Review
A paragon of self-tolerance: CD25+CD4+ regulatory T cells and the control of immune responses
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Abstract
The interest in naturally arising regulatory T (T_R) cells as a paradigm for maintaining immunological self-tolerance has undergone an explosive re-emergence in recent years. This renaissance was triggered by several key experimental observations and the identification of specific molecular markers that have enabled the isolation and experimental manipulation of these cells. Although their existence was once controversial, a large body of evidence now highlights the critical roles of T_R cells in maintaining immunological self-tolerance. Furthermore, abnormality of natural T_R cells can be a primary cause of autoimmune and other inflammatory diseases in humans.

Keywords: CD25+CD4+, Foxp3, regulatory cells, self-tolerance, suppression

Introduction
The random nature of T-cell receptor (TCR) generation inevitably leads to the appearance of deleterious autoreactive clones, but the vast majority of such cells are purged in the thymus during negative selection. However, there is abundant evidence showing that significant numbers of autoreactive cells can 'slip through the net' of central tolerance into the periphery and thereby potentially mediate autoimmunity. This phenomenon can be readily demonstrated by the experimental induction of autoimmunity when otherwise normal animals are injected with self-proteins plus a strong adjuvant [1].

The fact that healthy animals harbour such destructive cells implies the existence of mechanisms operating in the periphery that are able to effectively prevent their activation. Experimental evidence has indeed revealed numerous avenues by which this can occur, among them immune ignorance, peripheral deletion/anergy, and dominant suppression (reviewed in [2]). The existence of a specific T cell subset that could dominantly suppress immune responses was first proposed by Gershon and Kondo in 1970 [3]. The concept developed from experiments suggesting that tolerance was an active cell-mediated process and could be transferred into naïve animals. Elaborate circuits involving suppressor, contrasuppressor and veto cells were proposed to explain the maintenance of self-tolerance; however, the inability to clone any actual suppressor cells or identify critical molecules associated with them led to the decline of such a model. Furthermore, the subsequent emergence of the Th1–Th2 paradigm seemed largely able to subsume suppression phenomena by the patterns of regulatory cytokines that these cells could secrete, and the parsimony thus offered seemed much more attractive as a theory.

In contrast, accumulated evidence from the mid-1980s has shown that depletion of a particular T cell subset from normal animals can cause autoimmune disease similar to the counterparts in humans, and that reconstitution of this subset can prevent these diseases. Subsequent detailed phenotypic characterisation of such autoimmune preventative cells now leaves no doubt of the existence of T_R cells as crucial mediators of self-tolerance in both animal models and humans.

APC = antigen-presenting cell; DC = dendritic cell; GITR = glucocorticoid-induced tumour necrosis factor family related protein; IBD = inflammatory bowel disease; IL = interleukin; IL-2R, interleukin-2 receptor; TCR = T-cell receptor; TGF = transforming growth factor; Th = T helper cell; T_R cell = regulatory T cell.
Defining a ‘regulatory cell’

Broadly speaking, T cells with regulatory properties can be divided into two types: naturally occurring thymically generated regulatory cells, defined here as ‘T_R cells’, and those generated by antigenic stimulation under special conditions in the periphery, referred to variously as ‘Th3’, ‘Tr1’ cells or ‘adaptive regulatory cells’ (see, for example, [4]). This review will focus chiefly on the naturally arising suppressive T_R cells.

A discrete molecular description of T_R cells has proved to be a key issue in this field and was indeed one of the major stumbling blocks to their original exposition. Early clues hinting at the identity of regulatory cells emerged from experimental models of autoimmune disease. Many such models require the induction of lymphopenia in genetically susceptible strains of rodents, for example 3-day-old neonatal thymectomy or adult thymectomy coupled with an immunosuppressive treatment such as cyclophosphamide [5–7]. Depending on the strain background, experimental manipulations of this kind result in a variety of autoimmune diseases such as thyroiditis, gastritis, oophoritis and orchitis. It was subsequently shown that induction of such autoimmunity could be prevented by the transfer of normal CD4^+ splenocytes or CD4^+CD8^− thymocytes [7–10]. Collectively, such data strongly suggested that a cell population with a crucial role in maintaining self-tolerance was resident within the normal T lymphocyte pool.

Attempts were then made to phenotype putative T_R cells more specifically by isolating the T lymphocyte fraction that harvested regulatory activity. Sakaguchi and colleagues managed to first identify the CD5 molecule as a marker for T_R cells by demonstrating that otherwise normal lymphocytes depleted of CD5^+CD4^+ cells induced broad-spectrum autoimmunity when transferred into athymic nude mice [11]. Unfractionated CD4^+ cells (which contain CD5^+CD4^+ cells) prevented the induction of autoimmunity when transferred together with the CD5^−CD4^+ cells, implying that the T_R cells were contained specifically within the CD5^+CD4^+ compartment. Subsequent experiments aimed at homing in yet further on T_R cell-specific markers have identified a number of other potential candidate molecules. For instance, CD45RB seems to divide T cells into two distinct functional subsets [12]. Lymphopoenic mice transferred with CD45RB^high cells develop a lethal wasting disease characterised by severe inflammatory bowel disease (IBD), whereas unfractionated T cells or CD45RB^low cells alone cause no disease. Importantly, co-transfer of the CD45RB^low and CD45RB^high populations results in protection of the mice from colitis.

More recently, the most useful surface marker for T_R cells has proved to be the interleukin-2 (IL-2) receptor α-chain, CD25 [13]. About 5–10% of CD4^+ T cells and less than 1% of CD8^+ peripheral T cells constitutively express CD25 in normal naïve mice, and such cells are found in the CD5^+CD45RB^low T cell fractions. Indeed, transfer of CD25-depleted CD4^+ T cells to athymic mice results in a variety of autoimmune diseases, whereas transfer with CD25^+CD4^+ cells inhibits such disease development. Moreover, CD25^+CD4^+ cells in normal naïve mice exhibit clear immunosuppressive properties in vitro and in vivo [13,14]. It now seems that the naturally occurring CD25^+CD4^+ population could account for the regulatory effect of CD5^+CD4^+ cells in vivo.

A comprehensive characterisation of the surface profile of T_R cells has revealed them to be quite distinct from conventional naïve effector T cells. Aside from the constitutive expression of CD25, T_R cells show elevated levels of adhesion molecules such as CD11a (LFA-1), CD44, CD54 (ICAM-1), CD103 (αEβ7 integrin) in the absence of any apparent exogenous antigenic stimulation [14,15]. Naturally occurring CD25^+CD4^+ cells additionally express CD152 (CTLA-4), a molecule classically only expressed after T cell activation [16–18]. There is some evidence to suggest that T_R cells might also exhibit a characteristic chemokine receptor profile, with mouse CD25^+CD4^+ cells expressing elevated levels of CCR5 and their human counterparts expressing CCR4 and CCR8 [19,20]. Such a distinctive pattern of chemokine receptors suggests that T_R cells might be rapidly recruited to sites of inflammation and thereby efficiently control immune responses. Most recently, several groups have demonstrated that glucocorticoid-induced tumour necrosis factor family related protein (GITR) is predominantly expressed at both the RNA and protein levels by CD25^+CD4^+ cells [15,21,22]. Administration of the anti-GITR monoclonal antibody, DTA-1, in vivo elicits autoimmune disease, suggesting that this molecule has an important functional role in maintaining T_R cell suppression [22].

The surface marker profile of T_R cells is thus quite different from that of naïve T cells. However, it should be noted that most, if not all, of their apparently characteristic molecules are upregulated during conventional T cell activation. This similarity to otherwise normal but primed T cells is potentially problematic when trying to identify or isolate true T_R cells and precludes the use of CD25 alone (or any other surface molecule yet found) as an infallible marker. This caveat aside, several important distinctions still remain between the surface phenotype of T_R and primed T cells, but they are more relative than absolute. For example, although both primed T cells and T_R cells express CD25, the latter does so to a higher level and more stably. Indeed, when stimulation of normal T cells ceases, CD25 expression is lost, whereas T_R cells revert to their original constitutive expression level [23]. In addition, CD25^+ cells generated from originally CD25^−CD4^+ cells show no suppressive ability either in vitro or in vivo [23]. As a component of the high-affinity IL-2 receptor, CD25 itself is...
essential for the survival of T<sub>R</sub> cells, and the cells are exquisitely sensitive to an absence of signalling through this receptor [24]. Clear evidence for this can be seen by the almost total absence of CD25<sup>+</sup>CD4<sup>+</sup> cells in IL-2-deficient mice. In conclusion, the similarities between T<sub>R</sub> cells and primed T cells are therefore probably only a reflection of a shared activation state.

As noted above, the search for a definitive T<sub>R</sub> cell marker has been fraught with complications and an occasional lack of certitude regarding their undeniable existence as a functionally distinct population rather than simply another activation state of conventional T cells. However, some very recent data have gone some way to demonstrating conclusively that T<sub>R</sub> cells are a genuine T cell lineage, in the process identifying a seemingly unambiguous marker [25–27]. Studies with the Scurfy (sf) mutant mouse model provided the required breakthrough. The Scurfy mouse exhibits a fatal X-linked lymphoproliferative disease that is mediated by highly activated CD4<sup>+</sup> T cells and is akin to the phenotype of both CTLA-4 and transforming growth factor (TGFB) knockout mice [28–32]. Subsequent work mapped the sf mutation to a novel forkhead/winged-helix family transcriptional repressor termed Foxp3, which encodes the protein scurfin [33]. A mutation in the human orthologue, FOXP3, has also been identified as the underlying cause of the aggressive autoimmune syndrome IPEX (for Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) [33–35].

The overt immunological similarities seen with genetic defects of Foxp3 and the experimental depletion of CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells led several groups to investigate the potential role of Foxp3 in the development and function of T<sub>R</sub> cells. Three independent groups were able to demonstrate that Foxp3 mRNA [25–27] and the encoded protein [27] were specifically expressed only in naturally arising CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells and, critically, were never observed in normal T cells even after they had been activated and acquired the expression of CD25/GITR. However, a very low level of Foxp3 expression was observed in CD25<sup>−</sup>CD4<sup>+</sup> T cells; this appeared to be attributable to a small population of CD25<sup>−</sup>CD45RB<sup>low</sup>GITR<sup>high</sup> T<sub>R</sub> cells ([26], M Ono, manuscript in preparation). In addition, T<sub>R</sub> cells were unable to develop in the absence of Foxp3, as demonstrated by the use of sf mice or by the targeted deletion of Foxp3 [25,27]. Finally, and most convincingly, retroviral transduction of Foxp3 into conventional CD25<sup>−</sup>Foxp3<sup>−</sup> T cells converted them into phenotypical and functional T<sub>R</sub> cells capable of effectively suppressing both in vitro and in vivo [26,27]. Thus Foxp3 seems to be a ‘master gene’ controlling the normal development and/or function of naturally occurring T<sub>R</sub> cells.

As yet there are very few data detailing the role and expression patterns of FOXP3 in human cells. Some of the early indications, both published and unpublished, have shown FOXP3 expression in human CD25<sup>+</sup>CD4<sup>+</sup> T cells ([36], H Yagi and S Sakaguchi, unpublished results); however, it already seems that there are some discrepancies with the murine data. For example, there seem to be considerable differences in FOXP3 expression between individuals and, more significantly, FOXP3 might be inducible in human CD25<sup>−</sup>CD4<sup>+</sup> cells (which start off apparently FOXP3<sup>−</sup>) after anti-CD3/anti-CD28 stimulation [36]. It remains to be determined whether this simply represents an expansion to detectability of the tiny Foxp3<sup>+</sup>GITR<sup>+</sup>CD25<sup>−</sup>CD4<sup>+</sup> population described above ([26], M Ono, manuscript in preparation) or is a genuine property of human T cells radically different from that of mice.

The suppressive properties of T<sub>R</sub> cells can be modelled in vitro by mixing titrated numbers of highly purified CD25<sup>+</sup>CD4<sup>+</sup> cells and CD25<sup>−</sup>CD4<sup>+</sup> (or CD25<sup>−</sup>CD8<sup>+</sup>) responder cells plus a TCR stimulus such as anti-CD3, ConA or antigen-presenting cells (APCs) plus antigenic peptide. Under such conditions, the CD25<sup>+</sup> population suppresses both the proliferation and IL-2 production of the CD25<sup>−</sup> cells in a dose-dependent manner [37,38]. The T<sub>R</sub> cells require TCR stimulation to exert any suppressive effects, but once this condition has been satisfied the ensuing suppression is non-specific for antigen [37,38]. Suppression is therefore an active process and can be directed against bystander cells.

Curiously, the CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells themselves are anergic in vitro; that is, they do not proliferate or produce IL-2 in response to conventional T cell stimuli. However, this anergy can be broken by a sufficiently potent stimulus such as the addition of exogenous IL-2 or anti-CD28, or the use of mature dendritic cells as APCs [37,39]. Interestingly, anergy seems to be the default state for T<sub>R</sub> cells, because they revert to it once IL-2 is withdrawn [37,38]. However, the anergy in vitro is not reflected in vivo, wherein T<sub>R</sub> cells seem to have a highly active rate of turnover [24]. An anergic state also seems to be closely related to T<sub>R</sub> cells’ suppressive ability because if it is broken there is a concomitant loss of regulatory activity both in vitro and in vivo [37]. Table 1 summarises what is currently known about the T<sub>R</sub> cell phenotype.

**Development and origin**

CD25<sup>+</sup>CD4<sup>+</sup> T<sub>R</sub> cells are produced by the normal thymus as fully functioning suppressive cells, and such thymocytes exhibit apparently all the properties of their matured peripheral counterparts [14]. Itoh and colleagues showed that the adoptive transfer of CD25-depleted thymocytes to syngeneic nude mice recipients led to a similar spectrum of autoimmune disease to that with CD25<sup>+</sup>CD4<sup>+</sup> peripheral cells [14]. CD25<sup>+</sup>CD4<sup>+</sup> thymocytes are also anergic and suppressive in vitro and exhibit a classic T<sub>R</sub> cell surface phenotype, for example elevated levels of activa-
CD25–CD4+ cells, which predominantly express only classic co-stimulatory molecule CD28 seems to be important for the activation of suppressive functions, the programme involving T cells can develop in TCR transgenic mice specific for an extra-thymic generation of T R cells from conventional naïve T cells with regulatory properties and an anergic phenotype (such as the aforementioned Tr1 cells) can be generated in the periphery, but whether these are identical to naturally occurring T R cells remains to be established. Several approaches have led to the peripheral generation of regulatory cells. For instance, activation of conventional T cells in the presence of TGF-β/IL-10 or with the immunomodulatory agent 1-α-25-dihydroxyvitamin D₃ produces a suppressive T cell [44,45]. Also of potential interest is the induction of regulatory cells by immature or ‘tolerogenic’ dendritic cells (DCs) [46,47]. Additionally, in some now classic studies, Qin and colleagues were able to generate regulatory cells by the administration of non-depleting anti-CD4 monoclonal antibodies in vivo to thymectomised mice (reviewed in [48]). A final confirmation of whether such peripherally generated regulatory cells are contiguous with naturally occurring T R cells or are simply another T cell activation state will have to await the assessment of Foxp3 expression. A summary of T R cell developmental steps is shown in Fig. 1.

### Mechanisms of suppression

The suppression mechanism of activation-induced regulatory cells such as Tr1 cells is based primarily on the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (reviewed in [49]). The situation with naturally occurring T R cells is not nearly so clear-cut and despite intense interest remains strangely inconclusive. Potential T R cell suppression mechanisms can basically be divided into those mediated by secreted factors and those requiring intimate cell–cell contact. Most of the experiments in vivo examining T R cell suppression have been based on the murine IBD model described above and have, as with Tr1 cells, flagged the importance of IL-10 and TGF-β. By blocking IL-10 signalling in vivo with monoclonal antibodies against the IL-10 receptor, Asseman and colleagues were able to abrogate the normal IBD-preventative action of CD45RBlow T cells [50]. The same group was also able to show that CD45RBlow T cells from IL-10-deficient mice were unable to prevent colitis and, moreover, were even colitogenic themselves [50]. The importance of IL-10 in

### Table 1

Comparison of the phenotype of conventional naïve CD4+ T cells and CD4+ regulatory cells (T R)

| Conventional naïve helper T cell | Natural regulatory T cell (T R) |
|----------------------------------|---------------------------------|
| Foxp3−                          | Foxp3+                          |
| CD5low, CD11alow, CD25low, CD38low, CD44high, CD45RBhigh, CD54low, CD103low, GITRlow | CD5high, CD11ahigh, CD25high, CD38high, CD44high, CD45RBlow, CD54high, CD103high, GITRhigh |
| About 90–95% of splenic CD4+ T cells | About 5–10% of splenic CD4+ T cells |
| Responsive to conventional T cell stimuli | Anergic to conventional T cell stimuli |
| Non-suppressive | Suppressive |

Many of the distinctions are not absolute; for instance, activated non-regulatory effector T cells express cell surface markers with a pattern similar to that of T R cells, so such discrimination is possible only with constitutive expression. Currently, expression of Foxp3 seems to be the most accurate marker for T R cells because this does not vary with the activation state.
the control of IBD is also implied by the observation that IL-10−/− mice spontaneously develop IBD even though these mice are not lymphopenic [51].

Similarly, several groups have shown that a monoclonal-antibody-mediated blockade of TGF-β abrogates TR cell suppressive functions both \( \text{in vivo} \) and \( \text{in vitro} \) [52,53]. Interestingly, TGF-β does not necessarily have to act as a soluble factor but can be expressed exclusively on the surface of CD25+CD4+ cells after stimulation through the TCR and might therefore mediate its effects in a membrane-proximal manner [53]. The level at which these anti-inflammatory cytokines operate to maintain tolerance is also uncertain, but it might be through the inhibition of APCs or pathogenic T cells, by maintenance of the TR cell population and/or by enhancement of their function (reviewed in [54]).

Elucidation of the mechanism of TR cell suppression is complicated by the fact that most evidence \( \text{in vitro} \) shifts the emphasis of suppression to mechanisms solely based on cell contact. First, anti-IL-10 or anti-TGF-β monoclonal antibody fails to perturb the suppressive activity of CD25+CD4+ cells \( \text{in vitro} \) [54], although the use of soluble IL-10R seems to have a partial effect [55]. A study showing the successful neutralisation of suppression with anti-TGF-β monoclonal antibodies at the same time also demonstrated the TGF-β to be bound to the cell surface [53]. Second, culture supernatants from activated CD25+CD4+ cells show no inherent suppressive ability, nor is any suppression observed across a semi-permeable membrane [37,38]. Taken together, the data \( \text{in vitro} \) thus seem to obviate the role of not merely IL-10/TGF-β but also soluble factors in general, suggesting that TR cell suppression is dependent on close cell–cell contact, although it is still impossible to discount completely the possibility that suppression is mediated in an extreme paracrine fashion.

The membrane events that occur during cell contact-dependent suppression are entirely unclear, but presum-

Figure 1

A putative scheme for the development of regulatory T (Tr) cells. Tr and naïve conventional helper T cells (Th0) develop within a normal thymus through the processes of positive and negative selection. Precursor T cells of relatively high avidity trigger a Tr cell developmental programme involving the activation of Forkhead boxes (Foxp)3, whereas T cell receptors of intermediate avidity yield Th0 cells. Additionally, regulatory cells can be peripherally generated (for example, Tr1 cells) when activated under tolerogenic conditions (for example, with immature dendritic cells). As yet it is unclear whether de facto Foxp3+ Tr cells can be generated in the periphery or whether the Tr1 cells produced from conventional Th0 cells are equivalent to naturally present Tr cells. IL, interleukin; Tc cell, effector T cell; TGF, transforming growth factor.
ably an as yet uncharacterised inhibitory molecule is expressed on the surface of activated $T_R$ cells (see Fig. 2).

Another mechanism of suppression mediated by cell contact could proceed via simple competition for APCs and specific major histocompatibility complex-antigen gen- genic complexes. The high level of adhesion molecules and chemokine receptors present on the surface of $T_R$ cells would make them particularly well suited to homing to, and stably interacting with, APCs, thereby physically excluding normal CD25$^+$CD4$^+$ effector cells. Furthermore, constitutive expression of the high-affinity IL-2R would make $T_R$ cells into an effective sink for IL-2, depriving potential autoreactive cells of this essential growth factor. A final, conceptually attractive model of suppression would be $T_R$ cell-mediated inhibition or alter- ation of APC function. Supporting this model is the observation that CD25$^+$CD4$^+$ cells could alter the antigen-presenting function of DC by downregulating their expression levels of CD80/CD86 [56] or, as has recently been demonstrated, by triggering the immunosuppressive catabolism of tryptophan by DC [57]. Although APC perturb-utation might well occur in vivo, it is not essential because $T_R$ cells are able to suppress effectively even in the absence of any APCs [58].

**Conclusion**

Solid evidence now strongly supports the existence of the once controversial $T_R$ cells as key controllers of self-tolerance. Although limitations of space have forced this review to focus primarily on the role of $T_R$ cells and autoimmunity, there are ample data to suggest that this lineage might be crucial wherever immune reactions need to be regulated or tuned. For instance, $T_R$ cells might limit anti-tumour or microbial immune responses. A strategic manipulation of $T_R$ cells might thus be used either to enhance or to dampen immune responses as required. The identification of molecular markers, in particular Foxp3, has permitted the accurate isolation and study of these cells in ways not previously possible and will, it is hoped, facilitate therapeutic intervention with this potentially powerful immunological ally.

**Competing interests**

None declared.

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