REVIEW

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Control of immune responses by immunoregulatory T cells

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Abstract

Immunoregulatory T cells play a key role in modifying the immune responses to self antigens, tumor antigens, and pathogenic organisms. This review summarizes recent data on naturally occurring $CD4^+$ regulatory T cells that constitutively express CD25 ($CD25^+$ T_{reg}). We examine the markers that can be used to differentiate these cells from effector T cells, what is known about their mode of action in controlling the activity of effector T cells, the antigenic specificity of $CD25^+$ T_{reg}, and their ability to survive and to be selected *in vivo*. We also summarize specific information on the role of $CD25^+$ T_{reg} in controlling anti-tumor responses, an area were manipulation of this subset holds particular clinical promise.

Key words: regulatory T cells, tumor immunity, immunoregulation.

Abbreviations: $CD25^+$ T_{reg} – $CD4^+CD25^+Foxp3^+$ regulatory T cells, TGF-β. transforming growth factor β. CTLA-4 – cytotoxic T lymphocyte antigen-4, DC – dendritic cell, TCR – T cell antigen receptor, GITR – glucocorticoid-induced tumor necrosis factor receptor family-related, Rag – recombinase activating gene, NOD – non-obese diabetic, IBD – inflammatory bowel disease.

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The realization that CD25 could be used as a marker for a subset of $CD4^+$ T cells whose raison d'être is to suppress immune responses led to a resurgence of interest in immunoregulatory T cells. The regulatory ability of $CD4^+CD25^+$ T cells ($CD25^+$ T_{reg}) was first directly identified by the demonstration that these cells could prevent autoimmune disease caused by autoreactive T cells [69]. $CD25^+$ T_{reg} constitute approximately 7–10% of peripheral $CD4^+$ T cells in humans and mice and can suppress T cell function both *in vitro* and *in vivo*. They appear to influence immune responses to self antigens, tumors, and pathogenic organisms. In this review we examine the characteristics of T_{reg}, their mechanisms of action, and the roles they play in preventing immune responses to tumor antigens.

PHENOTYPE OF $CD25^+$ T_{REG}

$CD25^+$ T_{reg} express a TCR complex and share some of the features of activated $CD4^+$ T cells. They are, by definition, characterized by the surface expression of CD25, the α chain of the IL-2 receptor, though this protein is also expressed transiently on activated T cells. Generally, $CD25^+$ T_{reg} are also $CD45RB^{low}$, $CD5^{high}$, $CD44^{high}$, $GITR^{high}$, and $CTLA\text{-}4^+$ [35, 88], though $CD25^+$ T_{reg} are enriched by, rather than homogenous in, the expression of these markers. While none of the aforementioned surface markers are unique to $CD25^+$ T_{reg}, it has been proposed that Foxp3, a transcription factor gene, is a unique marker for regulatory activity amongst $CD4^+$ cells. Foxp3 is constitutively expressed by the majority of $CD4^+CD25^+$ T cells, unlike effector T cells whose Foxp3 expression is virtually undetectable [23, 31, 40], although there is a small $CD4^+CD25^-Foxp3^+$ regulatory cell population [100]. Crucially, activation of $CD4^+CD25^-$ T cells does not cause up-regulation of Foxp3, allowing activated and regulatory $CD4^+$ cells to be readily distinguished. The role of Foxp3 in $CD25^+$ T_{reg} is discussed in greater detail below.

ACTIVATION OF CD25+ T_{REG}

CD25+ T_{reg} are activated to suppress via stimulation of their T cell receptor (TCR) complex. *In vitro* it has been shown that this stimulation can be by recognition of their specific antigen (presented on MHC class II) or by polyclonal stimulation, such as anti-CD3 [81, 88]. However, unlike $CD4^+CD25^-$ effector T cells, TCR stimulation does not induce the proliferation of CD25+ T_{reg} or interleukin (IL)-2 production [81, 88]. *In vivo* it is assumed that CD25+ T_{reg} are activated by their specific antigen presented on MHC class II. Certainly, CD25+ T_{reg} of known specificity have been shown to respond to their specific antigen in a number of *in vivo* studies [12, 33, 36]. The presence of IL-2 also appears to be important in maintaining the ability of CD25+ T_{reg} to suppress the proliferation of $CD4^+CD25^-$ cells. Pre-activation of CD25+ T_{reg} by TCR stimulation alone does not suppress effector T cells unless IL-2 is also provided [87], while blocking the IL-2 receptor on CD25+ T_{reg} by antibody engagement prevents the suppression of proliferation *in vitro* [16]. As IL-2 is produced by activated effector T cells, but not by CD25+ T_{reg}, this suggests that effector T cells need to be activated before CD25+ T_{reg} can be activated.

SUPPRESSION OF T CELL PROLIFERATION

CD25+ T_{reg} have the ability to suppress the proliferation of effector T cells *in vitro* in a contact-dependent manner [81, 88]. However, the significance of these *in vitro* studies to the normal physiological actions of CD25+ T_{reg} are not clear and more recent studies have concentrated on the *in vivo* effects of CD25+ T_{reg} on proliferation, employing a wide variety of models. A number of studies have concluded that, similarly to their *in vitro* function, CD25+ T_{reg} suppress T cell proliferation *in vivo* [2, 44 53, 101]. Several studies have addressed the importance of antigen specificity for suppression to occur. Initially, *in vitro* studies found that the suppression of T cell proliferation was antigen non-specific, as antigen-stimulated TCR-transgenic CD25+ T_{reg} were able to suppress the antigen-stimulated proliferation of TCR-transgenic $CD4^+CD25^-$ T cells, even when their specificity differed [81, 88]. However, several studies using T cells from TCR-transgenic mice have suggested that efficient suppression of effector T cell activity requires that the effector and CD25+ T_{reg} must be of the same specificity [33, 84].

MECHANISMS OF IMMUNE SUPPRESSION

In vitro studies indicated that CD25+ T_{reg} suppression is cell contact dependent, as suppression did not occur when CD25+ T_{reg} and effector T cells were separated by a permeable membrane [57, 88]. A number of surface molecules expressed by CD25+ T_{reg} have been proposed to mediate their suppressive abilities. Cytotoxic T lymphocyte antigen-4 (CTLA-4), which interacts with the CD80 and CD86 ligands, has been proposed as a mechanism for mediating contact suppression. CD25+ T_{reg} express this molecule constitutively on their surface, while effector T cells up-regulate it upon activation [82]. Takahashi et al. [82] found that anti-CTLA-4 antibodies abrogate suppression by CD25+ T_{reg} *in vitro*, while *in vivo* CD25+ T_{reg} are unable to prevent graft-versus-host disease in lymphopenic mice receiving CD80- and/or CD86-deficient $CD4^+CD25^-$ cells [64], and injection of anti-CTLA-4 antibody abrogated the prevention of graft-versus-host disease by CD25+ T_{reg} [41]. Anti-CTLA-4 antibody has also been found to prevent CD25+ T_{reg}-mediated disease suppression in colitis disease models [48, 67], allow tumor rejection [44, 80], and increase graft rejection [41]. However, anti-CTLA-4 can also bind to CTLA-4 expressed on activated T cells. As engagement of CTLA-4 normally down-regulates T cell responses, blocking the receptor was unsurprisingly shown to directly increase the proliferation of T cells [87]. Therefore it is difficult to know whether the anti-CTLA-4 reagents prevent CD25+ T_{reg} action or instead stimulating effector T cells.

In addition to the direct action with target T cells, it has also been suggested that CD25+ T_{reg} may act through APCs such as dendritic cells (DCs). Cederbom et al. [10] found that the presence of CD25+ T_{reg} down-regulated the co-stimulatory molecules CD80 and CD86 on DCs *in vitro*, though these findings were contradicted by Thornton and Shevach [89]. Additionally, recent work tracking CD25+ T_{reg} in the lymph nodes of live animals revealed that CD25+ T_{reg} have little contact with activated $CD4^+CD25^-$ effector T cells, instead associating closely with DCs presenting their specific antigen [85], which suggests that they are acting on DCs rather than T cells. However, it should be noted that suppression of proliferation could still be observed *in vitro* in an antigen-presenting cell (APC)-free system that used peptide-MHC tetramers to stimulate the effector cells [66].

While initial *in vitro* studies concluded that CD25+ T_{reg} acted through contact-dependent mechanisms, it is now clear that cytokine production is very important to their function *in vivo*. Transforming growth factor (TGF)-β, an immunosuppressive cytokine expressed on the surface of $CD4^+CD25^+$ T cells, is one such molecule. TGF-β specific antibodies are able to prevent suppression *in vitro* [57], while CD25+ T_{reg} are not able to suppress the cytotoxicity of $CD8^+$ cells lacking a TGF-β receptor [12]. However, despite this, CD25+ T_{reg} from TGF-β-deficient mice are still able to cause suppression, and the proliferation of effector T cells lacking a functional TGF-β receptor may still be suppressed [65]. Fahlen et al. [19] suggested that CD25+ T_{reg} may not produce TGF-β themselves, but instead rely on other cells to produce the cytokine. CD25+ T_{reg} from TGF-β-deficient mice were able to prevent colitis in Rag-deficient mice induced by transfer of wild-type $CD4^+CD45RB^{high}$ cells, but not if $CD4^+CD45RB^{high}$

cells lacking a functional TGF-β receptor were used or if the mice were injected with anti-TGF-β antibodies. Therefore, while it is likely that TGF-β plays a role in inducing suppression, it may not be crucial in all cases and it is currently unclear whether $CD25^+$ T_{reg} directly produce the cytokine themselves or, instead, induce bystander cells to do so. IL-10, which is secreted by $CD25^+$ T_{reg} [62], is crucial to the controlling of many diseases by $CD25^+$ T_{reg}. In studies of inflammatory bowel disease (IBD), the ability of $CD25^+$ T_{reg} to prevent IBD can be abrogated by the injection of anti-IL-10 antibody [4] or by using $CD25^+$ T_{reg} from IL-10 knockout mice [1, 79]. IL-10 production is also required for $CD25^+$ T_{reg} to prevent graft rejection [41], prevent self-antigen-specific $CD4^+$ T cells from causing autoimmune disease [33], and control the immune response against a number of pathogens [6, 52]. However, $CD25^+$ T_{reg} from IL-10 knockout mice still prevent autoimmune gastritis [79], indicating that the importance of IL-10 production may differ depending on the particular disease.

It is still far from clear how $CD25^+$ T_{reg} biochemically alter signaling pathways in effector T cells and cause suppression, although suppression of IL-2 production appears to be important. $CD25^+$ T_{reg} suppress the transcription of IL-2 mRNA *in vitro*, thus preventing IL-2 production [88], which in turn leads to a reduction in the available IL-2 and reduced T cell proliferation. The reduction in available IL-2 is further increased by the ability of $CD25^+$ T_{reg} to consume IL-2 through their expression of the high-affinity IL-2 receptor CD25, thereby preventing the stimulatory cytokine from reaching effector T cells [5, 16]. However, prevention of IL-2 production is not the sole suppressive mechanism. For example, $CD4^+CD25^-$ T cells previously cultured with stimulated $CD25^+$ T_{reg} do not proliferate when subsequently cultured in the absence of $CD25^+$ T_{reg} with both TCR stimulation and IL-2 [18]. In addition, transferred $CD25^+$ T_{reg} can prevent autoimmunity in mice whose T cells lack an IL-2 receptor [50, 96].

ABROGATING SUPPRESSION

Effector T cells may be induced to become refractory to suppression. Engagement of the glucocorticoid-induced tumor necrosis factor receptor family-related (GITR) with the GITR ligand is thought to prevent effector T cell proliferation from being suppressed. GITR is expressed constitutively on $CD25^+$ T_{reg} and also on activated $CD4^+CD25^-$ T cells [55], though little is known of its function. *In vitro* proliferation assays found that antagonistic anti-GITR antibody prevented suppression of $CD4^+CD25^-$ T cells by $CD25^+$ T_{reg} [55], while injection of this antibody was capable of inducing autoimmune disease [76]. Initially it was proposed that engagement of GITR on $CD25^+$ T_{reg} was abrogating their ability to mediate suppression, but it was subsequently suggested that it is the engagement of GITR on effector T cells that prevents suppression, as the suppression of GITR-deficient effector T cells by GITR-sufficient $CD25^+$ T_{reg} could not be prevented by anti-GITR antibody [77]. GITR ligand is expressed on resting APCs and activated T cells, suggesting it has a role in modulating the immune response by preventing T cell suppression [77].

The cytokine IL-6 also causes effector T cells to become refractory to suppression [63]. Ligation of Toll-like receptors on splenic DCs by ligands such as lipopolysaccharide induces DCs to produce IL-6, which subsequently prevents $CD25^+$ T_{reg} from suppressing effector T cell proliferation. As ligands for Toll-like receptors are produced by microorganisms, this suggests that this is a mechanism designed to prevent the suppression of T cells responding to infections.

GENERATION OF $CD25^+$ T_{REG} AND THEIR ANTIGEN SPECIFICITY

T cell precursors are first generated in the bone marrow and then migrate to the thymus, where they receive stimuli that result either in their maturation or deletion due to apoptosis. The "strength" of interaction between the TCR and MHC on epithelial cells is considered to be important in determining the cells' fate, with a weak signal due to lack of MHC recognition or too strong a signal due recognition of self antigen resulting in apoptosis. This process results in T cells that can, in general, "recognize" foreign antigens, but not self antigens presented on MHC. A number of studies have examined the generation of $CD25^+$ T_{reg} in the thymus and periphery to determine how their developmental pathway and antigen specificity resemble and differ from those of "conventional" $CD4^+$ T cells.

$CD25^+$ T_{reg} are also derived from bone marrow cells [23] and, like effector T cells, mature in the thymus. A number of studies examining the thymus of adult mice have identified the presence of $CD8^-CD4^+CD25^+$ T cells, constituting approximately 5% of $CD4^+CD8^-$ cells, with similar expression of the surface markers CD62L, CD45RB, CD44, and CD5 to that of peripheral $CD25^+$ T_{reg} and the capability of causing T cell suppression *in vitro* [35, 61, 78]. They appear to be generated very early in ontogeny as they have been detected in the thymus of neonatal mice as early as two days after birth [35].

Currently, the most widely held theory for the selection of $CD25^+$ T_{reg} occurring in the thymus is via high-avidity TCR engagement with a specific peptide presented on MHC class II. It is envisaged that the avidity of the TCR engagement required for regulatory T cell selection is of sufficient, or nearly sufficient, strength to induce $CD4^+$ thymocyte death. Therefore, any self-reactive T cells that do escape negative selection are controlled by a relatively large number of $CD25^+$ T_{reg} specific for the same peptide. While the evidence is not conclusive, this theory is supported by the findings of a number of studies. $CD25^+$ T_{reg} of TCR-transgenic mice are more likely to possess a TCR comprised of an

endogenous α chain and transgenic β chain than the $CD4^+CD25^-$ T cells from the same mouse [30, 35], indicating that they need a different-strength signal from TCR/MHC engagement for their selection or survival than that of $CD4^+CD25^-$ cells. This is supported by the finding that while TCR-transgenic mice producing T cells specific for myelin basic protein do not develop autoimmunity, crossing these mice with mice that are Rag deficient (and therefore cannot perform endogenous TCR rearrangements) results in progeny that develop encephalomyelitis as they mature, due to a lack of $CD25^+$ T_{reg} [59]. The numbers of $CD25^+$ T_{reg} can be restored in TCR-transgenic mice lacking the ability to rearrange their TCR by the expression of their specific peptide in the thymus, indicating that a strong positive signal is required for $CD25^+$ T_{reg} to develop [21, 38, 39]. This is further supported by Jordan and colleagues, who compared transgenic TCRs of differing avidity and found a higher-avidity TCR increased $CD4^+CD25^+$ T cell number but a lower-avidity TCR did not. Continuing work with this model found that expressing antigen under different promoters also varied the degree of $CD25^+$ T_{reg} cell selection, indicating that selection was also based on how, or by what cell, the peptide was presented [45].

However, not all studies agree with the positive selection theory. Using a model which allowed variable expression of moth cytochrome c in the thymus of a TCR-transgenic mice specific for pigeon cytochrome c, van Santen et al. [92] reached conclusions different from those of Jordan and colleagues. Pigeon cytochrome c expression in the thymus increased the proportion, rather than the number, of pigeon cytochrome c-specific $CD25^+$ T_{reg} by deleting $CD4^+CD25^-$ T cells, with increased cytochrome c expression simply deleting more $CD4^+CD25^-$ T cells. This finding implies that while $CD25^+$ T_{reg} are resistant to apoptosis, either another signal is actually required to induce $CD25^+$ T_{reg} or they are a separate lineage from effector $CD4^+$ cells before they enter the thymus. The theory of positive selection was also contradicted by the finding that negative selection of $CD25^+$ T_{reg} can occur upon encountering antigen in the thymus [7] and by demonstrations that $CD25^+$ T_{reg} have a role in controlling the immune response against pathogens such as *Leishmania major* [29, 51], whose antigens would not be expressed in the thymus.

Co-stimulatory signals are important in the development of $CD4^+CD25^+$ T cells. Mice lacking B7, CD28 or CD80 and CD86 expression have a reduced but still detectable number of functional $CD25^+$ T_{reg} [70, 85], while antibodies against CD86 and CD80 block the ability of human thymus-derived DCs to induce the development of $CD25^+$ T_{reg} [95]. It has been suggested that IL-2 also plays a key role in the development of $CD25^+$ T_{reg}. IL-2 and IL-2 receptor-deficient mice have a reduced number of regulatory cells and develop lymphoproliferative autoimmune diseases [50, 61], which can be prevented by transfer of wild-type $CD25^+$ T_{reg} [50, 96]. Mice injected with neutralizing anti-IL-2 have reduced peripheral $CD4^+CD25^+$ T cell number [56]. However, $CD4^+$ T cells from IL-2-deficient mice are still protective in a spontaneous autoimmune encephalomyelitis model [25], while the generation of antigen-specific $CD25^+$ T_{reg} in the thymus was unaffected in IL-2-deficient mice, though their peripheral survival was reduced [15, 24]. Therefore it is likely that IL-2 receptor stimulation is required for $CD25^+$ T_{reg} survival rather than this thymic generation.

The particular thymic cells that are important in generating $CD25^+$ T_{reg} were first examined by Bensinger et al. [7]. It was found that mice that lack MHC class II expression do not develop $CD25^+$ T_{reg}. However, a normally sized and functioning $CD25^+$ T_{reg} compartment could be restored by the expression of MHC class II in the thymic cortical epithelium, while leaving the medullary epithelium absent of MHC class II, suggesting that it is the cortical epithelial cells that play an important role in $CD25^+$ T_{reg} selection. This conclusion was supported by Apostolou et al. [3] and Lerman et al. [45], who found that expression of hemagglutinin antigen in the thymic epithelium of hemagglutinin-specific TCR-transgenic mice was crucial in producing hemagglutinin-specific $CD25^+$ T_{reg}. However, Watanabe et al. [95] found that in the human thymus, DCs in the medullar appeared to be responsible for inducing $CD25^+$ T_{reg} from $CD4^+CD8^-$ cells. These DCs are localized with $CD25^+$ T_{reg} in the thymus and, when cultured together *in vitro*, are able to induce $CD25^+$ T_{reg} from $CD4^+CD8^-$ thymocytes, but not from $CD4^+CD8^-$ peripheral cells.

There is evidence that $CD25^+$ T_{reg} can also be generated in the periphery from naïve effector $CD4^+25^-$ T cells. This was first demonstrated *in vivo* by Thorstenson and Khoruts [90], who examined ovalbumin-specific TCR-transgenic mice on a Rag-2-deficient background, and therefore the mice had few $CD25^+$ T_{reg} due to lack of selection. However, an intravenous injection of ova peptide without adjuvant induced a large percentage of $CD4^+$ T cells to express CD25 and gain suppressive function *in vitro*. Induction appeared to be dependent on a relatively weak stimulation, as the addition of lipopolysaccharide to provide strong co-stimulation or the injection of a higher peptide dose no longer induced regulatory cells. This was further supported by Kretschmer et al. [43], who found that the administration of low, but not high, antigen doses induced $CD25^+$ T_{reg} from $CD4^+CD25^-Foxp3^-$ cells *in vivo*. TGF-β is thought to be a crucial cytokine in inducing peripheral $CD25^+$ T_{reg}. Stimulating $CD4^+CD25^-$ T cells *in vitro* with anti-CD3, APCs/anti-CD28, and TGF-β induces a fraction of the stimulated $CD4^+CD25^-$ T cells to express CD25, increase Foxp3 mRNA, and become suppressive, while without TGF-β this is not observed [13, 20]. Furthermore, low antigen doses do not induce $CD25^+$ T_{reg} development in $CD4^+CD25^-$ T cells lacking a TGF-β receptor [43], while the overexpression of TGF-β in T cells *in vivo* reduces peripheral $CD4^+$ T cell number, but increases the percentage that express CD25

and Foxp3, and mice whose T cells lack a TGF-β receptor have reduced numbers of $CD4^+$ cells expressing Foxp3 [72].

However, it has been suggested that $CD25^+$ T_{reg} are not actually being induced in the periphery. A small percentage of $CD4^+CD25^-$ cells express Foxp3, and many of these cells gain CD25 expression when transferred into a lymphopenic environment [100]. Those cells that gain CD25 expression have a higher level of Foxp3 mRNA than do $CD25^-$ cells, which, coupled to the finding that transferred $CD4^+CD25^+$ cells can lose CD25 expression in a lymphopenic environment, suggests that $CD25^+$ T_{reg} gain and lose CD25 expression. Therefore, the apparent induction of regulatory properties in effector T cells may instead be a consequence of an increase in CD25 expression by $CD4^+CD25^-Foxp3^+$ regulatory T cells.

ANTIGEN SPECIFICITY $CD25^+$ T_{REG}

$CD25^+$ T_{reg} have a polyclonal TCR repertoire, with Vα and Vβ usage which is the very similar to that of $CD4^+CD25^-$ cells and therefore suggestive of a broad antigen specificity [81]. However, there is some evidence that the repertoire of $CD25^+$ T_{reg} is skewed towards self reactivity. Seddon and Mason [73] found that the presence of thyroid tissue was necessary for peripheral $CD4^+$ cells to prevent thyroiditis when transferred into rats induced to develop the disease, suggesting that $CD25^+$ T_{reg} are self-antigen specific and must be maintained by encounter with their antigen in the periphery. Similarly, $CD25^+$ T_{reg} from female mice are considerably better at suppressing an autoimmune response against the ovary than cells from male mice [71]. Masteller et al. [54] were able to expand p31 (an antigen expressed in the pancreas) specific $CD25^+$ T_{reg} from non-obese diabetic (NOD) mice *in vitro*. After expansion, these $CD25^+$ T_{reg} were suppressive *in vitro* and able to delay diabetes when transferred into CD28--deficient NOD mice which, due to a lack of regulatory cells, are particularly vulnerable to diabetes. Both these studies demonstrate that at least some $CD25^+$ T_{reg} are specific for self antigen. Indeed, Hsieh et al. [32] suggested that the $CD25^+$ T_{reg} population is "enriched" in cells specific for self antigen compared with effector $CD4^+$ cells. Hsieh et al. [32] used transgenic mice with variable TRAV14 (Vα2) chains paired with a fixed Vβ chain "belonging" to a Clip-specific TCR, which allowed a more manageable repertoire to be examined, and created T cell clones from these mice. The Vα2 chains from both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell clones were transduced using retroviruses into $CD4^+$ helper cells with the same Vβ chain. When these cells were transferred into lymphopenic mice, cells containing Vα chains from $CD25^+$ T cells were able to expand to a greater extent than cells with a Vα chain derived from $CD25^-$ T cells. The greater expansion indicated that $CD25^+$ T_{reg} had TCRs that were more likely to recognize self peptide presented on MHC class II than were $CD4^+CD25^-$ T cells.

It has also been reported that the repertoire of $CD25^+$ T_{reg} is edited by negative selection to at least some self antigens, similarly to that of $CD4^+CD25^-$ T cells [7]. This study used H-2DM-deficient mice whose MHC class II complex almost exclusively presents the invariant chain peptide and therefore, due to a lack of negative selection, their $CD4^+CD25^-$ T cell population is highly self reactive. When placed in *in vitro* culture with wild-type APCs, which present self antigen on MHC class II, the $CD4^+CD25^-$ T cells proliferated. However, the addition of $CD25^+$ T_{reg} from H-2DM--deficient mice, but not wild-type mice, suppressed proliferation. This implies that $CD25^+$ T_{reg} specific for self antigen can be deleted when they encounter self antigen in the thymus. Studies on the immune response against infectious pathogens also gives rise to doubts that all $CD25^+$ T_{reg} are specific for self antigen. $CD25^+$ T_{reg} have been shown to prevent or modulate immune responses against a number of pathogens, such as *Leishmania major*, *Plasmodium yoelii*, and herpes simplex virus [68], which implies that a number of $CD25^+$ T_{reg} are specific for foreign antigens. It is unclear how specificity for foreign antigen fits with observations that a strong positive TCR signal is required for selection of $CD25^+$ T_{reg} in the thymus. One possibility is that molecular mimicry by self peptides with structural similarity to pathogen peptides may provide a strong positive signal for pathogen-specific $CD25^+$ T_{reg}.

SURVIVAL AND PROLIFERATION OF $CD25^+$ T_{REG}

While $CD25^+$ T_{reg} were first described as anergic to TCR stimulation, it has subsequently been found that they can be induced to expand both *in vitro* and *in vivo*. Perhaps unsurprisingly, many of the signals that result in expansion have also been shown to contribute to their thymic generation. Several studies using TCR-transgenic cells have shown the expansion of $CD25^+$ T_{reg} in lymph node draining tissues expressing their specific antigen [12, 22, 93], while it has been shown that $CD25^+$ T_{reg} only undergo homeostatic proliferation in lymphopenic mice if the mice express MHC class II [26]. However, while TCR stimulation is required for $CD25^+$ T_{reg} proliferation in the periphery, *in vitro* proliferation assays [81, 88] have indicated that various co-stimulatory signals are probably required to abrogate $CD25^+$ T_{reg} anergy.

Several experiments suggest that engagement of the IL-2 receptor appears to be crucial in both the survival and expansion of $CD25^+$ T_{reg}. Firstly, the addition of IL-2 allows the expansion of human and mouse $CD25^+$ T_{reg} *in vitro* when TCR stimulation is also provided [46, 61, 88]. Secondly, IL-$2^{+/+}$ $CD25^+$ T_{reg} die rather than expand in the periphery of IL-2-deficient mice [15, 24, 50]. Thirdly, $CD25^+$ T_{reg} lacking a functional IL-2 receptor

do not expand in the periphery of lymphopenic mice [50]. Thirdly, injection of neutralizing anti-IL-2 antibody reduces the peripheral $CD25^+$ T_{reg} number in wild-type mice without reducing effector T cell number [56, 74]. CD28 stimulation, which also causes $CD25^+$ T_{reg} proliferation *in vitro* [46, 47], may act through IL-2, as it induces the up-regulation of CD25, the IL-2 receptor, and increasing IL-2 production by effector T cells [86]. IL-2-mediated expansion of $CD25^+$ T_{reg} does not appear to abrogate their suppressive ability as, while IL-2 must be removed before expanded $CD25^+$ T_{reg} are able to suppress T cell proliferation *in vitro*, $CD25^+$ T_{reg} undergoing expansion still suppress IL-2 mRNA levels in target cells even when IL-2 is present [87].

It has also been suggested that the expansion of $CD25^+$ T_{reg} requires stimulation of the TGF-β receptor. $CD25^+$ T_{reg} lacking a functional TGF-β receptor are unable to prevent dextran sulfate-induced colitis in mice or expand to the same extent as wild-type $CD25^+$ T_{reg} during disease progression [34]. TGF-β may also be necessary to maintain $CD25^+$ T_{reg} number in the non-disease state. Mice whose T cells lack a functional TGF-β receptor have a reduced number of $CD25^+$ T_{reg} in the spleen, while mice which overexpress TGF-β have fewer $CD4^+$ cells, but a greater proportion are $CD25^+Foxp3^+$ [72]. $CD4^+CD8^-CD25^+$ cell number is not reduced in the thymus of TGF-β receptor-deficient mice, indicating that the lack of TGF-β is not affecting $CD25^+$ T_{reg} development.

Lipopolysaccharide can also induce $CD25^+$ T_{reg} proliferation. $CD25^+$ T_{reg} express a number of Toll-like receptors, including TLR-4, the receptor for lipopolysaccharide, and proliferate *in vitro* when lipopolysaccharide is combined with TCR stimulation [8].

All of the co-stimulatory signals required for $CD25^+$ T_{reg} proliferation would be expected to be generated during an active immune response. Activated effector T cells produce IL-2 and TGF-β, activated APCs express B7, the ligand for CD28, while microorganisms are the source of lipopolysaccharide. The expansion of $CD25^+$ T_{reg} may therefore be crucial for their function, with immunological insults that lead to the expansion of effector T cells also resulting in the expansion of $CD25^+$ T_{reg}, allowing $CD25^+$ T_{reg} to halt the progression of autoimmunity or prevent bystander tissue damage during infection.

$CD25^+$ T_{REG} AND FOXP3

Foxp3 is thought to have a crucial role in the development of $CD25^+$ T_{reg} and is a superior marker of regulatory activity to CD25. The *Foxp3* gene encodes a transcription factor also known as scurfin and is located on the X chromosome. Mutation of the *Foxp3* gene in male mice causes a $CD4^+$ T cell-mediated lymphoproliferative disease which results in wasting and lymphocytic infiltration into organs [49]. In humans, mutations in this gene result in a range of autoimmune diseases [11], though female heterozygous carriers are protected due to one functional copy of the gene. *Foxp3* mRNA is found in the majority of $CD4^+CD8^-CD25^+$ but not $CD4^+CD8^-CD25^-$ murine thymocytes, while in the periphery it is expressed largely by $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells [31]. Unlike CD25 expression, Foxp3 mRNA is not induced by T cell activation. The production of $CD4^+CD25^+Foxp3^+$ cells appears to begin early in life as they have been found in both the spleen and thymus of three-day-old mice, though with lower Foxp3 expression than that of adult mice [17].

Fontenot et al. [23] directly demonstrated the importance of Foxp3 in regulatory T cell development by creating, by gene deletion, male mice that were Foxp3 deficient. These mice developed an autoimmune disease characterized by inflamed skin, enlarged organs and lymph nodes, and lymphocytic infiltration in multiple organs and they died after approximately two weeks of life. These Foxp3-deficient mice could be rescued from morbidity and, to a large extent, from autoimmune disease by the injection of wild-type $CD25^+$ T_{reg} at 1–2 days of age. Bone marrow chimeras indicated that the requirement for Foxp3 was cell intrinsic.

It has been suggested that Foxp3 expression by $CD4^+CD25^-$ T cells, and subsequent regulatory cell development, is inducible by the cytokine TGF-β. *In vitro* the combination of anti-CD3, APCs/anti-CD28, and TGF-β causes a fraction of the stimulated $CD4^+CD25^-$ T cells to express CD25 and membrane-bound active TGF-β and also to increase the amount of *Foxp3* mRNA, while without TGF-β this does not occur [13, 20]. Mice which overexpress TGB-β in their T cells, due to insertion of a transgene, have fewer T cells, but a slightly greater percentage of their $CD4^+$ T cells express CD25 [72]. The $CD25^+$ cells have a much higher Foxp3 expression than wild-type $CD25^+$ T_{reg} and are more suppressive than wild-type $CD25^+$ T_{reg} *in vitro*. However, Zelenay et al. [100] suggests that these studies instead show that $CD4^+Foxp3^+$ regulatory T cells cycle between a $CD25^-$ and a $CD25^+$ state.

$CD25^+$ T_{REG} IN TUMOR IMMUNITY

$CD25^+$ T_{reg} are known to regulate immune responses against tumor cells. Their role was first investigated by depleting $CD25^+$ cells from the periphery of mice using a depleting antibody (PC61) and subsequently inoculating the mice with a range of tumor lines [60, 75], resulting in tumor regression in 6 of the 8 tumor lines tested. Mice that rejected the tumor cells were then able to reject subsequent inoculations of the same tumor line, indicating that depletion of $CD25^+$ cells had generated a memory response. This was demonstrated to be T cell mediated, as co-depletion of $CD8^+$ cells prevented $CD25^+$ cell depletion from causing tumor rejection of all the tumor lines tested, while $CD4^+$ cell depletion prevented rejection of some of the tumor lines. Additionally, the injection of athymic BALB/c mice

(which lack T cells) with PC61 antibody did not prevent tumor growth following inoculation with RL♂1 leukemia cells [60], while the transfer of spleen cells depleted of $CD25^+$ cells, but not whole splenocytes, caused tumor rejection [75]. Similar results have been found in a number of subsequent studies using a range of tumor lines [28, 37, 83]. Tumor rejection due to $CD25^+$ cell depletion results in long-term immunity, as subsequent tumor inoculations are rejected even though the $CD4^+CD25^+$ cell number recovers after several weeks. In one study, long-term protection (45 days after PC61 injection) was found to extend to tumor lines of different origin from the original inoculum, indicating that the immune response was generated against shared antigens between the tumor lines [28]. As an alternative to depletion of $CD25^+$ T_{reg}, tumor rejection can also be induced by blocking their function. Both CTLA-4 and GITR are expressed on $CD25^+$ T_{reg} and tumor rejection can be induced by CTLA-4 blockade using anti-CTLA-4 antibodies [80] or by engagement of GITR by injection of DTA-1 antibody [42, 91]. It has been proposed that anti-CTLA-4 acts by preventing CTLA-4 engagement with effector cells [82], while the engagement of the DTA-1 antibody with GITR prevents their ability to suppress proliferation [76]. However, it is possible that both anti-CTLA-4 antibody and DTA-1 antibody may instead be acting directly on effector T cells rather than the $CD25^+$ T_{reg} [77, 87].

Once it had been established that $CD25^+$ T_{reg} prevent an effective immune response against tumor cells, many studies attempted to better characterize their role in preventing immune responses. Several studies have examined the effect of $CD25^+$ T_{reg} on the proliferation of tumor-specific T cells and reached differing conclusions. Casares et al. [9] examined the effect of depleting $CD25^+$ cells on $CD4^+$ cells in lymph nodes draining a CT26 tumor. $CD4^+$ cells from $CD25^+$ cell-depleted mice proliferated when stimulated *in vitro* with tumor cell extracts, unlike $CD4^+$ cells from mice that were not depleted. One interpretation of these results is that the proliferation of tumor-specific $CD4^+$ T cells generated in the draining lymph nodes is normally suppressed by $CD25^+$ T_{reg}, though another is that the $CD25^+$ T_{reg} prevented tumor-specific $CD4^+$ T cells from being generated in the draining lymph nodes. The former interpretation is supported by Antony et al. [2], who found that the number of $CD8^+$ pmel-1 TCR-transgenic T cells (which are gp100 specific) responding to gp100-expressing B16 tumors in CD4-deficient mice could be increased by the co-transfer of $CD4^+CD25^-$ cells but not unfractionated $CD4^+$ T cells. Tumor rejection was also prevented by the whole $CD4^+$ T cells, but induced with $CD4^+CD25^-$ T cells. However, hemagglutinin-specific $CD25^+$ T_{reg} in mice inoculated with a hemagglutinin-expressing colon carcinoma line did not reduce the proliferation of hemagglutinin-specific TCR-transgenic $CD8^+$ T cells in the draining lymph node, even though they did prevent tumor rejection [12]. Instead it was suggested that rejection was prevented by suppression of cytotoxic activity by $CD25^+$ T_{reg} at the tumor site itself, which was indirectly supported by the finding that the $CD25^+$ T_{reg} reduced the ability of the $CD8^+$ T cells to lyse hemagglutinin-pulsed splenocytes in the draining lymph node.

There is strong evidence that $CD25^+$ T_{reg} act in some manner at the tumor site. Meth A tumors inoculated into BALB/c mice are infiltrated by, amongst other T cells, $CD25^+$ T_{reg} [42]. Yu et al. [99] observed that approximately seventy percent of the $CD4^+$ T cells infiltrating a murine fibrosarcoma were $CD25^+$ T_{reg}. The presence of $CD25^+$ T_{reg} may reduce effetor T cell infiltration into the tumor mass, as the injection of DTA-1 antibody, which may prevent regulatory T cell activity, 8 days after tumor inoculation led to an increase in $CD8^+$ and $CD4^+$ T cell infiltration into the Meth A tumor mass and lower Foxp3 mRNA levels in the $CD4^+$ cells [42]. Additionally, Yu et al. [99] found that intra-tumor depletion of $CD4^+$ cells fourteen days after tumor inoculation with a fibrosarcoma resulted in tumor rejection, characterized by $CD8^+$ T cell infiltration. The above studies employed experimental tumor cell lines in mice, but $CD25^+$ T_{reg} have also been observed in a number of naturally occurring human tumors [14, 94, 97]. Tumor-infiltrating T cells have a higher level of Foxp3 mRNA than their $CD4^+CD25^-$ counterparts. It appears likely that these cells suppress the local immune response, as $CD25^+$ T_{reg} from lung cancer patients were able to suppress the *in vitro* proliferation of peripheral blood lymphocytes [14, 98], while a study of malignant ovarian epithelial cancer found a correlation between increasing $CD4^+CD25^+$ cell number in the tumor mass and poor patient survival [14]. Wang et al. [94] suggested that the $CD25^+$ T_{reg} infiltrating the tumor masses are likely to be specific for self antigen, as they were able to establish a $CD4^+CD25^+$ cell line from T cells infiltrating a melanoma tumor that was specific for LAGE-1, an antigen normally expressed only in the testis and by some tumor cells. The entry and retention of $CD25^+$ T_{reg} in the tumor mass has not been extensively studied but may be due to migration towards chemokines produced by tumor cells or macrophages associated with the tumor mass [14].

Several studies have found that tumor cells may protect themselves from the immune system by inducing the proliferation of tumor-specific $CD25^+$ T_{reg}. Using a BD-IX rat model it was shown that a tolergenic colon carcinoma increased the percentage of $CD4^+$ cells expressing CD25 in the lymph nodes draining the tumor mass and in the spleen [27]. Transfer of $CD25^+$ cells from the spleens of these rats into rats inoculated with a closely related but immunogenic colon carcinoma cell line (REGb), which is normally rejected naturally, caused a delay in REGb rejection compared the control rats, suggesting the cells were enriched for tumour specific $CD25^+$ T_{reg}. Nishikawa et al [58] inoculated mice with plasmids encoding antigens from the sarcoma line CMS5m. Inoculation of mice with these plasmids appeared to generate $CD25^+$ T_{reg} as transfer of $CD25^+$ T_{reg} from these mice into mice inoculated with

CMS5m was found to increase the degree of lung metastasis, indicating the immune response was being suppressed. Expansion of tumor-specific $CD25^+$ T_{reg} was directly observed by Chen et al. [12], who found that hemagglutinin-specific TCR-transgenic suppressive $CD4^+$ cells, of which approximately 50% expressed CD25, injected into mice inoculated with a hemagglutinin-expressing colon carcinoma line proliferated, as assessed by carboxyfluoroscein succinimidyl ester (CFSE) labeling. They still retained regulatory function, despite undergoing expansion, as they were able to prevent tumor rejection by hemagglutinin-specific $CD8^+$ T cells. Ghiringghelli et al. [27] proposed that the proliferation of the $CD25^+$ T_{reg} was due to immature myeloid DCs, which were found to accumulate in the lymph nodes draining a melanoma and also in the spleen. Splenic immature myeloid DCs from tumor-bearing rats caused the expansion of $CD25^+$ cells when cultured together *in vitro*, and also *in vivo* when the two cell types were co-injected into tumor-free rats. Either activation state or antigen presentation was important in causing $CD25^+$ T_{reg} expansion, as immature myeloid DCs from tumor-free rats did not cause cell expansion. The ability of the immature myeloid DCs to expand $CD25^+$ T_{reg} appears to be dependent on TGF-β as anti-TGF-β antibody prevented *in vitro* expansion, while addition of TGF-β allowed immature DCs from tumor-free rats to expand $CD25^+$ T_{reg}.

CONCLUSION

Great progress has been made in uncovering the details of $CD25^+$ T_{reg} over the last 10 years. $CD25^+$ T_{reg} have gone from a cell type that was looked upon with some suspicion to one that is a respectable member of the immunological community. The ability to identify, manipulate, expand, and track these cells has opened a new vista on the control of immune responses. In the near future we expect that manipulating the activity of $CD25^+$ T_{reg} in a clinical setting to augment responses to vaccines or immunotherapies or to suppress unwanted responses to self antigens will become feasible and, eventually, commonplace.

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