CD4+CD25+ SUPPRESSOR T CELLS: MORE QUESTIONS THAN ANSWERS

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Several mechanisms control discrimination between self and non-self, including the thymic deletion of autoreactive T cells and the induction of anergy in the periphery. In addition to these passive mechanisms, evidence has accumulated for the active suppression of autoreactivity by a population of regulatory or suppressor T cells that co-express CD4 and CD25 (the interleukin-2 receptor α-chain). $CD4^+CD25^+$ T cells are powerful inhibitors of T-cell activation both *in vivo* and *in vitro*. The enhancement of suppressor-cell function might prove useful for the treatment of immune-mediated diseases, whereas the downregulation of these cells might be beneficial for the enhancement of the immunogenicity of vaccines that are specific for tumour antigens.

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T cells that were able to suppress immune responses were described first in the early 1970s[1,2]. Suppressor T cells were thought to be a specialized population, the effects of which were mediated by secreted antigen-specific factors. However, the failure to clone these factors led to the demise of this entire field of study in the early 1980s[3,4]. Sakaguchi and associates[5,6] rekindled interest in the concept of T-cell-mediated suppression in the mid-1990s by showing that a minor population (~10%) of $CD4^+$ T cells, which co-expresses the interleukin-2 receptor (IL-2R) α-chain (CD25), is crucial for the control of autoreactive T cells *in vivo*. Subsequent *in vitro* studies by several groups showed that $CD4^+CD25^+$ T cells are both hyporesponsive and suppressive[7–9]. $CD4^+CD25^+$ T cells were discovered originally in mice, but a population with identical phenotypic and functional properties has been defined recently in humans[10–16]. Although the term 'regulatory T cell' has replaced the term 'suppressor T cell' in the immunology literature, regulatory T cells might both enhance or suppress immune responses. As $CD4^+CD25^+$ T cells only downregulate immune responses, I refer to them here as suppressor T cells.

In addition to $CD4^+CD25^+$ T cells — which are best termed 'naturally occurring suppressor cells' — several *in vitro* and *in vivo* treatments have been shown to generate a spectrum of suppressor T cells (FIG. 1). The oral administration of antigen is the oldest approach used to induce suppressor T cells[17]. The relationship between these induced suppressor T-cell populations and the naturally occurring suppressor populations is unclear. Probably, the most intriguing question that must be addressed is whether any $CD4^+$ T cell in the normal peripheral lymphoid environment can develop into a suppressor cell? If so, what are the factors that promote the differentiation of such suppressor cells?

Although several reviews have been published recently on suppressor T cells, this is a rapidly evolving area of investigation[18–20]. Many of the issues that were raised ten years ago about the existence of suppressor T cells are still relevant today (BOX 1). I use a question-and-answer format in this review to address some of these issues and to emphasize important areas of agreement and controversy, as well as directions for future study.

$CD25^+$ T-cell-mediated suppression *in vitro*?

The first studies to define the suppressor function of $CD4^+CD25^+$ T cells *in vitro*[7–9] showed that the proliferation of $CD25^-$ T cells induced by CD3-specific antibodies was inhibited by 80–90% at a ratio of one $CD25^+$ T cell to four $CD25^-$ T cells. Suppression occurred only when the $CD25^+$ T cells were activated through their T-cell receptor (TCR)[21]. The main mechanism of suppression seemed to be inhibition of the transcription of IL-2 in the responder population. Suppression could be

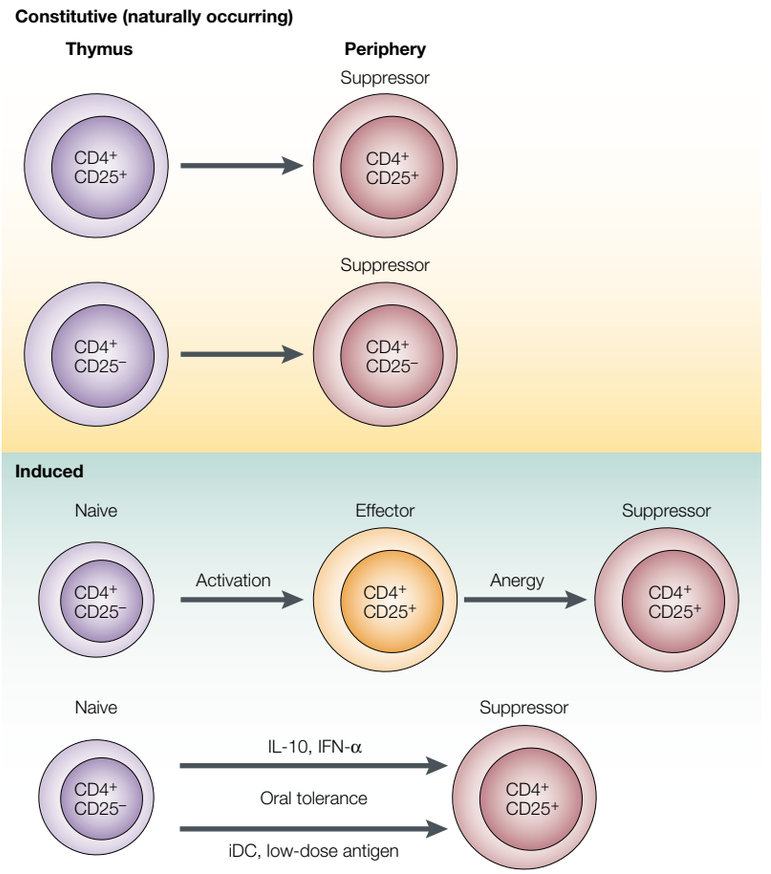

Figure 1 | **Many types of suppressor T cell exist in the normal host.** $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells are naturally occurring suppressor cells. Other types of suppressor might be generated by culture in the presence of suppressive cytokines (such as IL-10 and IFN-α), by the administration of antigen in a tolerogenic form or by exposing effector T cells to antigen under anergizing conditions. iDC, immature dendritic cell; IL-10, interleukin-10; IFN-α, interferon-α.

abrogated by the addition of exogenous IL-2 or by enhancing endogenous IL-2 production in the responder population by means of anti-CD28 antibody. This antibody mimics the potent stimulus for IL-2 production that is provided normally by the interactions of CD80 (B7.1) and/or CD86 (B7.2) on antigen-presenting cells (APCs) with CD28 on T cells[7,8]. However, the exact mechanism by which $CD25^+$ T cells exert their suppressive effects remains unknown. Although cell contact between suppressors and responders is required[7,8], it is not yet clear if the $CD25^+$ T cells target the responder $CD25^-$ T cells or the APCs.

A role for CTLA4? $CD4^+CD25^+$ T cells are the only lymphocyte subpopulation in both mice and humans that express cytotoxic T-lymphocyte antigen 4 (CTLA4) constitutively. Considerable controversy exists about the significance of this finding. Is the expression of CTLA4 merely consistent with the activated/memory phenotype of these cells, or does CTLA4 have an important functional role? Takahashi *et al.*[22] have shown that the addition of anti-CTLA4 antibody or its Fab (fragment of antigen binding) reverses suppression in co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Similarly, Read *et al.*[23] have shown that the treatment of recipients of $CD4^+CD45RB^{hi}$ and $CD4^+CD45RB^{lo}$ T cells with these agents abrogated the suppression of INFLAMMATORY BOWEL DISEASE (IBD). These studies indicate that signals that result from the engagement of CTLA4 by its ligands, CD80 or CD86, are required for the induction of suppressor activity (FIG. 2a). However, these *in vitro* studies have been difficult to reproduce[7], and no effects of anti-CTLA4 antibody or its Fab were observed in studies of human $CD4^+CD25^+$ T cells[10–16]. Under some circumstances, the engagement of CTLA4 on the $CD4^+CD25^+$ T cells by antibody or by CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity (FIG. 2b). One confounding variable in the interpretation of these studies is that CTLA4 is also expressed by activated $CD4^+CD25^-$ T cells. It remains possible that the effects of anti-CTLA4 antibody *in vitro* are the result of effects on the $CD25^-$ T cells (or the $CD45RB^{hi}$ T cells *in vivo*). Antibody-mediated blockade of the interaction of CD80 or CD86 with CTLA4 on activated effector populations might inhibit the normal downregulatory effects of CTLA4 on T-cell activation and raise the threshold that is required for $CD4^+CD25^+$ T cells to mediate suppression (FIG. 2c).

A role for TGF-β? Most studies have failed to identify a soluble suppressor cytokine[7–16]. The addition of neutralizing antibodies that are specific for IL-4, IL-10 or transforming growth factor-β (TGF-β) does not reverse suppression, and $CD25^+$ T cells from *Il4*$^{-/-}$ or *Il10*$^{-/-}$ mice are fully competent suppressors *in vitro*[7]. However, it is difficult to rule out the involvement of a cytokine that acts over short distances or a cell-bound cytokine. Indeed, Nakamura *et al.*[24] have raised the possibility that TGF-β produced by $CD25^+$ T cells — and bound to their cell surface by an as yet uncharacterized receptor — might be the main mechanism by which $CD25^+$ T cells mediate suppression. After activation *in vitro*, $CD25^+$, but not $CD25^-$, T cells react with a polyclonal antibody that is specific for TGF-β, and high concentrations of anti-TGF-β reagents are able to abrogate $CD25^+$ T-cell-mediated suppression completely. Normally, TGF-β is secreted in an inactive precursor form and must be converted to its active form to manifest biological activity[25]. Nakamura and colleagues hypothesize that latent TGF-β bound to the surface of the activated $CD25^+$ T cells is delivered directly to the responder $CD25^-$ T cells by a cell-contact-dependent delivery system. Presumably, in the milieu of this cell contact, the latent TGF-β that is bound to the cell surface would also be converted to its active suppressive form. High concentrations of antibody would, therefore, be required to reverse suppression because they must penetrate the interface between the $CD25^+$ and $CD25^-$ T cells.

These observations should be interpreted with caution, as numerous other studies with both mouse and human $CD25^+$ T cells have failed to find a role for TGF-β[7–16]. Recently, we have used a genetic approach to analyse the role of TGF-β in CD25-mediated suppression (C. Piccirillo *et al.*, unpublished observations).

INFLAMMATORY BOWEL DISEASE (IBD). A T-cell-mediated inflammatory response that affects the small and large bowel, resembling Crohn's disease in humans. In the mouse model, most of the inflammation is confined to the large bowel. The target antigen that is recognized by the pathogenic T cells is unknown.

Box 1 | **Suppressor T cells — unresolved issues**

- How many types of suppressor T cell exist?
- Are they generated in the thymus or periphery?
- Which markers reliably distinguish suppressor T cells from other T-cell populations?
- What is their physiological ligand(s)?
- What is their target cell(s)?
- What is their mechanism of action? Does it involve cytokine secretion or cell contact?
- What is their relationship to lymphopaenia?
- Are they beneficial (for the prevention of autoimmunity, allergy and graft rejection)?
- Are they harmful (in terms of their effects on tumour immunity, and the immune response to chronic infections and weak vaccines)?

Smad3 is required for TGF-β-mediated signalling in T cells[26], but Smad3-deficient $CD25^-$ T cells remain fully susceptible to suppression by $CD25^+$ T cells. In addition, $CD25^+$ T cells from Smad3-deficient mice are fully competent suppressors, which indicates that TGF-β has no role in the development of $CD25^+$ suppressor T-cell function (C. Piccirillo *et al.*, unpublished observations).

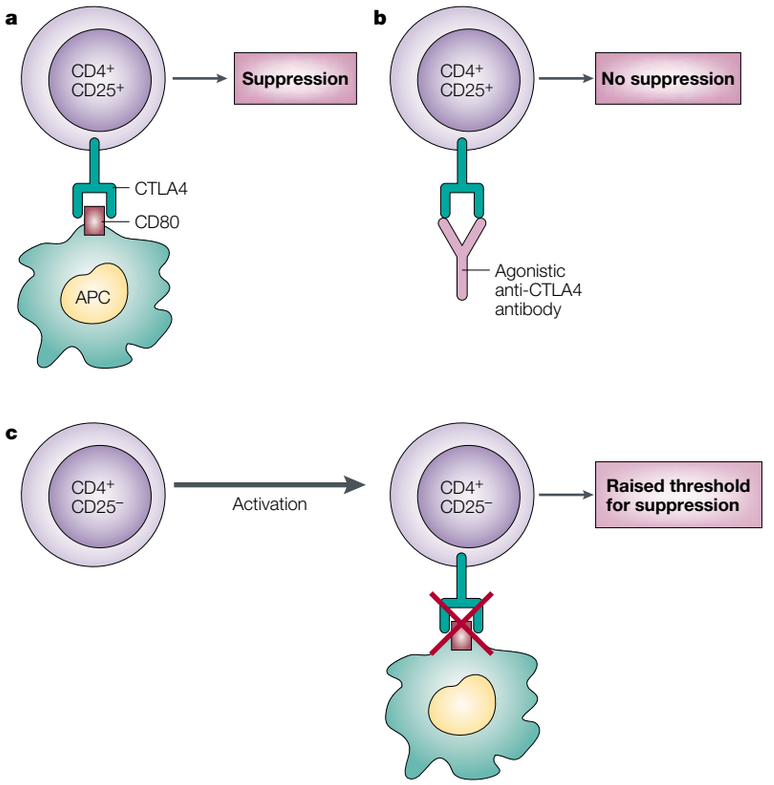

Figure 2 | **What role does CTLA4 have in $CD4^+CD25^+$ T-cell function?** Three possible alternatives are illustrated. **a** | Engagement of CTLA4 by CD80 (or CD86) is required for the induction of $CD4^+CD25^+$ suppressor T-cell function. Blockade of this interaction inhibits suppressor-cell function. **b** | Engagement of CTLA4 on $CD4^+CD25^+$ T cells by antibody or by CD80/CD86 inhibits the T-cell-receptor-derived signals that are required for the induction of suppressor-cell effector function. **c** | CTLA4 has no role on $CD4^+CD25^+$ T cells. Anti-CTLA4 antibody acts on activated $CD4^+CD25^-$ T cells to block the normal downregulatory signals that are mediated by CLTA4–CD80/CD86 interactions and, thereby, raises the threshold for suppression mediated by $CD4^+CD25^+$ T cells. APC, antigen-presenting cell; CTLA4, cytotoxic T-lymphocyte antigen 4.

Furthermore, we have used transgenic mice that express a dominant-negative form of the TGF-β receptor (TGFβRII) that cannot respond to TGF-β-derived signals[27]; again, $CD25^-$ T cells from these mice were fully suppressible. Finally, $CD25^+$ T cells isolated from young TGF-β-deficient mice[28] are fully competent suppressors when mixed with $CD25^-$ T cells from wild-type mice. So, the potential role of TGF-β in $CD25^+$ T-cell-mediated suppression remains controversial and deserves careful further study, particularly in view of the potential involvement of TGF-β in suppression *in vivo* (see below).

Effects on antigen-presenting cells. We proposed originally that $CD25^+$ T cells might target antigen-presenting cells (APCs) and inhibit their upregulation of expression of the co-stimulatory molecules that are required for IL-2 production by $CD25^-$ T cells (FIG. 3a). However, in co-cultures, the upregulation of expression of several co-stimulatory molecules on APCs occurred normally in the presence of $CD25^+$ T cells[21]. Suppression could not be overcome by the addition of an excess of fully competent, activated APCs. These observations should be compared with those of Cederbom *et al.*[29], who analysed the effects of $CD25^+$ T cells on relatively immature dendritic cells (iDCs) and described a modest decrease in the expression of CD86, but no downregulation of CD86 messenger RNA.

The most direct approach to determine whether $CD25^+$ T cells act on responder T cells rather than on APCs would be to assess the suppressor capacity of $CD25^+$ T cells in a system that is devoid of APCs. $CD4^+CD25^+$ T cells can suppress the proliferation of $CD8^+$ T cells and their effector cytokine production[30]. Furthermore, $CD8^+$ T cells can be activated readily by peptide–MHC tetramers in the complete absence of APCs. To determine directly whether $CD4^+CD25^+$ T cells suppress $CD8^+$ T-cell responders by modulating APC function or by direct T-cell–T-cell contact, we stimulated $CD8^+$ T cells from a TCR-transgenic mouse with their target peptide–MHC tetramer in the presence or absence of activated $CD25^+$ T cells[30]. Marked suppression of both proliferation and interferon-γ (IFN-γ) production was seen in the presence of the $CD25^+$ T cells. The results from this experiment show conclusively that $CD25^+$ T cells can mediate suppression by means of a T-cell–T-cell interaction, and that APCs are not required directly for the delivery of the suppressive signal to the responding $CD8^+$ T cells (FIG. 3b). However, this result does not exclude the possibility that $CD25^+$ T cells might also exert inhibitory/deactivating effects on APCs, or use the APC surface as a platform on which the suppressor cells interact physically with $CD4^+$ or $CD8^+$ effectors *in vivo*. Direct suppressive effects of $CD25^+$ T cells on B-cell activation, macrophage activation or natural killler (NK)-cell function have yet to be reported, but the possibility should be examined closely.

The main issue to be resolved by future studies of $CD25^+$ T-cell-mediated suppression is the identification of the molecular pathways that are responsible for mediating suppression. Logical candidates for these

pathways might include members of the tumour-necrosis factor/tumour-necrosis-factor receptor (TNF/TNFR) superfamily, as engagement of either the receptors or their ligands might lead to the inhibition of cytokine production and cell growth similar to that mediated by $CD25^+$ T cells[31]. However, antibodies that are specific for several members of this family have failed to reverse suppression when added to co-cultures of $CD25^+$ and $CD25^-$ T cells (A. Thornton and E.M.S., unpublished observations). One member of the TNFR family (the glucocorticoid-induced TNF receptor; TNFRSF8) has been shown recently to have an important role in the induction of the suppressor function of $CD4^+CD25^+$ T cells, but it does not mediate suppressor effector function directly[32,33]. A second candidate mechanism would be the engagement of a cell-surface molecule on the $CD25^-$ responders that contains an IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF (ITIM)[34] by a ligand on the $CD25^+$ suppressor cells. In a manner similar to that proposed for the regulation of NK-cell activity[35], such an interaction could result in the activation of a phosphatase that could mediate suppression. However, no evidence has been presented yet that this mechanism is operative in CD25-mediated suppression. There are, undoubtedly, other potential molecules involved.

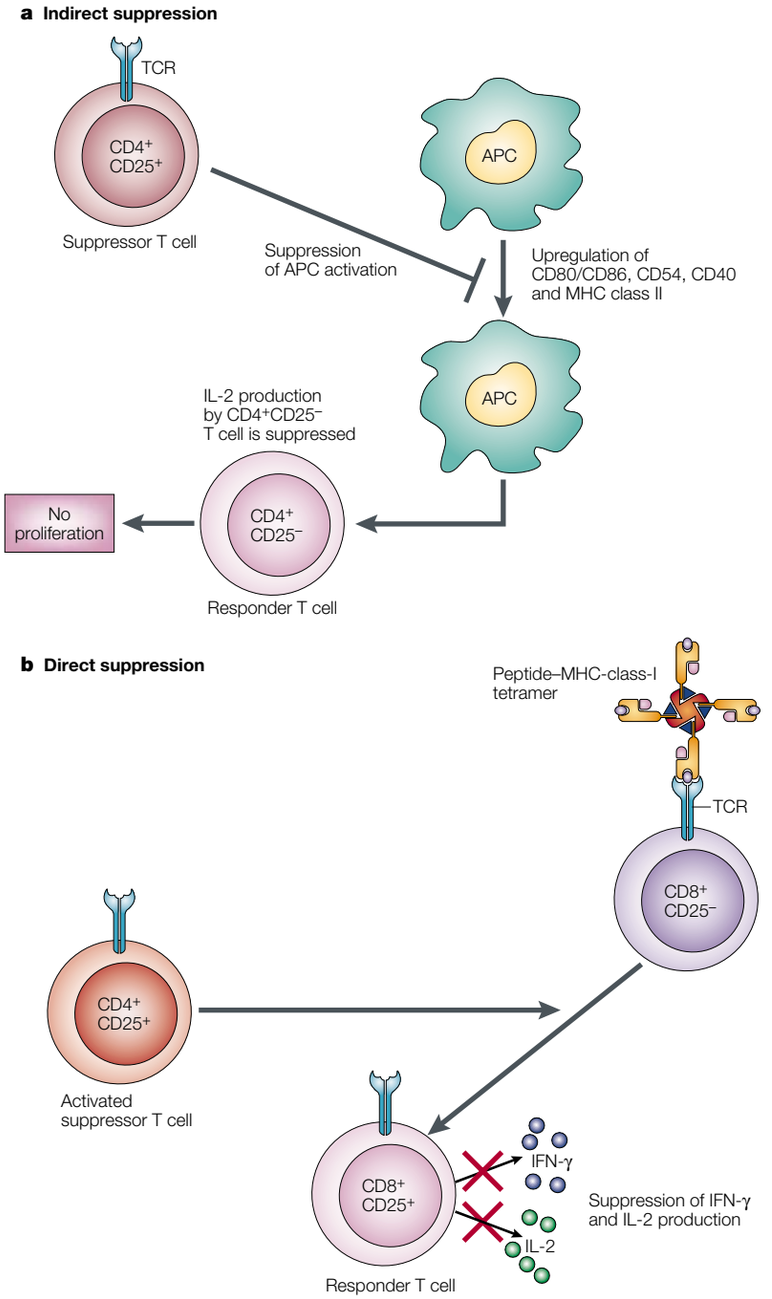

Figure 3 | **What is the target cell for $CD25^+$ T-cell-mediated suppression? a** | Indirect suppression. The $CD25^+$ T cell acts on the antigen-presenting cell (APC) to inhibit the upregulation of expression of co-stimulatory molecules that are required for the activation of $CD25^-$ T cells and, thereby, it indirectly inhibits the induction of interleukin-2 (IL-2) production and the proliferation of the $CD25^-$ responder T cells. **b** | Direct suppression. Studies carried out with $CD8^+$ responder T cells that are activated to proliferate and produce interferon-γ (IFN-γ) by peptide–MHC-class-I tetramers in the complete absence of APCs have shown that activated $CD25^+$ T cells are fully able to suppress the activation of the $CD8^+$ responders. So, $CD25^+$ T cells might mediate their inhibitory effects directly by acting on the responder T cells. It is also possible that, in some cases, both the direct and indirect pathways might operate. TCR, T-cell receptor.

$CD25^+$ T-cell-mediated suppression *in vivo*?

Suppressor cytokines. Although there is some agreement about the lack of involvement of suppressor cytokines *in vitro*, the mechanisms by which $CD25^+$ T cells suppress autoimmune diseases *in vivo* are more complicated, and several suppressor cytokines have been implicated as having crucial roles (FIG. 4). The evaluation of the role of cytokines in suppression in co-transfer studies of $CD25^+$ suppressors and $CD25^-$ effectors has been carried out directly by using $CD25^+$ T cells from cytokine-deficient animals or by treating reconstituted animals with neutralizing anti-cytokine antibodies[36–39]. In the latter situation, it remains possible that the suppressor cytokine was not produced by the $CD25^+$ T cells themselves, but was produced by host cells as a result of interaction with the suppressors. In IBD, IL-10 has been shown to be produced by $CD25^+$ T cells[39], but the source of TGF-β could be the $CD25^+$ T cells, other T-cell populations or, even, non-lymphoid cells, such as epithelium that is in the process of healing[40]. One important difference between AUTOIMMUNE GASTRITIS (AIG) and IBD is the requirement for intestinal bacteria for the induction of IBD, as the transfer of $CD25^-$ T cells to germ-free mice does not result in the induction of IBD. Although cell-contact-dependent inhibition might always be required for $CD25^+$ T-cell-mediated suppression, in the milieu of the inflamed bowel in IBD, IL-10 and TGF-β might also be required to suppress the inflammation. Although this model is appealing, the suppression of autoimmune thyroiditis in the rat[38] — a disease in which bacteria are much less likely to have a role in pathogenesis — by regulatory T cells is reversed by anti-IL-4 and anti-TGF-β antibodies. One possibility is that there are different subsets of $CD4^+CD25^+$ suppressor T cells that are programmed to inhibit either by a cell-contact-dependent mechanism or by the secretion of different suppressor cytokines. Alternatively, the inflammatory milieu in different autoimmune diseases might regulate the differentiation of $CD4^+CD25^+$ T cells to either suppress by cell

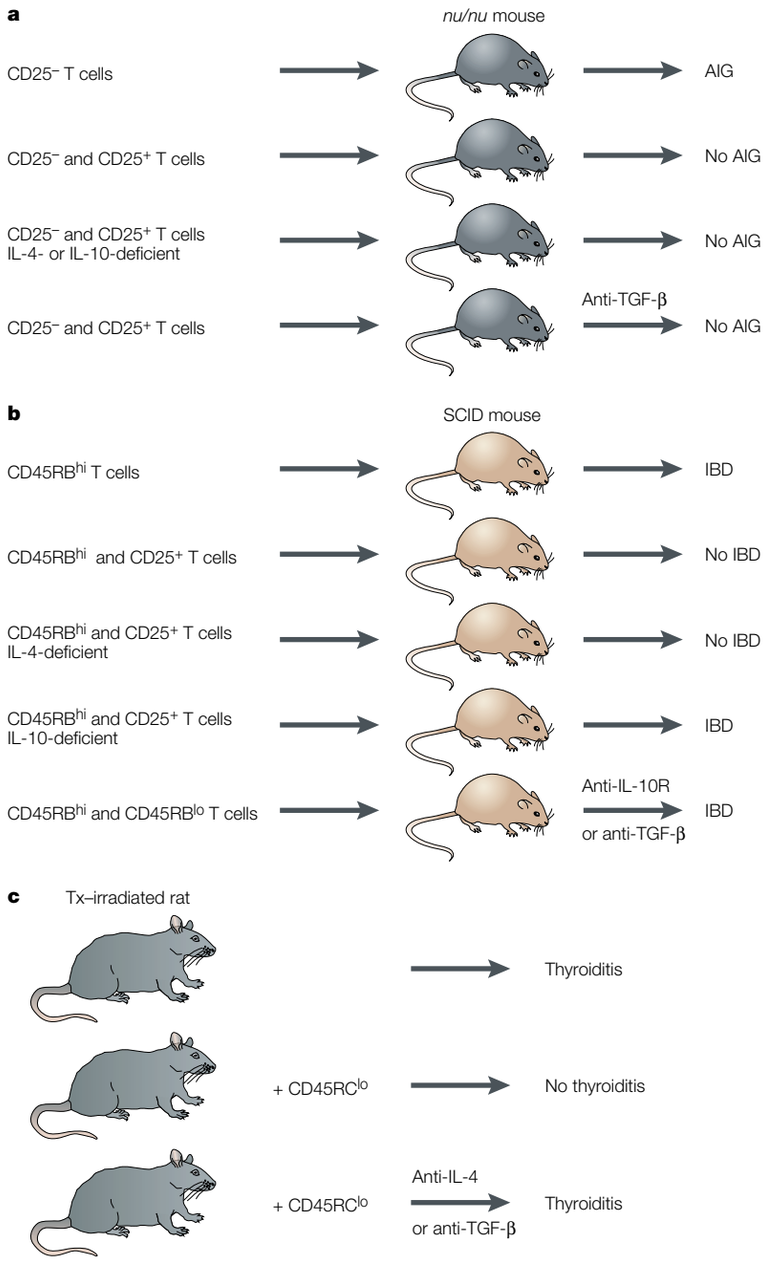

Figure 4 | **CD25⁺ T cells mediate protection from autoimmune disease by cytokine-dependent and -independent pathways.** The requirement for cytokines in CD4⁺CD25⁺ T-cell-mediated suppression of autoimmune disease has been evaluated in different disease models with disparate results. **a** | CD25⁺ T-cell-mediated protection from autoimmune gastritis (AIG) is cytokine independent. AIG was induced by the transfer of CD25⁻ T cells to *nu/nu* recipients, which lack all T cells. The co-transfer of CD25⁺ T cells prevented the induction of AIG. CD25⁺ T cells from interleukin-4 (IL-4)- or IL-10-deficient mice were as effective as CD25⁺ T cells from wild-type mice in preventing disease. The treatment of recipients of wild-type CD25⁺ T cells with transforming growth factor-β (TGF-β)-specific antibody did not abrogate suppression. **b** | Inflammatory bowel disese (IBD) was induced by the transfer of CD45RB^{hi} T cells to severe combined immunodeficient (SCID) recipient mice. The co-transfer of CD25⁺ (or CD45RB^{lo}) T cells prevented the induction of disease. CD25⁺ T cells from IL-4-deficient, but not IL-10-deficient, mice were protective. The treatment of recipients of wild-type CD45RB^{lo} T cells with anti-IL-10 receptor (R) antibody or with anti-TGF-β antibody abrogated suppression. **c** | Adult rats were thymectomized (Tx) and subjected to split-dose irradiation (four separate treatments of 250 rad). Reconstitution of the treated rats with CD45RC^{lo} T cells (containing CD25⁺ T cells) prevented the development of thyroiditis. The treatment of mice that had been reconstituted with CD45RC^{lo} T cells with anti-IL-4 or anti-TGF-β antibody abrogated suppression.

contact or to secrete suppressor cytokines. Furthermore, the contribution of suppressor cytokines to the *in vivo* function of CD4⁺CD25⁺ T cells in regulating autoimmunity emphasizes that considerable caution should be exercised in relying solely on *in vitro* studies for the analysis of regulatory-cell function.

Homeostatic control. Autoimmune diseases induced by CD25⁻ T cells are only seen after transfer of the CD25⁻ T cells to mice that lack CD25⁺ T cells. In most studies, CD25⁻ T cells are transferred to *nu/nu* mice, which lack all T cells, or to recombinase-activating gene (*Rag*)-deficient or severe combined immunodeficient (SCID) mice, which lack both T cells and B cells. It has been proposed that one of the main mechanisms that is used by CD25⁺ T cells to inhibit autoimmune disease is competition for space, cytokines or co-stimulatory signals in LYMPHOPAENIC MICE[41]. As CD25⁺ T cells have a phenotype that indicates previous activation, one extension of this concept is that any activated T cell would be as efficient as a CD25⁺ T cell in inhibiting autoimmune disease in the lymphopaenic environment. Although some studies[42] have shown that only CD25⁺ T cells, and not activated CD25⁻ T cells, can inhibit autoimmune disease after day-three thymectomy (d3Tx), one finding that is consistent with this hypothesis is that the depletion of CD25⁺ T cells from young BALB/c mice does not lead to the development of AIG[85]. However, when T cells from these same mice were transferred to *nu/nu* recipients, all of the recipients developed severe AIG. It is, therefore, possible that the CD25⁻ effector cells were unable to cause disease in a lymphocyte-sufficient environment because they were held in check by the normal population of activated T cells that are specific for the environmental or endogenous antigens that are present in the CD25⁺ T-cell-depleted host.

An alternative explanation for these observations is that a second signal is required to stimulate the CD25⁻ T cells to differentiate into autoreactive effectors in the absence of CD25⁺ suppressors (FIG. 5). When CD25⁻ T cells are transferred to a lymphopaenic environment, the second signal is provided by lymphopaenia-induced cell division. Similarly, in the d3Tx mouse that lacks CD25⁺ T cells, the lymphopaenic environment[43] would provide a stimulus for the proliferation of effectors. More importantly, we have been able to show that a second signal can also be provided by immunizing CD25⁺ T-cell-depleted animals with the target antigen for AIG, the parietal-cell gastric H/K ATPase[85]. Non-depleted animals failed to develop AIG, whereas 100% of the CD25⁺ T-cell-depleted animals developed severe AIG. Other sources of inflammation — for example, viral infections and others that activate the innate immune system — can probably also provide a second signal to push CD25⁻ T cells to develop into effectors in the absence of suppressors.

How are CD25⁺ T cells selected in the thymus?

Papiernik *et al.*[44] were the first to demonstrate the presence of CD25⁺ T cells in the thymus. In their studies, the expression of CD25 seemed to be induced at the CD4⁺

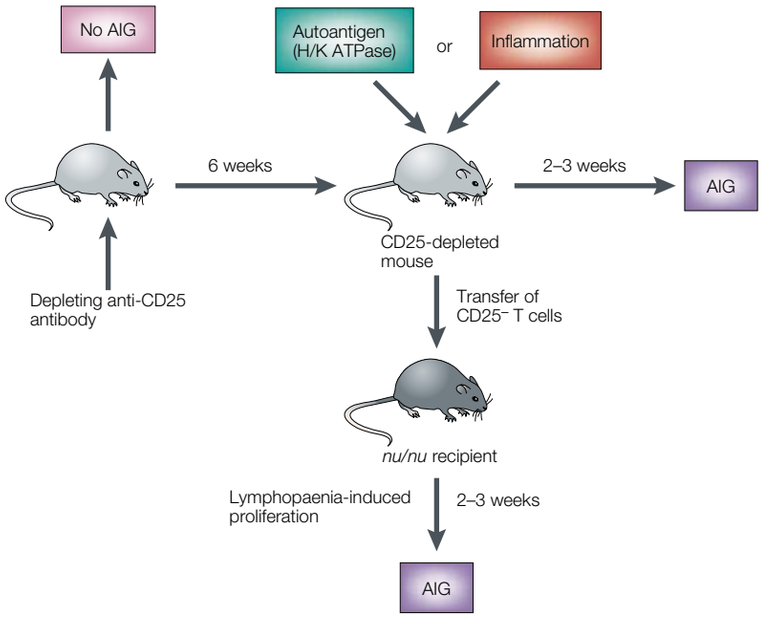

Figure 5 | **Depletion of CD25⁺ T cells is not sufficient for the induction of autoimmunity.** The treatment of young mice with anti-CD25 antibody is highly effective for depleting CD25⁺ T cells. However, treated animals fail to develop autoimmune disease. The transfer of CD25⁻ T cells from treated mice to *nu/nu* recipients readily induces autoimmune disease. Furthermore, immunization of the CD25-depleted mice with the H/K ATPase results in the development of autoimmune gastritis (AIG). It is probable that a non-specific inflammatory response could also supply the necessary second signal for the activation of the CD25⁻ T-cell effectors in the absence of CD25⁺ suppressors.

single-positive stage. Approximately 5% of CD4⁺CD8⁻ thymocytes expressed CD25 and, functionally, they were similar to CD25⁺ T cells in the periphery[45]. They did not seem to be cells that had re-circulated from the periphery. Fluorochrome labelling showed that CD25⁺ T cells emigrate from the thymus to populate the periphery. The identification of CD25⁺ T cells in the CD4⁺CD8⁻ T-cell pool gave rise to the hypothesis[18,45] that these cells might be educated on medullary DCs during the process of NEGATIVE SELECTION. CD25⁺ T cells would comprise a population that recognized self-antigens with an intermediate affinity — one that was insufficient to result in deletion, but too high to allow them to pass into the periphery. During this process of 'altered negative selection', it was proposed that the CD25⁺ T cells receive a signal that renders them anergic and suppressive (FIG. 6a). A test of this hypothesis was carried out by Jordan *et al.*[46] by crossing TCR-transgenic mice that expressed a receptor specific for influenza-virus haemagglutinin with transgenic mice that expressed the antigen. The most striking finding in this study was that 30% of TCR-transgene-positive thymocytes and 50% of lymph-node T cells were CD25⁺. Importantly, radio-resistant elements of the thymus (such as the thymic epithelium), and not bone-marrow-derived DCs, were both necessary and sufficient for the selection of the CD25⁺ T cells. When a distinct TCR-transgenic mouse that expressed a TCR with lower affinity for the haemagglutinin antigen was crossed with the haemagglutinin-expressing transgenic mouse, CD25⁺ T cells did not develop. The authors concluded from this study that CD25⁺ T cells are selected secondary to a high-affinity TCR interaction with target antigen, which is expressed most probably by medullary epithelial cells.

Bensinger *et al.*[47] analysed the development of CD25⁺ T cells in K14 TRANSGENIC MICE, in which the expression of MHC class II is under the control of the keratin promoter[48]; both medullary epithelium and bone-marrow-derived APCs are negative for MHC class II in this strain. As the development and function of the CD25⁺ T-cell population is normal in these mice, the authors concluded that positive selection on cortical epithelium is necessary and sufficient for the differentiation of CD25⁺ T cells from double-positive precursors (FIG. 6b). More importantly, the recognition of self-antigens in association with MHC class II in the periphery of these mice does not seem to be required for the survival/differentiation of CD25⁺ T cells in the periphery. CD25⁻ T cells from K14 transgenic mice on the C57BL/6 background do not undergo negative selection and proliferate when co-cultured with wild-type C57BL/6 cells. Similarly, CD25⁺ T cells in the K14 transgenic strain do not undergo negative selection, because CD25⁺ T cells from K14 trangenic mice, but not from wild-type C57BL/6 mice, readily suppress the response of CD25⁻ K14 transgenic cells to C57BL/6 cells. A subset of CD25⁺ T cells in normal C57BL/6 mice must, therefore, undergo negative selection on medullary bone-marrow-derived APCs.

Although it could be concluded from these studies that the normal selection of CD25⁺ T cells is similar to the selection of CD25⁻ T cells, there are still many unresolved questions about the differentiation of CD25⁺ T cells, including their relationship to cortical CD4⁺ CD8⁺ T cells. One potential confounding variable in the interpretation of studies that involve the transgenic expression of MHC or 'self'-antigen is that the level of expression might be much higher than is physiologically normal. The differentiation of CD25⁺ T cells in the presence of physiological levels of MHC class II and self-antigen might differ from what has been observed in these transgenic mice. In addition, it is worth noting that CD25⁺ T cells express a highly diverse TCR repertoire, at least in terms of TCR Vα and Vβ usage[45], but it is unknown if their receptor repertoire is as diverse as the repertoire of CD25⁻ T cells.

CD25⁺ T-cell maintenance in the periphery?

A role for IL-2. It was noted first that CD4⁺CD25⁺ T cells were absent from the periphery and from the CD4⁺CD8⁻ thymocyte pool of *Il2*$^{-/-}$ mice[44]. Several other studies have reported marked defects in the number of CD4⁺CD25⁺ T cells in other knockout strains of mice[49–53]. The common factor that characterizes all of these strains is that the products of all of the deficient genes have important roles in the production of IL-2, co-stimulation of the production of IL-2 or responsiveness to IL-2. As CD25⁺ T cells never produce IL-2, it should be pointed out that all of these defects relate to the production of IL-2 by CD25⁻ T cells. The IL-2 is required for the differentiation and/or survival of the

IMMUNORECEPTOR TYROSINE-BASED INHIBITORY MOTIF (ITIM). A structural motif containing tyrosine residues that is found in the cytoplasmic tails of several inhibitory receptors, such as FcγRIIB and PIRB. The prototype six-amino-acid ITIM sequence is (Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-(Leu/Val). Ligand-induced clustering of these inhibitory receptors results in tyrosine phosphorylation, often by SRC-family tyrosine kinases, which provides a docking site for the recruitment of cytoplasmic phosphatases that have an SH2 domain.

AUTOIMMUNE GASTRITIS (AIG). The destruction of gastric parietal cells by CD4⁺ T cells that recognize the proton pump, the H/K ATPase, that is expressed by parietal cells. It is an animal model of the human disease pernicious anaemia.

LYMPHOPAENIC MICE
A loss of both T and B cells, as is seen in SCID or Rag-deficient mice that lack an enzyme that is required for the generation of T- and B-cell receptors, or a loss of T cells only, as seen in *nu/nu* mice, which lack a thymus. A relative T-cell lymphopaenia can be seen when mice are thymectomized on day three of life.

NEGATIVE SELECTION
One step in the process of T-cell differentiation in the thymus in which T cells that express high-affinity receptors for self-antigens are eliminated from the repertoire by apoptosis after recognition of their target antigen on thymic medullary dendritic cells.

K14 TRANSGENIC MICE
First, mice that lack all MHC class II antigens are generated. Transgenic mice in which MHC class II antigens are expressed under the control of the keratin promoter are then generated from these deficient mice. MHC class II antigens are expressed solely by cells that can turn on expression of the keratin gene — primarily, epidermal cells and thymic cortical epithelial cells.

ACTIVATION-INDUCED CELL DEATH
(AICD). The normal physiological mechanism by which T cells that are specific for foreign antigen are eliminated from the T-cell repertoire.

CD25$^+$ T cells. Many, but not all, of these strains are also characterized by the presence of an autoimmune syndrome. Is autoimmunity solely the result of the deficiency of CD25$^+$ T cells? Wolf *et al.*[54] have shown that both CD25$^+$ and CD25$^-$ T cells can inhibit the development of disease in IL-2-deficient mice. The CD25$^+$ T-cell population might control autoimmunity in a manner similar to that observed in the d3Tx model at the level of activation of effectors, whereas CD25$^-$ T cells produce IL-2, which mediates the ACTIVATION-INDUCED CELL DEATH (AICD) of autoreactive T cells.

IL-2 receptor β-chain (*Il2rb*)$^{-/-}$ mice also develop an autoimmune syndrome. Malek *et al.*[49] have developed a transgenic mouse model in which the expression of

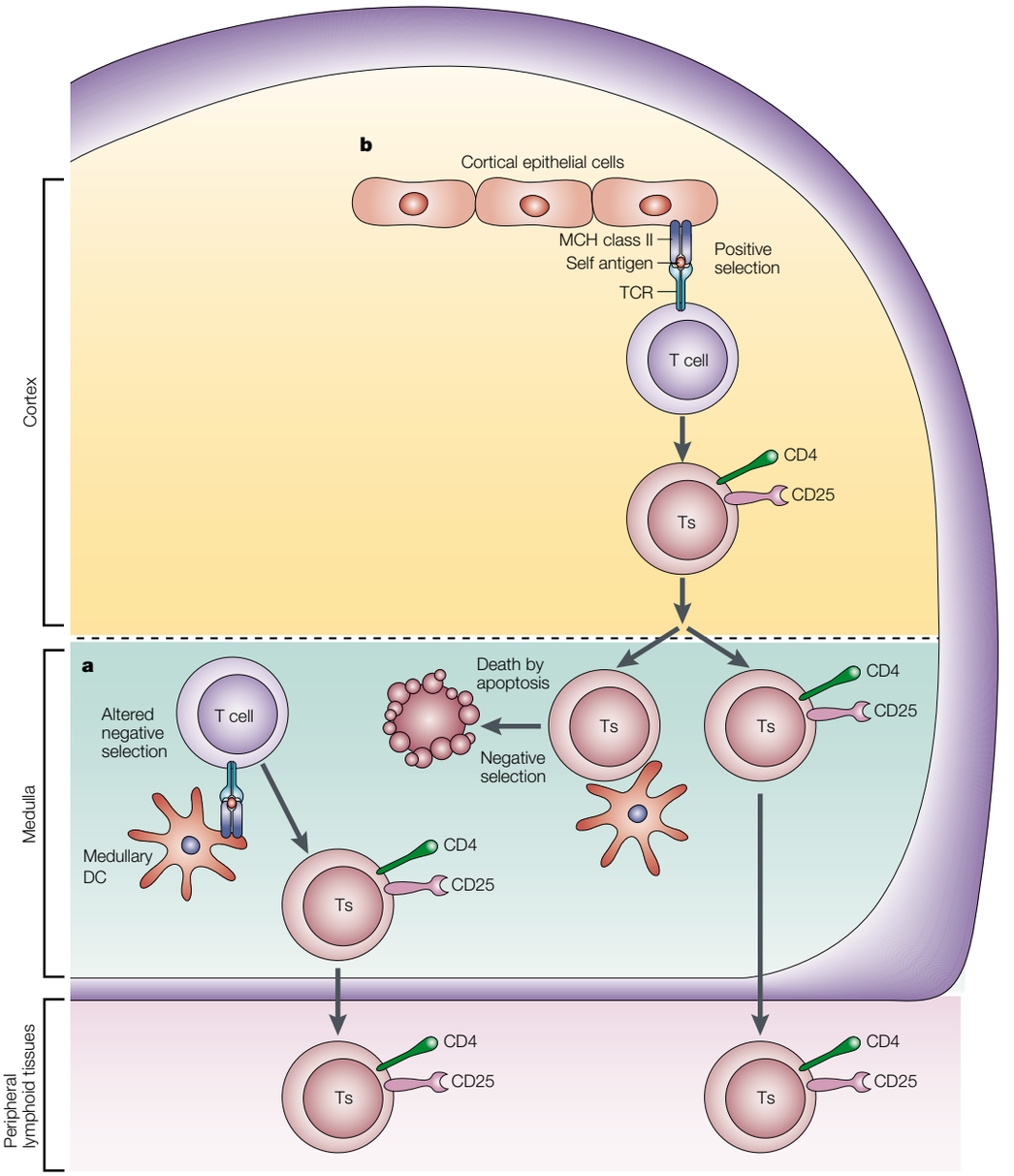

Figure 6 | **CD25$^+$ T cells differentiate in the thymus.** CD4$^+$CD25$^+$ T cells seem to be members of a unique lineage of T cells that are selected during the process of T-cell differentiation in the thymus. It remains unclear where and when this occurs. **a** | One possibility is that CD25$^+$ T cells acquire expression of CD25 and suppressor function in the thymic medulla, where they recognize self-antigens that are presented on MHC class II molecules by medullary dendritic cells (DCs) in a process that is known as 'altered negative selection'. They then migrate directly to peripheral lymphoid tissues. **b** | Studies with the K14 transgenic mouse have indicated that CD25 expression and suppressor function is acquired at a much earlier stage of differentiation in the thymic cortex during the process of positive selection on cortical epithelial cells. Some of these CD25$^+$ T cells then undergo a process of negative selection on bone-marrow-derived cells (such as DCs) in the medulla and die by apoptosis, but others are allowed to migrate to peripheral lymphoid tissues, according to the affinity of their TCR for self-antigens. TCR, T-cell receptor; Ts, suppressor T cell.

IL-2Rβ is targeted exclusively to the thymus of *Il2rb*$^{-/-}$ mice. The mature T-cell compartment of these animals was unresponsive to IL-2 *in vitro* and *in vivo*, but the mice did not develop autoimmunity. These results indicate that IL-2Rβ signalling in the thymus is required to regulate the development of crucial suppressor T cells, such as CD25$^+$ T cells. Indeed, Malek *et al.* (personal communication) have shown recently that the transgenic mice have numerous CD25$^+$ T cells in their thymus, as well as their peripheral lymph nodes and spleen. These results raise the possibility that IL-2 is required only for the development of CD25$^+$ T cells in the thymus and has no role in their maintenance in the periphery. However, these results disagree with the observations of Salomon *et al.*[53] that the short-term inhibition of co-stimulation in NON-OBESE DIABETIC (NOD) MICE resulted in a 4–5-fold decrease in the number of CD25$^+$ T cells and an increased incidence of diabetes. As CD25$^+$ T cells are long-lived — they persist for long periods of time after thymectomy[44] — this study is most compatible with a requirement for IL-2 or other cytokines for the survival of CD25$^+$ T cells in the periphery. One possibility is that CD25$^+$ T cells require a crucial IL-2 signal in the thymus for development, but can be maintained in the periphery by other cytokines, such as IL-4, the production of which is dependent on co-stimulatory signals.

Antigen specificity. Do the CD25$^+$ T cells that control organ-specific autoimmunity preferentially recognize autoantigens that are derived from the target organ? Several studies have shown that suppressor T cells from mice that lacked the organ that was the target of autoimmune attack were much less efficient than suppressor T cells from normal mice in preventing the development of autoimmunity[55–59]. These studies are not compatible with the results of Bensinger *et al.*[47] in K14 transgenic mice, which have normal numbers of CD25$^+$ T cells in the periphery in the absence of self-antigen presentation by MHC class II. However, the ability of CD25$^+$ T cells from K14 transgenic mice to protect against organ-specific autoimmunity has not been tested.

Taken together, these studies offer only limited insights into the physiological target antigens for CD4$^+$CD25$^+$ T cells. To make progress in this area, a genetic approach is required. We have developed TCR-transgenic mice that express a receptor that is specific for the parietal-cell gastric H/K ATPase[60]. T cells from the TCR-transgenic mice recognize a defined epitope on the autoantigen. Naive T cells from the TCR-transgenics readily induce AIG when transferred to *nu/nu* mice, and the transfer of disease is inhibited by CD25$^+$ T cells from normal BALB/c mice. Studies are now in progress to determine whether CD25$^+$ T cells from mice that lack different components of the H/K ATPase are able to suppress disease. Although these experiments should allow us to determine whether the autoimmune effectors and CD25$^+$ suppressors recognize the same autoantigen, they will not rule out the possibility that the suppressors recognize other antigens that are derived from the target organ.

NON-OBESE DIABETIC MICE (NOD mice). A strain of mice that normally develop idiopathic autoimmune diabetes that closely resembles type I diabetes in humans. The target antigen(s) that is recognized by the pathogenic CD4$^+$ T cells that initiate disease is expressed by pancreatic islet cells, but its identity has remained elusive.

How do CD25$^+$ T cells know where to go?

The recognition of organ-specific antigens by CD4$^+$CD25$^+$ T cells might be the most important factor that retains these cells in inflamed target organs or in the lymph nodes that drain those organs. However, if the receptor specificity of these cells is much broader and they recognize more-ubiquitously expressed antigens, specific signals to direct CD25$^+$ T cells to sites of inflammation would be required. Chemokines are the logical candidates to direct the recruitment of suppressor T cells. Iellem *et al.*[61] have shown that although some chemokines stimulate the migration of both resting human CD25$^+$ and CD25$^-$ T cells, CD25$^+$ T cells specifically express the chemokine receptors CC-chemokine receptor 4 (CCR4) and CCR8. One difficulty with the interpretation of these studies is that CCR4 and CCR8 are expressed also by activated CD25$^-$ T cells. Bystry *et al.*[62] have examined the expression of chemokine receptors on mouse CD25$^+$ T cells. They observed a selective response of mouse CD25$^+$ T cells to CCL4 and also showed enhanced expression of its receptor, CCR5, on CD25$^+$ T cells. The treatment of mice with anti-CCL4 antibody induced an increase in the number of activated germinal centres and autoantibody production similar to that seen when CD25$^-$ T cells were transferred to *nu/nu* recipients.

The selective expression of chemokine receptors on CD25$^+$ T cells is an important area for further study, and caution should be exercised in drawing conclusions from the limited data that are available. The studies of Iellem *et al.*[61] and Bystry *et al.*[62] have focused their attention on the chemokines that are produced by activated APCs. However, chemokine production by APCs could be induced by signals derived from the innate immune system or, alternatively, by signals induced by effector cells that recognize their target antigens during the initiation of autoimmune disease. Indeed, chemokine production by the effector T cells themselves might be as important as chemokine production by the APCs. Furthermore, one should not exclude the possibility that the CD25$^+$ T cells themselves might be able to produce chemokines after activation. Suppression might then be augmented by the influx of additional CD25$^+$ suppressors.

Do suppressor CD4$^+$CD25$^-$ T cells exist?

Athough most studies have shown that many of the T cells that are responsible for suppressing autoreactive effector cells are naturally occurring CD4$^+$CD25$^+$ T cells, several studies in both mice and rats have provided some evidence for a CD4$^+$CD25$^-$ suppressor T cell (FIG. 1). TCR-transgenic mice that express a receptor that is specific for the autoantigen myelin basic protein (MBP) do not develop EXPERIMENTAL ALLERGIC (OR AUTOIMMUNE) ENCEPHALOMYELITIS (EAE). However, when these mice are bred onto a *Rag*-deficient background, EAE develops spontaneously and rapidly in almost all mice[63]. The regulatory T-cell population that is present in the TCR-transgenic mice on a conventional background is CD4$^+$CD25$^-$ and expresses TCRs that are encoded by the endogenous TCR α- and β-chain loci[64].

Mouse $CD4^+CD25^-$ T cells have also been implicated in mediating protection from IBD[65]. Also, a population of $CD4^+CD25^-CD45RC^-$ suppressor T cells that can protect against autoimmune diabetes has been identified in rat peripheral lymphoid tissues after the removal of $CD4^+CD25^-CD45RC^-$ recent thymic emigrants[66]. These studies raise numerous questions for which we do not yet have answers. Does the thymus export $CD25^-$ T cells that are pre-committed to function as suppressor cells; do these cells acquire their suppressor function in the periphery; or are they derived from $CD25^-$ T cells?

$CD25^+$ natural and induced suppression

Several different *in vitro* protocols have been described over the past few years that result in the generation of suppressor T cells (FIG. 1). The activation of human or mouse $CD4^+$ T cells *in vitro* in the presence of IL-10 has been shown to result in the generation of T-cell clones with a cytokine profile that is different from that of T helper 1 (T_H1) or T_H2 cells. These T-cell clones produce high levels of IL-10, IFN-γ, TGF-β and IL-5, but only low levels of IL-2, and no IL-4. Functionally, these T-cell clones have inhibitory effects on the antigen-specific activation of naive autologous T cells that are mediated partially by IL-10 and TGF-β. These new T cells were termed T regulatory 1 (T_R1) cells[67]. In a model of IBD in SCID mice, the co-transfer of T_R1-cell clones together with pathogenic $CD4^+CD45RB^{hi}$ T cells prevented the induction of disease. Prevention of IBD was observed only in mice that also received the antigen that is recognized by the T_R1 cells, which shows that T_R1 cells must be activated *in vivo* through the TCR to exert their regulatory effects. Both human and mouse T_R1 cells are difficult to isolate under standard culture conditions. It has been reported that IFN-α, but not TGF-β, can act synergistically with IL-10 to facilitate the generation of immunosuppressive human T_R1 cells[68].

A related approach for the generation of suppressor T cells *in vitro* involves the stimulation of naive T cells with iDCs. Jonuleit *et al.*[69] repetitively stimulated naive cord-blood T cells with allogeneic iDCs and generated a population of poorly growing T cells that primarily produced IL-10. Surprisingly, although these cells produced IL-10, their suppressor phenotype resembled that of $CD25^+$ T cells, as it was contact-dependent, antigen non-specific and APC-independent. Furthermore, suppression could be overcome partially by the addition of IL-2. These cells also differ from T_R1 cells in that IL-10 is not required for their generation because iDCs do not produce IL-10. The precursors of these suppressor cells in cord blood do not express CD25 (H. Jonuleit, personal communication), so it is unlikely that they are derived from a $CD25^+$ T-cell population that has not fully differentiated. Immature DCs are the ideal population to prime regulatory T cells as they are deficient in co-stimulatory molecules, and priming with antigen–iDC complexes might even be able to downregulate pre-existing antigen-specific immune responses[70].

Exposure to TGF-β has also been reported to facilitate the differentiation/expansion of suppressor T-cell populations *in vitro*. After the culture of naive $CD4^+$ T cells with alloantigen in the presence of TGF-β, but not IL-10, $CD4^+CD25^+$ T cells with potent suppressor activity on the development of $CD8^+$ cytotoxic T lymphocytes (CTLs) could be isolated[71]. Inhibition of the generation of CTLs by these TGF-β-induced suppressors was not mediated by IL-10 or TGF-β. As the starting population was composed exclusively of $CD4^+CD45RA^+$ naive T cells, it seemed probable that TGF-β had stimulated $CD25^-$ T cells to develop into $CD25^+$ suppressors. However, when naive $CD4^+$ T cells were depleted of $CD25^+$ cells before culture with alloantigen and TGF-β, suppressor T cells could not be isolated from the cultures. These findings are consistent with the possibility that the regulatory T cells that are induced in the presence of TGF-β are the progeny of the few $CD4^+CD25^+$ T cells that are present in the starting population; alternatively, they could be derived from $CD25^-$ T cells that respond to signals that are produced by TGF-β-mediated stimulation of the small number of $CD25^+$ T cells that are present in the starting population. The ability of TGF-β to induce the differentiation of suppressor cells from $CD25^-$ T cells might explain the reversal of suppression that is seen after exposure to high levels of anti-TGF-β reagents in some *in vitro* studies[24].

An alternative approach to cellular immunotherapy with suppressor T cells might involve the pharmacological manipulation of APC function *in vivo* to generate a milieu that would promote the induction of suppressor T cells. Gregori *et al.*[72] treated animals with a combination of an activated form of vitamin D3 and mycophenolate mofetil — an immunosuppressive agent that inhibits T- and B-cell proliferation and the expression of co-stimulatory molecules on DCs[73,74]. These two agents inhibit the maturation/differentiation of DCs, downregulate their expression of co-stimulatory molecules, inhibit their production of IL-12, but enhance their production of IL-10. Short-term treatment with a combination of both agents led to donor-specific tolerance of heart and pancreatic-islet allografts. Most importantly, tolerant mice had a higher percentage of $CD4^+CD25^+$ T cells in their spleen and lymph nodes, and tolerance could be transferred by $CD4^+CD25^+$ T cells to naive recipients. It is not known whether the $CD25^+$ suppressor T cells in this model were derived from the population of naturally occurring $CD4^+CD25^+$ T cells or were induced from $CD4^+CD25^-$ T cells. $CD25^+$ suppressor T cells have also been generated *in vivo* in other organ-transplantation models by therapeutic manipulations that might also involve the inhibition of APC function[75,76].

Could fully mature T_H1 effector cells that are generated *in vivo* in response to immunization or exposure to infectious agents also acquire suppressive properties? Mouse, rat and human T-cell clones that have been stimulated under anergic conditions — for example, by T-cell–T-cell antigen presentation — can suppress the responses of non-anergic clones by a

EXPERIMENTAL ALLERGIC (OR AUTOIMMUNE) ENCEPHALOMYELITIS (EAE). An animal model of multiple sclerosis — a chronic demyelinating disease in humans. In animals, EAE is induced by the injection of several different antigens that are derived from the myelin sheath, including myelin basic protein, proteolipid protein and myelin oligodendrocyte glycoprotein, together with a potent adjuvant.

cytokine-independent mechanism[77,78]. It has been shown that such suppressive clones act by means of a cell-contact-dependent mechanism to inhibit the maturation of DCs, but fully mature DCs were not susceptible to the inhibitory effects of anergic T cells[77]. A fundamental difference between suppression mediated by anergic clones and suppression mediated by $CD25^+$ T cells is that in the former case, it is targeted to the APCs, whereas in the latter case, to effector T cells[30]. How can effector T cells be rendered anergic *in vivo*? During cessation of an inflammatory response, T cells might encounter antigen on cells that lack co-stimulatory molecules. Once rendered anergic, such suppressor T cells might be able to act on DCs that have been recruited to the inflammatory site, but have not undergone complete maturation, to create a milieu for further enhancement of suppressor activity.

Although the induction of expression of CD25 on $CD25^-$ T cells *in vitro* by TCR stimulation failed to convert them into suppressor cells, the possibility remains that exposure to antigen under other conditions can generate $CD25^+$ T cells that have suppressor activity. Thorstenson and Khoruts[79] exposed $CD25^-$ T cells derived from the DO11.10 TCR-transgenic mouse on a $Rag^{-/-}$ background to a low-dose antigen-tolerance protocol *in vivo*. Although this treatment led to a reduction in the number of transgenic T cells, those cells that remained were hyporesponsive to re-stimulation, and a small population of $CD25^+$ T cells could be detected in treated mice for as long as 23 days. More importantly, in limited functional studies *in vitro*, the $CD25^+$ T cells suppressed the production of IL-2 by naive T cells, and this suppression was not neutralized by anti-IL-10 or anti-TGF-β antibodies. Does the expression of CD25 by these cells merely indicate their activation status, or does it indicate that $CD25^-$ T cells have differentiated into a population that is identical to the naturally occurring $CD4^+CD25^+$ T cells? Resolution of this crucial question must await the availability of better cellular markers, molecular phenotyping and a complete understanding of the many potential mechanisms by which both the induced and naturally occurring $CD25^+$ T cells mediate suppression.

Are suppressor T cells clinically relevant?

Now that the concept of professional suppressor cells[76] is regaining acceptance among the immunological community, it is worth considering how the manipulation of $CD4^+CD25^+$ T cells and other suppressor populations might be used clinically. The main question is whether we wish to generate more or less suppressor T-cell activity? As tumour antigens are an important group of autoantigens, the depletion of $CD25^+$ T cells should result in an enhanced immune response to tumour vaccines. Several studies have shown that the antibody-mediated depletion of $CD25^+$ T cells facilitates the induction of tumour immunity[80–82]. The combined use of CD25 depletion and CTLA4 blockade was much more effective than either approach used separately for the enhancement of the immune response to a melanoma vaccine[82]. In normal mice, the effect of the vaccine was highly dependent on $CD8^+$ T cells, but in the CD25-depleted mice, the full efficacy of the therapy required T-cell help mediated by $CD4^+CD25^-$ T cells.

$CD25^+$ T-cell depletion followed by immunization might also prove to be useful for the enhancement of immune responses to conventional vaccines for infectious agents, particularly vaccines that are weakly immunogenic, such as HIV vaccines. Suppressor T cells have been implicated in the perpetuation of chronic indolent infectious diseases due to mycobacteria[83] or parasites[84]. A detailed investigation of the involvement of $CD25^+$ T cells in such diseases in both humans and experimental animals is required. The depletion of $CD25^+$ T cells combined with vaccination or vigorous antibiotic therapy might yield sterilizing immunity.

Other approaches to inhibit $CD25^+$ T-cell function *in vivo* should be explored also. A complete analysis of the molecular pathways that control the development of suppressor activity and suppressor effector function might allow the development of antibodies or low-molecular-weight compounds that inhibit these functions. $CD25^+$ T cells might also have other control mechanisms that prevent their activation during inflammatory responses, during which effector T-cell function must dominate[32,33].

Enhancement of the number and activity of $CD4^+CD25^+$ T cells is an obvious goal for the treatment of autoimmune and allergic diseases, and for the suppression of allograft rejection. However, our knowledge of the normal physiology of this population of suppressor T cells is still far from complete. $CD4^+CD25^+$ T-cell populations have proven difficult to grow, expand and clone *in vitro*. The molecular basis for the anergic state of the $CD25^+$ T cells remains unknown. A crucial area for future study is the identification of drugs, cytokines or co-stimulatory molecules that reverse anergy and enhance growth, but preserve the suppressor function of the $CD25^+$ T-cell population. Furthermore, the administration of large numbers of $CD25^+$ T cells might create a milieu that is conducive to the expansion of more $CD25^+$ T cells or the priming of other types of regulatory cell; this would result in INFECTIOUS TOLERANCE. The optimal stimulus for the expansion of $CD25^+$ T cells *in vitro* is the combination of TCR triggering and high concentrations of IL-2. Once the specific antigens that are recognized by $CD25^+$ T cells in organ-specific autoimmunity have been defined, the antigen could then be administered together with IL-2 to expand the $CD25^+$ T-cell population that is specific for the target organ. The administration of the target antigen on iDCs together with IL-2 might be a particularly effective method for the expansion of $CD25^+$ suppressors *in vivo*. So, the concept of a separate lineage of T cells that is equipped to mediate suppressor functions — which was all but abandoned by immunologists in the 1980s — has been resurrected and now awaits validation as a potential target for therapeutic approaches for immune-mediated disorders.

INFECTIOUS TOLERANCE
After the activation of suppressor T cells in one animal, suppression can often be transferred to a naive recipient. In some models, this results in the induction of recipient-derived suppressor T cells. Tolerance can then be transferred to a new recipient, leading to the further induction of recipient-derived suppressors. In many respects, this is the 'holy grail' of transplantation immunology.

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