REGULATORY T CELLS UNDER SCRUTINY

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Having been long debated, the notion of suppressor T cells — renamed regulatory T cells — is back on the map, but many questions remain regarding the nature of these regulatory cells. Are they specialized cells? What are their phenotype, antigen specificity, mode of action and, above all, biological (and immunopathological) relevance? The predominant role of naturally occurring $CD4^+CD25^+$ T cells has been emphasized recently. Other cell types, however, contribute to immunoregulation also, whether they arise spontaneously during ontogeny or during the course of an adaptive immune response.

REGULATORY LYMPHOCYTES

IMMUNOREGULATION
The regulation of immune responses mediated by either antibody or T cells, independently from the underlying effector mechanism.

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All immune responses develop within certain limits. Antibody concentration or the number of effector T cells progressively increases after antigenic stimulation, but the levels rapidly reach a plateau and eventually decrease. More generally, most immune responses are quantitatively and qualitatively adapted to produce the optimal result. Natural autoimmunity is kept under control and lymphocyte pools are maintained at constant levels through homeostatic processes. The mechanisms behind these observations are many and complex, but they probably involve IMMUNOREGULATION. Among the cells that might be responsible for this regulation, much attention has been focused, for more than three decades, on T cells (BOX 1). The concept of suppressor T cells emerged when it was shown that stimulation of the immune system by thymus-dependent antigens could give rise to the production of suppressor T cells that downregulate the differentiation of helper cells or antigen-specific effector cells[1]. A large number of studies were carried out with the aim of characterizing the phenotype of suppressor T cells and their mode of action. I do not intend to review the various conclusions that were reached at that time, with their many flaws in interpretation and, in many cases, patent errors, which led to the discrediting of the whole concept[2]. Instead, I discuss new evidence, in the context of the lessons that have been learnt from this 'black period', which indicates that T-cell-mediated suppression might indeed have a role in modulating immune responses. The questions to be discussed concern whether this function is mediated by specialized T cells (or whether it is one among several activities of non-specialized T cells) and the underlying mechanisms at the cellular and molecular levels. I discuss, in a critical manner, the possible alternative interpretations that might be given to some of the experimental models on which modern views of regulatory T cells are based.

Evidence for T-cell-mediated immunoregulation

Induction of autoimmunity by T-cell depletion. Normal individuals harbour autoreactive T cells that do not attack organs expressing the corresponding autoantigens. So, T-cell clones specific for myelin basic protein (MBP)[3], an autoantigen in multiple sclerosis, or for glutamic acid decarboxylase (GAD)[4], an important pancreatic β-cell autoantigen in type 1 diabetes, can easily be derived from the peripheral blood of healthy subjects. This situation is exemplified by double-transgenic mice in which pancreatic β-cells express large amounts of the lymphocytic choriomeningitis virus (LCMV) glycoprotein and most $CD8^+$ T cells express an LCMV-glycoprotein-specific T-cell receptor (TCR)[5]. These mice do not develop diabetes unless they are infected with LCMV, which activates the glycoprotein-specific T cells. This observation indicates that for an autoimmune response to develop, it is not sufficient that T cells express a TCR that is specific for an autoantigen. Autoreactive T cells must, in addition, be activated to

Box 1 | **Milestones**

- **1969** Y. Nishizuka and T. Sakakura[7] — induction of autoimmunity by day-3 thymectomy
- **1970** R. K. Gershon and K. Kondo[1] — T-cell-mediated suppression
- **1973** W. J. Penhale *et al.*[12] — spontaneous appearance of thyroiditis in sub-lethally irradiated adult thymectomized rats
- **1985** B. M. Hall *et al.*[26] — transfer of allograft tolerance by $CD4^+CD25^+$ T cells
- **1986** T. R. Mosmann and R. L. Coffman[101] — the T helper 1 (T_H1)/T_H2 paradigm
- **1990** F. Powrie and D. Mason[14] — induction of inflammatory wasting disease in athymic rats reconstituted with $CD45RC^{hi}$ T cells
- **1991** H. L. Weiner and colleagues[45] — bystander suppression
- **1993** H. Waldmann and colleagues[33] — infectious tolerance
- **1994** M. G. Roncarolo and colleagues[102] — interleukin-10-dependent T_R1 cells
- **1995** S. Sakaguchi *et al.*[9] — prevention of day-3 thymectomy-induced autoimmunity by $CD4^+CD25^+$ T cells
- **1998** E. Shevach, S. Sakaguchi and colleagues[77,88] — requirement for cell–cell contact for *in vitro* suppression by $CD25^+$ T cells

differentiate into pathogenic cells. Under normal conditions, such differentiation does not take place. Several mechanisms have been invoked to explain how such activation occurs in autoimmune diseases, including antigen mimicry and local inflammation of the target organ.

Pertinent to the concept of regulatory T cells, it is important to discuss experiments showing that, in the absence of deliberate T-cell activation by autoantigens or cross-reactive antigens, pathogenic autoimmunity occurs in certain experimental settings where selected T-cell subsets are depleted. Several experimental models have been used to obtain such data (FIG. 1).

In mice, neonatal thymectomy leads to impaired development of T cells when it is carried out within 24 hours after birth. Later on, thymectomy still has some effects on the composition of T-cell populations, but the main T-cell-dependent functions are maintained, notably delayed-type hypersensitivity, allograft rejection and help for antibody production[6]. Therefore, it is surprising that thymectomy carried out between day 3 and day 5 after birth in non-autoimmune-prone BALB/c mice induces the onset of a polyautoimmune syndrome, including gastritis, thyroiditis, oophoritis or orchitis and/or prostatitis (depending on sex), insulitis and, in some cases, diabetes[7,8]. Importantly, the occurrence of such a syndrome can be prevented by administration, in the days after thymectomy, of $CD4^+$ T cells — in particular, the minor subset of $CD4^+CD25^+$ T cells[8,9]. Also, thymectomy carried out at a later age (three weeks) in NON-OBESE DIABETIC MICE (NOD mice) markedly accelerates the onset of diabetes[10], a finding that is reminiscent of the earlier observation that thymectomy carried out at hatching in the obese chicken strain accelerates the development of autoimmune thyroiditis[11]. Similarly, adult thymectomy followed by sub-lethal irradiation in certain rat strains leads to the onset of thyroiditis and diabetes[12,13]. Autoimmunity can be prevented in these rats by the administration of $CD4^+$ T cells — in particular, of a subset defined by the expression of RT6 (a rat alloantigen that has been shown to be expressed by regulatory T cells) and low levels of CD45RC[13].

When nude (athymic mutant) or severe combined immunodeficient (SCID) mice are reconstituted with $CD4^+$ T cells that have been depleted of certain T-cell subsets, they can develop immune disorders. Restoration of nude mice with $CD4^+$ T cells depleted of interleukin-2 receptor α-chain (IL-2Rα; $CD25)^+$ T cells leads to the occurrence of the same polyautoimmune syndrome that is observed after day-3 thymectomy[8]. Athymic rats reconstituted with $CD4^+$ T cells depleted of $CD45RC^{low}$ T cells develop inflammatory wasting disease[14]. Similarly, SCID mice reconstituted with $CD45RB^{hi}$ T cells develop colitis[15].

Several models of transgenic mice that express an autoreactive TCR have been described. Mice that express a TCR specific for MBP derived from an encephalitogenic T-cell clone or mice that express a TCR derived from a diabetogenic T-cell clone have been produced. These mice show no disease symptoms when the transgene is expressed on the wild-type background, which leads to the co-existence of both transgenic and non-transgenic TCR^+ T cells[16] (L. Chatenoud, unpublished observations), but expression of the transgene on a $C\alpha^{-/-}$, recombination activating gene 1/2 $(Rag1/2)^{-/-}$ or SCID background, which prevents the generation of non-transgenic TCR^+ cells, leads to fulminant autoimmune disease[16,17]. Again, disease can be prevented by infusion of $CD4^+$ T cells from wild-type mice[16,18]. These data indicate that the small proportion (less than 10%) of T cells that undergo endogenous TCR rearrangements in the wild-type background contribute to the control of disease onset.

The interpretation that is most commonly proposed by the authors of these experiments is that absence of the T-cell subsets that are depleted by thymectomy or by cell sorting using the markers mentioned allows the activation or the differentiation of autoreactive T cells into pathogenic T cells, which indicates that the depleted T-cell subsets might have a direct regulatory role. Although this interpretation is plausible, two alternative explanations should be mentioned, which are difficult to prove but also impossible to exclude. First, the possibility cannot be ruled out that in such T-cell-depleted mice, which are presumably immunosuppressed, bacterial or viral infections develop and induce or boost an organ-specific autoimmune response. Second, it can be postulated that T-cell proliferation, and perhaps activation, is stimulated by the lymphopaenic environment in which all of these experiments are carried out, through a homeostasis-driven mechanism involving recognition of self-peptide–MHC and also some cytokines, such as IL-7 (REFS 19–21). This mechanism might apply, in particular, to immuno-reconstituted mice, which are more lymphopaenic than thymectomized mice. In support of this hypothesis is the observation that purified $CD4^+CD45RB^{hi}CD25^-$ T cells, which induce colitis when injected in low numbers into BALB/c SCID mice, lose this pathogenic effect when injected in large numbers[22]. Along the same lines, colitis induced by low numbers of

NON-OBESE DIABETIC MICE (NOD mice). An inbred strain of mice that spontaneously develop T-cell-mediated autoimmune diabetes.

CYCLOSPORINE A
An immunosuppressive drug that inhibits calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase that is necessary for nuclear translocation of the transcription factor NFAT.

INFECTIOUS TOLERANCE
The extension of tolerance to naive T cells that have not been exposed to the tolerizing antigen.

$CD4^+CD45RB^{hi}CD25^-$ T cells is inhibited by transfer of transgenic monoclonal $CD4^+$ or $CD8^+$ T cells (which are irrelevant to the disease process), depending on their capacity to proliferate[22]. Such a dose relationship was not observed with $CD25^-$ T cells, for which the higher the number of injected cells, the more severe are the autoimmune manifestations[9]. This latter result, which was obtained with partially purified $CD25^-$ T cells, should be confirmed using highly purified, sorted T cells. In any event, it is clear that $CD25^+$ or $CD45RB^{low}$ T cells contribute efficiently to homeostasis to the extent that this contribution could mediate, at least in part, their regulatory function. It is intriguing that one of the postulated mechanisms of homeostasis-driven proliferation is TCR-mediated recognition of self-peptides by autoreactive T cells. If the link between homeostasis and immunoregulation is correct, the possibility of competition for access to antigen-presenting cells (APCs) between regulatory T cells and pathogenic T cells should be addressed. It is worth noting, in this regard, that IL-2-deficient and *Cd25*$^{-/-}$ mice develop lymphoid hyperplasia and immune disorders, notably colitis. These abnormalities can be corrected by infusion of wild-type $CD25^+$ T cells[23] in an IL-10-dependent manner[24]. It can be postulated, in this context, that homeostasis-driven regulation explains, at least in part, the protection from allergic and autoimmune diseases that is afforded by infections[25], because immune responses to infection are associated with intensive lymphocyte proliferation. It will be important to determine how regulatory T cells operate *in vivo* under conditions that are not associated with lymphopaenia or infection-driven T-cell activation.

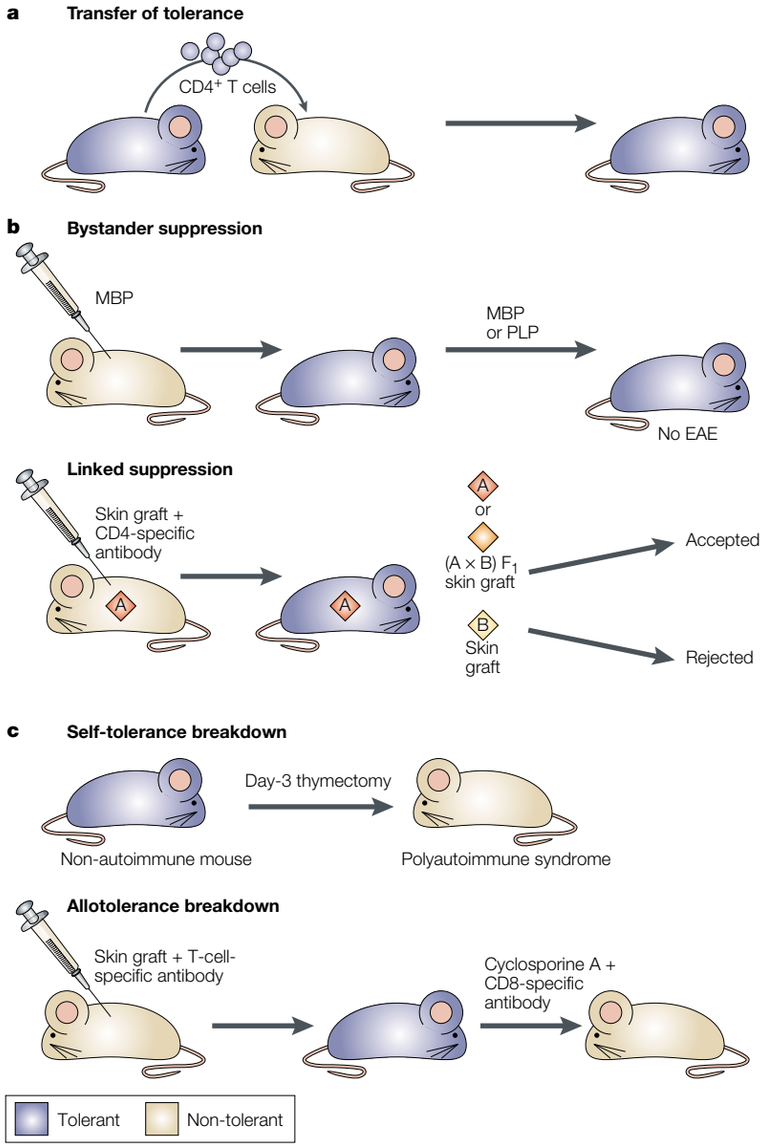

Figure 1 | **Experimental evidence for active tolerance involving regulatory T cells.** **a** | Transfer of tolerance by $CD4^+$ T cells. These experiments show that $CD4^+$ T cells suppress the differentiation and/or function of effector T cells in a dominant, and possibly infectious, manner. **b** | Tolerance can spread to other antigens. In bystander suppression, tolerance induced to one antigen, such as myelin basic protein (MBP), can spread to other antigens, such that mice are protected from subsequent induction of experimental allergic encephalomyelitis (EAE) by proteolipid protein (PLP). In demonstrations of linked suppression, mice rendered tolerant to a type-A allograft accept subsequent skin allografts from (A × B) F_1 donors. Both phenomena are demonstrations of antigen-nonspecific local immunoregulation, which is compatible with a mechanism of active, T-cell-mediated tolerance, but not of anergy or deletion. **c** | Tolerance breakdown by T-cell inactivation. T-cell depletion or inactivation of regulatory T cells induces or restores immune reactivity to self- or alloantigens.

Transfer of tolerance by $CD4^+$ T cells. Tolerance to cell-borne antigens can be induced in several ways, notably by administration of the relevant antigens to immunoincompetent hosts, such as neonatal mice or animals that have undergone various types of immunosuppressive regimen. Evidence has accumulated to indicate that in most of these experimental settings, tolerance is of the active type (also known as 'dominant') and can be transferred to naive recipients by $CD4^+$ T cells derived from the tolerant animal. Therefore, it can be postulated that tolerance is more readily explained by immunoregulation by these $CD4^+$ T cells than by deletion of helper or effector T cells.

Tolerance to allografts can be induced in mice or rats by the infusion of donor cells before graft implantation, or by the administration of various immunosuppressive agents. It has been shown convincingly that such protection against graft rejection can be transferred to naive recipients by the administration of $CD4^+$ regulatory T cells from tolerant mice, irrespective of whether tolerance was induced under the cover of CYCLOSPORINE A[26,27] or CD4-specific depleting or non-depleting antibodies[28–30]. The tolerance that is transferred in this manner is antigen specific. Furthermore, it was shown by H. Waldmann's group[31] that these $CD4^+$ regulatory T cells only appear after a lag of several weeks after tolerance induction, and that they require the presence of the specific antigen to appear and to persist, can target both naive and primed $CD4^+$ and $CD8^+$ T cells, and are found predominantly in the allograft[32].

In the case of non-depleting CD4-specific antibodies, it was shown that the transfer of tolerance was of the 'infectious' type (that is, transmissible to T cells that have not been exposed to the tolerizing antigen; INFECTIOUS TOLERANCE). Tolerance resists the transfer of syngeneic naive T cells[33]. Furthermore, in tolerant mice

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

CYCLOPHOSPHAMIDE
A DNA-alkylating agent that is used widely as an anti-tumour agent or an immunosuppressive agent. Cyclophosphamide has been shown to destroy certain subsets of lymphocytes preferentially, including B cells and regulatory cells.

transgenically expressing human CD2 that were infused with non-transgenic naive cells, tolerance was not broken after elimination of the originally tolerant cells by injecting a depleting monoclonal antibody specific for human CD2. The interpretation is that tolerance is 'infectiously' transmitted to the naive cells[33]. Similar results were obtained after grafting neonatal mice with allogeneic epithelium[34]. Such transplantation induced long-term tolerance to the allogeneic antigens, which could be transferred to non-grafted mice by $CD4^+$ T cells.

Transfer experiments in NOD mice have also provided evidence of immunoregulation by $CD4^+$ T cells. NOD mice only develop diabetes at 3 or 4 months of age; however, insulitis occurs long before the clinical expression of the disease (as soon as three weeks of age). Experiments in which $CD4^+$ T cells from pre-diabetic mice were co-transferred with diabetogenic T cells showed that this lag time is associated with the presence, in the thymus and spleen of these pre-diabetic mice, of $CD4^+$ T cells that can inhibit the diabetogenic potential of T cells derived from diabetic mice[35]. Recent data have indicated that this protective effect is confined to a subset of $CD4^+$ T cells expressing the markers CD62L (L-selectin)[36,37] and CD25 (L. Chatenoud, unpublished observations). This fits with the observation that $CD25^+$ T cells from wild-type NOD mice prevent the occurrence of fulminant diabetes in *Cd80*$^{-/-}$*Cd86*$^{-/-}$ NOD mice[38]. Interestingly, these markers allow the physical separation of effector T cells that transfer diabetes ($CD62L^-CD25^-$) from regulatory T cells that protect against diabetes ($CD62L^+CD25^+$ and, to some extent, $CD62L^+CD25^-$). Similarly, for EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE), it has been shown that the disease spontaneously remits after two or three weeks. This cure is associated with resistance to any further induction of the disease[39]. This resistance can be transferred to naive mice using purified T cells from remitting animals[40,41], and it can be broken by treatment with CYCLOPHOSPHAMIDE, which indicates a role for cyclophosphamide-sensitive suppressor cells[41]. That pre-diabetic NOD mice become diabetic after treatment with cyclophosphamide[42] and that they are resistant to the transfer of diabetes by T cells derived from diabetic mice are probably due to the same regulatory phenomenon. A similar interpretation might be given to the observation that pre-diabetic NOD mice become sensitive to diabetes transfer after adult thymectomy followed by $CD4^+$ T-cell depletion using a CD4-specific monoclonal antibody[43].

Transfer of tolerance in these various models can be interpreted as evidence that regulatory T cells exist in tolerant animals. The possibility cannot be excluded that antigen is transferred in a tolerogenic form together with the tolerant cells, as has been shown previously in other settings. However, this interpretation is unlikely, as tolerance can be transferred by purified T cells depleted of APCs. It could also be argued that more information is required about the fate of the transferred cells — where do they home to and for how long do they survive?

Tolerance can spread to other antigens. According to its initial definition, tolerance is an antigen-induced state of unresponsiveness specific for the initial tolerogen. So, in the classical Brent and Medawar model of allogeneic neonatal tolerance, unresponsiveness is limited strictly to alloantigens to which the neonate was exposed, or to cross-reactive alloantigens[44]. Therefore, it was surprising to observe that tolerance induced to soluble autoantigens can 'spread' to other autoantigens expressed by the same cell type, but that are devoid of any structural relationship with the initial autoantigen[45,46]. So, oral administration of MBP induces operational tolerance to MBP (resistance to MBP-induced EAE), but the tolerance

Box 2 | **Candidate regulatory T cells other than $CD25^+$, T helper 1 (T_H1)/T_H2 and T_R1 cells**

Natural killer T (NKT) cells
- Autoimmune-prone mouse strains, such as non-obese diabetic (NOD)[103], SJL[104] and $MRL^{lpr/lpr}$ (R. Singh, personal communication), have a numerical and functional NKT-cell deficiency.
- The progression of autoimmune disease is accelerated in *Cd1d*$^{-/-}$ NOD mice[105] and *Cd1d*$^{-/-}$ $MRL^{lpr/lpr}$ mice (R. Singh, personal communication).
- Diabetes is prevented in NOD mice that overexpress NKT cells (with a transgenic Vα14 T-cell receptor)[106] or that have been treated with α-galactosyl ceramide, a selective NKT-cell ligand[107,108].
- Most of the T cells that arise after total lymphoid irradiation (formerly known as natural suppressor cells) are $V\alpha14–J\alpha18^+$ NKT cells[109].

γδ T cells
- Administration of an insulin aerosol to NOD mice protects them from the onset of diabetes. This protection, which is of the active type (transferable by T cells), involves $CD8\alpha\alpha^+$ γδ T cells[54].
- The onset of systemic lupus erythematosus is accelerated in $MRL^{lpr/lpr}$ mice that are genetically deficient for γδ T cells[110].
- Regulation of cutaneous inflammation by local γδ T cells[111].

$CD8^+$ T cells
- *Cd8*$^{-/-}$ mice develop experimental allergic encephalomyelitis (EAE) in a more severe and chronic manner than wild-type mice[112].
- Treatment with CD8-specific antibody can induce acceleration of EAE[113], and can break tolerance to allogeneic islets induced under the cover of CD4-specific antibodies[53].

Box 3 | Phenotype of regulatory T cells in various experimental models

Models with lymphopaenia:

- Day-3 thymectomy[9] — $CD4^+CD25^+$ regulatory T cells
- Adult thymectomy plus sub-lethal irradiation[78,79] — $CD4^+CD25^+CD62L^+$ regulatory T cells
- Severe combined immunodeficient (SCID) mice recently reconstituted with $CD45RB^{hi}$ T cells[15] — $CD4^+CD25^+CD45RB^{low}$ regulatory T cells

Models without marked lymphopaenia:

- Non-obese diabetic (NOD) mice[35–37] — $CD4^+CD25^+CD62L^+$ regulatory T cells
- Experimental allergic encephalomyelitis (EAE)[39] — $CD4^+$ regulatory T cells
- Allograft tolerance[24,26,97] — $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory T cells

spreads to other myelin antigens also, such as proteolipid protein (PLP)[47]. Similar findings were reported for allogeneic tolerance induced under the cover of non-depleting CD4-specific monoclonal antibodies. CBA/Ca mice rendered tolerant to B10.BR alloantigens accept skin grafts from unrelated CBK mice (CBA/Ca mice transgenic for $H\text{-}2K^k$) when CBK antigens are presented on (B10.BR × CBK) F_1 cells (a phenomenon known as 'linked suppression')[48] (FIG. 1). These experiments indicate that tolerance can spread in an active fashion to antigens that are distinct from the tolerogen but expressed at the same cellular site. These results are incompatible with deletion or anergy, as these mechanisms would exclusively implicate T cells specific for the tolerizing antigen.

Tolerance break-down by T-cell inactivation. Several sets of data indicate an active role for T cells in tolerance irrespective of the mode of action in each experimental setting, including the possible induction of homeostasis-driven T-cell stimulation. Neonatal tolerance to alloantigens induced by intravenous infusion of allogeneic cells can be prevented by the administration of a neutralizing monoclonal antibody specific for IL-4 (REF. 49). These data indicate strongly that, at variance with initial interpretations, clonal deletion of alloreactive effectors is not the only explanation of tolerance in this setting. Immune-deviation mechanisms involving IL-4-producing T cells also have an important role. Long-term graft acceptance mediated by CD40 ligand (CD40L)-specific antibody and cytotoxic T-lymphocyte antigen 4 (CTLA4)–immunoglobulin fusion protein is prevented by calcineurin inhibitors such as cyclosporine A and FK506 (REFS 50,51). This supports the central role of T-cell activation and, more particularly, TCR-mediated signal transduction in the induction of long-term graft acceptance. As mentioned earlier, cyclophosphamide, a DNA-alkylating agent that is known to affect regulatory cells selectively[52], breaks $CD4^+$ T-cell-associated protection against EAE[39] and accelerates the onset of diabetes in NOD mice[42]. Finally, tolerance to islet allografts induced under the cover of a depleting antibody specific for CD4 can be broken by the administration of a depleting antibody specific for CD8 (REF. 53), raising the interesting possibility that there are regulatory T cells of the $CD8^+$ phenotype.

Conclusions. To recapitulate, converging experimental evidence indicates that many states of tolerance either to self- or alloantigens are associated with, and probably due to, $CD4^+$ T cells that can suppress the differentiation or function of helper and effector T cells. However, these experiments do not provide any clues about the precise nature, phenotype or mode of action of these putative regulatory T cells.

The diversity of regulatory T cells

The *in vivo* data described provide strong evidence for T-cell-mediated immune regulation. In addition to the well-established roles of $CD4^+$ T helper 1 (T_H1) and T_H2 cells and of $CD4^+CD25^+$ T cells, a large number of other candidate regulatory cells have been proposed (BOX 2). The definition of all of these regulatory cells has, for the most part, been based on their phenotype (BOX 3) and, to a lesser extent, on their cytokine usage. It is difficult to assess the autonomy of each cell subtype or the importance of each in the regulation of autoimmune responses.

At the risk of over-simplification, regulatory T cells that arise after deliberate administration of an antigen or an autoantigen can be distinguished from regulatory [T_H2 cells (which suppress T_H1-cell-related responses), T_H1 cells (which suppress T_H2-cell-mediated responses), T_R1 cells (a subset of IL-10-dependent, but not IL-4-dependent, antigen-specific regulatory T cells) and probably $CD8^+$ regulatory cells. Spontaneously appearing regulatory T cells include $CD25^+CD62L^+$ T cells and natural killer T (NKT) cells (BOX 2). This group should also probably include γδ T cells, although such cells seem to be induced by antigen sensitization[54]. This contrast between natural and induced regulation is reminiscent of the classical distinction made between innate and adaptive immunity. An important difference, however, is that some of the naturally occurring regulatory cells, such as $CD4^+CD25^+$ T cells and NKT cells, express a TCR, whereas cells of the innate immune system do not.

At present, it is difficult to determine whether the diversity of regulatory-cell types is due to variable expression of phenotypic markers or the production of cytokines by a limited number of T-cell subsets. In any event, there is a large overlap between the regulatory T-cell subsets that have been described, in terms of both phenotype and the cytokines involved. It will perhaps turn out that some of these regulatory T-cell types are related developmentally or associated functionally. It might also be shown that a given cell type can differ according to the stage of activation or to the micro-environment in which it develops. Particular attention should be paid to the nature of the APCs involved as it has been suggested that selective T_R1-cell generation follows antigen presentation by immature dendritic cells[55].

Cytokine dependency

Cytokines can downregulate immune responses, including autoimmune responses and, to a lesser extent, alloimmune responses. Transforming growth factor-β (TGF-β) and IL-10 inhibit both T_H1- and T_H2-cell responses *in vivo*[56,57]. IL-4 has been found to inhibit several T_H1-cell-mediated autoimmune diseases[58–61].

ALTERED PEPTIDE LIGAND (APL). A synthetic peptide homologous to a natural peptide that has a differential capacity to induce effector versus regulatory T cells. APLs can stimulate regulatory cells without inducing effector cells.

Similarly, interferon-γ (IFN-γ) inhibits T_H2-cell responses[62]. In addition, cytokine dependency has been shown in many of the *in vivo* models mentioned earlier, which provides evidence in favour of a role for cytokines in immune regulation (TABLE 1). IL-10-deficient mice develop severe EAE, whereas transgenic mice that overexpress IL-10 are protected from disease development[63]. Treatment with IL-4- or IL-10-specific antibodies prevents the induction of active tolerance after administration of soluble autoantigens or ALTERED PEPTIDE LIGANDS (APLs)[64–66]. IL-4-deficient NOD mice are resistant to tolerance induction after administration of the β-cell soluble autoantigen GAD[67]. Tolerance induction by oral administration of MBP is prevented by TGF-β-specific antibody and is associated with the generation of TGF-β-producing T-cell clones[46,68]. IL-10 and TGF-β have been implicated in the protection against colitis that is observed after the reconstitution of SCID mice with $CD45RB^{hi}$ T cells[69,70]. In NOD mice, protection from diabetes observed after the transfer of $CD4^+CD25^+CD62L^+$ T cells from pre-diabetic mice is inhibited by TGF-β-specific antibody (but not by IL-4-, IL-10- or IL-10-receptor-specific antibodies)[37] (L. Chatenoud, unpublished observations). However, NOD mice expressing a transgenic 'diabetogenic' TCR, which do not develop diabetes spontaneously, do so after treatment with an antibody specific for the IL-10 receptor[71]. The prevention of thyroiditis by the administration of $CD4^+CD25^+$ T cells in adult thymectomized sub-lethally irradiated rats is abrogated by IL-4- and TGF-β-specific antibodies[72].

Collectively, these data indicate that cytokines have a central role in most of the animal models on which the concept of immunoregulation is based. However, the diversity of cytokines produced in these models is of note. The difficulty in presenting a synthetic view of the role of cytokines in T-cell-mediated regulation raises the question of the diversity of regulatory cells, or at least of their suppressor activities, in various contexts. IL-4 has been implicated in the control of T_H1-cell responses by T_H2 cells. IL-10, another T_H2 cytokine, is central to the regulatory effects of $CD4^+CD45RB^{low}$ cells in the colitis model and of T_R1 cells, thereby indicating that these two cell types might be the same. A role for TGF-β has been implicated in a wide variety of *in vivo* models — including the colitis model[69], the rat thymectomy–irradiation model[72], NOD mice (L. Chatenoud, unpublished observations), rat mercuric-chloride-induced autoimmunity[73], oral MBP-induced tolerance[74] and donor-specific transfusion-induced allogeneic tolerance[75] — making this cytokine an important candidate for T-cell-mediated regulation. It is not clear, however, whether TGF-β acts as a mediator of regulation or as a growth and/or differentiation factor for regulatory T cells.

The elusive action of $CD25^+$ regulatory T cells

The experimental data discussed have led to the consideration of a new T-cell type, which is distinct from T_H2 cells, $CD4^+CD45RB^{low}$ T cells and IL-10-dependent T_R1 cells. These cells are commonly referred to as $CD4^+CD25^+$ T cells, since the report by Sakaguchi *et al.*[9] of the prevention of day-3 thymectomy-induced polyautoimmune syndrome by purified $CD4^+CD25^+$ T cells.

CD25, the expression of which has also been described for $CD4^+$ T cells mediating active allograft tolerance[26,76], is the best known marker of this unique regulatory T-cell population. CD25 might be related to regulatory T-cell function, because *Cd25*$^{-/-}$ mice show lymphoid hyperplasia and immune disorders. In addition, the proliferation of $CD25^+$ T cells is IL-2 dependent[77]. It should be mentioned, however, that spontaneously induced regulatory $CD4^+$ cells are not confined to $CD25^+$ T cells, as documented in the rat thymectomy–irradiation model[78] and in NOD mice (L. Chatenoud, unpublished observations). A cellular overlap has been described between $CD25^+$ and $CD45RB^{low}CD4^+$ T cells, but this overlap is not absolute. The problem is complicated by the fact that the $CD25^+$ T-cell compartment does not exclusively include regulatory cells, but also other cell types such as activated T cells. Another important marker for regulatory cells is CD62L, which is expressed by $CD4^+$ regulatory cells that protect against diabetes in NOD mice[36,37] and in the rat thymectomy–irradiation model[79].

Table 1 | **Cytokine dependency of regulatory T cells**

Model	IL-4 blockade		IL-10 blockade		TGF-β blockade	
	Antibody-mediated	*Deficient mice*	*Antibody-mediated*	*Deficient mice*	*Antibody-mediated*	*Deficient mice*
$CD25^+$ T-cell-mediated suppression (*in vitro*)	–	–	–	–	+/–	–
$CD25^+$ T-cell-mediated protection from diabetes in NOD mice (*in vivo*)	–	–	–	N.D.	+	N.D.
$CD45RB^{low}$ T-cell-mediated protection from colitis (*in vivo*)	–	N.D.	+	N.D.	+	N.D.
Autoantigen-induced autoimmune disease protection (EAE and NOD)	+	+	+	N.D.	+	N.D.
Control of spontaneous disease onset in NOD mice	N.D.	–	N.D.	–	N.D.	N.D.

Compiled from data in REFS 37,63–64,67,69,70,74,80,81,89. – indicates that the effect is maintained with cytokine blockade; + indicates that the effect is abrogated. EAE, experimental allergic encephalomyelitis; IL, interleukin; N.D., not determined; NOD, non-obese diabetic; TGF-β, transforming growth factor-β.

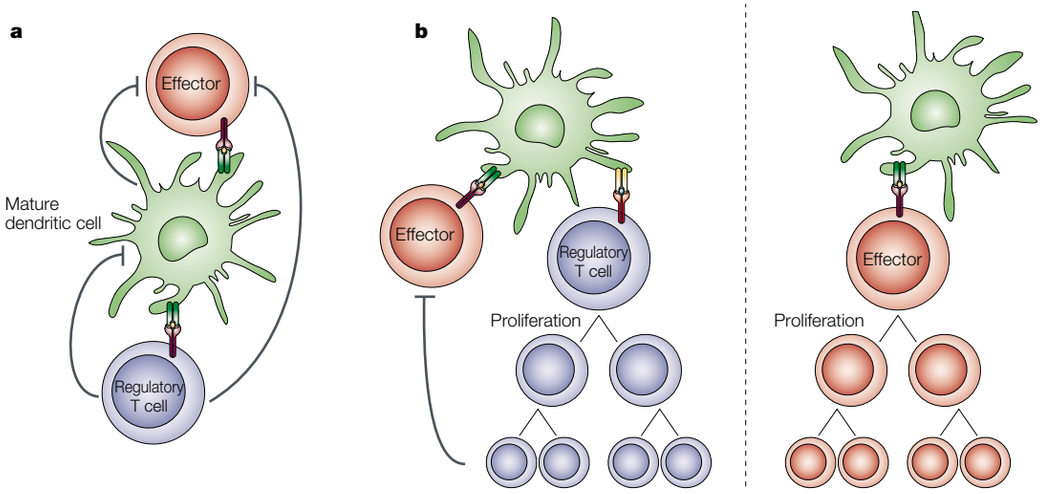

Figure 2 | **Contrasting models of the antigen specificity and function of regulatory T cells. a** | In the case of antigen-driven regulation, regulatory T cells recognize the same antigen as $CD4^+$ or $CD8^+$ effector T cells, although they are not necessarily specific for the same epitope. **b** | Alternatively, regulatory T cells cells might compete with effector T cells for homeostatic signals. So, effector T cells can only proliferate in the absence of regulatory T cells. In this model, regulatory and effector T cells are not specific for the same antigen, as homeostasis is polyclonally driven by recognition of self-peptide–MHC.

Much uncertainty remains regarding the mode of action of $CD25^+$ regulatory T cells. Do they target helper or effector T cells, or APCs? The first possible mechanism to be discussed involves cytokines, in particular TGF-β, as no role has been described so far for IL-4 or IL-10. As far as TGF-β is concerned, some emphasis has been placed on the role of membrane TGF-β, the expression of which has been demonstrated by one group on $CD25^+$ T cells[80], but these data have yet to be confirmed. Adding to the confusion are the conflicting data on the ability of antibodies specific for TGF-β to abrogate the *in vitro* suppressive ability of $CD25^+$ T cells[80,81].

Membrane-associated receptors have also been associated with suppression. Among these receptors is CTLA4, the expression of which is increased by $CD25^+$ regulatory cells[38]. In addition, CTLA4-specific antibody breaks the protection that is afforded by $CD25^+$ T cells in the gastritis model[82] and by $CD45RB^{low}$ T cells in the colitis model[83]. This is not, however, a definitive argument, as the possibility that CTLA4-specific antibody acts in an agonistic manner on the effector cells cannot be excluded. Another candidate molecule is glucocorticoid-induced tumour-necrosis factor receptor family-related protein (GITR; TNFRSF18)[84,85]. GITR was found to be the target of an antibody that is produced after immunization with $CD25^+$ T cells[86]. It remains to be determined whether GITR is indeed an effector molecule for regulatory T cells, and if so, for which category of regulatory T cells. Its mode of action needs be determined also.

Relevance of *in vitro* models

Some T-cell subsets can suppress the differentiation, proliferation or function of other T-cell subsets *in vitro*. Such suppression was first described in the 1970s, when it was shown that concanavalin-A-activated T cells could inhibit various T-cell-mediated reactions, notably the autologous mixed lymphocyte reaction[87]. More recently, a similar co-culture model was developed independently by the groups of Sakaguchi[77] and Shevach[88]. In brief, $CD4^+CD25^+$ T cells can inhibit the proliferation or cytokine production of CD3-specific antibody-stimulated $CD4^+CD25^-$ T cells, or of $CD8^+$ T cells. This suppression requires cell–cell contact as it is no longer observed when $CD25^+$ and $CD25^-$ T cells are separated by a cell-impermeable membrane. It is not inhibited by IL-4- or IL-10-specific neutralizing antibodies, and it can be mediated by cells from IL-4-deficient, IL-10-deficient[89] and TGF-β-deficient[81] mice. It is interesting to note that $CD25^+$ T cells do not proliferate *in vitro* in the presence of CD3-specific antibody alone (they are said to be anergic), except when CD28-specific antibody and IL-2 are added[77]. However, in the presence of IL-2, $CD25^+$ T cells lose their capacity to inhibit the proliferation of $CD25^-$ T cells. Recently, it has also been shown that $CD25^+$ antigen-specific T cells can inhibit the *in vitro* antigen-driven proliferation of $CD25^-$ T cells[90].

These *in vitro* models have the important merit of allowing a precise dissection of the cellular and molecular events that occur during the interaction between $CD25^+$ and $CD25^-$ T cells. The problem, however, lies in the relevance of the *in vitro* models to the *in vivo* observations described earlier. The fact that inhibition of $CD25^-$ T-cell proliferation by $CD25^+$ T cells does not require the presence of cytokines, but rather cell–cell contact, is worrisome, as it contrasts with the *in vivo* models discussed earlier, in which the role of cytokines has been demonstrated extensively. In addition, these *in vitro* models essentially relate to the inhibition of polyclonally activated T cells, without identification of organ-specific autoreactive cells, whereas data obtained *in vivo* indicate a role for $CD25^+$ T cells in the control of organ-specific autoimmunity. This calls for caution in extrapolating from these *in vitro* models to the *in vivo* setting.

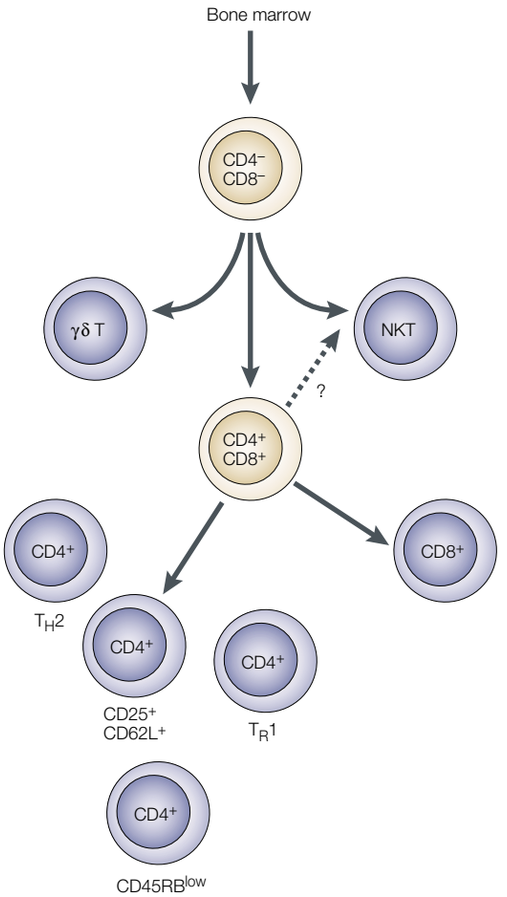

Figure 3 | **The diversity of regulatory T cells.** A wide range of T-cell subsets originate from T-cell precursors (yellow). Many of them can act as regulatory T cells (blue) in various settings. An important role is given to $CD4^+$ T cells — T helper 2 (T_H2), $CD25^+CD62L^+$, T_R1 and $CD45RB^{low}$ cells — but other T-cell subsets might also downregulate immune responses, notably γδ T cells, natural killer T (NKT) cells and $CD8^+$ T cells.

Antigen specificity

Few of the models that are described allow the antigen specificity of regulatory T cells to be assessed. Antigen specificity is apparent in experiments investigating T_H2- or T_R1-cell-mediated suppression, which is induced by the specific antigen. However, such specificity applies to the afferent limb of the response, namely sensitization, and not to the efferent limb, namely exertion of suppression. In fact, as discussed, suppression extends to immune responses other than those directed against the initial antigen, as shown by several models, which have given rise to the notion of BYSTANDER SUPPRESSION[46]. Such bystander suppression applies both to T_H2 cells and to the TGF-β-producing T_H3 cells that are induced during oral tolerance[68]. In mice, an ovalbumin-specific T_R1-cell clone was shown to protect against colitis, which indicates that bystander suppression applies to T_R1 cells also[91].

In the case of $CD25^+$ T cells, there is some indication of antigen specificity, but the evidence is still indirect. In the day-3 thymectomy model, it has been shown by Taguchi and Nishizuka[92,93] that oophoritis and orchitis are better prevented by spleen cells derived from animals of the same sex than by spleen cells from ovariectomized mice (in the case of oophoritis) or from female mice (in the case of orchitis). The interpretation of these data is not, however, completely clear, as there is still a regulatory effect of T cells derived from male mice in the case of oophoritis. Another indication of specificity is derived from the experiments carried out by McCullagh in sheep[94] and Seddon and Mason in rats[95], where prenatal thyroid ablation or destruction leads to a state of hyper-reactivity to self-thyroid in the post-natal period. It can be postulated that the lack of exposure of immature T cells to the thyroid prevents their differentiation to regulatory T cells.The specificity of regulatory T cells in transplantation tolerance is still debated as these cells have been less well defined than in models of autoimmunity. These regulatory cells include $CD4^+CD25^+$ T cells[27,96], but also $CD25^-$ T cells[97]. It will be important to determine the relationship between the naturally occurring $CD25^+$ T cells that control the onset of autoimmunity and the antigen-specific regulatory $CD25^+$ T cells that arise after deliberate sensitization in transplantation tolerance.

In any event, it is not clear from these experiments whether selection of the regulatory cells takes place in the thymus or the periphery. An important goal is to determine whether the TCRs of $CD25^+$ T cells are specific for the same antigens or epitopes as recognized by effector T cells (FIG. 2a), or whether they have a totally unrelated specificity for self-peptides that are expressed by the same (or other) APCs. In the latter scenario, suppression could be mediated through a homeostatic competition mechanism (FIG. 2b). In addition, it is to be hoped that the use of transgenic mice expressing a TCR with a well-defined specificity will help to establish whether regulatory cells use the same receptor as helper or effector T cells.

Another important and related question concerns $CD25^+$ T-cell thymic selection. $CD25^+$ T cells are found in the thymus (5% of $CD4^+CD8^-$ thymocytes)[98]. The mechanisms of their selection are still ill-defined. Using double-transgenic mice expressing influenza haemaglutinin and the specific TCR, Jordan *et al.*[99] showed that 30% of transgenic TCR^+ thymocytes and 50% of lymph-node T cells were $CD25^+$ T cells. Thymic stroma was mandatory for the selection of these $CD25^+$ T cells. Recent studies[90,100] have confirmed the role of MHC class-II-expressing thymic stroma.

Concluding remarks

At the end of this review of the main arguments in support of the concept of regulatory T cells, one is left with mixed feelings. On the negative side, the multiplicity of incriminated cellular subsets (FIG. 3) and the diversity of suggested molecular mechanisms, mainly involving cytokines, is problematic. The fragility of many of the experimental models, particularly those that have been developed in lymphopaenic environments or solely *in vitro*, is apparent and leaves room for alternative interpretations, mainly involving lymphocyte homeostasis.

BYSTANDER SUPPRESSION
The extension of tolerogen-induced suppression of an immune response to immune responses that are directed against antigens not structurally related to the tolerogen but expressed by the same target cell.

On the positive side, the convergent observations showing the active nature of many states of natural or induced tolerance involving increasingly well-defined T-cell subsets are impressive. It is to be hoped that experiments in progress in many laboratories will allow more direct confirmation of this concept and a better appraisal of the mode of action, antigen specificity and role of the various cellular subsets already described — in particular, of the still elusive $CD25^+$ T-cell subset. This is important at the fundamental level for a better understanding of the physiology of immune responses. It is also crucial for understanding the pathogenesis of autoimmune diseases and for the design of new therapeutic approaches for these diseases, as well as for organ transplantation. In terms of transplantation, the stimulation of regulatory T cells already seems to be an extremely attractive strategy, complementing and hopefully substituting for conventional antigen-nonspecific immunosuppression with all of its hazards.

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ERRATUM

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The author's name was presented incorrectly: the correct format is Jean-François Bach. The PubMed record for this article will be corrected.