How regulatory T cells work

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Abstract | Regulatory T (T_{Reg}) cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. However, they also limit beneficial responses by suppressing sterilizing immunity and limiting antitumour immunity. Given that T_{Reg} cells can have both beneficial and deleterious effects, there is considerable interest in determining their mechanisms of action. In this Review, we describe the basic mechanisms used by T_{Reg} cells to mediate suppression and discuss whether one or many of these mechanisms are likely to be crucial for T_{Reg}-cell function. In addition, we propose the hypothesis that effector T cells may not be 'innocent' parties in this suppressive process and might in fact potentiate T_{Reg}-cell function.

Peripheral tolerance
The lack of self-responsiveness of mature lymphocytes in the periphery to specific antigens. These mechanisms control potentially self-reactive lymphocytes that have escaped central-tolerance mechanisms. Peripheral tolerance is associated with suppression of the production of self-reactive antibodies by B cells and inhibition of self-reactive effector T cells, such as cytotoxic T lymphocytes. The actions of regulatory T cells constitute one mechanism of peripheral tolerance.

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Several sophisticated regulatory mechanisms are used to maintain immune homeostasis, prevent autoimmunity and moderate inflammation induced by pathogens and environmental insults. Principal among these mechanisms are the actions of regulatory T (T_{Reg}) cells, which are now widely regarded as the primary mediators of peripheral tolerance. Although T_{Reg} cells have a pivotal role in preventing autoimmune diseases, such as type 1 diabetes[1,2], and limiting chronic inflammatory diseases, such as asthma and inflammatory bowel disease (IBD)[3,4], they also block beneficial responses by preventing sterilizing immunity to certain pathogens[5,6] and limiting antitumour immunity[7]. A seminal advance in the analysis of T_{Reg} cells came with the identification of a key transcription factor, known as forkhead box P3 (FOXP3), that is required for their development, maintenance and function[8,9]. Mice (known as scurfy mice, BOX 1) and individuals that lack FOXP3 develop a profound autoimmune-like lymphoproliferative disease that graphically emphasizes the importance of T_{Reg} cells in the maintenance of peripheral tolerance[10–12]. Although FOXP3 has been proposed to be the master regulator of T_{Reg} cells that controls the expression of multiple genes that mediate their regulatory activity[13,14], this notion has recently been challenged, raising the possibility that other transcriptional events may operate upstream of and/or concurrently with FOXP3 to mediate T_{Reg}-cell development[15].

Although FOXP3 has proved to be an invaluable marker of mouse T_{Reg} cells, its role in human T_{Reg} cells is less straightforward (see BOX 2 for a discussion on T_{Reg}-cell markers). Humans that lack functional FOXP3 develop IPEX (immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome), which is a severe autoimmune disease that develops early in infancy. Although FOXP3 appears to be required for human T_{Reg}-cell development and function, expression of FOXP3 alone is clearly not sufficient for regulatory function, as a significant percentage of human activated T cells express FOXP3 but do not possess regulatory activity[16–20]. Furthermore, the induction of FOXP3 in human T cells by transforming growth factor-β (TGFβ) does not confer a regulatory phenotype, in contrast to their mouse counterparts[20]. Consequently, FOXP3 is not an adequate marker for human T_{Reg} cells (BOX 2). Whether this distinction is due to intrinsic differences between mouse and human FOXP3 and/or a requirement for additional co-factors or transcription factors is an important question that needs to be resolved.

Significant progress has been made over the past few years in delineating the molecules and mechanisms that T_{Reg} cells use to mediate suppression[21,22]. In this Review, we outline our current understanding of the mechanisms used by T_{Reg} cells to mediate suppression, and the challenges that lie ahead in defining their mode of action. We also discuss whether T_{Reg} cells are likely to depend on one, a few or many of these mechanisms. In addition, we propose that effector T cells may have a significant role in boosting and/or modulating T_{Reg}-cell function. Unless otherwise stated, we primarily focus on the mechanisms that are used by thymus-derived, naturally occurring $CD4^+CD25^+FOXP3^+$ T_{Reg} cells.

Basic mechanisms of T_{Reg}-cell function

Defining the mechanisms of T_{Reg}-cell function is clearly of crucial importance. Not only would this provide insight into the control processes of peripheral tolerance but it would probably also indicate several potentially important therapeutic targets. Although this quest has been ongoing

Box 1 | Scurfy mice: misplaced mechanistic expectations?

Mice that carry a spontaneous loss-of-function mutation (known as scurfy mice) or a deletion of forkhead box P3 (*Foxp3*) develop a fatal autoimmune-like disease with hyper-responsive $CD4^+$ T cells[9,12]. More recently, the generation of *Foxp3*–diphtheria-toxin-receptor (DTR) knock-in mice has allowed for the selective depletion of regulatory T (T_{Reg}) cells following treatment with diphtheria toxin[105]. These mice have been invaluable for dissecting the role of FOXP3 in T_{Reg}-cell function.

Given the profound phenotype of these mice, there is a general expectation that genetic disruption of any key T_{Reg}-cell inhibitory-dependent molecule or suppression mechanism would probably result in a scurfy-like phenotype. Of course, it is also possible that deletion of a key T_{Reg}-cell gene may be more synonymous with diphtheria-toxin-mediated T_{Reg}-cell depletion in which FOXP3 might still prevent expression of pro-inflammatory cytokines[105]. Nonetheless, this has led to the notion that if mutant mice don't have a scurfy-like or a T_{Reg}-cell-depleted phenotype, then the disrupted gene probably isn't important for T_{Reg}-cell function. However, this may not necessarily be correct. Indeed, it is possible that no mouse lacking a T_{Reg}-cell inhibitory molecule will ever be generated that develops a profound, spontaneous autoimmune disease[21]. It should be noted that mutant mice that are colonized by *Helicobacter* spp. and/or *Citrobacter rodentium* may have an exacerbated phenotype, as several studies have shown that opportunistic enteric bacteria can significantly exacerbate gut pathology[4].

Ultimately, the occurrence of disease in mice lacking a T_{Reg}-cell inhibitory molecule will depend on whether T_{Reg} cells rely on single or multiple suppressive mechanisms. Given the number of genes induced or modulated by FOXP3, it is probable that a programme of intrinsic and extrinsic regulation is induced that involves multiple proteins[9,13]. Therefore, it would not be surprising if deletion of a single molecule does not provoke the profound scurfy-like phenotype seen in mice that lack FOXP3.

since interest in T_{Reg} cells was re-ignited in 1995 (REF. 23), there has been significant progress in the past few years. From a functional perspective, the various potential suppression mechanisms used by T_{Reg} cells can be grouped into four basic 'modes of action': suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of dendritic-cell (DC) maturation or function (FIG. 1).

Suppression by inhibitory cytokines. Inhibitory cytokines, such as interleukin-10 (IL-10) and TGFβ, have been the focus of considerable attention as mediators of T_{Reg}-cell-induced suppression. There has also been significant interest in their ability to stimulate the development of induced (also known as adaptive) T_{Reg}-cell populations, either *in vivo* or experimentally as a potential therapeutic modality (BOX 3). Although the general importance of IL-10 and TGFβ as suppressive mediators is undisputed, their contribution to the function of thymus-derived, naturally occurring T_{Reg} cells is still a matter of debate[24]. This is partly due to the general perception that T_{Reg} cells function in a contact-dependent manner[25,26]. Indeed, *in vitro* studies using neutralizing antibodies or T cells that are unable to produce or respond to IL-10 and TGFβ suggested that these cytokines may not be essential for T_{Reg}-cell function[25–28]. However, this is in contrast with data from *in vivo* studies[29,30].

In allergy and asthma models, evidence suggests that both naturally occurring and induced antigen-specific T_{Reg} cells control disease in a manner that is, in part, dependent on IL-10 (REF. 29) and in some reports dependent on both IL-10 and TGFβ (REF. 31). Following allergen challenge, $CD4^+$ effector T cells were stimulated to produce considerable amounts of IL-10 in the lung by allergen-specific T_{Reg} cells that had been adoptively transferred, and thereby were able to control disease; this effect could be reversed with the administration of an antibody specific for the IL-10 receptor[32]. However, following the transfer of IL-10-deficient T_{Reg} cells, allergic inflammation and airway hyper-reactivity were still suppressed and the production of IL-10 was also increased, which suggests that the suppression of the T helper 2 (T_H2)-driven response to allergens *in vivo* by T_{Reg} cells is dependent on IL-10, but that the production of IL-10 by T_{Reg} cells themselves is not required for the suppression observed. This contrasts with a recent study suggesting that the T_{Reg}-cell-specific ablation of IL-10 expression resulted in increased lung allergic inflammation and hyper-reactivity[33].

This scenario might also occur in other disease models. For instance, the effects of IL-10 on the immune response to hepatitis B virus[34] and on the allograft tolerance response elicited by splenocytes exposed to non-inherited maternal antigens can only be partially attributed to T_{Reg}-cell-derived IL-10 (REF. 35). Recently, it was also shown that IL-10 is crucial for the control of

Type 1 diabetes
A chronic autoimmune disease that is characterized by the T-cell-mediated destruction of β-cells (which secrete insulin) in the pancreas. Individuals with type 1 diabetes develop hyperglycaemia and can develop diabetes-associated complications in multiple organ systems owing to a lack of insulin.

Inflammatory bowel disease
(IBD). A T-cell-mediated inflammatory response that affects the gastrointestinal tract. There are two forms of IBD in humans; Crohn's disease, which can affect any part of the gastrointestinal tract but usually descends from the terminal ileum, and ulcerative colitis, which mainly affects the colon. In the mouse model of IBD, most of the inflammation is confined to the large intestine. The target antigen for the pathogenic T cells is unknown.

Sterilizing immunity
An immune response that leads to the complete removal of the pathogen.

Box 2 | T_{Reg}-cell markers

Identifying discriminatory cell-surface markers for the characterization and isolation of regulatory T (T_{Reg}) cells has always been a crucial goal. Although there are excellent markers for mouse T_{Reg} cells, this goal has remained elusive for human T_{Reg} cells. Traditionally, mouse and human T_{Reg} cells have been characterized as $CD4^+CD25^+$ (also known as IL-2Rα). Indeed, mouse T_{Reg} cells can be effectively isolated based on staining for $CD4^+CD25^+CD45RB^{low}$ expression. However, the purity of isolated human T_{Reg} cells has always been an issue because T cells upregulate CD25 expression upon activation[106]. Indeed, during the influenza or allergy season, a substantial proportion of human $CD4^+$ T cells can express CD25.

Although the identification of forkhead box P3 (FOXP3) as a key regulator of T_{Reg}-cell development and function has facilitated their identification in mice[8], many activated (non-regulatory) human T cells also express FOXP3, precluding it as a useful marker for human T_{Reg} cells[16–20]. Consequently, the search for T_{Reg}-cell-specific cell-surface markers, particularly in humans, has continued in earnest with a growing number of candidates proposed (reviewed in REF. 107). For instance, it was shown that the expression of CD127 (also known as IL-7R) is downregulated by T_{Reg} cells, and that this could be used to increase the purity of human T_{Reg}-cell isolation. Indeed, there is a 90% correlation between $CD4^+CD25^+CD127^{low}$ T cells and FOXP3 expression[108,109]. In addition, it was recently found that T_{Reg} cells expressed a higher level of folate receptor 4 compared with activated effector T cells[110]. It is also important to recognize that T_{Reg} cells, like their T-helper-cell counterparts, may be heterogeneous and thus a collection of cell-surface markers could facilitate their isolation and functional characterization. Indeed, such heterogeneity has recently been described based on differential expression of HLA-DR or CC-chemokine receptor 6 (CCR6)[102,103]. However, the general use of both markers remains to be fully established. Therefore, it is quite probable that the search for better T_{Reg}-cell markers will continue for some time.

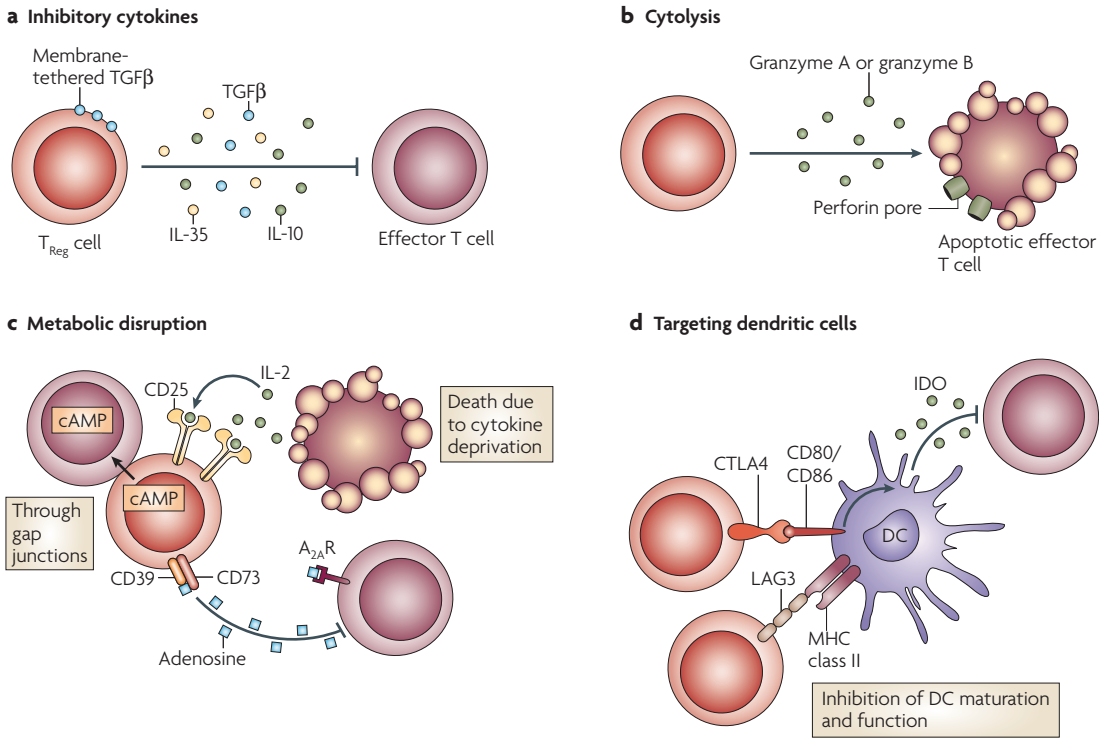

Figure 1 | **Basic mechanisms used by T_{Reg} cells.** Depiction of the various regulatory T (T_{Reg})-cell mechanisms centred around four basic modes of action. **a** | Inhibitory cytokines include interleukin-10 (IL-10), IL-35 and transforming growth factor-β (TGFβ). **b** | Cytolysis includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. **c** | Metabolic disruption includes high-affinity CD25 (also known as IL-2 receptor α)-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine receptor 2A ($A_{2A}R$)-mediated immunosuppression. **d** | Targeting dendritic cells (DCs) includes mechanisms that modulate DC maturation and/or function such as lymphocyte-activation gene 3 (LAG3; also known as CD223)–MHC-class-II-mediated suppression of DC maturation, and cytotoxic T-lymphocyte antigen-4 (CTLA4)–CD80/CD86-mediated induction of indoleamine 2,3-dioxygenase (IDO), which is an immunosuppressive molecule made by DCs.

various infections in which T_{Reg} cells have been reported to be involved, including *Mycobacterium tuberculosis*[36], *Toxoplasma gondii*[37], *Leishmania major*[38] and *Trichinella spiralis*[39]. However, T_{Reg} cells were not the source of IL-10 in these infection models.

In contrast to the previous studies, IL-10 production by T_{Reg} cells has been shown to be essential for the prevention of colitis in mouse models of IBD[40]. Moreover, it appears that the tumour microenvironment promotes the generation of FOXP3$^+$ T_{Reg} cells that mediate IL-10-dependent, cell-contact independent suppression[41]. Similarly, in UV-radiation-induced carcinogenesis, IL-10 production by T_{Reg} cells appears to be important for blocking antitumour immunity[42]. IL-10 produced by T_{Reg} cells also appears to be important for IL-10-mediated tolerance in a model of hepatitis induced by concanavalin A[43] and for tolerance to bacterial and viral superantigens[44]. In addition, recently published papers suggest new roles for T_{Reg}-cell-derived IL-10 in the induction of feto–maternal tolerance[45] and B-cell-enhanced recovery from experimental autoimmune encephalomyelitis (EAE)[46]. Interestingly, the T_{Reg}-cell-specific deletion of IL-10 did not result in the development of spontaneous systemic autoimmunity, but did result in enhanced pathology in the colon of older mice and in the lungs of mice with induced airway hypersensitivity, suggesting that the function of T_{Reg}-cell-derived IL-10 may be restricted to the control of inflammatory responses that are induced by pathogens or environmental insults[33]. Collectively, the picture that appears to be emerging is that the relative importance of IL-10 production by T_{Reg} cells as a mechanism of T_{Reg}-cell-mediated suppression is dependent on the target organism or disease and on the experimental system.

Although some early *in vitro* studies using neutralizing antibodies specific for TGFβ or using T_{Reg} cells that lack TGFβ (REFS 25,47) indicated that TGFβ was not required for the function of naturally occurring T_{Reg}-cells, other studies, both *in vitro* and *in vivo*, suggested a crucial role for T_{Reg}-cell membrane-tethered TGFβ (REFS 48,49). Therefore, the importance of TGFβ for naturally occurring T_{Reg}-cell function has also been a controversial topic. Indeed, there has been considerably more focus recently on the importance of TGFβ in the development of induced T_{Reg} cells and perhaps in T_{Reg}-cell maintenance in general (BOX 3). However, there are studies that suggest that TGFβ produced by T_{Reg} cells may directly participate in the suppression of effector T cells. For instance, effector T cells that are resistant to TGFβ-mediated suppression cannot be controlled by T_{Reg} cells in a mouse model of IBD[50]. In addition, TGFβ produced by

Airway hyper-reactivity
Initiated by exposure to a defined stimulus that is usually tolerated by normal individuals and that causes broncho-constriction and airway infiltration of inflammatory cells in allergic individuals.

Experimental autoimmune encephalomyelitis
(EAE). An animal model of the human autoimmune disease multiple sclerosis. EAE is experimentally induced in animals by immunization with myelin or with peptides derived from myelin. The animals develop a paralytic disease with inflammation and demyelination in the brain and spinal cord.

Box 3 | **Induced T_{Reg} cells: development and mode of action**

Naturally occurring forkhead box P3 (FOXP3)$^+$CD4$^+$CD25$^+$ regulatory T (T_{Reg}) cells develop in the thymus and display a diverse T-cell receptor (TCR) repertoire that is specific for self antigens[111,112]. However, T_{Reg} cells can also be 'induced', 'adapted' or 'converted' from effector T cells during inflammatory processes in peripheral tissues, or experimentally generated for therapeutic purposes[29,113,114]. For instance, T regulatory 1 (T_R1) cells and T helper 3 (T_H3) cells can be generated experimentally by and mediate their suppressive activity through interleukin-10 (IL-10) and transforming growth factor-β (TGFβ), respectively[114,115]. Typically, these regulatory populations do not express FOXP3.

In vivo, it has recently been suggested that stimulation of mouse effector T cells by CD103$^+$ dendritic cells (DCs) in the presence of TGFβ and retinoic acid induces the generation of FOXP3$^+$ T cells in the gut-associated lymphoid tissue (GALT)[116–121]. Furthermore, T_{Reg} cells can be preferentially induced in the periphery by exposure to $\alpha_V\beta_8$-integrin-expressing DCs[122] or DCs deficient in suppressor of cytokine signalling 3 (SOCS3) (REF. 123). Interestingly, independent of its role in generating induced T_{Reg} cells, TGFβ may also have an important role in helping to maintain FOXP3 expression by thymus-derived, naturally occurring T_{Reg} cells[124], a process that can be blocked by IL-4 or interferon-γ (REF. 125).

In contrast to mouse T cells, FOXP3 induction by TCR stimulation in the presence of TGFβ in human T cells does not confer a regulatory phenotype[20]. The mechanism of action of induced T_{Reg} cells may not necessarily be restricted to suppressive cytokines. Indeed, human induced T_{Reg} cells (CD4$^+$CD45RA$^+$ T cells stimulated with CD3- and CD46-specific antibodies) have also been shown to express granzyme B and killing target cells in a perforin-dependent manner[126]. In contrast to naturally occurring T_{Reg} cells, induced T_{Reg} cells often have a restricted specificity for particular cell types, tumours or foreign antigens[127]. Therefore, induced T_{Reg} cells may be ideally suited to respond to infectious agents. This may also be of particular importance in the GALT and in the tumour microenvironment where TGFβ drives the conversion of induced T_{Reg} cells[118,128]. A significant challenge in deciphering data from *in vivo* experiments is to assess the contribution of naturally occurring T_{Reg} cells versus induced T_{Reg} cells, and to determine whether inhibitory molecules, such as IL-10 or TGFβ, are derived from the former or the latter (or by other cells).

T_{Reg} cells has been found to be important in the control of the host immune response to *M. tuberculosis*[36], suppression of allergic responses[31] and prevention of colitis in an IBD model[51]. Interestingly, TGFβ produced by T_{Reg} cells has also been implicated in limiting antitumour immunity in head and neck squamous-cell carcinoma[52] and in follicular lymphoma[53], by rendering T cells unresponsive to the tumour. TGFβ also appears to limit the antitumour activity of cytokine-induced killer cells[54].

Membrane-tethered TGFβ can also mediate suppression by T_{Reg} cells in a cell–cell contact-dependent manner[48]. T_{Reg} cells can control the infiltration of CD8$^+$ T cells to pancreatic islets and delay the progress of diabetes through membrane-tethered TGFβ (REF. 49). However, experiments using mice in which the effector T cells were deficient in TGFβ-receptor (TGFβR) signalling or using TGFβ or TGFβR blocking reagents failed to show that membrane-tethered TGFβ is required for naturally occurring T_{Reg}-cell development or function[47]. More recently, however, interest in membrane-tethered TGFβ has re-surfaced with the description of a previously unappreciated role for it in the tumour microenvironment. TGFβ that is associated with tumour exosome membranes appears to enhance the suppressive function of T_{Reg} cells and skew T cells away from their effector functions and towards regulatory functions[55]. Furthermore, ovalbumin-induced airway inflammation can be attenuated by haeme oxygenase-1 through membrane-tethered TGFβ and IL-10 secretion by T_{Reg} cells[56], a process that activates the Notch1–HES1 (hairy and enhancer of split 1) axis in target cells[57]. Therefore, in light of the most current data, it now appears that soluble and/or membrane-tethered TGFβ may have a previously unappreciated role in the function of naturally occurring T_{Reg} cells.

Recently, a new inhibitory cytokine, IL-35, has been described that is preferentially expressed by T_{Reg} cells and is required for their maximal suppressive activity[58]. IL-35 is a new member of the IL-12 heterodimeric cytokine family and is formed by the pairing of Epstein–Barr virus-induced gene 3 (*Ebi3*; which normally pairs with p28 to form IL-27), and p35 (also known as *Il12a*; which normally pairs with p40 to form IL-12). Both *Ebi3* and *Il12a* are preferentially expressed by mouse FOXP3$^+$ T_{Reg} cells[58,59], but not resting or activated effector T cells, and are significantly upregulated in T_{Reg} cells that are actively suppressing[58]. As predicted for a heterodimeric cytokine, both *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ T_{Reg} cells had significantly reduced regulatory activity *in vitro* and failed to control homeostatic proliferation of effector T cells and resolve IBD *in vivo*. This precise phenotype suggested that IL-35 is required for the maximal suppressive activity of T_{Reg} cells. Importantly, IL-35 was sufficient for T_{Reg}-cell activity, as ectopic expression of IL-35 conferred regulatory activity on naive T cells and recombinant IL-35 suppressed T-cell proliferation *in vitro*[58]. Although IL-35 is an exciting addition to the T_{Reg}-cell portfolio, there is clearly much that remains to be defined about this cytokine and its contribution to T_{Reg}-cell function. For instance, it remains to be determined whether IL-35 suppresses the development and/or function of other cell types, such as DCs and macrophages.

It is now clear that three inhibitory cytokines, IL-10, IL-35 and TGFβ, are key mediators of T_{Reg}-cell function. Although they are all inhibitory, the extent to which they are used in distinct pathogenic and/or homeostatic settings differs, suggesting a non-overlapping function, but this hypothesis needs further refinement.

Exosomes
Small, lipid-bilayer vesicles that are released from activated cells. They comprise either plasma membrane or membrane derived from intracellular vesicles.

Notch
A transmembrane receptor involved in the pathway for direct cell–cell signalling that regulates cell-fate choice in the development of many cell lineages, and therefore is vital in the regulation of embryonic differentiation and development.

Suppression by cytolysis. Cytolysis mediated through the secretion of granzymes had long been considered the forte of natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) (reviewed in REF. 60). However, many human $CD4^+$ T cells exhibit cytotoxic activity. Consistent with this, activated human naturally occurring T_{Reg} cells have been shown to express granzyme A. Furthermore, T_{Reg}-cell-mediated target-cell killing was mediated by granzyme A and perforin through the adhesion of CD18 (REF. 61).

By contrast, as mouse $CD4^+$ T cells are not cytolytic, it was surprising that early gene expression arrays showed that the expression of granzymeB was upregulated in mouse T_{Reg} cells[62,63]. Noelle and co-workers were the first to report that granzyme-B-deficient mouse T_{Reg} cells had reduced suppressive activity *in vitro*, and that this granzyme-B-dependent suppression appeared to be a perforin-independent result of T_{Reg}-cell-induced apoptosis of effector T cells[64]. The notion that T_{Reg} cells might possess cytolytic activity was supported by studies showing that T_{Reg} cells can kill B cells in a granzyme-B-dependent and partially perforin-dependent manner that results in the suppression of B-cell function[65]. More recently, T_{Reg} cells were shown to suppress the ability of NK cells and CTLs to clear tumours by killing these cells in a granzyme-B-dependent and perforin-dependent manner[66]. In addition, effector T cells that overexpress the granzyme-B-specific inhibitor SPI6 are resistant to T_{Reg}-cell-mediated suppression (R. Noelle, personal communication). Using a transplantation model in which T_{Reg}-cell-mediated tolerance is induced by CD40–CD154 co-stimulatory blockade in conjunction with donor lymphocyte-specific transfusion, Noelle and colleagues have also shown that the T_{Reg} cells that mediate this tolerance also depended on granzyme B for their suppressive activity.

Although the majority of research to date regarding T_{Reg}-cell-induced cytolysis has focused on granzyme-B-mediated mechanisms, a recent study has suggested that activated T_{Reg} cells induce apoptosis of effector T cells through a TRAIL–DR5 (tumour-necrosis-factor-related apoptosis-inducing ligand–death receptor 5) pathway[67]. Furthermore, galectin-1 (also known as LGALS1), which can induce T-cell apoptosis, has been shown to be upregulated by mouse and human T_{Reg} cells and galectin-1-deficient T_{Reg} cells have reduced regulatory activity *in vitro*[68]. These studies emphasize that more work is required to define the cytolytic mechanisms that T_{Reg} cells use to mediate suppression.

Suppression by metabolic disruption. Recently, several intriguing suppressive mechanisms have been described that could collectively be referred to as mechanisms that mediate 'metabolic disruption' of the effector T-cell target. A long-standing debate in the T_{Reg}-cell field is whether the high expression level of CD25 empowers T_{Reg} cells to 'consume' local IL-2 and therefore starve actively dividing effector T cells by depleting the IL-2 they need to survive[26,69]. Although previous studies suggested that this was not a bona fide T_{Reg}-cell mechanism[70,71], a recent study has re-ignited interest in this question by suggesting that T_{Reg} cells induce cytokine (specifically IL-2)-deprivation-mediated apoptosis[72]. However, given that a recent report using human T_{Reg} cells suggested that IL-2 depletion alone is not required for T_{Reg} cells to suppress effector T cells[73], more work is clearly necessary to resolve this debate.

Two new T_{Reg}-cell mechanisms have recently been proposed that induce the intracellular or extracellular release of adenosine nucleosides. Concordant expression of the ectoenzymes CD39 and CD73 was shown to generate pericellular adenosine, which suppressed effector T-cell function through activation of the adenosine receptor 2A ($A_{2A}R$)[74–76]. Interestingly, binding of adenosine to $A_{2A}R$ appears to not only inhibit effector T-cell functions, but also to enhance the generation of induced T_{Reg} cells by inhibiting IL-6 expression while promoting TGFβ secretion[77]. In addition, adenosine has also been shown to modulate DC maturation and favour a tolerogenic phenotype (P. Ernst, personal communication). Although TGFβ induces FOXP3 expression and T_{Reg}-cell differentiation, IL-6 inhibits the generation of T_{Reg} cells and promotes the generation of pro-inflammatory T_H17-cell development[78]. Therefore, inhibiting IL-6 has important implications in the maintenance of T_{Reg} cells. T_{Reg} cells were also shown to suppress effector T-cell function directly by transferring the potent inhibitory second messenger cyclic AMP (cAMP) into effector T cells through membrane gap junctions[79]. Although these mechanisms represent interesting additions to the list of potential mechanisms used by T_{Reg} cells to mediate suppression, further studies will be required to corroborate these exciting findings and assess the relative use of these mechanisms by T_{Reg} cells.

Suppression by targeting dendritic cells. In addition to the direct effect of T_{Reg} cells on T-cell function, T_{Reg} cells might also modulate the maturation and/or function of DCs, which are required for the activation of effector T cells. Although this is an attractive idea, the data in support of this theory are limited[80]. Interestingly, studies using intravital microscopy have revealed direct interactions between T_{Reg} cells and DCs *in vivo*. These interactions were proposed to function in attenuating effector T-cell activation by DCs[81,82] in a process involving the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), which is constitutively expressed by T_{Reg} cells[25,83]. More specifically, the use of CTLA4-specific blocking antibodies or CTLA4-deficient T_{Reg} cells showed that in the absence of functional CTLA4, T_{Reg}-cell-mediated suppression of effector T cells via DCs was reduced[84,85]. Importantly, it was also shown that T_{Reg} cells could condition DCs to express indoleamine 2,3-dioxygenase (IDO), a potent regulatory molecule which is known to induce the production of pro-apoptotic metabolites from the catabolism of tryptophan, resulting in the suppression of effector T cells through a mechanism dependent on interactions between CTLA4 and CD80 and/or CD86 (REFS 86,87).

In addition to inducing DCs to produce immunosuppressive molecules, several studies have suggested that T_{Reg} cells may also downmodulate the capacity of DCs to activate effector T cells. Ivars and colleagues first

Granzymes
A family of serine proteases that are found primarily in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells. They enter target cells through perforin pores, and cleave and activate intracellular caspases, resulting in target-cell apoptosis.

Perforin
A component of cytolytic granules that participates in the permeabilization of plasma membranes, allowing granzymes and other cytotoxic components to enter target cells.

Adenosine nucleosides
Adenosine ($C_{10}H_{13}N_5O_4$) is a ribonucleoside (adenine linked to ribose) that is a structural component of nucleic acids. It is also the primary molecular component of cyclic AMP (an important intracellular second messenger), AMP, ADP and ATP (a key sourse of chemical energy for many enzymatic reactions).

Ectoenzymes
Enzymes that are outside the cell membrane and therefore can cleave extracellular substrates. These are typically tethered to the outside of the cell by a transmembrane domain.

T_H17 cells
(T helper 17 cells). A subset of $CD4^+$ T helper cells that produce interleukin-17 (IL-17) and that are thought to be important in inflammatory and autoimmune diseases. Their generation involves IL-6, IL-21 and IL-23, as well as the transcription factors RORγt (retinoic-acid-receptor-related orphan receptor-γt) and STAT3 (signal transducer and activator of transcription 3).

Intravital microscopy
This is used for examination of biological processes, such as leukocyte–endothelial-cell interactions, in living tissue. In general, translucent tissues are used, such as the mesentery or cremaster muscle, which can be exposed and mounted for microscopic observation.

reported that T_{Reg} cells could downregulate the expression of the co-stimulatory molecules CD80 and CD86 by DCs *in vitro*[88]. Several studies have also reported the immunomodulatory effects of T_{Reg} cells on DC maturation and/or function[85,89–92]. Studies with human T_{Reg} cells have also indicated that T_{Reg} cells may modulate the function of monocytes and macrophages[93,94]. Although the precise mechanism by which this is orchestrated is not known, this modulation may be mediated through cell-surface molecules such as CTLA4 and/or cytokines such IL-10 and TGFβ.

Recent studies have also suggested that lymphocyte-activation gene 3 (LAG3; also known as CD223) may block DC maturation. LAG3 is a CD4 homologue that binds MHC class II molecules with very high affinity, has a negative regulatory T-cell intrinsic function and is required for maximal T_{Reg}-cell suppression[95,96]. Binding of LAG3 to MHC class II molecules expressed by immature DCs induces an immunoreceptor tyrosine-based activation motif (ITAM)-mediated inhibitory signalling pathway — which involves FcγRγ and extracellular-signal-regulated kinase (ERK)-mediated recruitment of SRC-homology-2-domain-containing protein tyrosine phosphatase 1 (SHP1) — that suppresses DC maturation and their immunostimulatory capacity[97]. It is noteworthy that human MHC class II^+ T_{Reg} cells have been shown to be more suppressive than MHC class II^- T_{Reg} cells, raising the possibility that these cells suppress by ligating LAG3 on activated effector T cells[98]. Although more work is required to fully elucidate if and how T_{Reg} cells might suppress effector T-cell function through DCs, this mode of action is an attractive possibility, as it may be a more efficient way of suppressing immune responses *in vivo* given the ~1:8 ratio of T_{Reg} cells to effector T cells, compared with the ~1:0.8 T_{Reg}-cell to DC ratio found in the peripheral lymph nodes (as determined by flow cytometry and cell counting of pooled lymph nodes; C.J.W. and D.A.A.V., unpublished observations). Furthermore, it has recently been shown that neuropilin-1 promotes prolonged interactions with T_{Reg} cells and immature DCs[99]. Given that neuropilin-1 is differentially expressed by T_{Reg} cells, this may give them an advantage over naive T cells in modulating the function of DCs.

Finally, T_{Reg} cells can also influence immune responses by modulating the recruitment and function of other cell types. For instance, T_{Reg}-cell-derived IL-9 has been shown to recruit and activate mast cells, which were shown to be essential regulatory intermediaries in the establishment of peripheral allograft tolerance[100].

Complicating issues. It is the current opinion that a hallmark of T_{Reg} cells is their dependence on direct cell–cell contact to mediate their inhibitory activity. This has been supported by *in vitro* experiments showing that T_{Reg} cells could not suppress effector T-cell proliferation when the two populations were separated by a permeable membrane[25,26]. However, there are two important issues one should consider when evaluating the T_{Reg}-cell mechanisms outlined above in the context of contact dependency. First, these assays are really a measure of proximity rather than contact. Indeed, soluble mediators are most effective close to the source of their generation. The close proximity maintains high local cytokine concentrations, which has been shown to be important for the function of IL-2 (REF. 101). Therefore, the dilution effect of diffusion across the permeable membrane might render a soluble mediator ineffective. One should also consider the importance of proximity for labile mediators that might be very effective when T_{Reg} cells are close to their target cells but not when far apart. One example of a labile mediator is adenosine, which has a half life of less than 10 seconds.

Second, it is not yet clear how much of the regulatory potency of T_{Reg} cells is directed towards DCs or other antigen-presenting cells (APCs) versus effector T cells. Although several studies have shown that T_{Reg} cells can directly suppress effector T cells *in vitro* in the absence of APCs, there is no direct evidence that contact between T_{Reg} cells and effector T cells is required for suppression *in vivo*. Indeed, intravital microscopy experiments suggest that T_{Reg} cells are far more frequently found in contact with DCs[81,82] compared with effector T cells. Furthermore, it is still not clear what the primary target is for many of the mechanisms described above. For instance, suppression by cytolysis, adenosine or cAMP could be directed against DCs and/or effector T cells. Inhibitory cytokines could also influence both populations. For example, although IL-35 was shown to directly act on effector T cells, an effect on DCs has not been precluded. The one mechanism that might be considered to be effector-T-cell exclusive is IL-2-deprivation-mediated apoptosis. Clearly, more work is needed to determine the primary target of T_{Reg}-cell suppression, particularly *in vivo*.

How many mechanisms do T_{Reg} cells need?

Although efforts to define the suppressive mechanisms used by T_{Reg} cells continue, an important question looms large. Is it likely that all these molecules and mechanisms will be crucial for T_{Reg}-cell function? There are three broad possibilities.

The first possibility is that a single, overriding suppressive mechanism is used by all T_{Reg} cells. Until the entire mechanistic panoply of T_{Reg} cells is defined, one cannot completely rule this possibility out. However, this would seem unlikely as none of the molecules and/or mechanisms that have been defined to date result in the complete absence of regulatory activity when blocked or deleted — a consequence that one might predict would result in a 'scurfy-like' phenotype (BOX 1). So, although T_{Reg} cells that lack a single molecule, for instance IL-10, IL-35 or granzyme B, exhibit a significantly reduced suppressor function, a scurfy-like phenotype does not ensue. Given that none of the current T_{Reg}-cell mechanisms can exclusively claim this distinction, it seems unlikely that any 'unknown' molecules or mechanisms could do so either.

The second possibility is that multiple, non-redundant mechanisms are required for maximal T_{Reg}-cell function. In the studies conducted to date, T_{Reg} cells that lack various suppressive molecules have been shown

to be functionally defective. This favours a scenario in which there are multiple non-redundant mechanisms that can be used by T_{Reg} cells, with each molecule contributing to the mechanistic whole. At present, this possibility would seem plausible. Indeed, this is supported by the recent analysis of mice possessing a T_{Reg}-cell-specific ablation of IL-10 expression, in which enhanced pathology was observed following environmental insult[33]. One would predict that at some point we should be able to generate knockout mice that lack a particular set of genes, and have a complete loss of T_{Reg}-cell activity. For this to be truly non-redundant, the list of genes would probably be restricted (2–4 genes).

The third possibility is that multiple, redundant mechanisms are required for maximal T_{Reg}-cell function. With the plethora of regulatory mechanisms described to date and the possibility of more yet to be identified, it is conceivable that there are multiple mechanisms that function redundantly. Such a redundant system would help to mitigate against effector-T-cell escape from regulatory control. Also, given the very small size of the T_{Reg}-cell population, a sizeable arsenal may be required at the height of an effector T-cell attack to control the response. Of course, it is possible that a semi-redundant scenario exists.

These possibilities have been discussed from the perspective of there being a single homogeneous T_{Reg}-cell population. However, similar to helper-T-cell subsets, it remains possible that a few or even many different T_{Reg}-cell subsets exist[24]. Each of these may rely on one or multiple regulatory mechanisms. Several recent studies have provided support for both phenotypic and functional heterogeneity among T_{Reg} cells. For instance, it has recently been shown that a small subpopulation of T_{Reg} cells expresses CC-chemokine receptor 6 (CCR6), which is associated with T cells that possess an effector-memory phenotype[102]. $CCR6^+$ T_{Reg} cells appeared to accumulate in the central nervous system of mice with EAE, suggesting that they may have a prevalent role in controlling responses in inflamed tissues. Heterogeneous expression of HLA-DR has also been suggested to mark different subpopulations of functionally distinct human T_{Reg} cells[103]. Indeed, $HLA\text{-}DR^+$ T_{Reg} cells were found to be more suppressive than their $HLA\text{-}DR^-$ counterparts. One might speculate that their enhanced inhibitory activity is due to HLA-DR-mediated ligation of the inhibitory molecule LAG3 expressed by activated effector T cells[95,96].

So, if multiple suppressor mechanisms exist, how might these be integrated and used productively by T_{Reg} cells *in vivo*? We would propose the following possible models[21]. First, a 'hierarchical' model in which T_{Reg} cells have several suppression mechanisms that could be used but only one or two of these are really crucial and consistently important in various regulatory settings. Second, a 'contextual' model in which the background or context in which the T_{Reg} cells reside and the type of target cell that they have to repress dictate the appropriate suppression mechanism(s) that is used. For example, in some cases, cell types may be primarily inhibited by cytokines, whereas other cell types may be most effectively suppressed by T_{Reg}-cell-mediated lysis. Alternatively, different mechanisms may be more effective in different tissue compartments or in different disease settings. This notion is supported by the recent analysis of mice in which IL-10 expression was specifically ablated in T_{Reg} cells[33]. Whereas T_{Reg}-cell-derived IL-10 was not required for the systemic control of autoimmunity, it did seem to be required for the control of inflammatory events at mucosal interfaces, such as the lungs and colon. As a clear picture of the available T_{Reg}-cell suppressor arsenal emerges, an important challenge will be to determine their relative importance and contribution to T_{Reg}-cell function in different disease models.

Do effector T cells potentiate T_{Reg}-cell function?

Most cellular interactions in the immune system are bidirectional, with molecular signals moving in both directions even though the interaction has broader unidirectional intentions (for example, $CD4^+$ T-cell help). However, the general perception to date is that T_{Reg} cells suppress and effector T cells capitulate. We speculate that this is in fact an incomplete picture and that effector T cells have a very active role in their own functional demise. Three recent observations support this view. First, we have recently examined the molecular signature of activated T_{Reg} cells in the presence or absence of effector T cells and were surprised to find that it was strikingly different, with hundreds of genes differentially modulated as a consequence of the presence of effector T cells (C.J.W. and D.A.A.V., unpublished observations). Second, we have shown that *Ebi3* and *Il12a* mRNA are markedly upregulated in T_{Reg} cells that were cultured with effector T cells, supporting the idea that effector T cells may provide signals that boost IL-35 production in *trans*[58]. Third, we found that T_{Reg} cells were able to mediate suppression of effector T cells across a permeable membrane when placed in direct contact with effector T cells in the upper chamber of a transwell plate (L.W.C. and D.A.A.V., unpublished observations). Interestingly, this suppression was IL-35 dependent, as $Ebi3^{-/-}$ T_{Reg} cells were unable to mediate this 'long-distance' suppression. Collectively, these data suggest that it is the 'induction', rather than the 'function', of T_{Reg}-cell suppression that is contact-dependent and that effector T cells have an active role in potentiating T_{Reg}-cell-mediated suppression. Therefore, we propose that receptor–ligand interactions between the co-cultured $CD4^+$ effector T cells and T_{Reg} cells initiate a signalling pathway that leads to enhanced IL-35 secretion and regulatory activity (FIG. 2). Although the molecule that mediates this enhanced T_{Reg}-cell suppression is unknown, it is possible that IL-2 may serve this function[104]. Given the contrasting genetic profiles of activated T_{Reg} cells in the presence and absence of effector T cells, it seems possible that this interaction may boost the expression of other regulatory proteins. It may well be that effector T cells unwittingly perform the ultimate act of altruism.

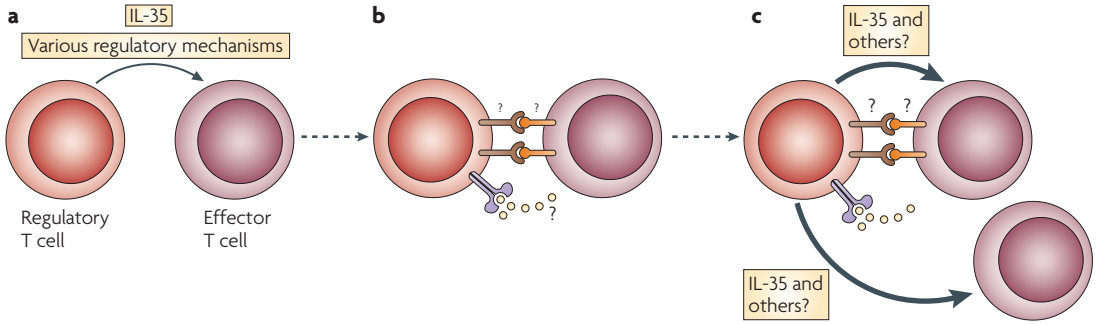

Figure 2 | **Model for how effector T cells might boost T_{Reg}-cell function.** This occurs in three stages. **a** | In addition to the constitutive production of interleukin-35 (IL-35) by regulatory T (T_{Reg}) cells, initial activation of these cells induces various regulatory mechanisms. **b** | T_{Reg} cells 'sense' the presence of recently activated effector T cells through a receptor–ligand interaction (cell surface or soluble). **c** | This in turn boosts or potentiates T_{Reg}-cell function resulting in the enhanced production of regulatory mediators, such as IL-35, and perhaps the induction of new mediators.

Concluding remarks

Although significant progress has been made over the past few years in defining the mechanisms that T_{Reg} cells use to mediate their suppressive function, there is clearly much that remains to be elucidated and many questions persist. First, are there more undiscovered mechanisms and/or molecules that mediate T_{Reg}-cell suppression? It is becoming clear that the transcriptional landscape of T_{Reg} cells is very different from naive or activated effector T cells, with literally thousands of genes differentially regulated. Although it seems unlikely that all or many of these will be important for T_{Reg}-cell function, it is quite possible that a few undiscovered genes might be important. It should be noted that although we are discussing mechanisms here, it is clear that some of these molecules may be essential in T_{Reg}-cell homing, homeostasis and other key functions, which might indirectly influence T_{Reg}-cell-mediated suppression *in vivo* without directly contributing to their inhibitory activity. It is also possible that some of these unknown molecules may represent more specific markers for the characterization and isolation of T_{Reg} cells, a particularly important issue for the analysis and use of human T_{Reg} cells (BOX 2).

Second, which mechanisms are most important? A significant but potentially complex challenge will be to determine if a few mechanisms are important in many T_{Reg}-cell settings or whether different mechanisms are required in different cellular scenarios. At present, it is difficult to assess this objectively as these mechanisms have predominantly been elucidated in different laboratories using distinct experimental systems and thus have not really been compared in side-by-side experiments. Furthermore, conditional mutant mice with a regulatory component specifically deleted in T_{Reg} cells[33] have only recently been examined.

It almost goes without saying that although defining the T_{Reg}-cell mode of action is of great academic importance, it is also essential to develop effective approaches for the clinical manipulation of T_{Reg} cells. Given the capacity of T_{Reg} cells to control inflammation and autoimmunity, and their role in blocking effective antitumour immunity and preventing sterilizing immunity, it seems probable that a clear understanding of how T_{Reg} cells work will present definitive opportunities for therapeutic intervention.

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DATABASES

Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene

CCR6 | CD25 | CD39 | CD73 | CTLA4 | *Ebi3* | FOXP3 | galectin-1 | granzyme A | granzyme B | IDO | IL-2 | IL-10 | IL-35 | IL-6 | LAG3 | neuropilin-1 | perforin | TGFβ | TRAIL

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