Review

Regulating the immune system: the induction of regulatory T cells in the periphery

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Abstract

The immune system has evolved a variety of mechanisms to achieve and maintain tolerance both centrally and in the periphery. Central tolerance is achieved through negative selection of autoreactive T cells, while peripheral tolerance is achieved primarily via three mechanisms: activation-induced cell death, anergy, and the induction of regulatory T cells. Three forms of these regulatory T cells have been described: those that function via the production of the cytokine IL-10 (T regulatory 1 cells), transforming growth factor beta (Th3 cells), and a population of T cells that suppresses proliferation via a cell-contact-dependent mechanism ($CD4^+CD25^+$ T_R cells). The present review focuses on the third form of peripheral tolerance – the induction of regulatory T cells. The review will address the induction of the three types of regulatory T cells, the mechanisms by which they suppress T-cell responses in the periphery, the role they play in immune homeostasis, and the potential these cells have as therapeutic agents in immune-mediated disease.

Keywords: interleukin-10, regulatory T cell, suppression, transforming growth factor beta, tolerance

Introduction

The ability of the immune system to distinguish between self-antigens and nonself-antigens, and between harmful and innocuous foreign antigens, is critical to the maintenance of immune homeostasis. Failure to maintain tolerance to self-antigens or innocuous antigen results in the development of autoimmune or allergic disease, respectively. To achieve this state of immune tolerance, the immune system has evolved a variety of mechanisms. These include deletion of self-reactive clones in the thymus through a process referred to as negative selection, or central tolerance [1]. Central tolerance is imperfect, however, and self-reactive T cells do appear in the periphery. Likewise, the immune system is continuously distinguishing between innocuous and pathogenic foreign antigens.

To deal with these situations the immune system has evolved a system of induced peripheral tolerance. Two well-characterized mechanisms of peripheral tolerance are the death of self-reactive T cells via negative selection and the induction of a state of nonresponsiveness, or anergy [2]. A third, less well-characterized, mechanism is the active suppression of T-cell responses. This latter mechanism involves a recently described T-cell subset, known as regulatory T cells, which are induced in the periphery in an antigen-specific fashion [3,4]. The present review will discuss the various types of regulatory T cells, as well as the mechanisms that have been described for their generation.

Natural versus acquired regulatory T cells

Several classes of regulatory T cells, capable of suppressing antigen-specific immune responses, have been identified and characterized. These subsets can be distinguished in a variety of ways; including whether suppression is cell-contact mediated or is mediated through soluble factors such as IL-10 and transforming

APC = antigen presenting cell; IL = interleukin; MHC = major histocompatibility complex; TCR = T-cell receptor; TGF-β = transforming growth factor beta; Th = T helper cell; Tr1 = T regulatory 1; T_R = $CD4^+CD25^+$ T regulatory cell.

Table 1

Cytokine expression profiles of the three classes of regulatory T cells

Cytokine expressed	Th3 cells	T regulatory 1 cells	$CD4^+CD25^+$ T_R cells
Interferon gamma	+/–	+	–
IL-4	+/–	–	–
Transforming growth factor beta	+++	++	+/–
IL-10	+/–	+++	+/–

The production of cytokine is indicated as absent (–) or present (+) with relative quantities of cytokine indicated by +/– < + < ++ < +++.

growth factor beta (TGF-β). These cells can also be distinguished based on where they originate, in the thymus or in the periphery.

One prevailing model is that a class of regulatory T cells that originate in the thymus, are self-reactive and are involved in protection from autoimmune responses [5]. This class of cells referred to as 'natural' T regulatory cells (T_R) are characterized by the expression of the IL-2 receptor α-chain (CD25), and more recently by the *forkhead*/winged-helix transcription factor FoxP3 [6–9]. These T_R have the ability to suppress the activation of conventional T cells in a cell-contact-dependent, IL-10-independent and TGF-β-independent, manner [10,11]. On the other hand, 'acquired' regulatory T cells arise in the periphery, either during an immune response or after encountering a tolerogenic dendritic cell. These regulatory T cells are believed to differentiate from naïve precursors and are specific for antigens not presented in the thymus, such as food antigens, bacterial flora, pathogens, and self-antigens such as insulin [3]. They suppress the activation of conventional T cells in a cytokine-dependent manner: TGF-β for Th3 cells, and IL-10 for T regulatory 1 (Tr1) cells (Table 1) [4,12,13].

Recent work has shown that this distinction between natural and acquired Tregs may be simplistic. As discussed in the following, Tregs with the properties of $CD4^+CD25^+$ (T_R) have been shown to be generated *in vitro* and *in vivo* in systems using both self-antigens and foreign antigens. The present review will summarize the recent findings on the development of acquired Tregs, and on their potential function in regulating immune responses to both self-antigens and foreign antigens.

Th3 cells

It has long been recognized that the oral administration of antigen can lead to immunological tolerance to that antigen. Recent work has begun to shed light on the mechanisms that underlie this process. Oral tolerance is established in the gut-associated lymphoid tissue, which consists of Peyer's patches, intraepithelial lymphoid cells, and scattered lymphoid cells in the lamina propria. Several lines of evidence have shown that oral tolerance is an active, ongoing process. For example, a high antigen dose leads to hyporesponsiveness mediated by anergy or deletion [14,15]. On the other hand, low doses of antigen lead to the generation of Th2 cells, as well as to active suppression through the generation of antigen-specific regulatory T cells known as Th3 cells [16].

Th3 cells produce TGF-β, but differ from classical Th2 cells in that TGF-β expression does not always correlate with IL-4 or IL-10 expression [12]. Importantly, these Th3 cells have been shown to transfer tolerance *in vivo*, and to suppress antigen-specific responses *in vitro* [16]. In both cases the suppression is mediated by TGF-β. Further support for the role of TGF-β in regulating immune responses comes from studies using mice that lack the ability to either produce or respond to TGF-β. In both instances the mice develop a fatal autoimmune lymphoproliferative disease [17]. As these mice have normal $CD4^+CD25^+$ T_R cells (see later), these data suggest that a defect in either Th3 or Tr1 cells is responsible for the observed autoimmunity [17,18].

Tr1 cells

In addition to TGF-β, IL-10 has been shown to be a potent immunoregulatory cytokine [13,19]. The mechanism by which IL-10 regulates immune responses involves both T cells and antigen presenting cells (APCs). IL-10 treatment of dendritic cells results in the downmodulation of the co-stimulatory molecules CD80 and CD86, as well as MHC class II, and decreases the ability of these dendritic cells to activate T cells [20,21]. IL-10 can also have direct effects on $CD4^+$ T cells. Constant antigen stimulation of T cells in the presence of IL-10, either in the presence or the absence of APCs, results in anergy [20,22–24]. Unlike other anergic $CD4^+$ T cells, however, anergy in IL-10-treated T cells is not reversed by the addition of IL-2 or IL-15 [23]. When these IL-10-anergized T cells are driven to proliferate, they have a unique cytokine expression profile, producing high amounts of IL-10 and TGF-β, lesser amounts of interferon gamma, and no IL-2 or IL-4 [13,22]. $CD4^+$ T cells with this phenotype are referred to as Tr1 cells.

In spite of the fact that Tr1 cells have poor proliferative capabilities, they express normal levels of T cell activation markers, including CD25, CD40L, and CD69, following TCR stimulation [20]. Tr1 cells have been shown to regulate immune responses both *in vitro* and *in vivo*. For example, co-culture of Tr1 cells with freshly isolated $CD4^+$ T cells in the presence of allogeneic APCs results in the suppression of the proliferative allo-response [25,26]. Neutralization of IL-10 and/or TGF-β reverses this suppression [22]. Tr1 cells have also been shown to be capable of suppressing antibody production by B cells [27], and to decrease the ability of monocytes and dendritic cells to act as APCs. Kemper and colleagues [28] very recently showed that human Tr1 cells can be derived by stimulating $CD4^+$ T cells through co-engagement of CD3 and the complement regulator CD46 in the presence of IL-2. These conditions resulted in IL-10-producing cells capable of inhibiting the activation of bystander T cells. Unlike the Tr1 cells described earlier, CD3/CD46-generated Tr1 cells exhibited strong and prolonged proliferation when stimulated. There thus appear to be multiple pathways capable of producing IL-10 secreting regulatory T cells.

Tr1 cells also have potent effects on *in vivo* immune responses. Studies with allograft systems have shown that long-term graft tolerance correlates with the presence of $CD4^+$ T cells that suppress naïve T cells via IL-10 and TGF-β [29–31]. In mouse systems, $CD4^+$ T cells with Tr1-like properties have been isolated following tolerance induction to allergens [32,33], as well as in models of autoimmunity [34,35] and in response to infectious pathogens [36,37].

As already described, IL-10-treated dendritic cells are capable of driving the generation of Tr1 cells *in vitro*. However, the nature of the *in vivo* dendritic cell subset responsible for Tr1 cell differentiation remains unclear. Several groups have isolated specific dendritic cell subsets from nonlymphoid peripheral tissues that are capable of inducing tolerance. These subsets have been isolated from a variety of tissues, including the liver, the lung, and the intestine, and they appear to function via IL-10 secretion [32,38–40]. Wakkach and colleagues [41] recently identified a subset of dendritic cells in the spleen and lymph node that appear to be a natural tolerizing dendritic cell subset. The cells have a plasmacytoid morphology and remain immature even after *in vitro* activation with lipopolysaccharide or CpG, they have an unusual cell-surface phenotype ($CD11c^{lo}$/$CD45RB^{hi}$), and they produce large amounts of IL-10 when stimulated. These cells are capable of directly generating Tr1 cells *in vitro* and *in vivo*, and may represent a naturally occurring dendritic cell subset involved in eliciting tolerance *in vivo* [41]. The identification of this dendritic cell subset, as well as the demonstration that Tr1 cells can regulate immune responses *in vivo*, thus enhances the possible therapeutic uses of Tr1 cells as a means to regulate immune responses in a variety of diseases.

$CD4^+CD25^+$, cell-contact-dependent T_R cells

A third regulatory T cell population has been identified, which is characterized by the expression of the cell surface markers CD4 and CD25 (referred to as T_R cells). These $CD25^+CD4^+$ (T_R) cells are anergic, but upon activation suppress the proliferation and IL-2 production of naive and memory $CD4^+$ T cells through a contact-dependent, cytokine-independent mechanism [10].

In mice, T_R cells are thought to represent a population of T cells that are thymically derived and suppress autoreactive $CD4^+$ T cells. This is supported by the finding that thymectomy of mice at day 3 of life leads to a lack of T_R cells and produces a spectrum of spontaneous organ-specific autoimmune manifestations including autoimmune gastritis, oophoritis, orchitis, and thyroiditis [42]. Mice that have undergone thymectomy are rescued by the infusion of $CD4^+CD25^+$ T cells [43,44], and the removal of $CD4^+CD25^+$ using depleting antibody leads to a similar autoimmune phenotype seen in mice after thymectomy [45]. Studies of experimental autoimmune encephalomyelitis have demonstrated the protective effect of these regulatory cells in the response to inflammation directed against self-antigens [46], and additional studies of thyroiditis [47], diabetes [48], and nerve injury [49] have suggested that the T_R responses are specific for self-antigens. It is thought that T_R cells in mice represent those thymocytes with the highest affinity for self-peptide but that are below the threshold of negative selection [50]. Only a small number of T_R cells are thus selected, all of which are more sensitive to self-antigens than other circulating autoreactive T cells.

The molecular basis for the development and function of T_R cells remains unclear. Work in mice with targeted mutations suggests a role for several molecules in the development and function of T_R cells. One gene clearly associated with the development and function of T_R cells is FoxP3. Mice carrying the X-linked scurfy mutation develop a lymphoproliferative disease, display a multi-organ autoimmune disease, and lack conventional $CD4^+CD25^+$ T_R cells [6,7,51–53]. In mice, FoxP3 has been shown to be expressed exclusively in $CD4^+CD25^+$ T_R cells and is not induced upon activation of $CD25^-$ T cells. When FoxP3 is introduced via retrovirus or enforced transgene expression, however, naive $CD4^+CD25^-$ T cells are converted to T_R cells [8]. Thus, in mice, FoxP3 is both necessary and sufficient for the development and function of $CD4^+CD25^+$ T_R cells.

T_R cells with properties similar to those described in the mouse are present in humans. These cells represent

1–3% of all $CD4^+$ T cells and require activation to induce suppressor function, which is mediated via cell–cell contact, and is abrogated by the addition of IL-2 [54,55]. In humans, T_R cells have been shown to regulate T-cell responses to both foreign antigen and self-antigen [56], including T_R cells specific for alloantigens [57]. T_R cells in humans, as in mice, express FoxP3 [58], and individuals with a mutation in the FoxP3 gene develop immunodysregulatory, polyendocrinopathy, enteritis X linked syndrome, a disease similar to that seen in scurfy mice [59].

However, the source of the $CD4^+CD25^+$ T_R cells found in the peripheral blood of humans, whether thymic or peripheral, is not known. $CD4^+CD25^+$ T_R cells have been identified in the human thymus [60], and T_R cells with a naïve phenotype have been identified in cord blood [61]. Those T_R cells isolated from adult peripheral blood are $CD4^+CD25^+$ $CD45RO^+$ $CD45Rb^{low}$ [62] and have a short telomere length, however, both of which suggest that this population of T_R cells is thought to be derived from highly differentiated memory cells [56].Yet, in humans, it is impossible to prove whether the T_R cells isolated from peripheral blood originate in the thymus, and are expanded in the periphery, or whether they have been generated in the periphery. The possibility exists that, due to differences between mouse and man in life expectancy, thymic involution, and antigen exposures, the development of $CD4^+CD25^+$ T_R cells may occur in the periphery in man.

Induction of $CD4^+CD25^+$ T_R cells in the periphery

The induction of T_R cells that resemble the 'natural' or thymically derived T_R cells described in mice has been described in man. The induction is based upon the ability to create $CD4^+CD25^+$ T cells from nonregulatory cells that suppress proliferation of T cells in a contact-dependent, cytokine-independent manner. In all cases, although the conditions under which these cells are induced differ, activation of $CD4^+$ T cells is required to generate a T_R cell. Both *in vivo* and *in vitro* studies in mice support the idea that these cells can arise outside of the thymus. T_R cells have been identified in the periphery of mice under conditions that do not favor T_R cell development in the thymus [63]. The administration of oral, subcutaneous, intravenous antigen [64–66] or a repeated [67–70] exposure to superantigen [67] have been reported to induce $CD4^+CD25^+$ T_R cells in the periphery of mice.

Induction of T_R cells from peripheral $CD4^+CD25^-$ T cells *in vitro* has been reported by several groups. Duthoit and colleagues have demonstrated that recently activated T cells (4 days post stimulation) are anergic, express FoxP3, and suppress the proliferation of naïve T cells via a cell-contact-dependent mechanism in co-culture experiments [68]. Additionally, *in vitro* induction of T_R cells by activation of $CD4^+CD25^-$ T cells in the presence of TGF-β has been reported by two groups [69,70]. In the most recent of these reports, Chen and colleagues have shown that the induction of both FoxP3 expression and T_R cell function in previously nonregulatory $CD4^+CD25^-$ T cells required both TCR activation and TGF-β exposure [70]. However, Piccirillo and colleagues [66] have found that T_R cell function is normal in the absence of either TGF-β production or responsiveness. We have also shown that T cells from mice expressing a T-cell-specific transgene encoding a dominant-negative TGF-βRII have normal levels of FoxP3 (K Newton, SF Ziegler, unpublished data).

In humans, $CD4^+CD25^+$ T cells with regulatory activity requiring only cell–cell contact have been induced via activation under several different conditions. Taams and colleagues [56] used T-cell clones to demonstrate that activation of these clones with peptide only, in the absence of co-stimulation, leads to T cells that are anergic and suppress proliferation of other T-cell clones via cell contact [56]. T_R cells specific to allogeneic antigens have been generated *in vitro* by activation with IL-10-treated allogeneic dendritic cells [20]. Induction of T_R cells from $CD4^+CD25^-$ T cells has also been successful by activation of $CD4^+CD25^-$ T cells with mature, allogeneic dendritic cells, and these T cells also expressed FoxP3. The specificity of the T_R cells was determined by the type of mature dendritic cells used: autologous dendritic cells generate T_R specific for self-antigens [71], and allogeneic dendritic cells produce alloreactive T_R cells (MR Walker, JH Buckner, SF Ziegler, unpublished data).

The induction of $CD4^+CD25^+$ T_R cells in the absence of APCs has also been achieved *in vitro*. Our group has recently demonstrated that activation of $CD4^+CD25^-$ T cells with plate-bound anti-CD3 and soluble anti-CD28 can induce a group of $CD4^+CD25^+$ T cells with regulatory function that express FoxP3. These T_R cells are derived from highly purified $CD4^+CD25^-$ cells; they become $CD25^+FoxP3^+$ within 4 days of activation and regulate in a contact-dependent, cytokine-independent manner. The function and cell surface markers of these cells are indistinguishable from the $CD4^+CD25^+$ T cells directly isolated from the peripheral blood that have been defined as 'natural' T_R cells [58]. Unlike that reported in the mouse, induction of FoxP3 in this system does not require the presence of TGF-β. However, the induction does require engagement of the TCR and co-stimulation through CD28. Similarly, induction of T_R cells with mature dendritic cells also required MHC II and CD80/86 co-stimulation to induce T_R cells [71]. The induction of T_R cells *in vitro* has also been shown using αCD3 and a novel antibody 4C8 [72] or exposure to staphylococcal enterotoxin B for 7 days in culture [67].

Each of these systems used to induce T_R cells have differences; however, several common factors are present. T_R cells can be generated from peripheral $CD4^+CD25^-$ T cells, but only in response to activation. The activation conditions required for that induction might differ between mouse and man. However, differences in culture conditions and assays used to measure suppression make these comparisons difficult, and more work is needed to clarify these apparent differences. For example, differences between the species in the expression of surface molecules on T cells may contribute. Human T cells, unlike those from rodents, express HLA class II and co-stimulatory molecules upon activation, which may allow induction of T_R cells to occur in the absence of an APC. In addition, if the differentiation and function of T_R cells are regulated by the expression of FoxP3 then, as the regulation of FoxP3 expression becomes better understood, the requirements for T_R cell induction will become more apparent and the differences between mouse and man will be better understood.

Our ability to generate T_R cells in the periphery suggests a larger question: Do T_R cells represent a lineage of T cells or a state of activation that may be achieved by any T cell under the appropriate conditions of activation? The induction of T_R cells in the periphery allows for a dynamic immune response when the body is threatened by infection or injury. In this setting T cells will become activated, and will recruit other T cells and other inflammatory cells and mediators to the site. As the response becomes mature, a group of regulatory T cells will develop locally as a result of the local milieu allowing for a resolution of inflammation and regulating the responses directed to self-antigens exposed during the inflammatory response.

Role of peripherally generated T_R cells in the immune response to foreign antigens

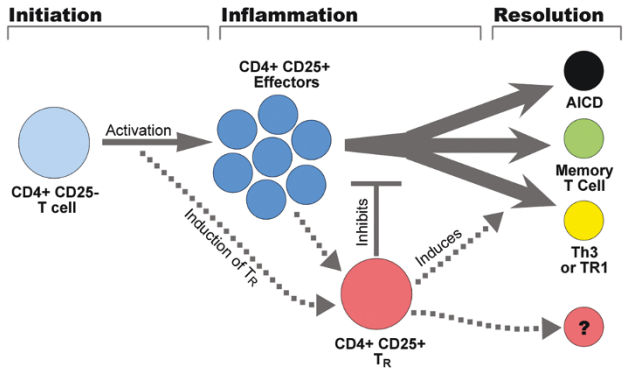
Regulation of immune responses is required to protect individuals from autoreactive T cells that have escaped into the periphery. Regulation of autoreactivity is present at the level of thymic selection, but also in the periphery. Those autoreactive cells that escape negative selection must be restrained in the periphery, and it is thought that $CD4^+CD25^+$ T_R cells generated in the thymus perform that role. In addition to regulation of autoimmune responses, the T-cell response to foreign antigens must be regulated as well. This regulation occurs in several forms: activation induced cell death once antigen or co-stimulation becomes limiting at the site of inflammation, the production of cytokines that lead to inhibition of T-cell responses, or the development of Tr1 or Th3 cells.

These regulatory phenomena are very important in the resolution of inflammation, to rein in the T cell response, to control bystander responses to self-antigens and to limit the resulting T cells for future responses, so as to avoid overwhelming immunologic reactions upon a repeated exposure to an antigen. In addition, regulation of the immune response may allow the fittest T cells to survive in a nutrient-limiting environment in order to proceed to become memory T cells. Evidence that this balancing act involves both cytokines and $CD4^+CD25^+$ T_R cells has been found with cutaneous infection of mice with *Leishmania major*, where the presence of T_R cells leads to a low-level persistence of the pathogen but allows for the development of long-term immunity, whereas animals that lack T_R cells or IL-10 are able to completely clear the infection but do not have any resistance to a second infection [73].

As we have already described, T_R cells may be generated at the site of inflammation through the activation of $CD4^+CD25^-$ T cells, inducing the expression of FoxP3 and CD25. In this way, activation itself would lead to a limitation of the extent to which the T-cell response could proceed. It is not yet known whether the $CD4^+CD25^-$ T cells capable of differentiating into T_R cells following activation are derived from a separate lineage of $CD4^+$ T cells, and whether their induction is a result of the initial activation early in the inflammatory process or occurs late upon depletion of growth signals (e.g. cytokines) in the local environment. We propose that these cells act by inhibiting further proliferation of T cells as the inflammatory process has peaked and nutrients are limiting. This allows a limited number of the most fit T cells to survive and become memory cells, while the remaining T cell response resolves through activation induced cell death (Fig. 1). In addition, $CD4^+CD25^+$ T_R cells leading to 'infectious tolerance', thus inducing the local induction of Th3 or Tr1 cells [74], extends this paradigm. The fate of the T_R cells induced at the site is not known – whether these cells exist only transiently then die, whether they persist in the body as T_R cells, or whether they return to their previous role as nonregulatory resting T cells remains unknown.

The balancing act required by the immune system to attack foreign invaders, to retain memory for future exposures to an antigen while reining in an inflammatory response once the danger has passed, and to retain tolerance to self probably combines many mechanisms both centrally and in the periphery. Our understanding of regulation is now expanding with the identification of peripherally generated Tr1, Th3 and T_R cells. The implication is that these cells may play a role in human disease. T_R cells have been isolated from tumors and could contribute to inadequacy of the immune response against these tumors. While inflammatory disease such as allergy and autoimmunity may occur when the T regulatory response is inadequate, a lack of T_R cell function has been demonstrated in autoimmune polyglondular syndrome II [75]. It is likely that more subtle defects in the generation

Figure 1



Schematic representation of the fate of CD4 T cells at a localized site of inflammation. Naïve or memory $CD4^+CD25^-$ T cells are recruited to the site, then become activated upon exposure to antigen and co-stimulation. These cells proliferate and become CD4 T effectors. Activation also induces a subset of $CD4^+CD25^-$ T cells to upregulate CD25 and FoxP3 and acquire $CD25^+CD4^+$ (T_R) cell function. These cells may result from activation at the initiation of the response or, more probably, as the response matures. As antigen and IL-2 is depleted, effector T cells undergo activation induced cell death, T_R cells lead to the induction of T regulator 1 (Tr1) cells and Th3 cells, which feed back to inhibit inflammation, and the T_R cells inhibit proliferation of antigen-specific and bystander T cells. This results in a small number of $CD4^+$ T cells surviving, which persist as memory T cells.

of regulatory response in the periphery could lead to manifestations of autoimmunity. Our ability to generate such regulatory cells holds promise for the development of new therapies to enhance regulation to treat autoimmune disease. A better understanding of how these forces work together will allow us to understand immunologic settings where either the immune response is inadequate, such as the response to tumors, or it is misdirected, as in the case of autoimmune disease and allergy.

Competing interests

None declared.

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