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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 anti-tumour 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 discuss 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 present the hypothesis that effector T cells may not be 'innocent' parties in this suppressive process and might in fact potentiate T_{reg}-cell function.

Several sophisticated regulatory mechanisms are used to maintain immune homeostasis, prevent autoimmunity and moderate inflammation induced by pathogens and environmental insults. Chief amongst these are regulatory T (T_{reg}) cells that are now widely regarded as the primary mediators of peripheral tolerance. Although T_{reg} cells play 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 anti-tumour immunity[7]. A seminal advance in the analysis of T_{reg} cells came with the identification of a key transcription factor, forkhead box P3 (FOXP3), that is required for their development, maintenance and function[8,9]. Mice and patients that lack FOXP3 develop a profound autoimmune-like lymphoproliferative disease that graphically emphasizes the importance of T_{reg} cells in maintaining peripheral tolerance[10-12] (BOX 1). Although FOXP3 has been proposed as the master regulator of T_{reg} cells that controls the expression of multiple genes that mediate their regulatory activity[13,14], this has been recently challenged raising the possibility that other transcriptional events may operate upstream of and/or concurrently with FOXP3 to mediate T_{reg}-cell development[15].

While Foxp3 has proven to be an invaluable marker for murine T_{reg} cells, its role in human T_{reg} cells is less straightforward (see BOX 2 for a discussion of T_{reg}-cell markers). Humans that lack FOXP3 develop immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a severe autoimmune disease that presents 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 as a significant percentage of human activated T cells express FOXP3 and yet do not possess regulatory activity[16-20]. Furthermore, induction of FOXP3 in human T cells by transforming growth factor-β (TGFβ) does not confer a regulatory phenotype, in contrast to their murine counterparts[20]. Consequently, FOXP3 is not a good 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 an additional cofactor/ transcription factor is an important question that needs to be resolved.

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Significant progress has been made over the last 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 stated, we focus here primarily on the mechanisms that are used by thymus-derived natural $CD4^+CD25^+$ $FOXP3^+$ 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 provide a number of potentially important therapeutic targets. Although this quest has been ongoing since interest in T_{reg} cells was reignited in 1995[23], there has been significant progress in the last few years. From a functional perspective, the various potential suppression mechanisms of 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 a mechanism of T_{reg}-cell-mediated suppression. There has also been significant interest in their ability to generate induced (also known as adaptive) T_{reg}-cell populations, either naturally *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, natural 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 contrasts with data from *in vivo* studies[29,30].

In allergy and asthma models, evidence suggests that both natural and antigen-specific T_{reg} cells control disease in a manner that is, in part, dependent on IL-10[29] and in some reports dependent on both IL-10 and TGFβ[31]. Adoptive transfer of allergen-specific T_{reg} cells induced significant IL-10 production by $CD4^+$ effector T cells in the lung following allergen challenge and this T_{reg}-cell-mediated control of disease was reversed by treatment with an IL-10-receptor-specific antibody[32]. However, suppression of allergic inflammation and airway hyper-reactivity, and increased production of IL-10 still occurred following transfer of IL-10-deficient T_{reg} cells, suggesting that T_{reg} cells can suppress the Th2-driven response to allergens *in vivo* through an IL-10-dependent mechanism, 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 hyperreactivity[33].

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

By contrast, several studies have shown that IL-10 production by T_{reg} cells is 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 anti-tumour immunity[42]. IL-10 produced by T_{reg} cells also appears to be crucial for IL-10-mediated tolerance in a model of hepatitis induced by concanavalin A[43] and tolerance to bacterial and viral superantigens[44]. In addition, recent 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[46]. Collectively, the picture that appears to be emerging is that the relative importance of T_{reg}-cell-derived IL-10 is very dependent on the target organism or disease and on the experimental system. Furthermore, 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 controlling inflammatory responses induced by pathogens or environmental insults[33].

While some early *in vitro* studies using neutralizing antibodies to TGFβ or T_{reg} cells lacking TGFβ[25,47] indicated that TGFβ was not required for natural T_{reg}-cell function, other studies, both *in vitro* and *in vivo* suggested a critical role for T_{reg}-cell surface bound TGFβ[48,49]. Therefore, the importance of TGFβ for natural 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 effector T-cell suppression. For instance, effector T cells that are resistant to TGFβ-mediated suppression cannot be controlled by T_{reg} cells in an IBD model[50]. In addition, TGFβ produced by 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 anti-tumour 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 anti-tumour 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 islet infiltration of CD8$^+$ T cells and delay the progress of diabetes through membrane-tethered TGFβ[49]. However, experiments using mice deficient in TGFβ-receptor (TGFβR) signalling in effector T cells or using TGFβ or TGFβR blocking reagents failed to show that membrane-tethered TGFβ is required for natural 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 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 heme 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]. Thus, in light of the most current data, it now appears that soluble and/or membrane-tethered TGFβ may have a previously unappreciated role in natural T_{reg}-cell function.

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 novel 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 murine Foxp3$^+$ T_{reg} cells[58,59], but not resting or active effector T cells, and are significantly upregulated in actively suppressing T_{reg} cells[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 and cure IBD *in vivo*. This precise phenocopy suggested that IL-35 is required for the maximal suppressive activity of T_{reg} cells. Importantly IL-35 was not only required but sufficient, 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 if 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 utilized in distinct pathogenic/homeostatic settings differs suggesting a non-overlapping function, which needs further refinement.

Suppression by cytolysis

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

By contrast, murine CD4$^+$ T cells are not cytolytic and therefore it was surprising that early gene expression arrays showed that granzyme B expression was up-regulated in murine T_{reg} cells[62,63]. Noelle and co-workers were the first to report that granzyme-B-deficient murine T_{reg} cells had reduced suppressive activity *in vitro*[64], but this T_{reg}-cell-induced apoptosis appeared to be perforin-independent. 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- and perforin-dependent manner[66]. In addition, Noelle and colleagues have unpublished data showing that effector T cells that overexpress a granzyme-B-specific inhibitor, Spi-6, are resistant to T_{reg}-cell-mediated suppression (Randolph Noelle, personal communication). Using a transplantation model in which T_{reg}-cell-mediated tolerance is induced by CD154–CD40 co-stimulatory blockade in conjunction with donor lymphocyte-specific transfusion, they have also shown that the T_{reg} cells that mediate this tolerance are also dependent on granzyme B for their suppressive activity.

Although the majority of research to date regarding T_{reg}-cell-induced cytolysis has been 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, it has been suggested that galectins can mediate cytolysis in a granzyme- and perforin-independent manner[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 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 *bone fide* T_{reg}-cell mechanism[70,71], a recent study has reignited 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 suggests that IL-2 depletion alone is not required for T_{reg} cells to suppress effector T cells[73], more work is clearly required 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 activating the adenosine A2A receptor[74-76]. Interestingly, binding of adenosine to the A2A receptor appears to not only inhibit effector T-cell functions but also to enhance the generation of adaptive 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 toleragenic phenotype (Peter Ernst, personal communication). Although TGFβ induces Foxp3 and T_{reg}-cell differentiation, IL-6 inhibits the generation of T_{reg} cells and promotes generation of pro-inflammatory Th17 cell development[78]. Thus, inhibiting IL-6 has important implications in T_{reg}-cell maintenance. T_{reg} cells were also shown to suppress effector T-cell function directly by transferring the potent inhibitory second messenger cyclic AMP into effector T cells via membrane gap junctions[79]. Although these mechanisms represent interesting additions to the T_{reg}-cell arsenal, further studies will be required to corroborate these exciting findings and assess their relative use by T_{reg} cells.

Suppression by targeting dendritic cells

In addition to directly affecting effector T-cell function, T_{reg} cells might modulate the maturation and/or function of dendritic cells (DCs) required for effector T-cell activation. This has long been considered an attractive idea but there has been only limited data in support[80]. However, intravital microscopy has revealed direct interactions between T_{reg} cells and DCs *in vivo*, which was proposed to attenuate effector T-cell activation by DCs[81,82]. So in what way might DCs be used as a conduit for T_{reg}-cell-mediated suppression? Some time ago, cytotoxic T-lymphocyte antigen 4 (CTLA4) was shown to be constitutively expressed by T_{reg} cells[25,83], and by using either CTLA4-specific blocking antibodies or CTLA4-deficient T_{reg} cells it was shown 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, through a mechanism dependent on interactions between CTLA4 and CD80 and/or CD86, to express indoleamine 2,3-dioxygenase (IDO), which is a potent regulatory molecule that induces the catabolism of tryptophan into pro-apoptotic metabolites that results in the suppression of effector T cells[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 reported that T_{reg} cells could downregulate the expression of the co-stimulatory molecules CD80 and CD86 on 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 also modulate the function of monocytes and macrophages[93,94]. Although the precise mechanism by which this is orchestrated remains elusive, 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 SH2-domain-containing protein tyrosine phosphatase 1 (SHP1), which suppresses DC maturation and 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 attractive, 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; CJW and DAAV, unpublished observations). Furthermore, it has recently been shown that neuropilin-1 (Nrp-1) promotes prolonged interactions with T_{reg} cells and immature DCs[99]. Given that Nrp-1 is differentially expressed on T_{reg} cells, this may give them an advantage over naïve T cells in modulating DC function.

Lastly, 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 allograph tolerance[100].

Complicating issues

Current dogma dictates that a hallmark of T_{reg} cells is their dependence on direct contact to mediate their inhibitory activity. This has been upheld by *in vitro* experiments where T_{reg} cells are unable to suppress effector T cell proliferation when the two populations are separated by a permeable membrane[25,26]. These data led to the notion that T_{reg}-cell-mediated suppression is contact-dependent. 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 interleukin-2 (IL-2)[101]. Therefore, the dilution effect of diffusion across the well may 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 away. For instance, adenosine has a half-life of less than ten seconds.

Second, it is not yet clear how much of the regulatory potency of T_{reg} cells is directed towards DCs/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]. 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 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.

One, a single, overriding suppressive mechanism is required by all T_{reg} cells

Until the entire mechanistic panoply of T_{reg} cells is defined, one cannot completely rule out this possibility. However, this possibility would seem unlikely as none of the molecules and/or mechanisms that have been defined to date, when blocked or deleted, result in the complete absence of regulatory activity — 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 significantly reduced suppressor function, a scurfy 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.

Two, 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 where there are multiple mechanisms that can be used by T_{reg} cells but they are non-redundant, 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 which results in a complete loss of T_{reg}-cell activity. For this to be truly non-redundant, this list would probably be restricted and small (2–4 genes).

Three, 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 sizable arsenal may be required at the height of an effector T-cell attack. 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, as for 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 amongst T_{reg} cells. For instance, it has recently been shown that a small sub-population of T_{reg} cells express the chemokine receptor CCR6, which is associated with T cells possessing an effector-memory phenotype[102]. $CCR6^+$ T_{reg} cells appeared to accumulate in the central nervous systems of mice with experimental autoimmune encephalomyelitis (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-DR positive T_{reg} cells were found to be more suppressive than their DR negative counterparts. One might speculate that their enhanced inhibitory activity is due to 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 possess many mechanisms that could be used but only one or two that are really crucial and consistently important in a variety of regulatory settings. Second, a 'contextual' model where different mechanisms become more or less important depending on the background or context in which the T_{reg} cells reside and the type of target cell that they have to repress. For example, some cell types may be inhibited primarily by cytokines, whereas others are most effectively suppressed through lysis by T_{reg} cells. 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 from the control of inflammatory events at mucosal interfaces such as the lungs and colon. As a clear picture of the available T_{reg}-cell weaponry emerges, an important challenge will be to determine their relative importance and contribution to T_{reg}-cell function in different disease models.

A hypothesis: effector T cells potentiate T_{reg}-cell function?

Most cellular interactions within 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, to date the general perception is that T_{reg} cells suppress and effector T cells capitulate. We hypothesize 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 and 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 co-cultured with effector T cells, supporting the idea that effector T cells may provide signals which 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 hypothesize 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). While 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.

Concluding remarks

Although significant progress has been made over the last 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? What is clear is that the transcriptional landscape of T_{reg} cells is very different from naive or activated effector T cells. There are

literally thousands of genes that are upregulated (or downregulated) in T_{reg} cells compared with effector T cells. Although it seems unlikely that all or many of these will be crucial 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 perform key T_{reg}-cell functions, such as T_{reg}-cell homing and homeostasis, which are likely to indirectly influence their suppressive capacity *in vivo* but don't directly contribute 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? An important 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 labs using distinct experimental systems and thus none have really been compared in side-by-side experiments. Furthermore, only recently have conditional mutant mice been examined that have a regulatory component specifically deleted in T_{reg} cells[33].

It almost goes without saying that although defining the T_{reg}-cell mode of action is of great academic importance, it is also essential in order 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 implication in blocking effective anti-tumour 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.

Box 1

Scurfy mice: misplaced mechanistic expectations?

Mice that carry a spontaneous loss-of-function mutation (known as *Scurfy* mice) or a deletion of *Foxp3* develop a fatal autoimmune-like disease with hyperresponsive $CD4^+$ T cells[9,12]. More recently Foxp3:diptheria toxin receptor (DTR) knockin mice have allowed for the selective depletion of T_{reg} cells following DT treatment[105]. These mice have been invaluable for dissecting the role of Foxp3 in T_{reg}-cell function. Given the profound phenotype in these mice, there is a general expectation that genetic disruption of any key T_{reg}-cell inhibitory molecule or 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 DT-mediated T_{reg}-cell depletion where Foxp3 may still serve to prevent expression of proinflammatory cytokines[105]. Nonetheless, this has lead 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. This may not necessarily be correct. Indeed, it is possible that no mouse lacking a T_{reg}-cell inhibitory effector molecule will ever be generated that develops a profound, spontaneous autoimmune disease[21]. It should be noted that mutant mice that are *Helicobacter* spp. and/or *Citrobacter rodentium* positive may have an exacerbated phenotype, as several studies have shown that opportunistic enteric bacteria can significant exacerbate gut pathology[4]. Ultimately, the occurrence of disease in knockout mice will depend on whether T_{reg} cells rely on a 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.

Box 2

T_{reg}-cell markers

Identifying discriminatory cell surface markers for the characterization and isolation of T_{reg} cells has always been a critical goal. Although excellent markers exist for murine T_{reg} cells, this goal has remained elusive for human T_{reg} cells. Traditionally, murine and human T_{reg} cells have been characterized as $CD4^+CD25^+$ (also known as interleukin-2 receptor α (IL-2Rα)). Indeed, murine 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 up-regulate CD25 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 the mouse[8], many activated (non-regulatory) human T cells 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 by Zhao and colleagues[107]). For instance, it was shown that the expression of CD127 (also known as IL-7R) is down-regulated on 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 (FR4) 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 CCR6[102,103]. However, the general use of both markers remains to be fully established so it is quite probable that the search for better T_{reg}-cell markers will continue for some time.

Box 3

Induced or adaptive T_{reg} cells: development and mode of action

Naturally occurring $FOXP3^+CD4^+CD25^+$ 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 as a possible therapeutic[29,113,114]. For instance, T regulatory 1 cells (Tr1) and T helper 3 cells (Th3) 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 suppressor of cytokine signalling 3 $(Socs3)^{-/-}$ DCs[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 in natural T_{reg} cells[124], a process that can be blocked by IL-4 or interferon-γ (IFNγ)[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 adaptive T_{reg} cells may not necessarily be restricted to suppressive cytokines. Indeed, human adaptive 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 natural 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 natural T_{reg} cells versus induced T_{reg} cells, and to determine whether inhibitory molecules, such as IL-10 or TGFβ, were derived from the former or the latter (or elsewhere).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

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 production of self-reactive antibodies by B cells and inhibition of self-reactive effector cells,

such as cytotoxic T lymphocytes. Regulatory T (T_{reg}) cells constitute one mechanism of peripheral tolerance.

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 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 affact any part of the gastrointestinal tract but usually desends from the terminal ileum, and ulcerative colitis (UC), which mainly affects the colon. In the mouse model, most of the inflammation is confined to the large intestines. The target antigen for the pathogenic T cells is unknown.

Airway hyper-reactivity, Initiated by exposure to a defined stimulus that is usually tolerated by normal individuals and that causes bronchoconstriction and inflammatory-cell infiltration in allergic individuals.

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

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

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

Ectoenzymes, An enzyme that is outside the cell memebrane and thus can cleave extracellular substratetes. These are typically teathered to the outside of the cell by a transmembrane domain.

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.

Granzymes, A family of serine proteinases that are found primarily in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells. They enter target cells through perforin pores, then cleave and activate intracellular caspases and lead to 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.

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

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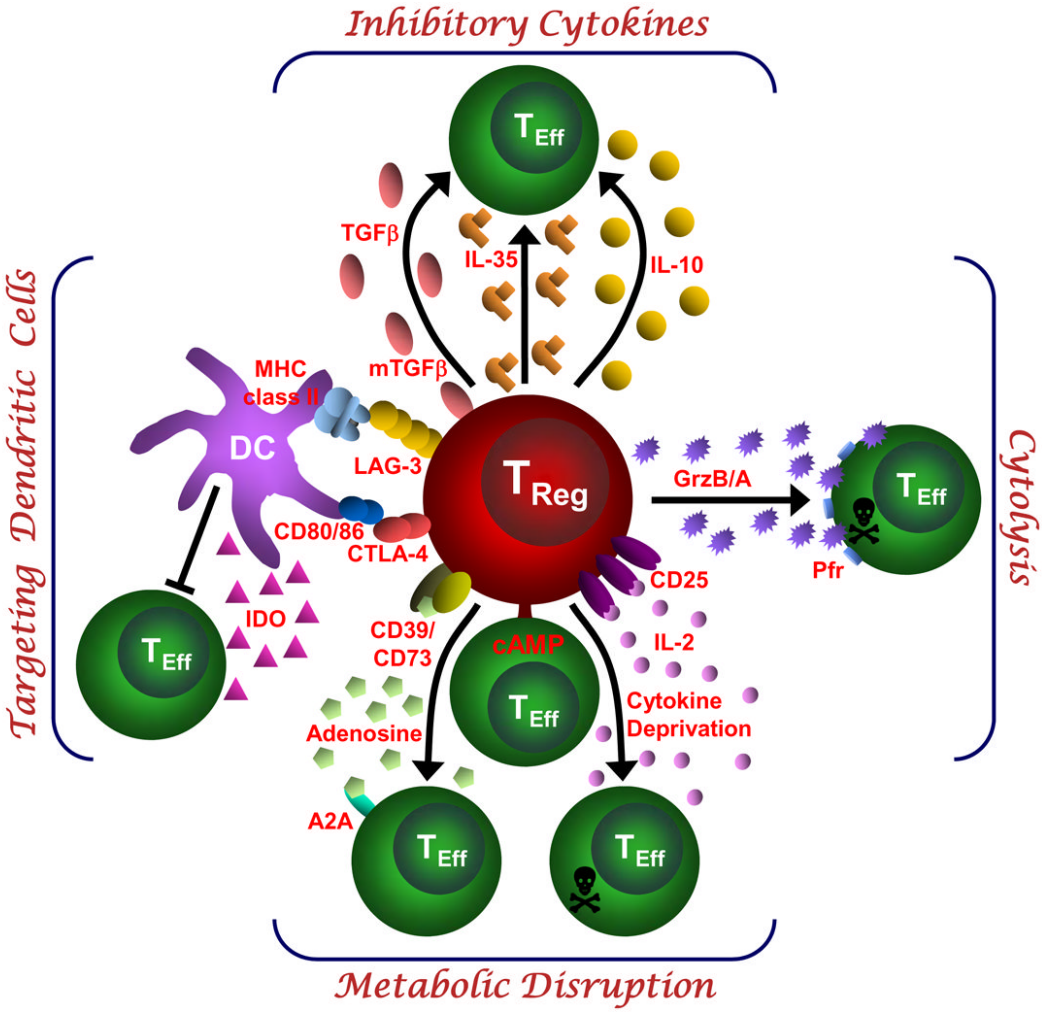

Figure 1. Basic mechanisms used by T_{reg} cells

This schematic depicts the various regulatory T (T_{reg})-cell mechanisms arranged into four groups centred around four basic modes of action. 'Inhibitory cytokines' include interleukin-10 (IL-10), interleukin-35 (IL-35) and transforming growth factor-β (TGF-β). 'Cytolysis' includes granzyme-A- and granzyme-B-dependent and perforin-dependent killing mechanisms. 'Metabolic disruption' includes high affinity IL-2 receptor α (CD25)-dependent cytokine-deprivation-mediated apoptosis, cyclic AMP (cAMP)-mediated inhibition, and CD39- and/or CD73-generated, adenosine–purinergic adenosine receptor (A2A)-mediated immunosuppression. 'Targeting dendritic cells' 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, by DCs.

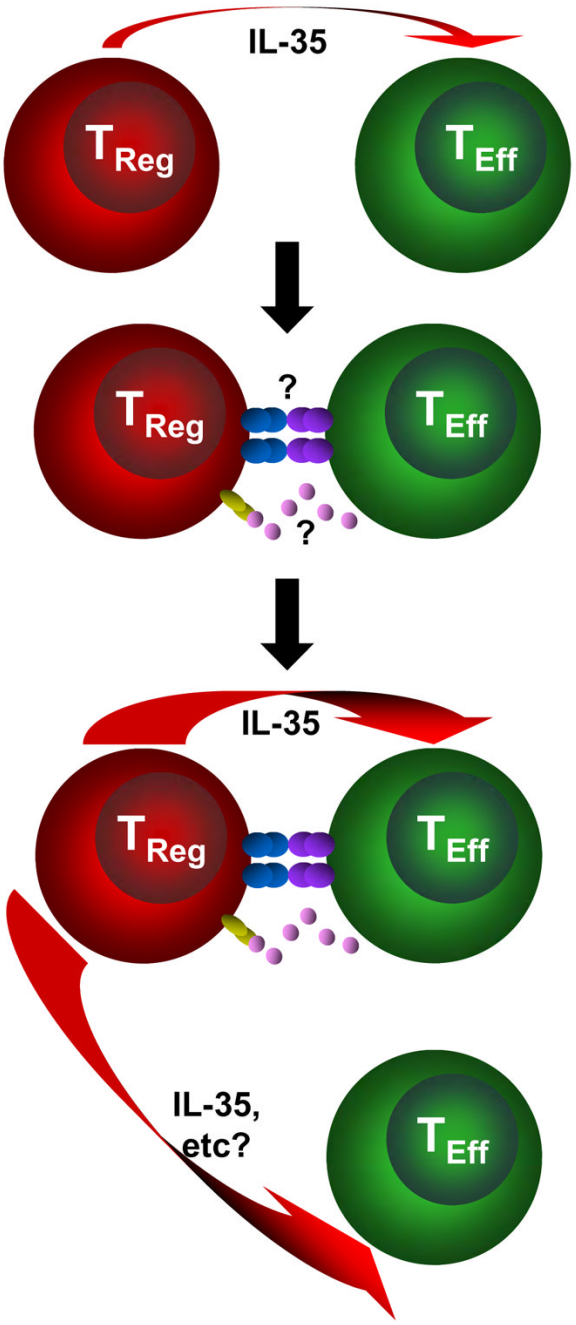

Figure 2. Model for how effector T cells might boost T_{reg}-cell function

This occurs in three stages. (a) Initial regulatory T (T_{reg})-cell activation induces production of regulatory factors such as interleukin-35 (IL-35). (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.