

# Regulation of immunity by self-reactive T cells

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**A basic principle of immunology is that lymphocytes respond to foreign antigens but tolerate self tissues. For developing T cells, the ability to distinguish self from non-self is acquired in the thymus, where the majority of self-reactive cells are eliminated. Recently, however, it has become apparent that some self-reactive T cells avoid being destroyed and instead differentiate into specialized regulatory cells. This appears to be beneficial. Subpopulations of self-reactive T cells have a strong influence on self tolerance and may represent targets for therapeutic intervention to control a variety of autoimmune diseases, tumour growth and infection.**

The immune system has evolved to recognize and combat infectious agents<sup>1</sup>. From a teleological point of view, an ideal set of immune receptors would recognize pathogens but ignore the proteins, DNA and other components that make up our own bodies. Self-reactive cells pose an immediate threat of autoimmunity. In higher organisms, multiple mechanisms of immunological tolerance eliminate or inactivate lymphocytes that bear receptors specific for autoantigens (see the review in this issue by Goodnow *et al.*, page 590). Nevertheless, some autoreactive lymphocyte clones escape these mechanisms and are present within the peripheral lymphocyte pool. One mechanism by which the pathogenic potential of these autoreactive clones is kept in check is through a dedicated lineage of regulatory T ( $T_R$ ) cells.

The coexistence of autoreactive and protective T cells was revealed by the multi-organ autoimmunity observed in lymphopenic (immune-deficient) recipient mice upon adoptive transfer of naive  $CD4^+$  T cells, and by the protection from autoimmune pathology upon co-transfer of a subset of  $CD4^+$  T cells expressing interleukin (IL)-2 receptor  $\alpha$ -chain (CD25) (ref. 2). Current evidence suggests that the  $CD25^+CD4^+$  T cells are themselves self reactive (Fig. 1), and that this property plays an essential role in the commitment to a  $T_R$ -cell lineage. Thus, self reactivity can be beneficial as part of a dedicated cellular mechanism preventing autoimmunity.

In addition to  $CD25^+CD4^+$   $T_R$  cells, other important self-reactive T-cell sublineages have been identified. Prominent among these are cells that express a semi-invariant T-cell receptor (TCR) specific for conserved self ligands (Fig. 1). These ligands, which are normally present at a low level, might be induced and serve as molecular signs of stress or infection. The best-characterized such T-cell sublineage is the CD1d-dependent natural killer T (NKT) cell. Mucosal invariant T cells, which are reactive with the major histocompatibility complex (MHC) class-I-like molecule MR-1, are a second example of this type of T cell<sup>3,4</sup>. In contrast to  $CD25^+CD4^+$   $T_R$  cells, which have a dedicated suppressor function, NKT cells in some situations facilitate autoimmune pathology, but in others they are part of a protective mechanism.

In this review, we discuss the origin and biology of two distinct lineages of naturally arising self-reactive T cells:  $CD25^+CD4^+$   $T_R$  cells and CD1d-dependent NKT cells. Recent evidence suggests that during thymic differentiation, beneficial self reactivity instructs the develop-

ment of specialized populations of T cells. The properties of these different T-cell sublineages, described below, are summarized in Table 1. Here we highlight recent advances including elucidation of the role of the transcription factor Foxp3 as a dedicated  $T_R$ -cell lineage specification factor and the identification of natural self and bacterial glycolipids that are recognized by NKT cells.

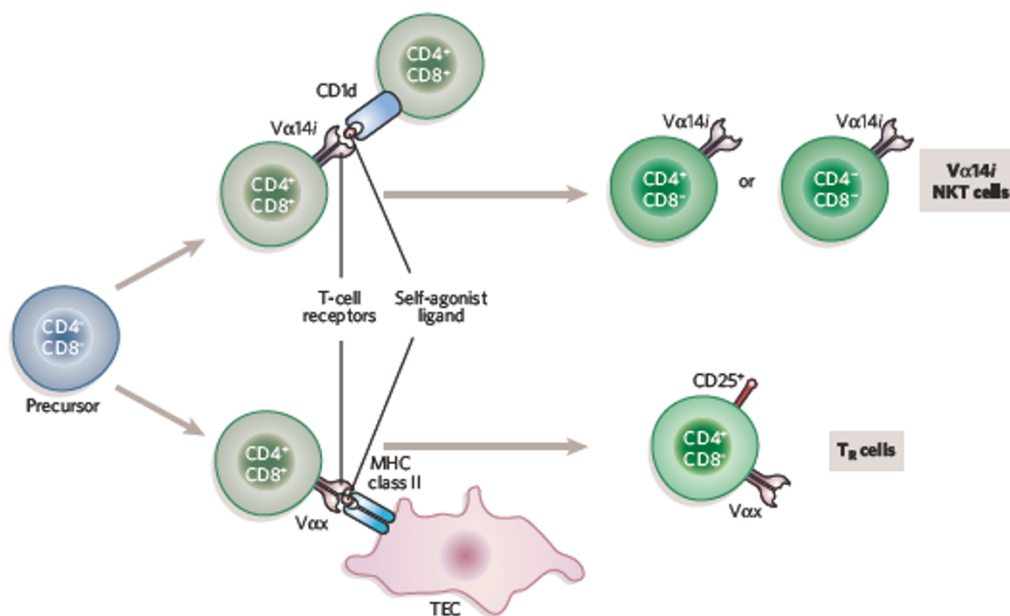
## The $T_R$ -cell lineage

The widespread acceptance of the existence of a dominant tolerance-inducing mechanism that can suppress the response of other immune cells should not be taken for granted. The idea of a suppressive T-cell lineage was out of favour for many years until results from a few key experiments overcame resistance to this notion. This was thanks largely to a functional *in vivo* screen using adoptive cell transfers and anti-CD25 antibody-mediated *in vivo* depletion experiments<sup>2,5,6</sup>.

**Table 1 | Comparison of  $T_R$  and iNKT cells**

	$T_R$ cells	iNKT cells
TCRs	Diverse	Invariant V $\alpha$
Co-receptors	Mostly CD4	CD4 or double negative (in mice)
Unique genetic requirements	Foxp3, partial for CD28, IL-2	Many, including IL-15 pathway, transcription factors, SAP and genes involved in CD1d antigen presentation
Specificity	Diverse autologous peptides bound to MHC class II Capable of recognizing non-self peptides	Autologous and bacterial glycosphingolipids presented by CD1d
Distribution	Thymus, lymph nodes, spleen, circulation, sites of inflammation	Thymus, spleen, circulation, liver, bone marrow, sites of inflammation
Effector functions	IL-10, TGF- $\beta$ , CTLA 4, cytotoxicity? other?	Diverse $T_H1$ and $T_H2$ cytokines
Regulation of autoimmunity	Required to prevent a plethora of autoimmune conditions	Activation of $T_H1$ and $T_H2$ responses may prevent or exacerbate autoimmunity

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**Figure 1** | Recognition of self-agonist ligands in the thymus can create at least two different sublineages of self-reactive T cells. They probably branch off from the mainstream pathway of development at the double-positive stage of differentiation. Thymic  $T_R$  precursors can also branch off at the CD4 single-positive stage of differentiation. MHC class II<sup>+</sup> bone-marrow-derived cells may also participate in  $T_R$ -cell selection. TEC, thymic epithelial cell.  $V\alpha$ , diverse  $V\alpha$  regions.

$CD25^+CD4^+$  T cells, which develop naturally in uninfected healthy individuals, are readily detectable in the thymus and secondary lymphoid organs in mice, rats and humans, where they make up 2–10% of the total  $CD4^+$  T cells.

Importantly,  $T_R$  cells are functionally competent when isolated *ex vivo*. Upon TCR crosslinking, thymic and peripheral  $CD25^+CD4^+$   $T_R$  cells suppress proliferation and IL-2 production by responder  $CD25^-CD4^+$  or  $CD8^+$  T cells in a contact-dependent manner.  $T_R$  cells themselves have a reduced capacity to proliferate and produce IL-2 or pro-inflammatory cytokines under these conditions<sup>2,6</sup>. However, this apparent anergy (hyporesponsiveness) ascribed to naturally arising  $T_R$  cells is probably an *in vitro* artefact. Adoptive cell transfer and labelling of cycling cells revealed that these cells proliferate *in vivo* and survive over extended periods of time, thereby showing a capacity for self renewal<sup>7–9</sup>. The robust proliferation of  $CD25^+CD4^+$  T cells expressing a transgenic TCR upon stimulation with the cognate ligand is in agreement with these results<sup>10–12</sup>. Importantly, upon expansion,  $T_R$  cells maintain and even enhance their suppressive capacity after proliferation. These results, in combination with the thymic origin of  $T_R$  cells, suggest that these cells are a dedicated lineage. Although other T-cell populations may play an analogous suppressive role, the genetic mechanisms underlying their generation and function have not been ascertained, and their dedicated suppressive function has not been proved. We focus our attention on the so-called  $CD25^+CD4^+$   $T_R$  cells because of their proven *in vivo* role in suppressing autoimmunity, tumour immunity, allergy and immunity to chronic infections.

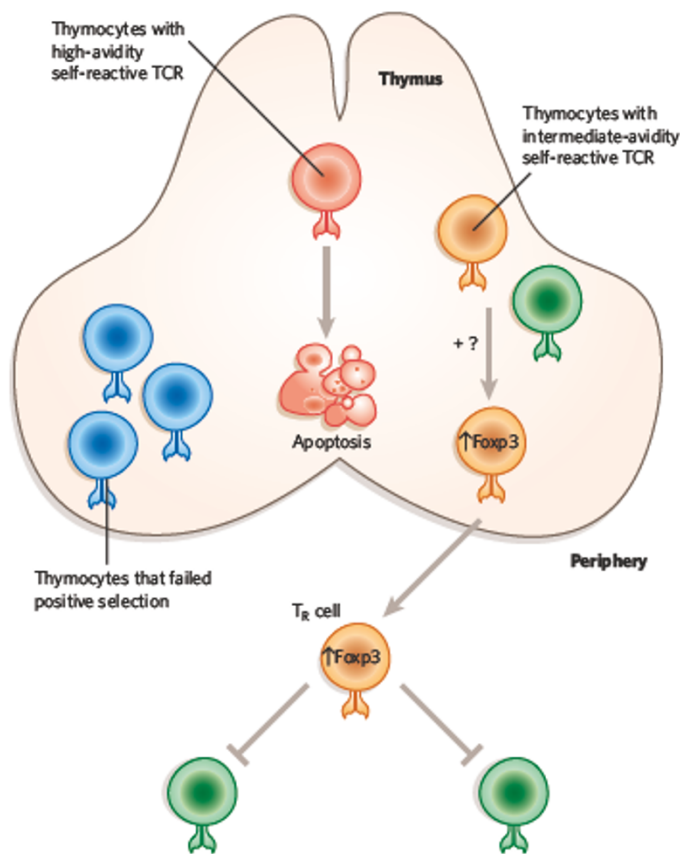
### Control of immune responses by $T_R$ cells

Several mechanisms might explain  $T_R$ -cell-mediated suppression *in vivo*. In particular, expression of the high-affinity trimeric IL-2 receptor by  $T_R$  cells might result in growth-factor competition and soak up IL-2 (refs 13, 14). This is unlikely to be a major mechanism because, even in the presence of exogenous IL-2,  $T_R$  cells inhibit the upregulation of IL-2 messenger RNA (mRNA) in responder T cells<sup>15</sup>. Unlike passive IL-2 deprivation, two major immunosuppressive cytokines (IL-10 and TGF- $\beta$ ) have been implicated as an active effector mechanism of  $T_R$ -cell-mediated suppression *in vivo*<sup>2,16,17</sup>. However, other cell types, including non-regulatory T cells, produce these cytokines. Thus, the relative contribution of TGF- $\beta$  and IL-10 derived from  $T_R$  cells or from other cellular sources to the overall control of autoimmune inflammation in a particular organ and genetic background is currently not known.

*In vitro* studies show that  $T_R$  cells can suppress  $CD4^+$  and  $CD8^+$  T-cell responses by an undefined contact-dependent, but IL-10/TGF- $\beta$ -independent, mechanism<sup>6</sup>. One such mechanism may involve 'reverse' signalling by B7 molecules upon crosslinking on the surface of dendritic cells or activated T cells by CTLA4, the high-affinity receptor for B7 that is expressed at a high level by  $T_R$  cells<sup>18,19</sup>. In dendritic cells, B7 crosslinking induces indoleamine-2,3-dioxygenase, resulting in local tryptophan depletion. In T cells, the biochemical consequences of B7 engagement by CTLA4 remain unclear<sup>19,20</sup>. In addition, recent reports suggest that pre-activated murine  $T_R$  cells induce granzyme-B-dependent, yet perforin-independent, apoptosis in responder T cells<sup>21</sup>. In humans, similarly activated  $T_R$  cells are capable of killing responder T cells in a perforin- and granzyme-dependent fashion<sup>22</sup>. These and other putative contact-dependent mechanisms have been illuminated by *in vitro* studies using antibody-mediated crosslinking of  $T_R$  TCR either before or during the suppression assay. The latter may not accurately reflect the physiological level of activation of  $T_R$  effector function. Furthermore, the significance of B7 reverse signalling and granzyme/perforin-induced apoptosis/killing of responder T cells as  $T_R$ -cell suppression mechanisms operating *in vivo* is not known. Thus, the interplay between cell-contact-dependent and cytokine-dependent mechanisms, the extent of their redundancy and their relative contribution to prevention of autoimmune disease in a specific tissue or organ need to be explored further. In addition,  $T_R$  cells can suppress tumour immunity and immune responses that are associated with chronic exposure to infectious agents or environmental antigens<sup>2</sup>.

### Control of $T_R$ -cell lineage specification by Foxp3

Although cell-surface expression of CD25 has been instrumental in the isolation and enumeration of  $T_R$  cells, its use as a  $T_R$ -cell marker during an ongoing immune response is very limited, as all activated  $CD4^+$  and  $CD8^+$  T cells transiently upregulate CD25 expression. The search for a definitive  $T_R$ -cell marker in mice resulted in the identification of the transcription factor Foxp3, which is expressed in  $T_R$  cells but not in recently activated or resting T cells<sup>23–25</sup>. Several years ago, mutations in the X-chromosome-encoded Foxp3 gene were identified as the cause of the early-onset fatal autoimmune disorder observed in human IPEX patients (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and in *scurfy* mutant mice that spontaneously develop autoimmune disease<sup>26–28</sup>. In both humans and mice, the various manifestations of autoimmunity are observed in mutant



**Figure 2 | Alternative fates for developing thymocytes.** During development, thymocytes with a high affinity for self-peptide–MHC complexes are deleted (red cells), whereas thymocytes with TCRs that do not react to self undergo death by neglect (blue cells). Thymocytes with a low avidity for self-peptide–MHC are positively selected into the conventional CD4<sup>+</sup> and CD8<sup>+</sup> lineages. Some self-reactive thymocytes with an intermediate avidity for self-peptide ligands upregulate Foxp3 in response to increased strength or duration of a TCR signal in combination with an unknown signal (yellow cells). Upon Foxp3 induction, thymocytes commit to the T<sub>R</sub>-cell lineage and are therefore capable of keeping other T-cell responses in check, thereby preventing autoimmunity.

males, but not in heterozygote female carriers, although the X chromosome undergoes random inactivation in T cells. Therefore approximately 50% of T cells in females lack Foxp3 expression. The resistance of females to autoimmunity is consistent with the ability of T<sub>R</sub>-cell-mediated control of immune tolerance to act *in trans* on cells that lack Foxp3. Furthermore, examination of chimaeric mice containing a mixture of Foxp3-deficient and wild-type haematopoietic precursor cells unequivocally demonstrated that CD25<sup>+</sup>CD4<sup>+</sup> T cells fail to develop from Foxp3-deficient progenitors. Wild-type precursors, however, gave rise to a normal T<sub>R</sub>-cell population that could prevent development of overt autoimmune symptoms<sup>29</sup>. As a corollary to these results, an expanded CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> subset is found in mice with T-cell-specific expression of a Foxp3 transgene. Even the CD25<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells in these mice exhibit some suppressive capacity, as assessed by an *in vitro* assay<sup>25</sup>. Similarly, retroviral Foxp3 gene transfer into peripheral mouse and human CD25<sup>+</sup>CD4<sup>+</sup> T cells results in acquisition of suppressive function by at least some of the transduced T cells<sup>23,24,29,30</sup>. Together, these studies reveal a principal role for Foxp3 in guiding regulatory CD25<sup>+</sup>CD4<sup>+</sup> T-cell development and function (Fig. 2).

Recent analysis of mice expressing green fluorescent protein 'knocked into' the Foxp3 locus suggests that Foxp3 is a T<sub>R</sub>-cell-lineage specification factor. This idea stems from the observation that Foxp3 expression is uniquely restricted to a subset of peripheral and thymic

αβ T cells primarily composed of CD25<sup>+</sup> and CD25<sup>+</sup>CD4<sup>+</sup> T cells with potent suppressive activity. These cells also share a transcriptional signature that is distinct from either CD25<sup>+</sup>Foxp3<sup>+</sup> or CD25<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup> T cells<sup>31</sup>. This suggests that Foxp3 is a dedicated genetic mechanism for the generation of T cells that can promote dominant tolerance.

#### Autoreactivity and T<sub>R</sub>-cell lineage commitment

The idea of T<sub>R</sub> specificity for self antigens, one of the major tenets in our current understanding of the biology of T<sub>R</sub> cells, can be traced to early studies of protection against day-3 thymectomy-induced autoimmunity in specific target tissues, such as the ovaries or thyroid gland<sup>2,32,33</sup>. Important clues suggesting the requirement for a specific TCR signal for thymocyte commitment to the T<sub>R</sub>-cell lineage came from studies of mice expressing a TCR specific for a peptide derived from myelin basic protein (MBP). MBP is a major antigen in the pathogenesis of experimental allergic encephalomyelitis (EAE). This MBP peptide ligand has a low affinity for the MHC class II molecule that presents it, and it is not expressed in the thymus at a level sufficient to induce negative selection of MBP-reactive TCR transgenic T cells. Spontaneous disease was observed only in TCR transgenic mice on the *Rag1*<sup>-/-</sup> background. (Recombination activating genes 1 and 2 (*Rag1*, *Rag2*) encode proteins that mediate recombination in pre-B cells and thymocytes, leading to the production of antibodies and TCRs, respectively). Protection against EAE in these experiments was provided by a regulatory CD4<sup>+</sup> T-cell subset that was dependent on the rearrangement of endogenous TCR genes for its development<sup>34</sup>. These and other TCR transgenic mice lack CD25<sup>+</sup>CD4<sup>+</sup> T cells in the absence of RAG function. This suggests that only certain TCR specificities support T<sub>R</sub>-cell development. An important role for high-affinity TCR engagement in the thymus for T<sub>R</sub>-cell development was suggested by an increase in the proportion of CD25<sup>+</sup>CD4<sup>+</sup> T cells when a transgenic TCR was paired with its cognate ligand, encoded by another transgene<sup>35,36</sup>.

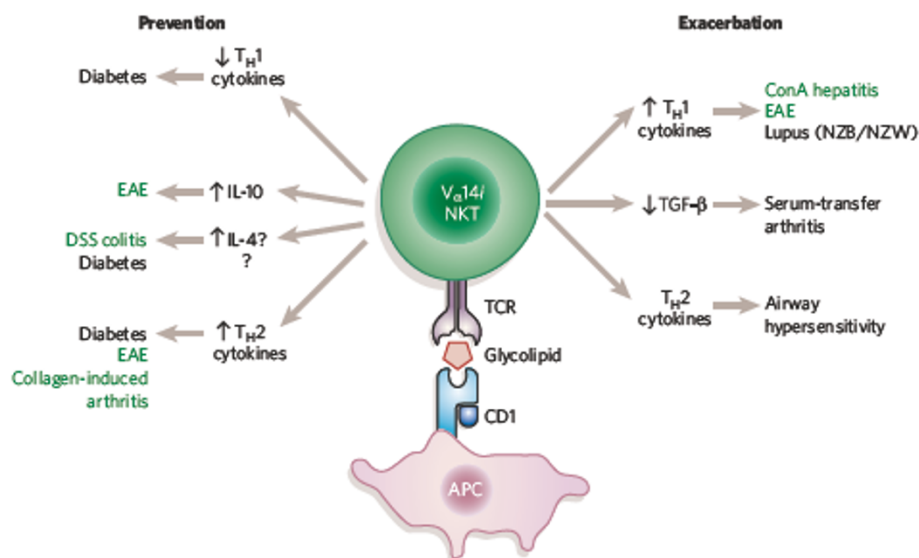
For naturally arising CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells, an increased avidity of TCRs for self-peptide–MHC class II complexes was revealed by the analysis of TCRs from T<sub>R</sub> cells compared with non-T<sub>R</sub> TCRs upon their transduction into TCR transgenic RAG-deficient T cells of unrelated specificity<sup>37</sup>. Increased avidity of the TCR for self-peptide–MHC results in a high level of expression of several proteins that attenuates TCR signalling (such as CTLA4, PD-1 and CD5) and increases the level of proteins that promote survival in response to TCR ligation (such as glucocorticoid-induced TNFR-family-related receptor (GITR), TNF-RII, OX40 and 4BB-1; refs 2, 6, 8). The highly diverse TCR repertoire displayed by naturally arising CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells<sup>37</sup> suggests that they recognize a great variety of ligands.

A noticeable skewing of CD4<sup>+</sup> T-cell development towards the CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub>-cell lineage observed upon transduction of bone-marrow stem cells with CD25<sup>+</sup> T<sub>R</sub>-derived, but not CD25<sup>-</sup> non-T<sub>R</sub>-cell-derived, TCRs suggests that self-reactive TCRs instruct T<sub>R</sub> lineage commitment<sup>37</sup>. An alternative model that suggests that self-reactive TCRs are not responsible for T<sub>R</sub> cell lineage commitment has recently challenged this view. Instead, self-reactive TCRs may control the relative size of the T<sub>R</sub> subset by affecting expansion or survival of these cells and enabling them to compete with non-T<sub>R</sub> cells<sup>38</sup>. Nevertheless, recent demonstration of an absolute MHC dependence of Foxp3 expression in the Foxp3<sup>+</sup> subset of immature CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes, similar to that observed in mature single-positive thymocytes, supports an instructive role for TCR signalling in T<sub>R</sub>-cell lineage commitment.

The nature of the antigen-presenting cells (APCs) involved in self-ligand presentation to developing T<sub>R</sub> cell precursors in the thymus is not known. However, MHC class II ligand expression by thymic stromal cells is sufficient for the thymic generation of CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells of a particular specificity<sup>39</sup>.

It is likely that an additional factor(s) cooperates with TCR signals to induce Foxp3 expression in developing thymocytes. Such a factor may be developmentally regulated, as suggested by the inefficient gen-

**Figure 3 | Illustration of some of the effects of the activation of V $\alpha$ 14i NKT cells on animal models of autoimmunity and inflammation. Green text indicates that the effect was seen only after addition of exogenous glycolipids such as  $\alpha$ GalCer. The black text indicates a role detected in the absence of such stimulation. Autoimmunity can be prevented by T<sub>H</sub>2 cytokines by an unknown mechanism, by inhibiting T<sub>H</sub>1 cytokine secretion and T<sub>H</sub>1-cell expansion in some diabetes experiments or by inducing IL-10 in one EAE (animal model of multiple sclerosis) study. V $\alpha$ 14i NKT cells also exacerbate autoimmunity by stimulating secretion of either T<sub>H</sub>1 or T<sub>H</sub>2 cytokines or by decreasing TGF- $\beta$  levels.**



eration of CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells in the thymus before day 3 after birth. The absence of these cells is responsible for the aforementioned autoimmunity in day-3 thymectomized mice<sup>40</sup>. CD28 and IL-2R are unlikely to play an indispensable role in thymic Foxp3 induction, despite their major effect on the size of the T<sub>R</sub>-cell subset<sup>41–43</sup>. Therefore, we favour a model of Foxp3 induction and T<sub>R</sub>-cell lineage commitment in developing thymocytes in response to an unidentified factor and TCR engagement by self-peptide–MHC complexes within a certain increased range in TCR avidity above that required for conventional T cells (Fig. 2).

In addition to the thymic generation of T<sub>R</sub> cells, peripheral non-T<sub>R</sub> cells can acquire Foxp3 expression and convert to T<sub>R</sub> cells *in vivo* upon chronic antigenic stimulation or under lymphopenic conditions<sup>44,45</sup>. Thus, acquisition of Foxp3 expression by peripheral non-T<sub>R</sub> cells *in vivo* is also facilitated by chronic TCR stimulation and probably by the cytokine environment.

#### Is autoimmunity associated with Foxp3 mutation due to T<sub>R</sub>-cell deficiency?

Lack of T<sub>R</sub> cells in Foxp3-deficient mice is the cause of the lymphoproliferative syndrome. Evidence for this is provided by the substantial reduction in this syndrome upon neonatal transfer of a small number of wild-type T<sub>R</sub> cells<sup>23</sup>. It is noteworthy that the antigen-specific responses of mouse non-regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing or lacking the Foxp3 gene are indistinguishable when measured by clonal expansion and cytokine production *in vivo* and by cognate peptide-dose response and co-stimulation dependence *in vitro*. Importantly, temporal Foxp3 upregulation is not observed in these cells during the course of conventional antigen-specific immune responses. These observations, combined with the identical onset and progression of autoimmune disease in mice with germline and  $\alpha\beta$  T-cell-specific ablation of the Foxp3 gene, provide further proof that T<sub>R</sub>-cell deficiency in mice results in a major breakdown of self tolerance and autoimmune pathology affecting multiple organs<sup>31</sup>.

Because the human and murine Foxp3 genes have a high level of sequence conservation throughout the coding and non-coding regions, it is likely that results obtained in mouse studies can be extrapolated to humans. Indeed, human CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells have high levels of Foxp3 expression. As a word of caution, we should mention the conflicting reports as to whether human non-regulatory CD25<sup>+</sup>CD4<sup>+</sup> or CD8<sup>+</sup> T cells are capable of transient or prolonged Foxp3 upregulation upon TCR stimulation<sup>30,46</sup>. One difficulty with the interpretation of this type of study is our current inability to analyse Foxp3 expression at a single-cell level. Expansion of Foxp3-expressing CD25<sup>+</sup> T cells or acquisition of Foxp3 expression by small numbers of T cells 'pre-

committed' to the T<sub>R</sub>-cell lineage may account for these observations. Development of a method to detect Foxp3 expression at the single-cell level would help to address this issue and would permit the monitoring of the dynamics of T<sub>R</sub> cells in clinical settings.

The lack of T<sub>R</sub> cells in IPEX patients, a result of a Foxp3 deficiency, probably leads to autoimmune pathology in a variety of organs. So, manipulation of the number of T<sub>R</sub> cells and their suppressive activity could be tailored to develop novel therapeutic approaches for treatment of the more common forms of autoimmunity that are under polygenic control (see below). Experiments in a variety of models of experimental autoimmunity in mice support this possibility. In this regard, a better understanding of the signals that induce Foxp3, identification of Foxp3 downstream targets and further elaboration of T<sub>R</sub>-effector mechanisms are of immediate importance.

#### Natural killer T cells are a distinct T-cell sublineage

A subset of T cells express receptors found on natural killer cells and are known as NKT cells<sup>3</sup>. Like T<sub>R</sub> cells, NKT cells are a self-reactive T-cell sublineage generated in the thymus (Fig. 1), and they may regulate autoimmunity. However, their developmental pathway is distinct from T<sub>R</sub> cells. The specificity of NKT cells is focused on a few antigens and, unlike the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells, their roles in autoimmunity include both protection from pathogenesis and enhancement of disease<sup>47</sup>.

In mice, the majority of NKT cells are CD4<sup>+</sup> or double-negative (CD4<sup>+</sup>CD8<sup>-</sup>) T cells that recognize glycolipids presented by CD1d<sup>3</sup>, a non-classical MHC class I-like antigen-presenting molecule. Most of these cells express a V $\alpha$ 14/J $\alpha$ 18 TCR rearrangement with an invariant CDR3 region. Therefore, they are sometimes referred to as V $\alpha$ 14 invariant (V $\alpha$ 14i) NKT cells or *i*NKT cells<sup>48</sup> to distinguish them from other T cells that express NK receptors. Most studies indicating a role for NKT cells in autoimmunity have implicated V $\alpha$ 14i NKT cells.

#### Specificity of *i*NKT cells

The ligand most widely used for activating V $\alpha$ 14i NKT cells is the glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ GalCer), which was originally isolated from a marine sponge in a screen for compounds that could prevent tumour metastasis<sup>49</sup>.  $\alpha$ GalCer binds to CD1d, and the resulting complex is a very strong agonist that binds avidly to the V $\alpha$ 14i TCR<sup>50–52</sup>. Humans and monkeys also have T cells that are reactive for  $\alpha$ GalCer presented by CD1d, and these cells express the TCR V regions orthologous to mouse V $\alpha$ 14 and V $\beta$ 8, including an invariant V $\alpha$ 24/J $\alpha$ 18 (V $\alpha$ 24i) rearrangement. *i*NKT cells are cross-reactive for APCs expressing either mouse or human CD1d. The strict conservation of this specificity is indicative of its fundamental importance.

Glycosphingolipids closely related to  $\alpha$ GalCer<sup>53</sup> are abundant in *Sphingomonas* bacteria, which are Gram-negative organisms that lack lipopolysaccharide (LPS). These bacteria are ubiquitous in the environment, and their glycolipids provide a unique example of a microbial glycolipid that can activate the majority of mouse and human *i*NKT cells. Moreover, mice lacking  $V\alpha 14i$  NKT cells have a reduced ability to clear the bacteria<sup>54,55</sup>. This information led to the speculation that the rapid immune response of  $V\alpha 14i$  NKT cells to *Sphingomonas* glycolipids might be analogous to the Toll-like receptor 4 (TLR4)-mediated response to LPS in providing an innate-type reaction important for host defense to bacteria that lack LPS.

#### Selection of $V\alpha 14i$ NKT cells

Although  $V\alpha 14i$  NKT cells are derived from a double-positive precursor<sup>56</sup>, they branch off from the mainstream pathway of thymic differentiation. Unique features of this pathway include positive selection mediated by CD1d<sup>+</sup> double-positive thymocytes<sup>57</sup> (Fig. 1), rather than the cortical epithelial cells required for selection of conventional T cells. A distinct set of factors required for  $V\alpha 14i$  NKT-cell development is also required. These include cytokines such as IL-15, transcription factors such as NF- $\kappa$ B family members and T-box expressed in T cells (T-bet), and signalling molecules such as the Src family kinase Fyn<sup>50</sup> and the adaptor signalling lymphocyte activation molecule-associated protein (SAP) that interacts with Fyn<sup>58–60</sup> (Table 1). Thymus differentiation programmes the unique properties of  $V\alpha 14i$  NKT cells, including expression of activation markers and the ability to produce cytokines such as IL-4 and interferon (IFN)- $\gamma$ <sup>50</sup>. Mature  $V\alpha 14i$  NKT cells express inhibitory receptors of the Ly49 family, and these receptors are acquired at a late stage of thymus ontogeny or after export from the thymus. Both the differentiation and immune response of  $V\alpha 14i$  NKT cells might be regulated by the interplay of TCR signals and inhibitory signals from NK receptors<sup>61</sup>, with the inhibitory NK receptors perhaps blocking uncontrolled autoreactive responses.

Isoglobotrihexosylceramide (iGb3), a glycosphingolipid with the structure Gal $\alpha$ (1,3)Gal $\beta$ (1,4)Glu $\beta$ (1,1)ceramide, is required for the positive selection of  $V\alpha 14i$  NKT cells<sup>62</sup>. iGb3 can also stimulate mature *i*NKT cells, indicating that a self agonist can positively select  $V\alpha 14i$  NKT cells. In contrast to  $T_H$  cells, which react with a wide range of self antigens, the invariant TCR of *i*NKT cells is selected to recognize a very limited set of antigens presented by CD1d. The focused self reactivity of *i*NKT cells could have a dual purpose. Presentation of iGb3 in the periphery signals for cellular stress or provides a signal that is important for the immune system to recognize. At the same time, the selection for this specificity might provide an important component of host defence from certain types of bacteria, through recognition of unusual glycosphingolipids.

#### Regulation of *i*NKT-cell cytokine production

Once activated through their TCR with  $\alpha$ GalCer,  $V\alpha 14i$  NKT cells produce a mixture of  $T_H1$  cytokines, such as TNF and IFN- $\gamma$ , and  $T_H2$  cytokines, including IL-4 and IL-13, within hours<sup>47</sup>. The rapid production of cytokines, and the speed and intensity of the ensuing activation of dendritic cells and NK cells and other cell types are reminiscent of innate responses. Despite producing a mixture of  $T_H1$  and  $T_H2$  cytokines after TCR activation,  $V\alpha 14i$  NKT cells can in some cases polarize the immune response in either a  $T_H1$  or  $T_H2$  direction<sup>47</sup>. The modulation of  $V\alpha 14i$  NKT-cell cytokine production is crucial for their regulation of autoimmunity, although the mechanisms that determine the cytokine polarity are not well understood. The immediate response of  $V\alpha 14i$  NKT cells to TCR stimulation with agonists such as  $\alpha$ GalCer is clearly less easily polarized in either a  $T_H1$  or a  $T_H2$  direction than the response of conventional CD4<sup>+</sup> T cells to peptide antigen stimulation. The sustained cytokine response of  $V\alpha 14i$  NKT cells may be influenced, however, by the nature of the glycolipid antigen and by the context in which it is presented. For example, presentation of  $\alpha$ GalCer pulsed on dendritic cells favours IFN- $\gamma$  over IL-4

production<sup>63</sup>, and the use of altered glycolipid ligands related to  $\alpha$ GalCer may help to polarize the  $V\alpha 14i$  NKT-cell response in either a  $T_H1$  or a  $T_H2$  direction<sup>64</sup>. Additionally, cytokine production by  $V\alpha 14i$  NKT cells may be determined by the integration of signals from different types of receptor. For example, IL-12 can selectively stimulate IFN- $\gamma$  production by  $V\alpha 14i$  NKT cells, in conjunction with the recognition of self antigens<sup>65</sup>.

#### *i*NKT cells and autoimmunity

Several animal models indicate that  $V\alpha 14i$  NKT cells prevent autoimmunity and inflammation, either when activated naturally or when using  $\alpha$ GalCer or related compounds. Some of these results are summarized in Fig. 3. Stimulation of  $V\alpha 14i$  NKT cells was beneficial in murine models of diabetes, EAE (which is an animal model of multiple sclerosis), and collagen-induced arthritis<sup>47</sup>. Stimulating these cells was also beneficial in a chemically induced model of colitis. Moreover, the number of  $V\alpha 14i$  NKT cells is reduced in diabetes-prone non-obese diabetic (NOD) mice, and increasing the number of NKT cells by adoptive transfer, or by expression of a  $V\alpha 14i/\alpha 18$  transgene, reduced this disease<sup>47</sup>. In some studies,  $V\alpha 14i$  NKT-cell-deficient, CD1<sup>-/-</sup> NOD mice developed accelerated disease, but this was not always the case<sup>66</sup>, illustrating the controversy surrounding some of the findings on  $V\alpha 14i$  NKT cells and autoimmunity. In EAE and diabetes, the beneficial effect of stimulating  $V\alpha 14i$  NKT cells could be attributed in some studies to the induction of IL-4 synthesis by autoantigen-reactive T cells<sup>47</sup>. This was not always observed<sup>67</sup>, and in one study activation of  $V\alpha 14i$  NKT cells could induce IL-10 synthesis by the autoreactive T cells<sup>68</sup>. Moreover, some protocols for stimulating  $V\alpha 14i$  NKT cells with synthetic glycolipids exacerbated EAE rather than diminished it<sup>69</sup>. An additional example of  $V\alpha 14i$  NKT cells promoting autoimmunity is provided by the spontaneous model of systemic lupus erythematosus that arises in (NZB  $\times$  NZW)<sub>F1</sub> mice<sup>70</sup>.  $V\alpha 14i$  NKT cells apparently stimulated pathogenic anti-DNA antibody production, even without activation by exogenous glycolipids. Furthermore, in airway hypersensitivity experiments that model asthma, two reports indicate that mice lacking  $V\alpha 14i$  NKT cells are resistant to developing hypersensitivity, and IL-4 or IL-13 production by these cells was required for susceptibility<sup>71,72</sup>.

In parallel with some of the mouse studies, there is evidence that a decrease in  $V\alpha 14i$  NKT cells in human peripheral blood is correlated with a variety of organ-specific and systemic autoimmune diseases<sup>73</sup>. Also, when *in vitro* expanded  $V\alpha 14i$  NKT cells were studied, increased  $T_H2$  cytokine release by these cells correlated with disease that was in remission<sup>74</sup>. In diabetics, however, these conclusions are controversial<sup>75</sup>, and the intrinsic problems of these studies include the variability in the number of  $V\alpha 14i$  NKT cells in normal humans and assessment of the peripheral blood rather than the site of disease.

In summary, although there are exceptions in which  $V\alpha 14i$  NKT cells induces IL-10 synthesis<sup>76</sup> or anergy, in most studies they activate rather than suppress the immune response. This is exemplified by recent results in the K/BxN transgenic mouse model of arthritis in which transfer of serum from these donors causes an arthritis in which deposition of glucose-6-phosphate-isomerase-specific autoantibodies leads to an inflammatory cascade that includes mast cells and complement activation<sup>77</sup>. Recent work implicates  $V\alpha 14i$  NKT cells in arthritis pathogenesis in this animal model: the level of IFN- $\gamma$  and IL-4 mRNA decreased in the joints of CD1d<sup>-/-</sup> mice given the arthritogenic serum, and the level of mRNA for the immunosuppressive cytokine TGF- $\beta 1$  increased<sup>78</sup>. This is the opposite of the effect that would be expected for the  $T_H$  cells, and immune activation may reflect the true physiological role mediated by  $V\alpha 14i$  NKT cells in response to infection and in other contexts. Therefore, in many of the cases in which the activity of *i*NKT cells was beneficial in autoimmunity, it might have been through the induction of immune deviation or a cytokine response highly skewed in either a  $T_H1$  or a  $T_H2$  direction, rather than through the induction of a truly unresponsive state.

## Conclusions

It has long been believed that the thymus dictates one of three fates to developing T cells: death by neglect, positive selection or negative selection. Recent evidence hints at a 'fourth way'<sup>79</sup>, in which selection by self agonists leads to the differentiation of T-cell subsets with an activated phenotype and specialized functions in the maintenance of self tolerance. The two populations discussed here, however, exhibit different phenotypes, localization and roles in immune regulation. Self recognition is common to both cells, but the factors in the thymus that imprint the distinct fates of T<sub>R</sub> cells and V $\alpha$ 14i NKT cells remain to be fully defined.

There are some potential advantages to using T<sub>R</sub> cells or V $\alpha$ 14i NKT cells in immune therapy for autoimmune disease, including the fact that the difficult identification of specific autoantigens involved in disease pathogenesis would not be required. Human V $\alpha$ 14i NKT cells can be expanded dramatically by stimulation of their TCR in the presence of cytokines<sup>80,81</sup>, and the same is true for mouse T<sub>R</sub> cells<sup>82</sup>. It may be possible to carry out *ex vivo* expansion of these types of T cell followed by re-infusion of the expanded cells back to patients. These strategies are likely to be cumbersome and expensive, and their practicality remains to be demonstrated. Realization of the therapeutic potential of T<sub>R</sub> cells and V $\alpha$ 14i NKT cells probably depends upon devising ways to augment their activity *in vivo* or, in some cases for V $\alpha$ 14i NKT cells, to antagonize their activity.

For V $\alpha$ 14i NKT cells, antagonist or activating glycolipids might have clinical applications, as  $\alpha$ GalCer is not toxic<sup>83</sup>. Antagonist ligands have not been identified, however, and glycolipids that skew the immune response mediated by activated mouse V $\alpha$ 14i NKT cells in a T<sub>H</sub>1 or a T<sub>H</sub>2 direction have not been extensively tested in humans. An even more daunting problem is the unpredictable nature of the contrasting effects NKT cells have on different autoimmune diseases. Therefore, despite some promise, significant obstacles must be overcome before the therapeutic potential of this cell type can be realized.

The latest clinical studies suggest that treatment of type 1 diabetes patients with non-depleting anti-CD3 antibody induces differentiation of some peripheral T cells into T<sub>R</sub> cells or expands the pre-existing T<sub>R</sub> subset. The latter may account for the beneficial therapeutic effect of this experimental treatment<sup>84</sup>. In addition to these approaches, recent experimental work has suggested that *Foxp3* gene expression in human T cells specific for a particular autoantigen, or induction of *Foxp3* expression upon activation of autoantigen-reactive, conventional CD4<sup>+</sup> T cells in the presence of TGF- $\beta$ , may provide an additional way of generating antigen-specific T<sub>R</sub> cells<sup>23,24,29,30,85,86</sup>. Finally, understanding the critical role of *Foxp3* in T<sub>R</sub> biology suggests that this transcription factor can be used as a target for drug development to modulate its expression *in vivo* and thereby affect numbers and activity of T<sub>R</sub> cells. Nevertheless, major work is also required to reduce the wealth of knowledge accumulated in experimental models of T<sub>R</sub>-mediated inhibition of autoimmune, tumour- and pathogen-specific immune responses to clinical practice. ■

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