Immunity
Review

Mechanisms of Foxp3$^+$ T Regulatory Cell-Mediated Suppression

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Foxp3$^+$ T regulatory (Treg) cells control all aspects of the immune response. Here, I will review the in vitro model systems that have been developed to define the mechanisms used by Treg cells to suppress a large number of distinct target cell types. These mechanisms can be broadly divided into those that target T cells (suppressor cytokines, IL-2 consumption, cytolysis) and those that primarily target antigen-presenting cells (decreased costimulation or decreased antigen presentation). Although multiple mechanisms for Treg cell suppression have been shown in vitro, it is unclear whether the same or different mechanisms are used by Treg cells in vivo. An increase in our understanding of Treg cell suppressor mechanisms will offer an insight into how Treg cell function can be manipulated either positively or negatively in vivo.

Introduction

The field of T regulatory (Treg) or T suppressor cells was reborn by the observations of Sakaguchi et al. (1995) that depletion of the minor population of CD4$^+$ T cells that coexpress CD25 from a population of normal adult CD4$^+$ T cells generated a population of cells that induced a spectrum of autoimmune diseases when transferred to an immunocompromised recipient. Cotransfer of the CD25$^+$ cells prevented the development of autoimmunity. The critical question that needs to be addressed is the mechanism of action of these Treg cells in vivo. It was thought that the development of an in vitro model system (Thornton and Shevach, 1998; Takahashi et al., 1998) for the analysis of Treg cell function would offer major insights into the mechanism of action of Treg cells in vivo. However, the in vitro model systems have identified a long list of molecules and processes that contribute to Treg cell-suppressive activities and it remains unclear whether any of the conclusions drawn from these studies shed light on how Treg cells function in vivo. A detailed analysis of Treg cell function is further confounded by the large number of different cell types that are purported to be directly targeted by Foxp3$^+$ Treg cell (Box 1). Although multiple T cell subsets (e.g., Tr1 cells, Th3 cells, Th1 and Th2 cells, etc.) can certainly exert negative immunoregulatory effects by producing immunomodulatory cytokines (IL-10, TGF-β), I will confine this discussion to CD4$^+$Foxp3$^+$ Treg cells that develop in both the thymus and periphery and represent the major Treg cell populations that are critical for immune homeostasis.

Lessons to Be Learned from In Vitro Suppression Assays: Responder T Cells as Targets

Box 2 summarizes some of the major conclusions drawn from the in vitro suppression assays performed with both human and mouse Foxp3$^+$ Treg cells that are agreed to by most workers in the field. Numerous controversial issues remain regarding the interpretation of these assays and I will discuss several of the ones that I regard as most critical. First, what is the cellular target for Treg cell-mediated suppression in vitro? Suppression assays are performed in two distinct ways. In some studies, a source of antigen-presenting cells (APCs, most often irradiated T-depleted spleen cells or unirradiated dendritic cells [DCs]) is added and the stimulus for activation is soluble anti-CD3, whereas in other studies bead-coupled or plate- bound antibodies together with anti-CD28 are used in the complete absence of APCs. In the former case, the target of Treg cell suppression can be the APC, the responder cells, or both, whereas in the latter situation the responder T cells are the only targets. This section will focus on potential mechanisms of suppression that target responder T cells (Figure 1), whereas the next section will deal with mechanisms that potentially target APCs. As is apparent from the discussion to follow, this division is somewhat arbitrary and some mechanisms can target both cell types.

Murine Treg cells are potent suppressors of T cell proliferation in the presence of soluble anti-CD3 and APCs and are very inefficient suppressors (requiring a high ratio of suppressors to responders) when anti-CD3 is coupled to a solid phase in the presence or absence of anti-CD28. Furthermore, in the presence of anti-CD28 and soluble anti-CD3, suppression was frequently abrogated secondary to induction of high amounts of IL-2 production by the responder cells (Thornton and Shevach, 1998, 2000; Thornton et al., 2004b). These results raised the possibility that the APC is the primary target of the Treg cells, but suppression of the response of transgenic CD8$^+$ T cells to stimulation with MHC class I peptide tetramers by preactivated Treg cells was readily observed in the absence of a source of professional APC, suggesting that Treg cells could also efficiently target responder T cells (Piccirillo and Shevach, 2002).

Many studies of human Treg cell function have used bead-coupled reagents as the stimulus in cocultures and again substantial suppression was observed only at high suppressor to responder ratios. It is unclear which assay system should be used, but it is obvious that the two assays may be measuring different components of Treg cell-suppressor function. This issue is most relevant in interpreting studies claiming abnormal Treg cell function in patients with autoimmune diseases, where several studies have concluded that there is a fundamental defect in Treg cell function based on the results obtained in a particular in vitro assay system. An additional caveat in the analysis of many of the results measuring human Treg cell

Box 1. Cellular Targets of Foxp3$^+$ Treg Cell-Mediated Suppressor Function

- CD4$^+$, CD8$^+$ T cells
- Dendritic cells
- B cells
- Macrophages
- Osteoblasts
- Mast cells
- NK cells
- NK T cells

Box 2. Properties of Foxp3$^+$ Treg Cell In Vitro

- -Do not proliferate when stimulated via the TCR
- -Nonresponsiveness is not overcome by costimulatory signals
- -Will proliferate in the presence of a TCR signal and IL-2
- -Do not produce effector cytokines such as IL-2, IL-4, or IFN-γ
- -Require activation via the TCR to suppress
- -Once activated, do not require restimulation via the TCR to suppress
- -Activated Treg cell mediate "bystander" suppression
- -Once activated, suppression is not MHC restricted
- -Suppress both CD4$^+$ and CD8$^+$ responder cells
- -Suppression is not observed when responder and stimulator cells are separated by a semipermeable membrane
- -Suppression is not mediated by IL-10 or TGF-β

function is that many studies have used CD25$^+$ T cells purified with anti-CD25-coupled magnetic beads rather than CD25^{hi} cells purified by cell sorting. These studies did not control for the percentage of Foxp3$^+$ T cells in their preparations and the bead-purified cell populations were frequently contaminated with CD25$^+$Foxp3$^-$ activated effector cells. When the suppression assays are performed with high concentrations of bead-coupled reagents, suppression is incomplete and upon stimulation in culture, the activated CD25$^+$Foxp3$^-$ T effector cells can respond to IL-2 produced by the responder cells, rapidly divide, and deplete IL-2 from the culture, so that marked suppression of responder T cell proliferation is observed when the cultures are assayed after 4–5 days.

Most studies have demonstrated that Treg cells mediate suppression by inhibiting the induction of IL-2 mRNA (and mRNA for other effector cytokines) in the responder Foxp3$^-$ T cells (Thornton and Shevach, 1998; Takahashi et al., 1998; Oberle et al., 2007). Furthermore, the addition of exogenous IL-2 had no effect on the Treg cell-mediated suppression of IL-2 mRNA production (Thornton et al., 2004a; Oberle et al., 2007). The role of IL-2 consumption in the suppressive mechanism of Treg cells is under dispute. Treg cells express all three components of the high-affinity IL-2R—CD25, CD122, and CD132—and IL-2 is essential not only for Treg cell homeostasis in vivo (Yu et al., 2009), but also for their efficient suppressor function in vitro (Thornton et al., 2004a). One study (Pandiyan et al., 2007) has raised the possibility that Treg cells may compete with Foxp3$^-$ T cells for IL-2, consume it, and inhibit the proliferation of Foxp3$^-$ T cells, resulting in a form of apoptosis dependent on the proapoptotic factor Bim. Curiously, this study is the only one to claim that Treg cells do not inhibit IL-2 production by responder T cells. Although it has been widely assumed that Foxp3$^+$ Treg cells express high numbers of high-affinity IL-2 receptors that would render them efficient competitors, quantitation of the number of high-affinity IL-2 receptors on Foxp3$^+$ T cells has never been determined and is dependent not on the expresssion of CD25, the low-affinity IL-2 receptor, but on the expressions of CD122 and CD132 that are required to form the high-affinity IL-2 receptor complex. In a hybrid system where human Treg cells were shown to be capable of efficiently suppressing the proliferation of mouse responder cells, the addition of antihuman CD25 that blocks IL-2 binding also had no effect on the function of the human Treg cells (Tran et al., 2009a). Taken together, these studies argue against IL-2 consumption as a major pathway of Treg cell suppression in vitro, but IL-2 consumption may influence the results of suppression assays when activated conventional T cells contaminate Treg cells used in the assays.

Could a soluble suppressor cytokine mediate Treg cell suppressor function in vitro? It should be emphasized that the failure to observe suppression when Treg cells are separated from the responder cells by a membrane does not rule out the possibility that Treg cells secrete an as yet uncharacterized cytokine that functions in a gradient fashion and requires proximity between suppressor and responder. It is also possible that the production of a suppressor cytokine by either the Foxp3$^+$ Treg cell or by the responder cell might require cell contact between the Treg and the responder T cell. One such candidate is IL-35, a new inhibitory cytokine, which may contribute substantially to the function of Treg cells by directly acting on responder T cells (Collison et al., 2007). IL-35 is a member of the IL-12 heterodimeric cytokine family and constitutes a pairing between Epstein-Barr virus-induced gene 3 (*Ebi3*, which normally pairs with p28 to form IL-27) and *Il12a* (also known as p35 and

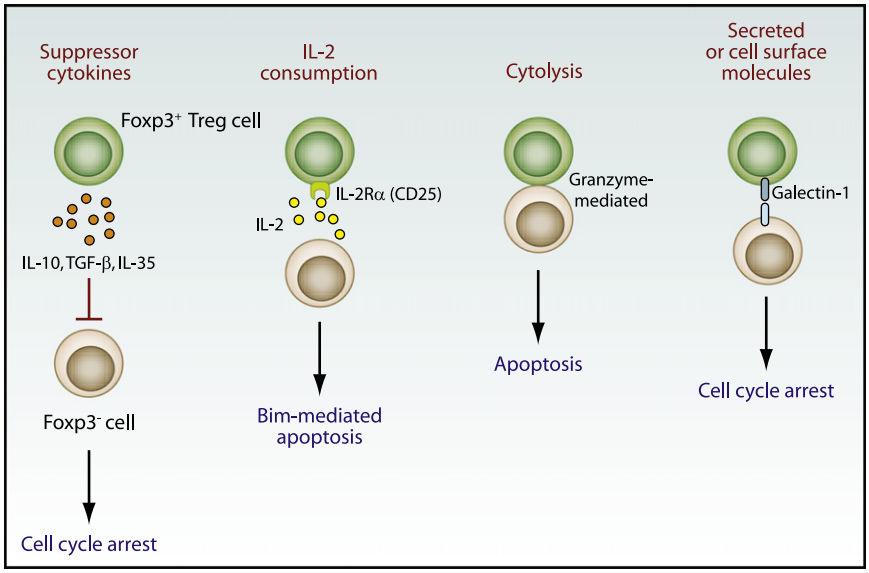

Figure 1. Major Mechanisms by Which Treg Cells Can Directly Suppress Responder Foxp3$^-$ T Cells

Treg cells may secrete suppressor cytokines that can directly inhibit the function of responder T cells and myeloid cells. Treg cells express high CD25, the IL-2 receptor α chain, and have the capacity to compete with effector T cells for IL-2 resulting in cytokine-mediated deprivation of the effector cells and Bim-mediated apoptosis. Activated Foxp3$^+$ Treg cells may function as cytotoxic cells and directly kill effector cells in a manner similar to CD8$^+$ cytotoxic cells. Activated Treg cells may express known (e.g., galectin-1) or unknown molecules on their cell surface that can interact with receptors on effector T cells resulting in cell cycle arrest.

All of these mechanisms may also be utilized by Treg cells to inhibit the function of antigen-presenting cells or other cells of the innate immune system.

normally pairs with p40 to form IL-12 p70). Murine Treg cells express both *Ebi3* and *Il12a*. *Ebi3* is a Foxp3 target gene as shown by the fact that conditional ablation of Foxp3 in mature peripheral Treg cells resulted in the downregulation of *Ebi3* expression. Both *Ebi3* and *Il12a* mRNA are upregulated in Treg cells that are actively suppressing effector cells, raising the possibility that cell contact between suppressor and responder is required for maximal IL-35 production. Importantly, both *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Treg cells have substantially reduced regulatory activity in vitro when T responder cells are stimulated with bead-coupled anti-CD3 and anti-CD28, but the *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Treg cells have not been tested in an in vitro system where DCs are used as stimulators. Treg cells from *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ animals also were unable to control homeostatic proliferation and cure inflammatory bowel disease (IBD) in vivo. Neutralizing antibodies to IL-35 are not available and the nature of its receptor or its cellular distribution remains unknown. In contrast to the results reported for mouse IL-35, Bardel et al. (2008) demonstrate that *Ebi3* is expressed in human plancental trophoblasts and activated DCs, but is undetectable in normal resting CD3$^+$ T cells, or resting or activated Treg cells, whereas human p35 is constitutively expressed at low amounts in many cell types. It is therefore unlikely that human Treg cells are able to express substantial amounts of the two Ebi3-associated heterodimeric cytokines, IL-35 or IL-27. Stimulation of human Foxp3$^-$ T cells with anti-CD3 and anti-CD28 does induce Ebi3 expression and activated human Foxp3$^-$ T cells can express p35 transcripts, indicating that activated human T cells may produce IL-35, but coassociation of the two chains was not observed in immunoprecipitation studies. Further analysis of the contribution of IL-35 to Treg cell function will require the development of neutralizing antibodies and characterization of its receptor.

Another secreted molecule that potentially may play a role in Treg cell-DC or Treg cell-T cell interactions is galectin-1, a member of a highly conserved family of β-galactoside binding proteins (Garin et al., 2007). Galectin-1 is secreted as a homodimer, and binds to many glycoproteins including CD45, CD43, and CD7. The consequences of galectin binding correlate with those induced by Treg cells in responder cells including cell cycle arrest and apoptosis and inhibition of the production of proinflammatory cytokines. It is not clear whether galectin-1 acts as a soluble cytokine or mediates it effects via cell-cell contact. Galectin-1 is preferentially expressed in Treg cells and is upregulated upon T cell receptor (TCR) activation. Blocking of galectin-1 markedly reduced the inhibitory effects of human and mouse Treg cells and Treg cells from galectin-1-deficient mice had reduced Treg cell activity. Another member of the galectin family, Galectin-10, was identified by a proteomics approach as being selectively expressed by human Treg cells (Kubach et al., 2007). Galectin-10 is exclusively expressed intracellularly and is probably not directly involved in the contact-dependent suppression mediated by Treg cells. However, galectin-10-specific siRNA treatment reversed the anergic state of human Treg cell in vitro resulting in increased proliferation upon activation and a partial abrogation of their suppressive activity. The intracellular ligands for galectin-10 have not been identified.

One other potential mechanism for Treg-mediated suppression of responder T cells would be cytolysis of target cells. Human CD4$^+$CD25$^+$Foxp3$^+$ Treg cells can be activated by a combination of antibodies to CD3 and CD46 to express granzyme A and kill activated CD4$^+$ and CD8$^+$ T cells and other cell types in a perforin-dependent, Fas-FasL-independent manner (Grossman et al., 2004). Activation of mouse Treg cells also results in upregulation of granzyme B expression and one report has claimed that Treg cells kill responder cells by a perforin-independent, granzyme B-dependent mechanisms (Gondek et al., 2005) and that granzyme B-deficient Treg cells had reduced suppressive activity in vitro. Other studies have failed to confirm responder cells lysis by activated murine Treg cells, but demonstrated that highly activated Treg cells could kill antigen-presenting, but not bystander B, cells (Zhao et al., 2006). It has been difficult to demonstrate granzyme B expression by Treg cells in vivo, but Cao et al. (2007) demonstrate that 5%–30% of Treg cells in a tumor microenvironment express granzyme B and these Treg

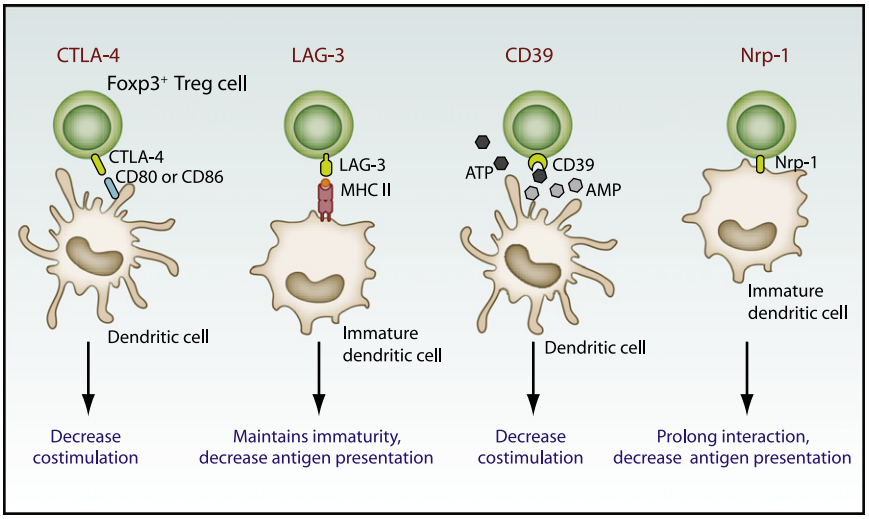

Figure 2. Major Mechanisms by which Treg Cells Can Suppress the Function of APC and Indirectly Block the Activation of Foxp3$^-$ T Cells

CTLA-4 on the surface of Treg cells downregulates or prevents the upregulation of CD80 and CD86, the major costimulatory molecules on antigen-presenting cells. Similarly, LAG-3 on Treg cells can interact with MHC class II on antigen-presenting cells, and binding of LAG-3 to MHC class II molecules expressed by immature DCs results in an inhibitory signal that suppresses DC maturation and immunostimulatory capacity. Extracellular ATP functions as an indicator of tissue destruction and exerts inflammatory effects on DCs. Catalytic inactivation of extracellular ATP by CD39 represents an anti-inflammatory mechanism that may be used by Treg cells to prevent the deleterious effects of ATP on antigen-presenting cell function. In contrast, Nrp-1 promotes long interactions between Treg cells and immature DCs and restricts access of the effector cells to antigen-presenting cells. Some of these mechanisms may also be used by Treg cells to suppress responder T cells.

cells are lytic for NK cells and CTLs in a granzyme B- and perforin-dependent manner. Thus, Treg cells in certain contexts can differentiate to become what was once termed "cytotoxic-suppressor" cells. Although killing of APCs in vivo represents a potentially potent mechanism by which Treg cells can control both primary and secondary immune responses, no studies to date have been able to document Treg cell-mediated cytolysis of DC or B cells in an in vivo model.

Lessons to Be Learned from In Vitro Suppression Assays: APC as Targets

One of the major effects of Treg cells both in vitro and in vivo is to inhibit the priming and differentiation of effector T cells, so the APC is an obvious target. A number of mechanisms for Treg cell-mediated suppression in vitro have been proposed that appear to primarily affect the function of APC (Figure 2). The observation that Treg cells are the only lymphocyte subpopulation that constitutively expresses CTLA-4 together with the profile of Treg cell suppression in vitro (hypoproliferation, low cytokine production by responder cells) that closely resembles that seen in the presence of reagents that block costimulation have raised the possibility that the interaction of CTLA-4 on Treg cells with CD80 and CD86 on DCs is an important pathway by which Treg cells could mediate their suppressive functions in vitro. A number of early studies demonstrated that Treg cells could downregulate the expression of costimulatory molecules on both human and mouse DC in vitro (Misra et al., 2004; Serra et al., 2003). It was also claimed that Treg cell-mediated suppression of proliferation could be reversed by anti-CTLA-4 or its Fab fragment in vitro, but the inhibitory effects of anti-CTLA-4 were not seen in all studies (Thornton et al., 2004b). Very few studies have examined the effects of anti-CTLA-4 in vivo, but it has been shown that treatment of mice with anti-CTLA-4 abrogates suppression of IBD mediated by Treg cells (Read et al., 2006).

The function of CTLA-4 on Treg cells has been somewhat clarified by the recent demonstration that animals with a selective deletion of expression of CTLA-4 develop systemic autoimmunity at 7 weeks of age (Wing et al., 2008). Thus, CTLA-4 deficiency in Treg cells alone is sufficient to cause fatal disease, and maintenance of its expression in activated effector T cells is insufficient to prevent this outcome. Selective CTLA-4 deficiency does not alter the development or homeostasis of Treg cells or render them pathogenic. These cells remain anergic, but are less suppressive in vitro than are their wild-type counterparts when DCs were used as stimulator cells. CTLA-4-deficient Treg cells are less suppressive in vivo as indicated by the fact that immunodeficient mice reconstituted with total CD4$^+$ T cells from these mice show enhanced immune responses to transplanted tumors. It has been proposed that the interaction of CTLA-4 on Treg cells with its ligands, CD80 and CD86, on DCs blocks the subsequent increase of CD80 and CD86 expression or even downregulates CD80 and CD86 expression induced by antigen-specific effector cells (Onishi et al., 2008). Treg cells from mice lacking CTLA-4 are defective when compared to Treg cells from wild-type mice in preventing increased expression of CD80 and CD86 in DCs, and the addition of anti-CTLA-4 Fab fragments into cultures of wild-type Treg cells and DCs partially inhibited the suppression of CD80 and CD86 expression (Wing et al., 2008). The conclusions drawn from these studies are that inhibition of CD80 and CD86 expression by Treg cells limits the capacity of the DCs to stimulate naive T cells through CD28 resulting in immune suppression. The biochemical nature of the extrinsic signal transduced to the DCs by the interaction of CTLA-4 with CD80 and CD86 remains to be characterized. One mechanism that may mediate the downregulation of CD80 and CD86 expression is trogocytosis, a process in which lymphocytes extract surface molecules through the immunological synapse from the antigen-presenting cells to which they are conjugated (Joly and Hudriser, 2003; Qureshi et al., 2008). It is also possible that CTLA-4 on Treg cells may interact with CD80 and CD86 that are expressed on activated Foxp3$^-$ T cells and in some manner downregulate effector T cell functions (Paust and Cantor, 2005).

A number of other mechanisms have been proposed by which Treg cells either abrogate the antigen-presenting activity of DCs

or promote the secretion of suppressive factors by the target DC population. Treg cells can condition DCs through a mechanism dependent on interactions between CTLA-4 and CD80 and CD86 to express indoleamine 2,3-dioxygenase (IDO), which is a potent regulatory molecule that induces the catabolism of tryptophan into proapoptotic metabolites that result in the suppression of activation of effector T cells. IDO induction was found to depend on high expression of CTLA-4 on the Treg cells (Grohmann et al., 2002). However, there is no clear evidence of the involvement of IDO in Treg cell function in vivo or in vitro. One other cell surface antigen that may play a role in Treg cell suppression of DC function is LAG-3 (CD223), a CD4 homolog that binds MHC class II molecules with very high affinity. Binding of LAG-3 to MHC class II molecules expressed by immature DCs induces an ITAM-mediated inhibitory signal that suppresses DC maturation and immunostimulatory capacity (Liang et al., 2008). Because activated human T cells can express MHC class II, Treg cell-mediated ligation of LAG-3 on effectors might also result in suppression.

In the immune system, extracellular ATP functions as an indicator of tissue destruction and may exert its effects on DCs. The intracellular ATP concentration is high so that substantial amounts of the nucleotide are released upon cell damage. The presence of extracellular ATP can be sensed by purinergic P2 receptors. CD39 is the dominant ectoenzyme in the immune system that hydrolyzes ATP or ADP to AMP and is expressed by B cells, DCs, all mouse Treg cells, and about 50% of human Treg cells (Borsellino et al., 2007). Thus, catalytic inactivation of extracellular ATP by CD39 is another anti-inflammatory mechanism that may be used by Treg cells. Retroviral transduction of $CD25^-$ mouse T cells with Foxp3 induced the expression of CD39. Freshly isolated Treg cells do not hydrolyze ATP, but activated Treg cells can mediate active hydrolysis. ATP can upregulate CD86 expression on DCs. Pre-exposure of Treg cells to ATP containing medium reduced ATP-driven DC maturation. Removal of extracellular ATP by CD39 may allow the Treg cell to enter inflamed regions and permit the Treg cell to quench ATP-driven proinflammatory processes on multiple cell types, particularly DCs. CD39-deficient Treg cells are dysfunctional because they are not anergic and proliferate in response to anti-CD3 and anti-CD28 stimulation in the absence of exogenous IL-2. CD39-deficient cells are also about 50% less effective at suppressing stimulation of T cells from A2A-deficient mice when compared with the ability of WT T cells to suppress WT T cell proliferation. The immunomodulatory effects of removal of ATP by CD39 can be amplified by the generation of adenosine. Adenosine can be generated by CD39 in concert with the 5′-ecto-nucleoside CD73, which dephosphorylates the CD39 product, AMP (Deaglio et al., 2007). Adenosine signals via the A2A adenosine receptor and may inhibit the functions of DCs as well as act directly on activated T cells.

One other molecule that is secreted by Treg cell and that may affect DC function is fibrinogen-like protein 2 (FGL2), a member of the fibrinogen superfamily. $CD25^+$ T cells express 6-fold higher FGL2 mRNA than do $CD25^-$ T cells. Recombinant FGL2 inhibits T cell proliferation in response to anti-CD3 and anti-CD28 and alloantigen stimulation. Treg cells from *Fgl2* $^{-/-}$ mice are less effective at suppressing the proliferation of WT T cells and a polyclonal anti-FGL2 completely blocked the suppressive activity of Treg cells in a dose-dependent manner. Recombinant FGL2 binds to DCs via the inhibitory FcγRIIB receptor (Shalev et al., 2008). Treatment of DCs from wild-type mice with FGL2 results in decreased expression of CD80 and CD86, whereas FGL2 treatment had no inhibitory effect on DCs from FcγRIIB-deficient mice. Thus, FGL2 may represent an important Treg cell product that primarily downregulates DC function.

Neuropilin (Nrp-1) has been proposed to play a role in the interaction of Treg cells with DCs. Nrp-1 is a receptor for class III semaphorins and a coreceptor for vascular endothelial growth factor. Nrp-1 is preferentially expressed on Treg cells and can be induced by ectopic expression of Foxp3 in $Foxp3^-$ T cells (Sarris et al., 2008). Nrp-1 promotes long interactions between Treg cells and immature DCs. Blocking of Nrp-1 decreases the frequency of long interactions, whereas ectopic expression of Nrp-1 in $Foxp3^-$ T cells increases the number of long interactions. Anti-Nrp-1 completely abrogates suppression of proliferation mediated by Treg cells when the responder T cells are stimulated with low concentrations of antigen. These data suggest that the role of Nrp-1 is to give Treg cells a head start over naive responder T cells under conditions in which antigen is limiting.

The Pleiotropic Role of TGF-β in Treg Cell Development, Homeostasis, and Effector Function

Although TGF-β plays a critical role in the induction of $Foxp3^+$ Treg cell in vivo and in vitro (Chen et al., 2003; Liu et al., 2008) and in Treg cell homeostasis (Marie et al., 2005), its role as a suppressor effector molecule remains controversial. The majority of studies with either human or mouse Treg cells have failed to demonstrate that Treg cell function in vitro could be reversed by anti-TGF-β. Nakamura et al. (2001) first raised the possibility that TGF-β produced by Treg cells is bound to the cell surface by an as yet uncharacterized receptor and would mediate suppression in a cell contact-dependent fashion. In their studies, TGF-β is detected on the surface of resting and activated $CD25^+$ T cells, and suppression could be reversed by high concentrations of anti-TGF-β. They postulated that latent TGF-β, bound to the cell surface of activated Treg cells, is delivered directly to responder $CD25^-$ T cells and is then locally converted to its active form. In contrast to these studies, Piccirillo et al. (2002) were unable to show a requirement for either the production of TGF-β or responsiveness to TGF-β in Treg cell-mediated suppression. High concentrations of anti-TGF-β did not reverse suppression, nor did anti-TGF-β or a soluble form of the TGF-βRII inhibit suppression mediated by activated Treg cells.

More recent studies have re-evaluated the cell surface expression and function of TGF-β on both human and mouse $Foxp3^+$ Treg cell. Neither active nor latent (TGF-β bound to latency associated peptide [LAP]) TGF-β could be detected on the cell surface of resting human or mouse $Foxp3^+$ Treg cells. However, after activation via the TCR, a very high percentage of activated mouse and human Treg cells could be stained with anti-LAP (and presumably expressed latent TGF-β), whereas none were reactive with anti-TGF-β (Andersson et al., 2008; Tran et al., 2009b). Latent TGF-β is likely produced by the Treg cells, as indicated by the fact that expression of LAP on activated Treg cells is inhibited by the addition of monensin to the activation cultures and that treatment of Treg cells with an siRNA specific for

TGF-β inhibited the expression of LAP. What then is the functional significance of the selective expression of latent TGF-β on both activated human and mouse Treg cell? The simplest interpretation of this finding that would be consistent with the model proposed by Nakamura et al. (2001) would be that the cell surface latent TGF-β is activated and plays a major role in Treg cell suppression by acting directly on responder T cells or on the DCs. However, siRNA knock down of TGF-β in human Treg cells resulted in only a slight reduction in their suppressor capacity in vitro (D. Tran and E.M.S., unpublished data), and soluble recombinant LAP (the most potent neutralizer of TGF-β) had no effect on the ability of activated mouse Treg cells to inhibit the activation of naive T cells. We have recently demonstrated (Andersson et al., 2008) that the major role of the expression of latent TGF-β on the surface of murine Treg cells is to convert $Foxp3^-$ T cells into $Foxp3^+$ Treg cells by a mechanism of infectious tolerance when both populations are activated via their TCRs in a DC-independent manner. It is also possible that TGF-β on the cell surface of activated Treg cells may be delivered to DCs and downregulate their function.

The contribution of TGF-β to the suppressive capacity of Treg cells in vivo has also been difficult to dissect. A number of in vivo experiments have demonstrated that autocrine production of TGF-β by Treg cells is not essential for Treg cell suppression in vivo. For example, transfer of *Tgfb1*$^{-/-}$ splenocytes to *Rag2*$^{-/-}$ mice induced disease with features similar to those of the *Tgfb1*$^{-/-}$ mice, disease transfer was accelerated by the depletion of *Tgfb1*$^{-/-}$ Treg cells, but cotransfer of *Tgfb1*$^{-/-}$ Treg cells attenuated disease (Mamura et al., 2002). Although TGF-β appears to play a nonredundant role in control of intestinal inflammation, Treg cells from *Tgfb1*$^{-/-}$ mice or from mice expressing a dominant-negative form of the TGF-βRII are capable of inhibiting IBD induced by $Foxp3^-$ T cells in vivo (Fahlen et al., 2005). Importantly, the function of *Tgfb1*$^{-/-}$ Treg cells is abrogated by anti-TGF-β, indicating that TGF-β is absolutely required for protection from IBD, but can be derived from a non-Treg cell source. In contrast, mice with a T cell-specific deletion of TGF-β production (Li et al., 2007) or processing (Pesu et al., 2008) do develop an autoimmune syndrome including IBD. The differences between these studies are unclear, but may relate to the flora in different animal colonies. It is also possible that Treg cell-mediated Foxp3 induction may be required at sites of significant inflammation such as the gastrointestinal tract. TGF-β may also play a role in the development of Treg cells that are capable of producing IL-10, as shown by the fact that treatment of mice with anti-TGF-β prevented the conversion of $CD4^+Foxp3^-$ T cells to $CD4^+Foxp3^+$IL-10 producers in intestine-associated lymphoid tissues (Maynard et al., 2007).

In Vivo Veritas: Mechanisms of Treg Cell Suppression In Vivo

Although multiple mechanisms for Treg cell suppression have been shown in vitro, it is unclear whether the same or different mechanisms are used by Treg cells in vivo. Analysis of the potential mechanisms of Treg cell suppression in vivo is even more complex. In contrast to the fixed environment of the culture dish, in vivo the Treg cell must be able to home to various parts of the body and to physically interact directly with effector T cells or indirectly via the APCs. Put simply, there is ample room in vivo for the effector T cells to evade suppression by Treg cells. It is unknown whether the suppressive activity of Treg cells is executed in secondary lymphoid organs or at the site of inflammation or both. There are also a number of major differences between the properties of Treg cells in vivo compared to in vitro. First, although Treg cells are nonresponsive or anergic to stimulation via the TCR in vitro, Treg cells expand after engagement of their TCR by cognate antigen in a manner indistinguishable from conventional $CD4^+$ T cells in vivo (Walker et al., 2003). Second, as discussed above, most studies of Treg cell suppression in vitro have failed to define a role for Treg cell-produced suppressor cytokines such as IL-10, and the role of TGF-β production by Treg cells remains controversial. However, multiple studies in vivo have shown that secretion of IL-10 by Treg cells constitutes an important component of their suppressive effects (Belkaid, 2007; McGeachy et al., 2005). The major effect of Treg cells on T cell activation in vitro is to inhibit priming by blocking IL-2 production and effector cell expansion. However, the contribution of IL-2 in the antigen-driven expansion of $CD4^+$ T cells in vivo remains unknown, as indicated by the fact that antigen-specific T cells from *Il2*$^{-/-}$ mice expand normally after stimulation by antigen in vivo. A further confounding factor in the analysis of Treg cell function in vivo is that many of the studies on Treg cell suppression of autoimmunity have been performed after transfer of Treg cells and effectors into immunodeficient mice, an environment that promotes lymphopenia-induced proliferation of either the Treg cells or the effectors. The results of these studies may not be readily transferable to studies performed in normal mice. Lastly, it remains unclear at what stage of activation or differentiation effector T cells are subject to Treg cell control.

The original studies documenting the ability of $Foxp3^+$ T cells to suppress autoimmune disease in vivo were all performed with polyclonal populations of Treg cells. In vitro studies strongly suggest that Treg cells have to be activated via their TCR to suppress, so one question that remains to be addressed is whether polyclonal Treg cells have to be activated via their TCR to suppress in vivo? The source of the TCR signal during in vivo activation of polyclonal Treg cells is not known. It is unlikely that suppression is secondary to the presence of the very small number of autoantigen-specific Treg cells present in the polyclonal population. A more likely scenario is that polyclonal Treg cells are able to control various responses because they are continuously being activated via their TCR by complexes of MHC class II and ubiquitous self-peptides. Additional signals (IL-2?) provided by the responding effector T cells may be needed to fully activate their suppressive activity in situ.

A detailed analysis of which components of the development and activation of effector T cells are inhibited by polyclonal Treg cells has not been performed. Kohm et al. (2002) supplemented normal mice with polyclonal Treg cells (potentially doubling the number of Treg cells), immunized the mice with an encephalitogenic peptide in adjuvant, and observed a downregulation of the severity of experimental autoimmune encephalomyelitis (EAE). Suppression of disease was accompanied by a decreased migration of cells into the target organ, but cytokine production by effector T cells was not inhibited. In preliminary studies with a similar model, we also failed to observe an inhibition of cytokine production, but did observe a marked

enhancement in the numbers of effectors cells in the draining lymph node, raising the possibility that polyclonal Treg cells might trap effector cells at the site of immunizaton and prevent their egress to the target organ (T. Davidson and E.M.S., unpublished data). In a model of autoimmune gastritis, when a small numbers of autoreactive T cells were transferred into *nude* mice expressing a tissue-specific autoantigen, polyclonal Treg cells did not inhibit the migration of the autoreactive T cells into the gastric lymph or into the target organ, nor did they inhibit the antigen-driven expansion of autoreactive T cells in the gastric lymph node. The polyclonal Treg cells appeared to block the differentiation of the autoreactive T cells to Th1 effector cells (DiPaolo et al., 2005). Sarween et al. (2004) reported that polyclonal Treg cells inhibited the development of diabetes induced by transfer of OVA-specific transgenic T cells into *Rag1*$^{-/-}$ mice expressing OVA driven by the rat insulin promoter. They also observed a minimal effect on the expansion of the transgenic T cells in the draining lymph node accompanied by a reduction in the amount of IFN-γ production. In contrast to the studies on gastritis, they did observe an inhibition of T cell infiltration into the pancreatic islets.

Very few studies have dealt with the effectiveness of polyclonal Treg cells in treating established autoimmune disease. One advantage of the IBD model is that Treg cells can cure ongoing colitis 4 weeks after initiation of disease (Uhlig et al., 2006). Treg cells proliferate and accumulate in the mesenteric lymph node and also in the colonic lamina propria. At both sites, the progeny of the Treg cells are found to be in direct contact with CD11c$^+$ DCs as well as effector T cells. These findings suggest that regulation of an active immune response by Treg cells occurs in the draining lymph node as well as at the site of inflammation. The majority of the Foxp3$^+$ T cells were IL-10-secreting cells and the IL-10-producing cells were selectively enriched in the colonic lamina propria suggesting that the gut environment may condition the Treg cells to differentiate into IL-10-producing Treg cells in the colon. It was impossible in this model to directly examine the effects of the Treg cells on effector cell numbers or expansion.

Two studies have used two-photon laser scanning microscopy to examine potential interactions between antigen-specific Treg cells and DCs in lymph nodes (Tang et al., 2006; Tadokoro et al., 2006). In the absence of antigen-specific Treg cells, an arrest of antigen-specific effector T cells interacting with DCs was observed, whereas in the presence of Treg cells, the arrest of effector T cells was markedly diminished. Treg cells were also capable of suppressing the formation of stable clusters of diabetogenic TCR transgenic T cells in isolated pancreatic lymph nodes. In both of these studies, the conventional T cells showed diminished cytokine production in the lymph nodes. Importantly, stable Treg cell-T effector cell interactions or simultaneous interactions between Treg cell-T effector cell and the DCs were not seen. Although one might conclude from these studies that Treg cells exert their suppressive actions on DCs in secondary lymphoid organs by diminishing the activation of the DCs, by inhibiting the ability of DCs to physically interact with effectors cells, or by blocking the capacity of DCs to present antigen, no direct effects of the Treg cells on DC function were seen in these studies.

The majority of studies have shown that antigen-specific Treg cells are more potent at suppressing the induction of autoimmune disease than polyclonal populations. Klein et al. (2003) attempted to model the mechanisms by which antigen-specific Treg cells inhibit effector T cell activation by cotransferring antigen-specific Treg cells and naive T cells followed by priming with antigen in incomplete adjuvant. Treg cells were stimulated by antigen to proliferate almost as strongly as naive CD4$^+$ T cells and a marked accumulation of Treg cells was observed in antigen-draining lymph nodes. Both Treg and non-Treg cells expanded in an antigen-dependent manner and produced typical patterns of cytokines, the Treg IL-10 and the naive T cells IL-2 and IFN-γ. At later time points, effector cell expansion ceased, but when the few effector cells remaining were restimulated, they appeared to be fully competent producers of both IL-2 and IFN-γ. It was concluded from these studies that, in contrast to the in vitro studies, no influence of Treg cells on the differentiation of naive T cells could be observed; the responder cells may have died or migrated from the draining nodes.

Very different conclusions were drawn from a study in which antigen-specific TGF-β-induced Treg cells were used to prevent gastritis (DiPaolo et al., 2007). The induced Treg cells were long lived in vivo, maintained Foxp3 expression, and protected mice from disease. The major effect of the Treg cells was to inhibit the expansion of the cotransferred effector cells on day 5 after transfer. Most importantly, DCs that had been exposed to Treg cells in vivo had a reduced capacity to present the endogenous autoantigen compared to those from noninjected mice. It thus appears that one mechanism by which Treg cells exert their function in vivo is by reducing the ability of DCs to prime autoreactive T cells, hence stopping the autoimmune response before it even starts. This study supports the experiments that demonstrate that the presence of Treg cells results in fewer long-lasting interactions between effector T cells and DCs, but also demonstrates that the Treg cells exert their negative effects by decreasing the stimulatory capacity of DCs rather than by competing with the effectors for antigen or by acting directly on the effector cells to prevent their interaction with DCs.

In contrast to the effects of antigen-specific Treg cells on CD4$^+$ effector T cells, Chen et al. (2005) have shown that antigen-specific Treg cells do not inhibit the expansion of CD8$^+$ effector cells or their differentiation to cytotoxic T lymphocytes (CTLs), but do block the ability of the activated CD8$^+$ T cells to kill antigen-expressing target cells. Impaired TGF-β signaling in the CD8$^+$ effectors conferred resistance to Treg cell-mediated suppression of cytolytic ability in vivo. Further analysis of this model (Mempel et al., 2006) by multiphoton microscopy demonstrated that in the presence of Treg cells, the CTLs underwent normal differentiation, migrated normally, and responded to antigen-presenting target cells by forming stable conjugates. Ex vivo analysis of CTLs show that their failure to kill target cells in vivo is correlated with impaired release of lytic granules, whereas cellular granule content is unchanged. Suppression is reversible upon in vivo removal of Treg cells after CTL priming, but did not require prolonged physical interaction of CTLs with Treg cells. Thus, Treg cells can modulate a terminal effector function of CD8$^+$ T cells.

Taken together, these studies indicate that Treg cells can suppress immune responses in vivo at multiple levels and that distinct or even contradictory mechanisms may predominate in different experimental models. A number of critical questions

remain to be addressed. What are the functional mechanisms used by Treg cells in lymphoid versus nonlymphoid tissues? What are the signals that direct Treg cell migration to nonlymphoid tissues? Do Treg cells need to reprogram their homing and chemokine receptor profiles in order to enter nonlymphoid sites? Is Treg cell suppression in nonlymphoid sites primarily mediated by the production of anti-inflammatory cytokines, whereas suppression in lymphoid sites is exclusively secondary to inhibition of priming? Can polyclonal Treg cells migrate to sites of autoantigen expression or are signals generated by activated effector T cells required to recruit the polyclonal Treg cells? Do polyclonal Treg cells also target DCs and suppress later steps in the differentiation of pathogenic Th1 or Th17 cells by blocking the production of IL-12 or IL-23 by DCs? Lastly, are the effects of Treg cells in vivo reversible upon removal of the Treg cells as suggested by a number of studies (Samy et al., 2005; Mempel et al., 2006) or do Treg cells delete effectors or render them permanently anergic?

Reversal of Suppression

Although the mechanism of Treg cell-mediated inhibition of T cell activation remains unknown, one approach to determining potential cell surface antigens involved in this process has been to reverse suppression with antibodies to candidate antigens. One member of the tumor necrosis receptor superfamily (TNFRSF), the GITR (TNFRSF18), has been claimed to play an important role in regulation of T cell suppressor activity. Both a polyclonal antiserum and a mAb to the GITR were initially reported to reverse suppression mediated by freshly isolated Treg cells (Shevach and Stephens, 2006). Because the GITR is rapidly upregulated on Foxp3$^-$ T cells and Treg cells constitutively express the GITR, it was impossible to conclude that the anti-GITR mediated its effects by acting solely on the Treg cells. When combinations of WT and *Tnfrsf18*$^{-/-}$ Foxp3$^-$ T cells and Treg cells were used in coculture experiments, ligation of the GITR on the responders, not the Treg cells, was required to abrogate suppression. Thus, engagement of the GITR on effector cells by its ligand on APC early during the course of an immune response rendered the responder cells resistant to suppression by Treg cells. This model is also compatible with the observations of Pasare and Medzhitov (2003) that soluble factors such as IL-6 released by activated DCs can act directly on effector cells, but not Treg cells, to render them resistant to suppression.

A second member of the TNF receptor superfamily, OX40 (CD137), is also constitutively expressed on mouse Treg cells and transiently expressed on Foxp3$^-$ lymphocytes upon antigen stimulation. It has been claimed that engagement of OX40 reverses the suppressive function of Treg cells by acting on the Treg cells rather than by rendering the responder cells resistant to suppression (Valzasina et al., 2005). If Treg cells are preincubated with an agonist anti-OX40, they lose the ability to suppress effector T cells in an in vivo mouse model of graft versus host disease. Reversal of Treg cell suppression by anti-OX40 also resulted in complete rejection of already established tumors (Piconese et al., 2008). However, it is difficult to rule out that persistence of the agonistic antibody also costimulated effector T cells that expressed OX40 after activation. It remains possible that engagement of OX40 in some manner abolishes Treg cell suppression, but the effects of anti-OX40 in vivo could also be secondary to partial depletion of Treg cells by the antibody. Curiously, a recent study has demonstrated that Treg cells can inhibit the release of allergic mediators from mast cells and that inhibition was mediated by OX40 expressed on the Treg cell interacting with OX40L expressed on the mast cells (Gri et al., 2008). Thus, OX40 in some cases can function as a suppressor effector molecule.

Engagement of Toll-like receptor 2 (TLR2) on Treg cells has also been claimed to reverse the suppressive effects of mouse Treg cells in some studies (Liu et al., 2006; Sutmuller et al., 2006). In contrast, the suppressive function of human Treg cells is enhanced by engagement of TLR5 by its ligand, flagellin (Crellin et al., 2005). The major problem with the interpretation of these studies is that the TLR are also expressed at low amounts on Foxp3$^-$ T cells and at much higher amounts on multiple cell types in the innate immune system. Contamination of the preparations of Treg cells used in these studies by non-Treg cells may have contributed to the discrepant results. The other major problem with the interpretation of studies on reversal of suppression is that we have very little understanding of the biochemical pathway used by the Treg cells and even less understanding of the signals induced by those agents that are claimed to reverse suppression. On the brighter side, it is clear that Treg cell-mediated suppression can be overcome by costimulation of effector cells (e.g., via the GITR or OX40). Reagents that stimulate these pathways may be valuable adjuncts to enhance responses both to tumor vaccines and responses to weak vaccines to infectious agents.

Concluding Comments

The in vitro models of Treg cell function certainly suggest that Treg cells may use multiple mechanisms to suppress immune responses. Yet, how many of the mechanisms are actually operative in vivo? Have we now exhausted the list of potential suppressor mechanisms or is a critical suppressor pathway yet to be discovered? One argument against the existence of an as yet to be discovered "major player" is that one would have expected that a mutation in such a pathway would have led to the development of a severe autoimmune syndrome similar to that seen with mutations of Foxp3. A more important question is whether our current understanding of Treg cell suppressor mechanisms offers any insight into how Treg cell function can be manipulated in vivo. For example, can we transiently and selectively downregulate Treg cell function prior to the administration of a tumor vaccine? Alternatively, how can we enhance Treg cell numbers or effectiveness without inducing systemic immunosuppression? Even if we can increase Treg cell numbers by using cellular biotherapy with expanded polyclonal or antigen-specific Treg cells to treat autoimmune disease, will the expanded Treg cells survive in vivo? How often would such therapy have to be administered? Will Treg cells induce long-lasting antigen-specific tolerance via an infectious tolerance mechanism? Some of these questions are likely only to be answered by well-designed and well-controlled clinical trials.

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