway [111]. Subsequently, it was shown that granzyme B was upregulated in Tregs in the tumor environment and that, in the presence of granzyme B-deficient Tregs, an active tumor response was generated and the tumor cleared [112]. These data suggest that Tregs can suppress the anti-tumor response via a cytolysis-mediated event.

Taken together, it is apparent that Tregs utilize a variety of mechanisms to suppress the beneficial anti-tumor response. While it is clear that suppressive cytokines are an important part of the Treg arsenal, Tregs also suppress APC function as well as kill their targets in a granzyme B-dependent manner. Interestingly, the tumor environment is the only disease state in which Tregs have been shown to utilize their cytolytic capabilities to control the immune response. It is important to note, that this was demonstrated in murine models and further studies are required to determine if it is the same in humans. However, cytolysis does represent a potentially novel mechanism to target Tregs in a-disease specific manner.

Infections

It is clear that Tregs play a significant role in the immune response to infections as well as the clearance of the invading pathogen. This has been demonstrated in both humans and murine models [200]. In particular, the importance of Tregs has been shown in parasitic, fungal and bacterial infections such as *Leishmania major* [201], *Plasmodium yoelii* [202], *Candida albicans* [203], *Listeria monocytogenes* [204] and *Mycobacterium tuberculosis* [205,206]. Viral clearance is also altered by the presence of Tregs. This has been seen during infection with herpes simplex virus (HSV) [207,208], hepatitis B and C virus [209,210], cytomegalovirus (CMV) [211] and human immunodeficiency virus [212,213].

The consequences of Treg mediated suppression of the immune response during infection are controversial. There are studies that suggest Tregs, while limiting local tissue damage, prevent sterilizing immunity against the pathogen and thus allow for a persistent infection [201,214]. In turn this persistent infection results in protective immunity against a subsequent challenge with the pathogen [201]. While this ‘symbiotic relationship’ may be beneficial to the host, there

is also evidence to suggest that Tregs can be detrimental to the host. For instance, Treg suppression of the immune response to *Plasmodium yoelli* allows the parasite to escape clearance [202]. In support of this, sterilizing immunity in a *Leishmania major* model can be achieved by depletion of Tregs [201]. However, depletion of Tregs may not always be beneficial to the host, as a recent study suggests that Tregs are critical in the early stages of infection by HSV and depletion of Tregs accelerated the time to death as well as caused an increase in viral loads [215].

The mechanisms by which Tregs suppress or alter the immune response against viral, parasitic, fungal and bacterial infections is still unclear [216]. This is primarily clouded by the fact that the immune response to foreign antigens results in the production of a milieu of cytokines and inhibitory factors, especially IL-10 and TGFβ. While it has been demonstrated that the majority of inhibition of the immune response generated against certain pathogens may be mediated by IL-10, this is not necessarily produced by Tregs [205,217]. However, there is some evidence to suggest that TGFβ may be an important cytokine utilized by Tregs in the suppression of the immune response against *Mycobacterium tuberculosis* [205].

It is clear that there is a need for further analysis to understand the mechanisms involved in Treg-mediated suppression of effective anti-pathogen responses in humans. As the immune system is under constant assault from pathogens, infection is the most common of all disease states. Infection is also the only disease state in which Treg activity can be both deleterious, because it prevents sterilizing immunity, and helpful, by limiting rampant immune responses that could be harmful to the surrounding tissue. Therefore, it is possible that Tregs utilize all mechanisms at their disposal to achieve a balanced immune response so that proper pathogen clearance can be achieved. Understanding the mechanisms and determining if certain mechanisms are over utilized in certain infections may help in the development of effective treatment and immunization strategies against a wide variety of infectious agents.

Summary

Since the discovery of suppressor T cells, and their subsequent ‘reinvention’ as regulatory T cells, research has firmly established a role for Tregs in controlling immune homeostasis and modulating a wide variety of disease states. While a great deal of progress has been made in understanding the development and mechanisms of suppression of both nTregs and iTregs, there remain a number of questions that need to be addressed. First, while it is clear there are several mechanisms used by Tregs to mediate their suppression, it remains to be determined if additional mechanisms are used and whether any new or previously described mechanism may be specific to a particular disease state. Second, while the analysis of Tregs in murine models have been indispensible in characterizing their role in immune homeostasis, tolerance and a variety of diseases, it is clear that additional research needs to be performed on human Tregs, particularly with regard to specific markers with which to isolate and study them. Answering these questions will not only bring us closer to understanding how this unique population of cells develop and function, but also how to exploit or mitigate their suppressive activity for targeted therapy against a wide variety of diseases.

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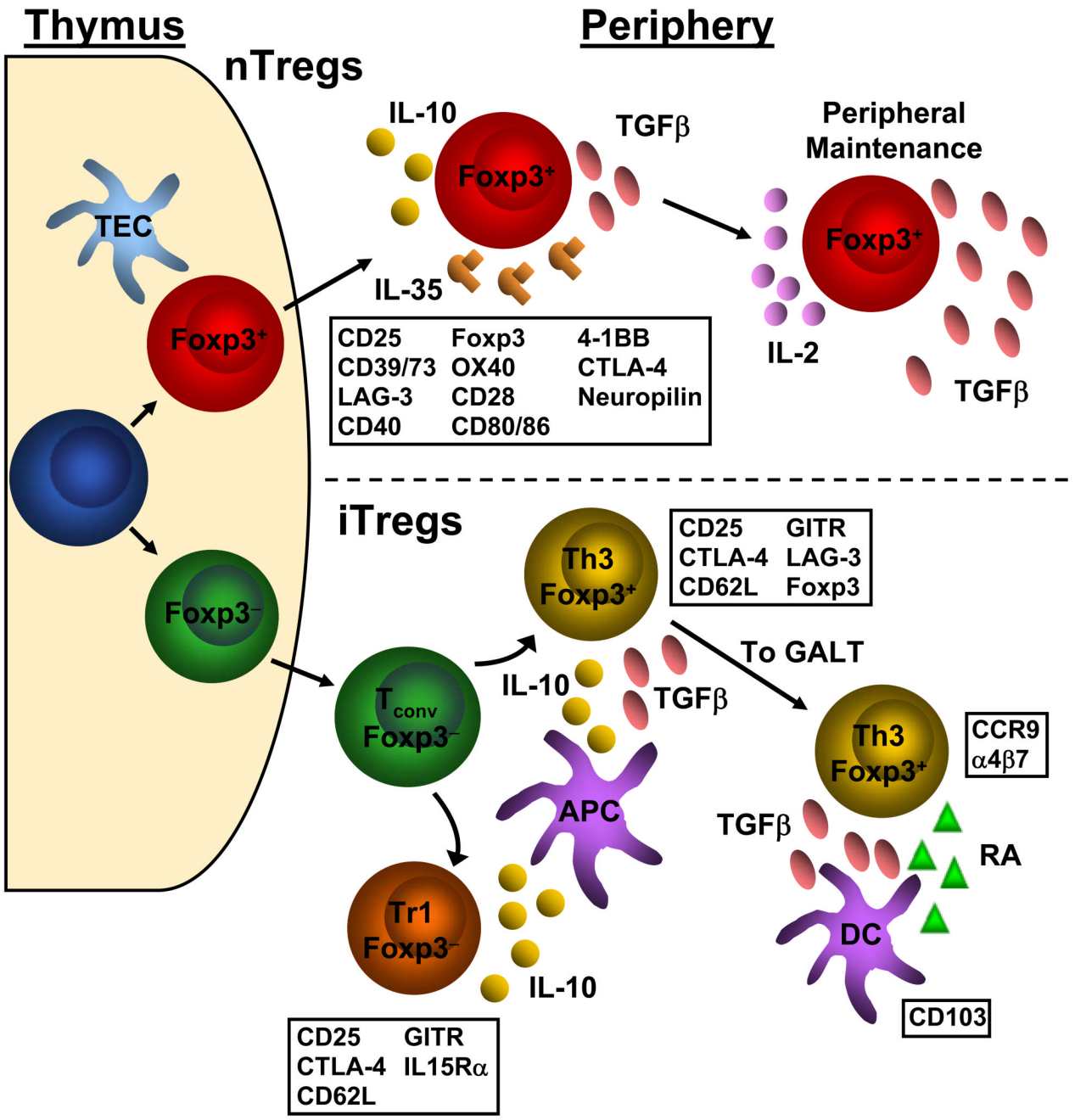

Figure 1. Development of nTregs and iTregs and the relevant markers associated with each.

nTregs (top) differentiate from naïve conventional T cells to Foxp3$^+$ Tregs in the thymus. In the periphery, natural Tregs express a number of cell surface markers, indicated in the box below the depiction of the natural Treg. However, none of these cell surface markers are unique to Tregs as they are also found on activated conventional T cells. Natural Tregs utilize the cytokines IL-10, IL-35 and TGFβ to exert their suppressive effects upon conventional T cells. TGFβ and IL-2 have also been shown to be important to the maintenance and fidelity of the Treg signature. iTregs (bottom) can be generated from conventional T cell precursors. Once in the periphery, naïve conventional T cells can be induced to become Foxp3$^-$ Tr1 cells or Foxp3$^+$ Th3 cells via IL-10 and/or TGFβ secreted by APCs such as dendritic cells and

macrophages. These induced Tregs share similar cell surface markers as natural Tregs. Foxp3$^+$ induced Tregs can accumulate in the gut through upregulation of CCR9 and α4β7 via TGFβ and retinoic acid produced by CD103$^+$ dendritic cells. TEC= thymic epithelial cell; T_{conv}= conventional T cell; DC= dendritic cell; RA= retinoic acid

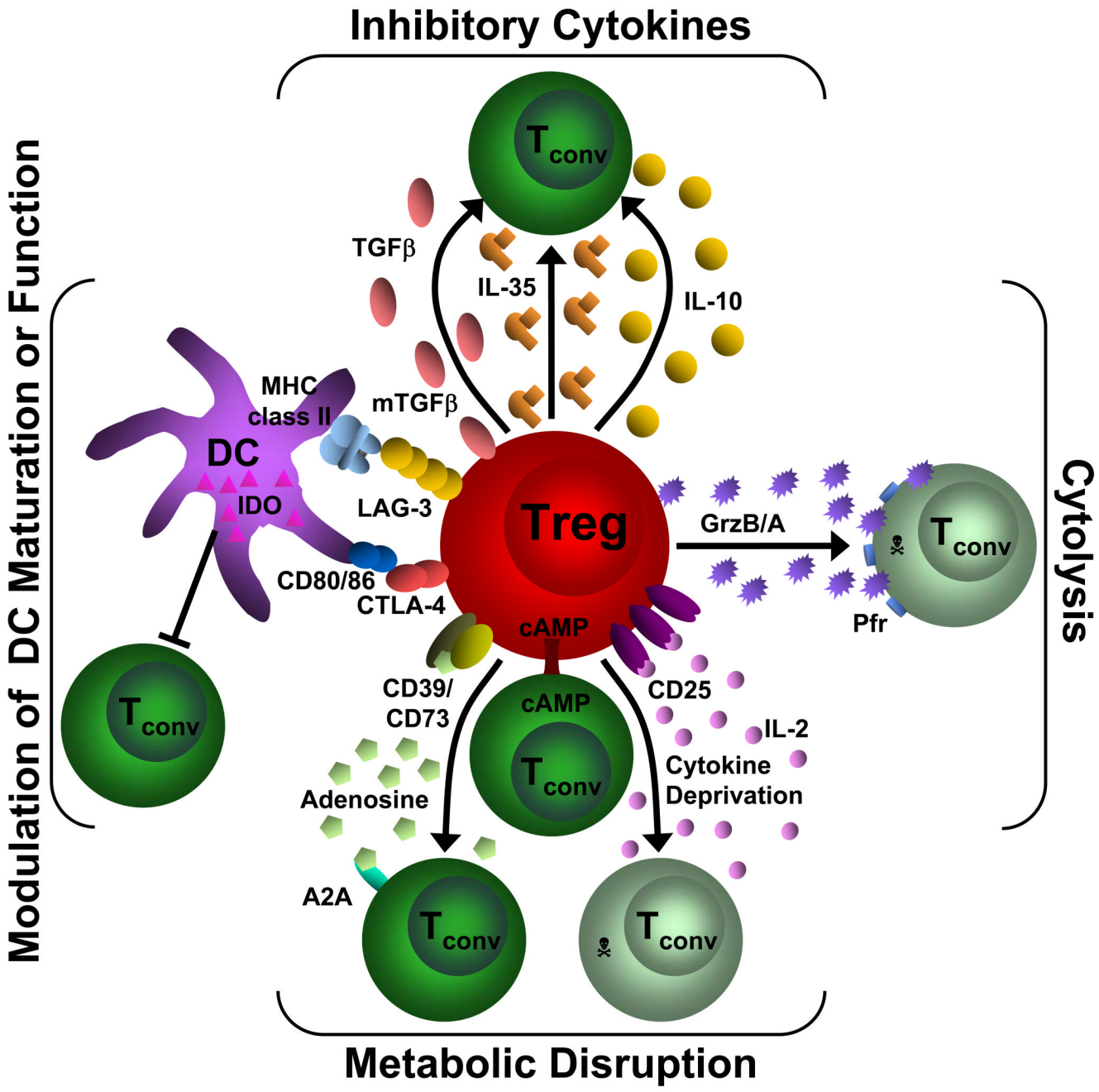

Figure 2. Mechanisms of Treg suppression.

This diagram depicts the four basic modes of Treg suppression. A primary mode of Treg suppression is mediated through the inhibitory cytokines IL-10, IL-35 and TGFβ. Tregs also induce cytolysis through granzyme A/B (GrzB/A) and perforin (Pfr). They can disrupt metabolic function by IL-2 deprivation which results in apoptosis, cAMP inhibition or by CD39/CD73- generated A2A-mediated immunosuppression. Tregs can also modulate DC maturation or function via a CD80/86 and CTLA-4 interaction or through a LAG-3 and MHC class II interaction. In addition, they can induce the upregulation of IDO in DCs. T_{conv}= conventional T cell; GrzB/A= granzyme B or A, Pfr= perforin, cAMP= cyclic adenosine

monophosphate, A2A= adenosine-purinergic adenosine receptor, IDO= indoleamine 2,3-dioxygenase, DC= dendritic cell