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From Vanilla to 28 Flavors: Multiple Varieties of T Regulatory Cells

Perspective

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Summary

Numerous T cell subpopulations have now been claimed to exhibit regulatory activity. Shevach discusses the current understanding of the different subsets of T regulatory cells and provides a perspective on the current areas of uncertainly and controversy in the field.

Introduction

The resurgence of interest in regulatory or suppressor T cells can be compared to the development of the ice cream industry in the United States. Howard D. Johnson was a struggling businessman in the 1920s when he invented a high-butterfat vanilla ice cream and started a chain of ice cream shops in Massachusetts. His ice cream rapidly became very popular and he increased his repertoire from a single flavor to 28 flavors. Suppressor T cells, or as they are now more commonly called, T regulatory (Treg) cells, were rediscovered in the mid-1990s by Shimon Sakaguchi (Sakaguchi et al., 1995), who was the first to demonstrate that a minor population of $CD4^+$ T cells that coexpressed the CD25 antigen functioned as Treg cells in adult mice. Interest in Treg cells has exploded over the past 10 years, and numerous other T cell populations have now been claimed to exhibit regulatory activity. Although we have probably not yet reached 28 different "flavors" of Treg cells, we are rapidly approaching that number (Figure 1). I will attempt to summarize our current understanding of the different types of Treg in both animals and man and to emphasize the current areas of uncertainly and controversy in the field.

Treg Cells in Mice and Men

The importance of $CD4^+CD25^+$ T cells in immune regulation was evident from the initial demonstration that the depletion of this subset (~10%) from a population of $CD4^+$ T cells from a normal adult mouse resulted in the development of a spectrum of autoimmune diseases when the remaining $CD4^+CD25^-$ T cells were transferred to immunoincompetent recipients. The subsequent demonstration that $CD4^+CD25^+$ T cells also manifest regulatory activity in vitro in a simple and highly reproducible assay (Thornton and Shevach, 1998) formed the foundation of the resurgence of interest in T cell-mediated immunoregulation. Human $CD4^+CD25^+$ T cells with functional properties very similar to those of mouse $CD4^+CD25^+$ T cells were subsequently identified by multiple groups (Shevach, 2001). Any doubts about the importance of this subset of Treg cells were erased by the observations that the Foxp3 transcription factor is selectively expressed in $CD4^+CD25^+$ T cells, that it is required for their development, and that the lethal autoimmune syndrome that develops in scurfy mice and in humans with the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome is secondary to a mutation in Foxp3 with a resultant deficiency in Treg cells (Hori et al., 2003).

The development of Foxp3 monoclonal antibodies (mAb) that are capable of detecting their target intracellularly by flow cytometry and the development of knockin mouse strains (Fontenot et al., 2005) where Foxp3 is coexpressed with a fluorescent label have greatly enhanced our ability to define and characterize this Treg cell subset. In the mouse, there is an excellent correlation between the expression of Foxp3 and CD25, but a minor population (10%) of $Foxp3^+$ cells is $CD25^-$; conversely, about 10% of $CD25^+$ T cells are $Foxp3^-$ and likely represent activated effector cells. In humans, the situation is considerably more complex. Almost all $CD4^+CD25^{hi}$ cells are $Foxp3^+$, whereas a variable percentage of $CD25^{int}$ cells express lower, but substantial, amounts of Foxp3. $CD4^+CD25^-$ cells are uniformly $Foxp3^-$. Although flow-cytometric isolation of the cells expressing the highest amounts of CD25 does allow one to obtain an almost pure population of human Treg cells, it should be pointed out that a substantial subset of $Foxp3^+$ cells will also be lost (the $CD25^{int}Foxp3^+$ cells).

The other important difference between the regulation of Foxp3 expression in mouse and humans is that Foxp3 expression is readily induced in a majority of both human $CD4^+CD25^-Foxp3^-$ and $CD8^+CD25^-Foxp3^-$ T cells by activation via the T cell receptor (TCR), whereas Foxp3 expression is not induced in mouse $CD25^-$ T cells under similar activating conditions. The expression of Foxp3 after TCR stimulation of human $CD25^-$ T cells can approach that of the resting $CD4^+CD25^+Foxp3^+$ population. However, the induced $Foxp3^+$ population is neither anergic nor suppressive in vitro (Gavin et al., 2006), and the expression of Foxp3 appears to be transient, declining to baseline amounts with prolonged culture. In many respects, the expression of Foxp3 after activation of human T cells resembles that of CD25—rapid induction and decline. In any case, one must question whether Foxp3 expression is an absolute marker of T regulatory cells in humans. More importantly, one must certainly raise the possibility that some of the $Foxp3^+$ cells isolated from healthy individuals or from patients with autoimmune and inflammatory diseases represent activated T effector cells. Appropriate caution should therefore be used in the evaluation of claims of purported defects of T regulatory function in disease even when the studies were performed with cells that were $CD25^+Foxp3^+$.

"Adaptive" or "Induced" Treg Cells

$CD4^+CD25^+Foxp3^+$ T cells develop in the thymus, and many investigators have termed these cells "natural" Treg (nTreg) cells in contrast to Treg cells that develop in peripheral lymphoid tissues, which are frequently $Foxp3^-$ and have been termed "adaptive" or "induced"

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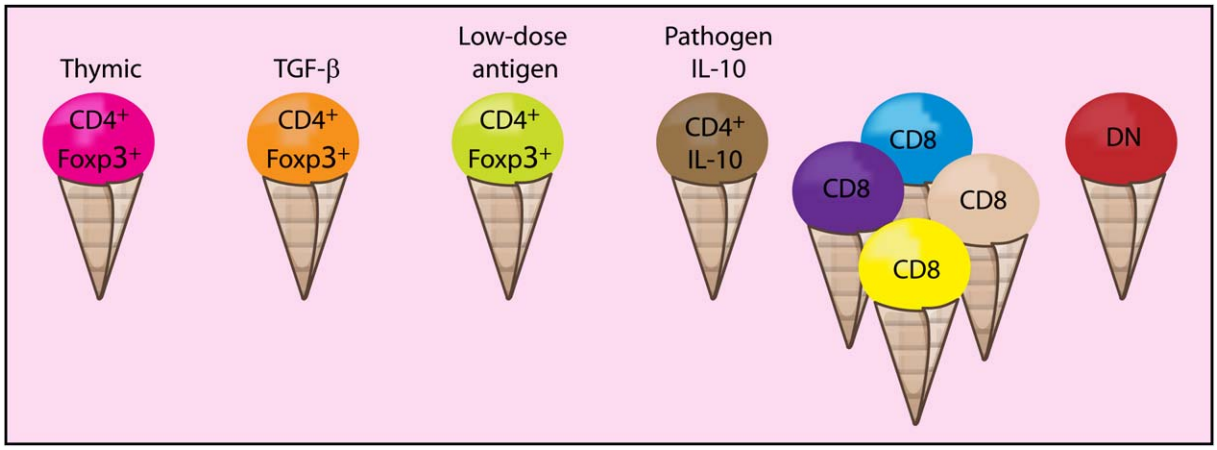

Figure 1. Multiple Flavors of T Regulatory Cells

Several different types of T regulatory cells have been described in the literature. In addition to the "classic" $CD4^+Foxp3^+$ thymic-derived T regulatory cells, a similar population can be induced extrathymically in the presence of TGF-β or other stimuli. $CD8^+$ T regulatory cells have been poorly characterized. It is not clear how the multiple types are related.

Treg cells. Several studies have raised the possibility that $CD4^+Foxp3^+$ cells might also be generated in peripheral lymphoid tissues from naive $CD4^+Foxp3^-$ progenitors. Two major factors appear to be involved in the induction of Foxp3 expression in the periphery—the cytokine TGF-β and the mode of antigen presentation. A role for TGF-β in the induction of Treg cells was first described by Horwitz and colleagues (Yamagiwa et al., 2001), who demonstrated that TGF-β can induce naive human $CD4^+$ T cells to develop powerful contact-dependent suppressor activity that is mediated by TGF-β or IL-10. Other studies (Chen et al., 2003; Fantini et al., 2004) demonstrated that mouse $CD4^+CD25^-$ T cells could be converted to $CD4^+CD25^+Foxp3^+$ T regulatory cells by stimulation via the TCR in the presence of TGF-β. The TGF-β-converted $CD4^+$ T cells exhibited cell-contact-dependent suppressor activity in vitro when cocultured with normal $CD4^+$ T cells. In addition, antigen-specific TGF-β-converted cells could inhibit antigen-driven $CD4^+$ T cell expansion in vivo. Fantini et al. (2004) also postulated a positive autoregulatory loop in which Smad 7, a transcription factor that is normally induced by TGF-β and limits TGF-β signaling, is downregulated by Foxp3. Foxp3 downregulation of Smad7 then renders $CD25^-$ T cells highly susceptible to the further inductive effects of TGF-β signaling via Smad3 and Smad4.

Although these studies strongly suggested that costimulation of T cell activation in the presence of TGF-β may induce the expression of Foxp3 and regulatory activity, it was unclear at the time these studies were performed what percentage of the $CD25^-$ T cells were actually converted. The possibility remained that TGF-β was not inducing Foxp3 expression, but was facilitating the selection of the minor population of $CD25^-Foxp3^+$ cells that were present in the starting populations. More recent studies analyzing the expression of Foxp3 on the single-cell level by intracellular staining have, in fact, confirmed these initial studies and indicated that 50%–100% of $CD25^-Foxp3^-$ cells can be induced to express Foxp3 by costimulation in the presence of TGF-β (Bettelli et al., 2006). Some of these studies have been performed with $CD4^+$ T cells from TCR transgenic mice on a RAG-deficient background that are as $Foxp3^-$ as a starting population. Again, there appears to be a difference between murine and human T cells. Whereas the TGF-β-induced murine $Foxp3^+$ T cells manifest suppressor activity both in vitro and in vivo, it has yet to be shown that the TGF-β-induced human T cells behave in similar fashion. As noted above, Foxp3 expression in human T cells does not uniformly correlate with suppressor activity, and in our hands (D. Tran and E.M.S., unpublished data), TGF-β-induced $Foxp3^+$ human T cells are neither anergic nor suppressive. This result may indicate that Foxp3 is necessary, but not sufficient, for human Treg function. Other explanations including the stimulatory conditions, the stability of Foxp3 expression, and the absolute amount of Foxp3 induced may also account for the species difference.

The second major factor that appears to induce Foxp3 expression in $Foxp3^-$ peripheral T cells is the mode of antigen presentation. When peptide-specific T cells from TCR transgenic mice on RAG-deficient background are stimulated in vivo for 2 weeks by the continuous delivery of low doses of antigen via a mini-osmotic pump, the antigen-specific cells expressed Foxp3 and could suppress proliferation and cytokine production in vivo (Apostolou and von Boehmer, 2004). Similar results were observed when animals were primed with minute amounts of peptide coupled to the mAb DEC205 that targeted the antigen to dendritic cells (DCs) (Kretschmer et al., 2005). Exposure of TCR transgenic T cells to their target antigen expressed endogenously in soluble form also resulted in the induction of Foxp3 expression and Treg cell activity (Knoechel et al., 2005). It is unclear whether TGF-β plays a costimulatory role in these in vivo models, although peptide-specific T cells from mice expressing a dominant-negative form of the TGF-β-receptor II developed many fewer $Foxp3^+$ T cells in vivo than T cells from wild-type mice when primed with peptide-DEC205 complexes. Taken together, these studies suggest that antigen presentation under certain conditions, presumably in the absence of DC activation, results in the generation of $Foxp3^+$ suppressor rather than effector T cells.

The major issue that remains to be addressed is the physiologic relevance of the peripheral induction of Foxp3$^+$ Treg cells. TGF-β is a ubiquitously expressed cytokine produced by many cell types whose expression is increased at local sites of inflammation. Although some studies (Cobbold et al., 2004; Ochando, et al., 2006) suggest that certain protocols used for the induction of transplantation tolerance in vivo mediate their effects by the TGF-β-dependent induction of Foxp3$^+$ Treg cells, numerous questions regarding the induction of Foxp3$^+$ Treg cells remain to be addressed: What percentage of the CD4$^+$CD25$^+$Foxp3$^+$ T cells present in the periphery of a normal adult is thymus-derived, and what percentage is induced in the periphery? If TGF-β is constantly promoting the induction of Treg cells, why does the absolute number of Treg cells remain relatively constant throughout the lifetime of an animal? Is there a parallel death pathway for certain subsets (thymic or peripheral) of Foxp3$^+$ Treg cells? Are the effects of TGF-β continuously antagonized in vivo by the presence of IL-6 in the periphery, resulting in the induction of proinflammatory rather than anti-inflammatory T cells (Bettelli et al., 2006)? How is the delicate balance between TGF-β induction of Foxp3$^+$ Treg cells and the combined effects of TGFβ and IL-6 in the induction of IL-17-producing T cells regulated? Are human TGF-β-induced Foxp3$^+$ cells fully competent Treg cells? If not, what component is missing? Lastly, can the limited antigen dose and nonactivated DC strategy for the induction of Treg in animal studies be developed for use as a vaccine to generate organ-specific Treg for the treatment of autoimmune disease?

Identification and Isolation of Foxp3$^+$ Treg

One problem that has plagued the Treg cell field is the identification of cell-surface markers that can be used to reproducibly and reliably isolate pure populations of Treg cells. Homogenous populations of Treg cells are needed for functional studies and for in vitro expansion for potential therapeutic purposes. More importantly, for studies of abnormalities in Treg cell function in disease, methods are needed to obtain not only pure Treg cells, but also a population that is representative of the entire population of Treg cells in vivo. Put simply, a population of CD4$^+$CD25$^+$ T cells that are 100% Foxp3$^+$ might be quite suitable for many studies. However, if CD25 expression is downmodulated in disease or during the course of an inflammatory response (Fontenot et al., 2005), an analysis of the frequency and function of only the CD4$^+$CD25$^+$ population might be misleading.

The expression of Foxp3 protein intracellularly as detected by antibodies or in animals that express a Foxp3-GFP fusion protein is now accepted as the "gold standard" for defining either thymic-derived Treg cells or Treg cells that might be generated in the periphery. The availability of mice that express a Foxp3-GFP fusion protein allows one to clearly define and isolate nTreg. These mice are being rapidly bred onto multiple backgrounds to facilitate analysis of nTreg function in disease. It would also be advantageous to have a mouse that expressed a cell-surface marker (e.g, human CD2) under the control of the Foxp3 promoter so magnetic bead-separation technologies could be used. Other markers that have been proposed as being useful for the identification of Treg have proven to be less useful. Many of these identify activation antigens, including CTLA-4, GITR, and other members of the TNF-receptor superfamily, that are also expressed by activated effector T cells. Some, including neuropilin, CD38, and CD5 (Knoechel et al., 2006), appear to be shared with Foxp3$^-$ anergic T cells that do not exert suppressive functions.

Identification and isolation of human Treg cells continues to be a dilemma, because one must rely on expression of cell-surface antigens. Although CD25 expression has been used to identify human Treg cells, CD25 is expressed on a high percentage of human T cells, and only the highest CD25 expressors (~2% of CD4$^+$ T cells) have been claimed to exert significant suppressive effects (Baecher-Allan et al., 2001). However, a higher percentage of human CD4$^+$ T cells express Foxp3, but lower amounts of CD25 (Liu et al., 2006). It was also proposed that almost all of the human Foxp3$^+$ T cells can be identified as expressing low IL-7 receptor (CD127) and that CD127^{lo} expression can be used to isolate human Treg cells. There is, however, a major theoretical problem with the choice of CD127^{lo} as a Treg cell marker. CD25 is not ideal because it is expressed on some activated effector cells. Similarly, expression of CD127 is also downregulated early in the course of T cell activation, so the CD127^{lo} phenotype is also unlikely to be Treg cell specific during an ongoing immune or inflammatory response. In addition, only about 40% of the CD127^{lo} population is Foxp3$^+$, and even purified CD4$^+$CD127^{lo}CD25$^+$ cells were only 85%–90% Foxp3$^+$. Thus, the utility of using the differential expression of CD127 for the isolation of human Treg cells requires further study.

Lastly, one must consider the possibility of subpopulations of Foxp3$^+$ Treg cell with different functional properties. The most interesting marker of a potential Treg cell subpopulation is the integrin ($\alpha_E\beta_7$, CD103) that is expressed on approximately 25% of mouse Treg cells, and that controls the adherence of conventional T cells to epithelium in the gut by binding to E cadherin. The subset of CD25$^+$CD103$^+$ T cells appears to exert more potent suppressive effects in vitro and to be expressed on Treg cells that circulate preferentially to inflammatory sites (Huehn et al., 2004). Certain chemokine receptors may also be preferentially expressed on Treg subpopulations, but more study is needed to determine whether their differential expression defines functionally distinct populations.

Antigen-Induced CD4$^+$ IL-10-Producing Treg Cells

One of the first procedures used for the induction of Treg cells in vivo was administration of antigen by the oral route. Oral administration of antigen can result in deletion of antigen-specific T cells after high-dose antigen delivery or the induction of Treg cells at low concentrations of antigen. The selective induction of Treg cells via the oral route is thought to be secondary to poorly characterized properties of gut-resident antigen-presenting cells (APCs). In some studies, clones derived from mice that have been orally tolerized with low antigen dose primarily produced TGF-β, and these cells were termed Th3 cells. Progress in the further characterization of Treg cells induced by oral tolerance has been slow, and Treg cells that exclusively produce TGF-β have not been frequently observed in other models.

However, the important lesson to be learned from the oral-tolerance studies is that the milieu in which T cells are primed plays an important role in determining whether effector cells or suppressor cells are generated.

One systematic approach to the analysis of the priming milieu was to culture murine or human $CD4^+$ T cells with antigen or alloantigen in the presence of exogenous IL-10 (O'Garra and Vieira, 2004). Clones derived from this protocol produced large amounts of IL-10, some TGF-β, IFN-γ, and IL-5, but no IL-2 or IL-4. $CD4^+$ T cells generated in this manner have been termed T regulatory 1 (Tr1) cells. IL-10-producing Treg cells can also be generated in vitro by culture in a combination of vitamin D, dexamethasone, and cognate antigen. Homogeneous populations of Tr1 cells do not express Foxp3, can arise in the absence of nTreg cells, but inhibit T cell proliferation with comparable efficiency to nTreg cells. In contrast to nTreg cells whose antigenic specificity is frequently unknown, Tr1 cells are by definition antigen specific. However, in some models, $Foxp3^+$ nTreg cells have also been shown to be both antigen specific and IL-10 producers (Suffia et al., 2006), blurring the distinction between the Treg cell subsets. Although most of the suppressive activity of Tr1 cells is secondary to the production of IL-10 and perhaps TGF-β, some IL-10-producing Tr1 cells suppress the proliferation of naive $CD4^+$ T cells in vitro by an IL-10-independent, cell-contact-dependent mechanism (Vieira et al., 2004). Here again, the distinction between the various flavors of Treg cells is not at all clear.

The capacity of IL-10 to induce Tr1 cells in vitro has a direct in vivo correlate in the immune response to certain infectious agents. Pathogen-specific Tr1 cells can be generated in vivo during the course of bacterial, viral, fungal, or parasitic infections. The major purpose of these cells is to control inflammation and collateral tissue damage. Chronic infectious states may result if this mechanism is subverted by the pathogen. It has been proposed that a unique cytokine environment with enhanced IL-10 production and decreased IL-12 production is created during the course of infection (Mills, 2004). For example, the filamentous hemagglutinin antigen derived from *Bordetella pertussis* induces IL-10 production and inhibits IL-12 production by DCs and macrophages. These pathogen-derived products may also modulate costimulatory molecule expression by DCs, thereby creating a favorable environment for induction of Tr1 cells rather than Th1-Th2 effector cells. Pathogen products may also directly interact with their cellular receptors on T cells, resulting in the generation of Tr1 populations. For example, IL-10-producing Tr1 cells have been generated in vitro by antibody-mediated coligation of CD46 and CD3 on human T cells, and this process may mimic ligation of CD46 by a pathogen-derived ligand (Kemper et al., 2003).

$CD8^+$ T Regulatory Cells

Although the most prominent suppressor T cell populations of the 1970s and 1980s were $CD8^+$ T cells, there has been a reluctance to re-explore the role of $CD8^+$ T cells as Treg cells. More importantly, because $CD4^+CD25^+Foxp3^+$ T cells are potent suppressors of the activation of $CD8^+$ T cells (Piccirillo and Shevach, 2001), there has been no need for a unique CD8-specific regulatory cell. Nevertheless, there have been a number of reports of $CD8^+$ T cells with regulatory functions. $CD8^+$ Treg cells indeed come in many "flavors," and most of them have been characterized in a limited number of experimental models by only one or two laboratories.

One class of $CD8^+$ Treg cells appears to recognize peptides derived from cell-surface antigens in association with classical or nonclassical MHC class I antigens. $CD8^+$ T cells play a prominent role in protection from experimental autoimmune encephalomyelitis (EAE) as depletion of $CD8^+$ T cells from mice that have recovered from EAE renders them susceptible to the development of EAE upon reimmunization with myelin basic protein (MBP). In addition, $Cd8^{-/-}$ PL/J mice develop more chronic EAE than wild-type PL/J mice. $CD8^+$ T cells from mice that had recovered from EAE downregulated or killed some, but not all, $CD4^+$ neuroantigen-specific T cell clones. Inhibition was blocked by anti-TCR and anti-CD8, and by antibodies to the MHC class Ib molecule Qa-1, but not by antibodies to MHC class Ia molecules. It has been proposed that Qa-1 self-peptide complexes expressed by activated $CD4^+$ T cells trigger the TCR on $CD8^+$ T cells. These $CD8^+$ cells then differentiate into suppressor effector cells that in turn suppress $CD4^+$ T cells expressing the same Qa-1-peptide complex (Jiang and Chess, 2000). It remains unknown whether the self-peptides recognized by the $CD8^+$ T cells are derived from the $CD4^+$ TCR or from activation molecules induced by antigen-specific stimulation of the antigen-specific $CD4^+$ T cells. The mechanism of suppression by these $CD8^+$ T cells is also unknown, although it may involve differentiation of the $CD8^+$ T cells to cytotoxic T cells or the secretion of suppressor cytokines. Some studies suggest that human $CD8^+$ T cells can be induced to differentiate into regulatory cells whose function is restricted by HLA-E, the human homolog of Qa-1 (Li et al., 2001).

$CD8^+$ Treg cells that recognize determinants on immunoglobulin molecules have also been detected. Hahn et al. (2005) used an IgG-V_H-region-peptide-based tolerizing regimen that delays autoantibody production and nephritis in a murine model of spontaneous lupus-like disease. Treated mice developed $CD8^+$ T cells that decreased autoantibody production both in vitro and in vivo. The action of the induced $CD8^+$ suppressors is probably on helper $CD4^+$ T cells, because $CD8^+$ T cells from tolerized mice suppressed IFN-γ production in cocultures of $CD8^+$ and $CD4^+$ T cells plus B cells, but did not have direct effects on B cells unless $CD4^+$ T cells were present in culture. The tolerized $CD8^+$ T cells secreted large amounts of TGF-β, and neutralization of this cytokine abrogated suppression of DNA antibody production.

The second class of $CD8^+$ Treg cells appears to resemble the murine $CD4^+$ Tr1 cells subset because their suppressive effects are primarily mediated by IL-10. Gilliet and Liu (2002) were the first to demonstrate that stimulation of $CD8^+$ T cells with plasmacytoid DCs resulted in the induction of IL-10-producing $CD8^+$ T cells that inhibited the proliferative response of naive $CD8^+$ T cells when stimulated with allogeneic monocytes, immature DCs, or mature DCs. The generation of $CD8^+$ IL-10-producing DCs was also IL-10 dependent. Wei et al. (2005) have isolated plasmacytoid DCs from

malignant ascites of patients with ovarian carcinoma. After CD40L activation and pulsing with tumor-associated peptide antigen, these plasmacytoid DCs could induce antigen-specific $CD8^+CCR7^+$ IL-10-producing Treg cells that suppressed the responses of tumor-antigen-specific $CD4^+$ T cells. $CD8^+CCR7^+$ IL-10-producing T cells were found in blood, malignant ascites, and tumor-draining lymph nodes in patients with ovarian cancer.

A second population of $CD8^+$ IL-10 producing Treg has been defined as expressing high amounts of CD122, the IL-2 receptor β-chain. The percentage of $CD122^+CD8^+$ T cells is high (~50%) in young mice, but decreases to ~10% at 7–10 weeks of age, and increases again in older mice (Rifa'i et al. 2004). Most of the studies have been done with cells from 6-week-old mice. Cd122-deficient mice develop an autoimmune syndrome that can be prevented when neonates are injected with $CD8^+CD122^+$ T cells. RAG-deficient mice that received $CD8^+CD122^-$ cells die from an autoimmune like syndrome within 10 weeks after cell transfer, indicating that $CD8^+CD122^-$ cells become activated in the absence of $CD8^+CD122^+$ T cells. Interestingly, $CD4^+CD25^+$ T cells do not control the disease induced in RAG-deficient mice by transfer of $CD8^+CD122^-$ cells, and $CD8^+CD122^+$ Tregs cells are $Foxp3^-$ and cannot be induced by activation of $CD8^+CD122^-$ T cells in vitro. $CD8^+CD122^+$ Tregs inhibit the activation of both $CD8^+$ and $CD4^+$ T cells in vitro to plate bound anti-CD3 in the absence of APCs. IL-10 produced by the $CD8^+CD122^+$ T cells appears to be the factor responsible for suppression of proliferation and IFN-γ production. Thus, $CD8^+CD122^+$ T cells from IL-10-deficient mice were incapable of suppressing T cell activation in vitro, but did show some activity in vivo in suppressing the activity of wild-type $CD8^+CD122^-$ T cells, suggesting that $CD8^+CD122^+$ T cells may use additional suppressor mechanisms (Endharti et al., 2005).

A distinct type of $CD8^+$ Treg cells develops after repeated stimulation of human T cells in vitro with xenogeneic APCs or with antigen-pulsed APCs. These $CD8^+$ T cells are $CD28^-$ and express Foxp3 mRNA. The most interesting property of the $CD8^+CD28^-$ regulatory T cells is that they target APCs and render them tolerogenic. Exposure of monocytes and DCs to this subset of regulatory T cells results in increased expression of genes encoding Ig-like transcripts, ILT3 and ILT4, members of the NK-cell inhibitory-receptor family. Both ILT3 and ILT4 display long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motif. Expression of these receptors is associated with inhibition of NF-κB activation and with a reduced capacity of the APCs to transcribe NF-κB-dependent costimulatory molecules. It has been postulated that antigen-specific MHC class I restricted $CD8^+CD28^-$ T regulatory cells first induce ILT-expressing tolerogenic DCs, which in turn generate $CD4^+$ regulatory T cells (Vlad et al., 2005).

Is there a $CD8^+$ counterpart of the $CD4^+CD25^+Foxp3+$ nTreg? Cosmi et al. (2003) have characterized a population of $CD8^+CD25^+$ human thymocytes that were noncytolytic, had reduced expression of perforin and granzyme A, and mediated suppression by a cell-contact-dependent mechanism that could be reversed by anti-CLTA-4 or anti-TGF-β. This population, therefore, in some respects resembles human nTreg although the suppressive effects of the latter are usually not blocked by anti-CTLA-4 or anti-TGFβ. The expression of Foxp3 by this thymocyte subpopulation was not determined, and it also unknown whether a similar subpopulation is present in normal human peripheral lymphoid tissues.

$CD4^+CD25^+Foxp3^+$ T cells are thought to display an enhanced affinity for self-MHC class II. Another approach to search for the $CD8^+$ homolog of the $Foxp3^+$ nTreg is to isolate a population of $CD8^+$ T cells that are self-MHC class I restricted. Indeed, repeated stimulation of human $CD8^+$ T cells with LPS-activated DC, followed by cloning, resulted in the identification of a number of $CD8^+$ T cell clones that were not anergic, but responded to stimulation with DCs in an HLA-restricted manner, produced IL-4 and IL-13, but not IFN-γ, and expressed CTLA-4 and Foxp3 (Jarvis et al., 2005). They suppressed IFN-γ production and proliferation by $CD4^+$ T cells in a contact-dependent manner that could be reversed by anti-CTLA-4, but not by anti-cytokine mAb. A similar population of cells has been described in the rat, where they suppressed the transfer of graft versus host disease (Xystrakis et al., 2004). $CD8^+CD25^+Foxp3^+$ T cells have also been seen in MHC class II-deficient, but not in wild-type or MHC class I- and class II- double-deficient mice (Bienvenu et al., 2005; G. Stephens and E.M.S., unpublished data). These cells are likely to be restricted by MHC class I molecules and functionally appear to be very similar to nTreg in their suppressive capacity in vitro.

Double-Negative Treg

$CD4^-CD8^-$ double-negative (DN) Treg cells compose 1%–3% of peripheral T lymphocytes in rodents. DN Treg cells isolated from mice that have permanently accepted allo- or xenografts can specifically suppress and kill syngeneic antidonor $CD4^+$ and $CD8^+$ T cells in vitro. DN Treg cells, upon expansion in vitro with allogeneic donor lymphocytes, can specifically suppress proliferation of syngeneic $CD4^+$ and $CD8^+$ T cells in vitro and prolong donor-specific allogeneic-skin-graft survival when infused into syngeneic naive mice (Chen et al., 2005). Human DN Treg cells have also been isolated and characterized (Fischer et al., 2005). These cells compose 0.8%–1% of total human peripheral blood $CD3^+$ T cells and 2.5% of lymph node T cells. Human DN Treg cells can also suppress immune responses mediated by syngeneic $CD8^+$ T cells in an antigen-specific and dose-dependent manner. A rather unique mechanism has been proposed for the fine specificity of the DN Treg. Both mouse and human DN Treg cells are cytotoxic to syngeneic $CD8^+$ T cells that express the same TCR specificity as the DN Treg cells. DN T cells can acquire via their TCR allo-MHC peptides from antigen-presenting cells and use them to specifically trap and kill $CD4^+$ and $CD8^+$ T cells that recognize the same allo-MHC peptides through a process that requires cell-cell contact and Fas-FasL interaction.

In summary, it is difficult to draw solid conclusions about the biologic significance of any of the $CD8^+$ Treg or the DN subsets. Do they represent the "flavor of the month" that never achieves lasting popularity or do they represent important targets for therapeutic manipulation?

Concluding Remarks

One of the troubles with having multiple flavors of ice cream (when I was growing up, the 28 available at

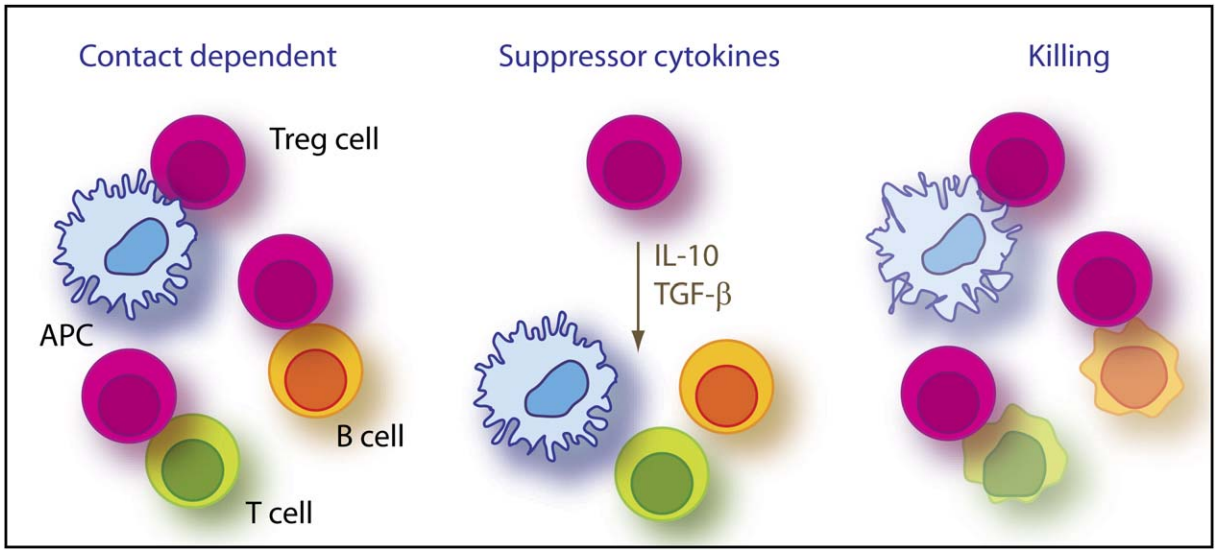

Figure 2. Potential Suppressor-Effector Mechanisms Utilized by Treg Cells

Three potential pathways by which T regulatory cells can mediate their suppressive effects on a number of different types of target cells including effector T cells, antigen-presenting cells (APCs), and B cells. Two of the pathways involve direct suppression of the target cells, whereas the secretion of suppressor cytokines may lead to bystander suppression.

Howard Johnson's, and today, with a few more—31—at Baskin-Robbins) is that it was always difficult to make a selection, and in reality, many of flavors were not easily distinguishable from one another. Selection of the most important and biologically relevant Treg cell population is easy. Vanilla remains the most popular flavor of ice cream in the U.S. today, and there is little doubt that the critical role of the Foxp3$^+$ nTreg cells in the immune system is firmly established. The relative importance of thymic-derived versus peripherally generated Foxp3$^+$ nTreg cells still needs to be established, and major differences may exist between species in the usage of these pathways. One very important issue that remains unresolved is that no progress has been made in elucidating the biochemical and molecular basis of nTreg-mediated suppression or firmly defining the target cell(s). The literature is filled with the term "contact-mediated suppression," yet this conclusion is based on a negative result, the failure to see suppression across a semipermeable membrane. So, a soluble molecule that acts in a concentration-dependent fashion at short range has never been ruled out. Alternatively, if suppression is mediated by a cell-surface antigen interacting with its counter-receptor on another cell, identification of this receptor-ligand pair would greatly facilitate manipulation of nTreg function positively for the control of autoimmune disease, allergy, and graft rejection or negatively for the augmentation of the immune response to tumors or weak vaccines.

The role of Foxp3$^-$ IL-10-producing Treg cells is also reasonably well established, particularly in the immune response to pathogens. Yet, the factors that control the generation of populations of T cells that selectively produce IL-10 and not Th1- or Th2-type cytokines in vitro and in vivo need to be much better defined. The availability of an immunization protocol using an adjuvant that would facilitate the selective priming of antigen-specific IL-10-only producers would be a major advance in the development of a vaccine for organ-specific autoimmune diseases or for infectious diseases with a substantial inflammatory component. However, one aspect of the Tr1 cell subset that has been ignored by most immunologists is that Tr1 populations frequently produce small to moderate amounts of other cytokines, particularly IFN-γ. We need to consider the possibility that such dual producers actually play an important role in vivo as a type of helper and suppressor cell (or "hermaphrocyte" [Gershon et al., 1976]) whose function would be regulated by the signals derived from the microenvironment.

Lastly, it is well worth considering the possibility that the Treg field need not involve 28 or 31 different subsets with distinct mechanisms of suppression, but can easily be explained by three (vanilla, chocolate, strawberry) suppressive mechanisms that may be used separately or together (neapolitan) by different cell types: (1) a novel cell-contact pathway, (2) suppressor cytokines—IL-10, TGF-β, etc., and (3) killing (Zhao et al., 2006) (Figure 2).

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