
CD4⁺CD25⁺ regulatory T cells:

I. Phenotype and physiology

Review article

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Holm TL, Nielsen J, Claesson MH. CD4⁺CD25⁺ regulatory T cells: I. Phenotype and physiology. APMIS 2004;112:629–41.

The immune system protects us against foreign pathogens. However, if fine discrimination between self and non-self is not carried out properly, immunological attacks against self may be launched leading to autoimmune diseases, estimated to afflict up to 5% of the population. During the last decade it has become increasingly clear that regulatory CD4⁺CD25⁺ T cells (T_{reg} cells) play an important role in the maintenance of immunological self-tolerance, and that this cell subset exerts its function by suppressing the proliferation or function of autoreactive T cells. Based on human and murine observations, this review presents a characterization of the phenotype and functions of the Treg cells *in vitro* and *in vivo*. An overview of the surface molecules associated with and the cytokines produced by the Treg cells is given and the origin, activation requirements and mode of action of the Treg cells are discussed. Finally, we address the possibility that Treg cells may play a central role in immune homeostasis, regulating not only autoimmune responses, but also immune responses toward foreign antigens.

Key words: T_{reg} cells; T cells; review.

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The immune system employs several strategies to maintain its own homeostatic balance. The thymus plays an important role in this regulation, because of its particular role in clonal deletion of self-reactive T cells, but other mechanisms preventing immunological self attacks are also involved in the maintenance of self tolerance. These latter mechanisms occur in the periphery and include T cell anergy, T cell ignorance and the effects of regulatory cells. Regulatory T cells can be divided into two groups, those that are anergic and require cell-cell contact for their function, and those, including Th1

and Th3 cells, functioning by the secretion of inhibitory cytokines (reviewed in 1).

In this review we will focus on CD4⁺CD25⁺ T cells (T_{reg} cells), which are characterized by their constitutive expression of CD4 and CD25. In the early 1970s it was discovered that some T cells are able to suppress the development of autoimmune diseases (2, 3). Unfortunately, this research was almost abandoned because of the failure to find and clone cells or factors mediating the suppression (4). Subsequently, a new marker for the regulatory T cells was discovered (5): CD4⁺ T cells with constitutive expression of the interleukin (IL)-2 receptor α -chain (CD25) were shown to mediate suppression of autoimmune diseases in mice.

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Recently, several groups have reported that CD4⁺CD25⁺ T cells functionally similar to their murine counterpart, T_{reg} cells, can be identified in humans. Human T_{reg} cells have been found in the thymus (6), in peripheral blood (6–12), in lymphoid organs (tonsils and spleen) (7) and in umbilical venous blood (12). The human studies carried out so far show many similarities to murine *in vitro* studies, indicating that similar mechanisms are involved in both man and mice. Scientists working with autoimmune diseases, transplantations and cancer are looking into the mechanisms controlling suppression, hoping that modulation of T_{reg} cells might be applied in future treatments of these conditions. In this review we will primarily concentrate on the human T_{reg} cells and focus on their surface and cytokine profile, activation requirements, interplay with other cells and mechanisms of suppression *in vitro*. Finally, we will also mention similarities and differences compared to murine studies.

FUNCTIONAL STUDIES OF T_{reg} CELLS IN VITRO

Obtaining the T_{reg} cells

In mouse and man the CD4⁺CD25⁺ T cell population comprises 5–10% of all peripheral CD4⁺ T cells (5–10). In human peripheral blood mononuclear cells (PBMC) the CD4⁺CD25⁺ T cell population can be further divided into CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells (11), demonstrating that the actual suppressor cells (CD25^{high}) represent only 1–2% of the total number of CD4⁺ T cells. Human peripheral blood CD4⁺CD25⁺ T cells were also divided into cells expressing or not expressing CD45RO (9). Only CD4⁺CD25⁺ CD45RO⁺ were found to possess suppressive properties. Despite this latter observation, the majority of studies dealing with regulatory CD4⁺ T cells, including those presented here, focus on the presence or absence of CD25 expressing CD4⁺ T cells.

Proliferation

To identify the responsiveness or proliferation of human CD4⁺CD25⁺ and CD4⁺CD25[−] T cells, monocultures of the two subpopulations have been investigated. T cells were activated

through their T-cell receptor (TCR) by anti-CD3 monoclonal antibody (mAb) and anti-CD28 mAb or by alloantigen-presenting cells (APC). Thereafter, proliferation was monitored in the different combinations of T cell subpopulations, through incorporation of [³H] thymidine.

Separated CD4⁺CD25[−] T cells showed a high potential for proliferation, whereas no proliferation was seen when CD4⁺CD25⁺ T cells were cultured alone. A similar pattern was obtained when the cells were stimulated repeatedly, indicating that the CD4⁺CD25⁺ T cell subset is anergic (7, 8, 12). Apoptosis and necrosis measured by annexin V and propidium iodide staining did not reveal cell death in the stimulated CD4⁺CD25⁺ T cells (8, 9). Proliferation of the CD4⁺CD25⁺ T cell subset could be induced by addition of high amounts of IL-2, IL-4, IL-15 to the antibody-exposed cell cultures (6–10, 12). The response to IL-2 is very likely mediated through the high-affinity IL-2 receptor complex CD25 CD122 CD132 (6). Exposure of CD4⁺CD25⁺ T cells to cross-linked anti-CD28 mAb also induces endogenous IL-2 production and proliferation of CD4⁺CD25⁺ T cells (10). In general, murine studies also show that addition of IL-2 together with anti-CD3 mAb can abolish the anergic state of CD4⁺CD25⁺ T cells (13). The lack of proliferation of anergic human and murine T_{reg} cells is probably due to their arrested cell cycle (9).

Suppression mediated by T_{reg} cells

Co-culture of activated and titrated numbers of human CD4⁺CD25⁺ T cells with CD4⁺ or CD8⁺ T cells resulted in a dose-dependent suppression of the proliferation of CD4⁺ or CD8⁺ T cells, and in a 1:1 mixture almost total inhibition occurred (6–8, 10–11). Limiting dilution assays with mixed lymphocyte cultures set up with human CD4⁺CD25⁺ T cells and CD4⁺CD25[−] T cells as responders, and irradiated allogeneic PBMCs as stimulator cells, demonstrated suppression at a clonal level. This suggests that only a minor subset of the CD4⁺CD25⁺ T cells possesses suppressive properties (12). This finding also supports other data. (9, 11) showing that not all CD4⁺CD25⁺ T cells express the functional activity of T_{reg} cells.

Importantly, both human and murine T_{reg}

cells need to be activated through their TCR to be suppressive (8, 13, 14). The diminished proliferation of murine CD4⁺CD25⁻ T cells in co-cultures with T_{reg} cells is apparently due to cell cycle arrest, since there is no evidence of cell death (13). It was also observed that activated T_{reg} cells do not become blast cells as they do not enter the cell cycle (15).

Suppression after proliferation

As stated above, T_{reg} cells are suppressive only when they become activated and they can proliferate if certain cytokines are added. It was therefore important to find out whether T_{reg} cells were still suppressive after a period of proliferation. T_{reg} cells induced to proliferate by anti-CD3 mAb and IL-2 were subsequently assayed for suppression of CD4⁺CD25⁻ T cells (10, 12). This experiment showed that proliferating CD4⁺CD25⁺ T cells are still capable of suppressing CD4⁺CD25⁻ T cells. These data are of importance in the light of clinical use, where *in vitro* expansion of the T_{reg} cells might well be necessary.

CYTOKINE PROFILE

Secretion of cytokines by freshly isolated and activated T_{reg} cells

Initially, no inhibitory cytokines were found in the culture supernatants of freshly isolated T_{reg} cells (6). In culture supernatants from T_{reg} cells activated through allogeneic APCs or anti-CD3 mAb, IL-2 and IL-4 were absent and only low levels of IFN- γ , TGF- β and IL-10 were detected (Table 1). In contrast, activated CD4⁺CD25⁻ T cells secreted IL-2, IL-10 (low to medium), IFN- γ and low levels of TGF- β (9, 10) (Table 1).

With respect to *in vitro* studies in mice the data are conflicting. In some agreement with the human data, it was found that anti-CD3 mAb-

stimulated mouse T_{reg} cells secreted low levels of IL-10, whereas IL-2, IL-4, TGF- β and IFN- γ could not be detected (13, 14). In general it can be seen that T_{reg} cells do not secrete IL-2. DNA microarray experiments in mice showed that three molecules involved in suppression of cytokine signalling (SOCS) were highly induced in activated T_{reg} cells (16). This might explain why T_{reg} cells do not make enough IL-2 to induce their own proliferation in an autocrine fashion.

The mRNA levels in activated cells

The mRNA levels of cytokine production in allostimulated human T_{reg} cell populations were investigated using the reverse transcriptase-polymerase chain reaction (RT-PCR). The results showed that only IL-10 and TGF- β mRNA were detectable in both activated and nonactivated T_{reg} cells. In murine T_{reg} cells, IL-10 mRNA increased after activation of the cells (13). In contrast, activation of human CD4⁺CD25⁻ T cells showed detectable mRNA levels of a number of cytokines, including IL-2, IL-4, IL-10, IFN- γ and TGF- β (9).

Regulation of secretion in co-cultures

IFN- γ and especially IL-2 might be target cytokines for the suppression induced by T_{reg} cells. Thus, the synthesis of these two cytokines by human CD4⁺CD25⁻ T cells was markedly decreased in co-cultures of APC-activated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells (6, 9, 10). Baecher-Allan et al. (11) have also shown that IFN- γ might be linked to suppression. Measurements of IFN- γ and IL-2 mRNA were carried out in co-cultures having different ratios of anti-CD3 mAb-exposed CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. The results showed a dose-dependent inhibition of IFN- γ secretion and IL-2 mRNA expression. It is thus very likely that there is a connection between the suppression and the inhibition of production of IL-2 and IFN- γ in co-cultures. Murine studies

TABLE 1. Cytokine production by human CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells. The results are collected from seven different studies to establish a general profile (6–12)

Stimulation	Cell type	IL-2	IL-4	IL-10	IFN- γ	TGF- β
APC	25 ⁺	Negative	Negative	Low/Medium	Low	Low
APC	25 ⁻	High/Medium	Negative	Low/Medium	High	Low
pICD3/CD28	25 ⁺	Negative	Negative	Low/Medium	Low	Low
pICD3/CD28	25 ⁻	High	Low	Low/Medium	High	Medium

have also clearly demonstrated that anti-CD3 mAb-stimulated CD4⁺CD25⁺ T cells inhibit IL-2 production by CD4⁺CD25⁻ T cells (13). Finally, the IL-10 concentration is not downregulated to the same extent as IL-2 and IFN- γ (6).

Cytokines and suppression

The lack of IL-2 in the co-culture systems will most likely influence the proliferation. Several groups have used anti-IL-4 mAb, anti-IL10 mAb, anti-TGF- β mAb or combinations of these antibodies in an attempt to block the suppression mediated by human T_{reg} cells (6, 7, 9, 10, 12). None of these antibodies, alone or in combination, was capable of blocking suppression. Neither could an effect be observed in co-culture studies with murine T_{reg} cells. Furthermore, supernatants from co-cultures could not induce suppression when added to cultures of anti-CD3-activated CD4⁺CD25⁻ T cells (14). However, there exists no common agreement in this area concerning a possible role of TGF- β and IL-10 (see below).

The suppression mediated by Treg cells is contact-dependent

In general the above *in vitro* experiments have not found a primary role for cytokines in the induction of the suppression mediated by T_{reg} cells, and the lack of suppression when the target and the T_{reg} cells are separated in transwell systems has excluded cytokines as effector molecules in suppression. Thus, activated human or mouse T_{reg} cells, separated from activated CD4⁺CD25⁻ T cells by a semipermeable membrane, did not suppress proliferation of the latter cells. These experiments demonstrated that suppression is not mediated by a soluble factor (6–9, 12, 13).

T_{reg} CELL SURFACE PHENOTYPE

Surface profile of T_{reg} cells

FACS analyses have shown that T_{reg} cells express high surface levels of CD25, HLA-DR, CD122 (IL-2 receptor γ -chain), CD45RO, and intracellular cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Table 2). The surface phenotype is in many ways similar to that of memory CD4⁺ T cells. Also, the short telomere length measured in T_{reg} cells is comparable to

the length in CD4⁺ memory T cells (17). The difference is that T_{reg} cells, in contrast to memory T cells, express CD25 and CTLA-4 constitutively.

When comparing surface profiles of human T_{reg} cells and CD4⁺CD25⁻ T cells after activation through the TCR, differences can also be seen, although they are less distinct (Table 2). After a short period of activation, high levels of surface CTLA-4 will be expressed by T_{reg} cells but not by CD4⁺CD25⁻ T cells. However, activated CD4⁺CD25⁻ T cells increase their expression of intracellular CTLA-4, surface HLA-DR, surface CD122 and surface CD45RO, and express 10-fold higher levels of CD25, HLA-DR and CD122 compared with T_{reg} cells (8). Furthermore, FACS analyses have not shown any difference between CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells regarding expression of CD28, CD62L, CD69, CD95 and CD154 (CD40L) (8).

In general, it is difficult to compare data obtained with different activation protocols. Some groups have observed differences between CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T cells in their expression of CD38, CD62L, CD69, CD 95 and CD154, but there is no general trend. In mice, expression of CD5, CD25, CD38, CD45RB^{low} (similar to CD45RO⁺) and CD62L is associated with the suppressor phenotype (reviewed in 18). Thus, in many ways human and mouse T_{reg} cells express similar surface phenotypes.

CTLA-4 and suppression

The function of the CTLA-4 receptor is of considerable interest on account of its properties in negative regulation of activation of mouse T cells *in vivo* (19). CTLA-4, which is a receptor for APC-B7, is expressed following T cell activation. Upon binding of B7, CTLA-4 negatively regulates the IL-2 production of the newly activated T cell and inhibits further T cell proliferation. Interestingly, CTLA-4 has also been shown to play a role in induction of TGF- β secretion by murine CD4⁺ T cells (20). However, anti-CTLA-4 mAb added to co-cultures of human CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells did not block suppression (9, 10). Thus, *in vitro* experiments with T lymphocytes from human peripheral blood have not been able to show any role for CTLA-4 in T_{reg} cell function. Meanwhile, murine *in vitro* studies have shown

TABLE 2. Surface profile of human CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, both activated and non-activated. The results are collected from six different studies to make a general profile (6–11)

Marker	Naïve (freshly isolated) CD4 ⁺ CD25 ⁺	Naïve (freshly isolated) CD4 ⁺ CD25 ⁻	Activated (pICD3/CD28) CD4 ⁺ CD25 ⁺	Activated (pICD3/CD28) CD4 ⁺ CD25 ⁻
Int.* CTLA-4	High	Low	High	High
Surface CTLA-4	Low	Low	High	Low
CD45RO	High	Low	High	Low/medium
CD45RA	Low	High	Low	Medium
HLA-DR	High	Low	High	High/medium
CD25	High	Low	High	High
CD122	High	Low	High	High
CD134 (OX40)	High	No data	No data	No data
LFA-3	Medium	No data	No data	No data

* Intracellular.

conflicting results in this respect. Thus, anti-CTLA-4 mAb was shown to block suppression by murine T_{reg} cells (14, 21), an observation which was not confirmed by another group of workers (13). Taken together, there appears to be no role for CTLA-4 in suppression mediated by T_{reg} cells derived from human PBMC, whereas the picture in mice is unclear.

CD25 and suppression

Several groups have shown that addition of IL-2 and anti-CD3 mAb can to some extent abrogate suppression by human and murine T_{reg} cells (7, 11–14). Thornton & Shevach (13) originally suggested the CD25⁺ population was exerting its inhibitory effect by binding IL-2 and functioning as an “IL-2 sink”, a hypothesis contradicted by the transwell experiment mentioned above. As CD25 is also expressed transiently on newly activated CD4⁺ T cells, it is important to confirm that the suppressive properties are restricted to the T cells that constitutively express CD25. Two groups (7, 10) have demonstrated that CD25⁺ T cells derived from activated CD4⁺CD25⁻ T cells never gained suppressor functions. This was also shown for mouse cells (13). Thus, the suppressive properties have to be special to cells constitutively expressing CD25. However, these data do not rule out the possibility that CD25 has a smaller and less direct role in suppression. This idea is supported by *in vivo* studies showing that mice deficient for IL-2, CD122 and CD25 develop autoimmune diseases (22–24). Furthermore, it was shown that T_{reg} cell-mediated suppression of CD8⁺ T cells cannot be abrogated

by the addition of IL-2. It was therefore suggested that T_{reg} cells inhibit the CD8⁺ T cell by blocking the IL-2 production and additionally by lowering CD8⁺ T cell responsiveness to exogenous IL-2 (25).

HLA-DR and suppression

HLA-DR is – as earlier mentioned – upregulated in human T_{reg} cells. It was therefore speculated (11) that T_{reg} cells present a not yet identified molecule associated with their HLA-DR which is recognized by CD4⁺CD25⁻ T cells. This would render the CD4⁺CD25⁻ T cells anergic because of lack of co-stimulation. However, there have been no data in support of this idea.

CANDIDATE SUPPRESSOR MOLECULES

GITR, PD-1, Notch

Studies of surface molecules which can abrogate cell contact-dependent suppression have pointed out some promising candidates, such as GITR (11), PD-1 (26) and Notch (12, 27, 28).

Glucocorticoid-induced tumor necrosis factor receptor (GITR) is a possible target molecule in contact-dependent suppression. In mice it was shown that high expression of GITR is restricted to CD4⁺CD25⁺ T cells in the non-activated state (29). In addition, *in vitro* studies with murine cells have shown that exposure to anti-GITR mAb abrogates suppression in co-cultures of CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells. Ligation of GITR leads to enhanced levels of NFκB in T_{reg} cells (29), prob-

ably resulting in modulation of suppressive signals and counteracting suppression. Recently, blocking of GITR on human T_{reg} cells was shown to diminish suppression in co-cultures of T_{reg} and CD4⁺CD25⁻ T cells, although to a lesser degree than in the murine experiments (30). Other groups working with mice have also found suppressor effects associated with GITR. McHugh *et al.* (16), who employed DNA microarrays to compare activated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, found that GITR is upregulated in CD4⁺CD25⁺ T cells at both the mRNA and the protein level. Furthermore, antibodies against a number of surface molecules, including GITR, CD103, CTLA-4, 4-1BB, OX40 and CD2, showed that only anti-GITR polyclonal antibody was able to reverse suppression. Interestingly, abrogation of suppression was only seen when anti-GITR polyclonal antibody was added prior to activation, indicating that GITR affects the activation process of CD4⁺CD25⁺ T cells. GITR may act as a co-stimulatory molecule for TCR stimulation (29) and as such modulate cell activation. Interestingly, GITR has also been shown to inhibit apoptosis after ligation of TCR (31).

The PD-1 molecules expressed by T_{reg} cells

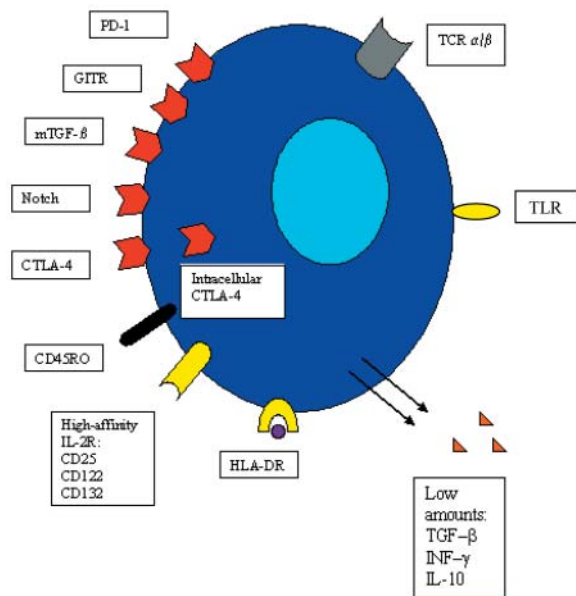


Fig. 1. Phenotype of the activated T_{reg} cell. The red and yellow surface molecules are the candidate players involved in T-cell/T-cell suppression, and the orange triangles are the cytokines produced.

are critical for the regulation of peripheral tolerance and autoimmunity. Thus, PD-1-deficient mice develop lupus-like glomerulonephritis, arthritis and autoimmune-dilated cardiomyopathy (26). In mice, activation of the Notch pathway in T cells by antigen-presenting cells over-expressing Notch ligands favours differentiation of T_{reg} cells responsible for antigen-specific tolerance (27). In streptozotocin-induced autoimmune diabetes, Notch transgenic mice fail to develop disease and this is associated with an increase of T_{reg} cells, accumulating in lymphoid organs, in pancreas infiltrates, and paralleled by increased expression of IL-4 and IL-10. Accordingly, CD4(+) T cells from Notch3-transgenic mice inhibit the development of hyperglycemia and insulinitis when injected into streptozotocin-treated wild-type mice and display *in vitro* suppressive activity (27). These observations therefore suggest that Notch3-expression regulates the expansion and function of T regulatory cells, leading to protection from experimental autoimmune diabetes.

TGF-β and CD4⁺CD25⁺ regulatory thymocytes

CD4⁺CD25⁺ purified human thymocytes exhibit suppressive properties similar to those of peripheral T_{reg} cells (32). Following activation, thymic T_{reg} cells express both CTLA-4 and membrane-bound TGF-β antibodies to these molecules abrogated suppression.

The role of membrane-bound TGF-β and CTLA-4 of CD4⁺CD25⁺ human thymocytes is supported by some murine experiments *in vitro*. Thus, activated CD4⁺CD25⁺ T cells express membrane-bound TGF-β and the presence of anti-TGF-β mAb blocks cell contact-dependent suppression (33). In contrast, recent studies on human cells (34) have shown that membrane-bound TGF-β is downregulated on activated T_{reg} cells, and no abrogation of suppression was found in the presence of anti-TGF-β mAb, indicating that membrane-bound TGF-β has no role in cell contact-dependent suppression by human T_{reg} cells. The role of TGF-β in T_{reg} cell-mediated suppression is, however, still unclear since mice transgenic for a dominant-negative form of the TGF-β receptor, and unable to respond to TGF-β derived signals, are fully susceptible to the suppression mediated by T_{reg} cells (35). Taken together, the data concerning

the role of TGF- β point in different directions both *in vitro* and *in vivo*.

Fig. 1 is based on the data discussed above and illustrates the phenotype of activated T_{reg} cells with regard to surface molecules and cytokine secretion.

T_{reg} CELL ACTIVATION

Antigen specificity

Experiments with transgenic mice have provided convincing evidence that T_{reg} cells need to be activated through their TCR to gain functional activity. Thus, T_{reg} cells from wild-type mice cannot suppress TCR transgenic CD4⁺CD25⁻ T cells in co-cultures if only the antigen for the transgenic TCR is added (14). As mentioned above, activation is also needed for human T_{reg} cells to be suppressive (8). To show that the CD4⁺CD25⁺ T cells need a specific Ag for activation Takahashi et al. (14) have used T_{reg} cells from TCR transgenic mice specific for the OVA-antigen. The experiment showed that when OVA-peptide was added to co-cultures of transgenic CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, suppression clearly occurred. In contrast, no suppression was seen when the T_{reg} cells from non-transgenic BALB mice were co-cultured with CD4⁺CD25⁻ T cells from the transgenic mice and OVA-peptide was added. When anti-CD3 mAb was added, both transgenic and non-transgenic T_{reg} cells were able to mediate suppression.

Activation through foreign and self-antigens

A number of experiments have tested whether T_{reg} cells can be activated by self and foreign Ag. Taams et al. (17) stimulated human peripheral blood-derived CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells with irradiated autologous PBMCs and different Ags: heat shock proteins (=self Ag), tetanus toxin (=foreign Ag) and cows' milk proteins (=dietary Ag). In all the experiments CD4⁺CD25⁺ T cells were activated and suppressed the proliferation of CD4⁺CD25⁻ T cells, indicating that human CD4⁺CD25⁺ T cells can be activated and suppress proliferation induced by self Ag as well as by foreign Ag. It was speculated whether CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells express comparable and oligoclonally distributed TCR- $\alpha\beta$

repertoires. However, as demonstrated by PCR analyses, the TCR- $\alpha\beta$ repertoires of T_{reg} cells and the responding T cells are highly diverse both in mouse and man (14, 17).

Different activation requirements for T cell subsets

Baecher-Allan et al. (28) have shown with human T_{reg} cells that the level at which the T cells are activated is of crucial importance. Experiments were set up where CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were activated for variable time periods using immobilized anti-CD3 mAb and soluble anti-CD3 mAb. Proliferation was measured in CD4⁺CD25^{high}, CD4⁺CD25⁻ T cells and in co-cultures. The results showed that a weak activation signal of CD4⁺CD25⁺ T cells led to the highest and longest lasting (60 h) suppressive activity, whereas a strong activation signal (immobilized anti-CD3 + anti-CD28 mAbs) of CD4⁺CD25⁺ T cells resulted in suppression which only lasted for a short period (15 h). The activation requirements for CD4⁺CD25⁻ T cells were quite different. When strongly activated, these cells became unresponsive to suppression, whereas they were responsive to suppression after a weak activation stimulus, but only for time periods lasting 38–60 h. Studies *in vivo* partly supported these observations by showing that the Ag concentration needed to activate T_{reg} cell-mediated suppression is lower than the Ag concentration needed to stimulate proliferation of CD4⁺CD25⁻ T cells (14).

T_{reg} CELLS AND APC

Suppression and APCs

It has been suggested that the suppression could be mediated through downregulation of co-stimulatory molecules on APCs or by competition for signal molecules on the APC (14).

Several groups have shown *in vitro* that suppression clearly is present without APCs. Thus, anti-CD3/CD28 mAb activated co-cultures of human T_{reg} cells and CD4⁺CD25⁻ T cells, the suppression being just as effective as in APC-activated co-cultures (9). In contrast, murine studies show conflicting results concerning the role of APCs in the mechanism of suppression. Takahashi et al. (14) showed that the addition of high numbers of APCs or CD86-transfected

APCs lowered the suppression when added to co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. However, when APCs were fixed with paraformaldehyde and then added in high numbers to co-cultures, they had no effect on the suppression. This latter observation indicates that the suppression is not mediated through alterations on the APC surface. It was therefore suggested that the role of APCs in suppression is to bring $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells together (14). In contrast to (14) other authors have shown that only smaller alterations in suppression are detectable when an excess of APCs was added to co-cultures (15). Piccirillo & Shevach (25) used peptide-MHC-tetramers to stimulate murine TCR-transgenic $CD8^+$ T cells and the activated $CD8^+$ T cells were then co-cultured with anti-CD3mAb-activated $CD4^+CD25^+$ T cells. A marked suppression of both proliferation and IFN- γ production was observed. The suppression of $CD8^+$ T cells was therefore concluded to be APC-independent. Taken together, suppression can occur *in vitro* without APCs. Meanwhile, this does not mean that APCs are passive bystanders. Especially *in vivo*, the cellular interactions and cytokine concentrations and requirements will be very different from the *in vitro* conditions.

SPECIFICITY OF SUPPRESSION

Specific or non-specific suppressor functions

Transgenic (Tg) mice have been used to find out whether the suppressor effector function is Ag specific or nonspecific (15). $CD4^+CD25^+$ T cells with a Tg TCR specific for a MHC-II/hemagglutinin (HA) peptide were co-cultured with $CD4^+CD25^-$ T cells having a Tg TCR specific for a MHC-II/pigeon cytochrome C (PCC) peptide complex. The results showed a clear inhibition of the $CD4^+CD25^-$ (PCC) T cell population when the two Ags were presented on different APCs. This proves that the suppression mediated by activated $CD4^+CD25^+$ T cells is Ag non-specific as also shown by other authors (14). Furthermore, the data show that suppression is not mediated through competition for Ags or costimulatory molecules on the same APC. Similarly, studies with human cells have shown that suppression is Ag non-specific (9).

DIRECT AND INDIRECT SUPPRESSION – A MODEL

Suppression through cytokines IL-10 and TGF- β

So far we have described experiments showing that suppression is mediated exclusively through cellular contact. However, recently a new hypothesis suggested that there might be both a primary cell-dependent and secondary secretion-dependent suppression (34, 36). The primary suppression is, as discussed above, mediated through cell-to-cell contact by T_{reg} cells and $CD4^+CD25^-$ T cells inducing anergy in the latter subset. The secondary suppression is mediated through the cytokines IL-10 and/or TGF- β secreted from the anergized $CD4^+CD25^-$ T cells, which thereby suppress other $CD4^+CD25^-$ T cells. The anergized $CD4^+CD25^-$ T cells in this model are termed Th_{sup} cells (Fig. 2). This model illustrates the phenomenon of “infectious tolerance” (34).

Two independent research groups have contributed to this new model by performing a number of very similar experiments on human T_{reg} cells. They have focused in particular on IL-10 and TGF- β , respectively (34, 36). They observed that markedly higher amounts of IL-10 (34) or TGF- β (34) were secreted in activated co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells as compared with monocultures of $CD4^+CD25^+$ T cells (36). After certain time periods, cells from the co-cultures were separated into T_{reg} cells and anergized Th_{sup} cells.

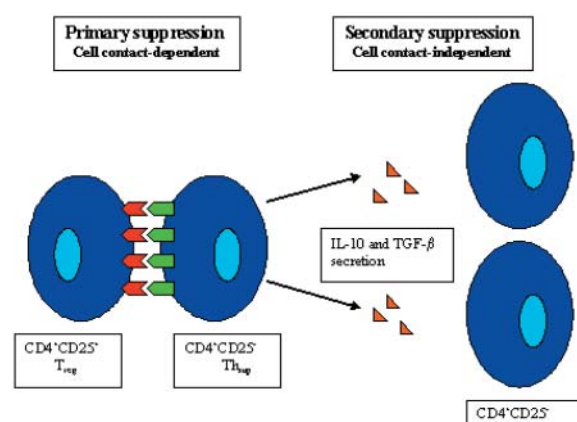


Fig. 2. Primary and secondary suppression. The model illustrates how T_{reg} cells through cell-cell contact induce $CD4^+CD25^-$ T cells to become Th_{sup} cells. The Th_{sup} cells then suppress other $CD4^+CD25^-$ T cells through secreted cytokines (32).

The experiments showed that both T_{reg} cells and Th_{sup} cells had the ability to mediate suppression of CD4⁺CD25[−] T cells. Further experiments with ELISA showed that both IL-10 and TGF-β (36) or only TGF-β (34) were secreted from the Th_{sup} cells. Unfortunately, the authors did not comment on the need for CD4⁺CD25[−] T activation in order for the cells to become Th_{sup} cells. Suppression in some of these experiments (36), but not in all of them (34), was abrogated when anti-IL-10 mAb was added to the activated Th_{sup}/CD4⁺CD25[−] T cell co-cultures. In contrast, T_{reg} cell inhibition by anti-IL-10 mAb was not seen in co-cultures of T_{reg} cells and CD4⁺CD25[−] T cells (36). Addition of anti-TGF-β mAb, however, could partly inhibit suppression (34). Thus, even though the results are conflicting, it appears that human Th_{sup} cells are capable of suppressing CD4⁺CD25[−] T cells through secretion of IL-10 and/or TGF-β.

To confirm that suppression mediated by the Th_{sup} cells is different from suppression mediated by T_{reg} cells, experiments were set up with T_{reg} cells and Th_{sup} cells, which were fixed with paraformaldehyde prior to or after reactivation. T_{reg} cells and Th_{sup} cells were then separately co-cultured with syngeneic activated CD4⁺CD25[−] T cells. The results showed that only the T_{reg} cells, which had been activated prior to fixation, were able to mediate suppression and no suppression was seen in co-cultures of fixed Th_{sup} cells and CD4⁺CD25[−] T cells (34, 36). Thus, human T_{reg} cells can mediate suppression through fixed surface molecules, whereas Th_{sup} cell function depends on secretion of cytokines, which in transwell culture systems can diffuse through a semipermeable membrane (34, 36).

Given the rather low proportion of CD4⁺CD25⁺ T cells and their dependency on cell contact to mediate suppression, it was speculated how these cells could regulate immune homeostasis *in vivo*. These new *in vitro* results dealing with infectious tolerance might help to explain how low numbers of T_{reg} cells through induction of Th_{sup} cells can induce high levels of suppression, including bystander suppression. In addition, IL-10 and/or TGF-β not only affect T cells but also influence the function of DCs, Langerhans cells, macrophages and other haematopoietic cells.

T_{reg} CELLS IN AUTOIMMUNE DISEASE

In vivo studies on the role of T_{reg} cells in autoimmune diseases have been carried out in different murine model systems. One of the most thoroughly investigated autoimmune diseases is autoimmune gastritis (AIG). Here the gastric parietal cells are destroyed by CD4⁺ T cells, which recognize the H⁺/K⁺-ATPase expressed by the parietal cells (35). Another example is chronic colitis resembling inflammatory bowel disease (IBD) in humans. This disease is T cell-mediated, and the antigens recognized by these cells are probably derived from the commensal enterobacterial flora, since germ-free mice do not develop colitis (37). It should be noted that IBD is not an autoimmune disease per se.

In general the *in vivo* studies of AIG confirm the *in vitro* observations that neither the cytokines IL-4, IL-10, IFN-γ and TGF-β nor CTLA-4 are necessary for the suppressive function of T_{reg} cells. IL-4 is not necessary for the prevention of colitis either. In contrast, the *in vivo* studies on T_{reg} cells and colitis indicate that IL-10, TGF-β and CTLA-4 are necessary for T_{reg} cell-mediated prevention of colitis. It is not known why there are these discrepancies in the *in vivo* results. It might reflect the fact that the disease-inducing antigenic load is so much higher and qualitatively more diverse in colitis than in AIG. Enterobacterial antigens also signal through Toll-like receptors (TLR), which have been found on murine T_{reg} cells, which selectively express TLR-4, -5, -7 and -8 (38). Signalling through TLRs might affect T_{reg} cells in such a way that they overexpress CTLA-4 and induce Th_{sup} cells secreting IL-10 and TGF-β. In our accompanying APMIS review: “CD4⁺CD25⁺ regulatory T cells: II. Origin, disease models and clinical aspects” the autoimmune and infectious mouse models will be described in detail.

A MODEL FOR T_{reg} CELL-MEDIATED SUPPRESSION

It is clear that T_{reg} cells are capable of regulating the proliferation of other T cells, but how are the T_{reg} cells themselves regulated by other cells? A unifying model should include both aspects.

T_{reg} cells in regulation of AIG and IBD

Above, a model involving primary and secondary suppression was described. This model was based on *in vitro* results. Here we will try to link this model to *in vivo* data. As mentioned previously, the model describes how T_{reg} cells through cell-cell contact induce CD4⁺CD25⁻ T cells to become Th_{sup} cells. The Th_{sup} cells then suppress other CD4⁺CD25⁻ T cells through secreted cytokines. *In vivo* it was clear that in some disease models (e.g. IBD) cytokines were needed for T_{reg} cells-mediated protection, whereas other models did not require inhibitory cytokines for protection (e.g. AIG). Thus, both Th_{sup} cells and T_{reg} cells might be involved in the first situation, whereas only the T_{reg} cells are involved in the latter situation. What determines this difference? We suggest that it is the strength of combined “signals” to the T_{reg} cells that determines the outcome. These “signals” would include the strength of the TCR stimulation, co-stimulatory signals and cytokine repertoires. It is noteworthy that the cytokine requirement was restricted to IBD, which involves the presence of bacteria. As suggested earlier, T_{reg} cells could be informed of the presence of bacteria or other foreign pathogens through TLRs. Indeed, exposure of murine T_{reg} cells to the TLR-4 ligand lipopolysaccharide (LPS) increases the suppressor activity of these cells 10-fold (38) and various activation markers were upregulated and their survival prolonged. Hence, it could be imagined that signalling through TLRs modifies the T_{reg} cells in such a way that they are able to induce Th_{sup} cells. Another way to modify T_{reg} cells would be through APCs. The presence of foreign pathogens upregulates co-stimulatory molecules on APCs, thereby influencing the activation level of T_{reg} cells.

T_{reg} cells and regulation of immune responses towards non-self

In the above we have outlined a model of how T_{reg} cells might function in the protection against AIG and IBD. However, it is very possible that these cells have a much more general role in immune homeostasis. They might regulate not only immune responses towards self, but also against foreign antigens, where they could be of importance in tuning the strength of the response.

Recently, the role of T_{reg} cells in controlling infections has been explored by Belkaid *et al.* (39). They showed that T_{reg} cells influence the development of immunity towards *Leishmania major* infection in C57BL/6 mice. Both IL-10-independent and IL-10-dependent mechanisms are involved since anti-IL-10 receptor Ab could not restore the proliferation of lesion-derived CD4⁺CD25⁻ T cells in co-cultures with lesion-derived T_{reg} cells. On the other hand, IL-10-deficient mice, as opposed to wild-type mice, attained a strong sterilizing immunity to *Leishmania major* infection. These mice, however, showed a complete loss of memory to reinfection, an observation which suggests that lack of IL-10-secreting T_{reg} cells in the primary stage of infection leads to effective elimination of microbial antigens, thereby influencing the generation of memory T cells. These data are in line with recent data from Xu *et al.* (40) showing that the inflammation induced by local injection of *Leishmania major* bacteria in SCID mice transplanted with CD4⁺CD25⁻ effector T cells was downmodulated by co-injection of CD25⁺ T_{reg} cells.

Thus, it appears that T_{reg} cells can be activated by both non-self and self antigens. If reactivity against foreign antigens is controlled by T_{reg} cells, it is necessary to consider how T_{reg} cells are regulated. Several proposals have been made. Based on their hypothesis respecting differential activation it was suggested (30) that the stimulation exerted by foreign pathogens is so strong that T_{reg} cells cannot function optimally. Given that addition of IL-2 to co-cultures of T_{reg} cells and CD4⁺CD25⁻ T cells counteracts suppression, another explanation could be that the high levels of IL-2 associated with infection render the T_{reg} cells non-suppressive. Infection might also lead to the secretion of a ligand for GITR and signalling through this receptor might impair the activity of T_{reg} cells. A ligand for GITR could be produced by cells in the infected area, e.g. by endothelial cells (29) or DCs (41).

As mentioned above, T_{reg} cells might also be regulated through TLRs. Addition of LPS, APCs and anti-CD3 mAb increases their proliferation 20-fold. After proliferation, T_{reg} cells are even more suppressive (38). We speculate that under *in vivo* conditions, the T_{reg} cells are proliferating, while CD4⁺CD25⁻ T cells are

fighting the infection. Later on, the now highly suppressive T_{reg} cells will ensure the downregulation of the immune response. It might very well be that all of these hypothetical regulatory mechanisms function together, although different mechanisms might predominate in different situations. Regulation of T_{reg} cells in relation to infection has recently been reviewed (41, 42)

CONCLUDING REMARKS

Evidence presented in this review shows that T_{reg} cells are suppressive both *in vitro* and *in vivo*. In addition, these cells are characterized by a distinct anergic phenotype upon activation. T_{reg} cell function requires activation which can be induced unspecifically *in vitro* but is antigen specific *in vivo*. T_{reg} cell-mediated suppression is both antigen-specific and non-specific and might involve both primary cell contact-dependent and secondary cell contact-independent suppressor cells. T_{reg} cells are clearly involved in protecting the individual against autoreactive T cells, but this is most likely not their only task. Thus, more recent data indicate a central role for these cells in the regulation also of immune responses against foreign antigens. The properties of the T_{reg} cells make them attractive for clinical use, especially because the cells can be expanded *in vitro* without losing their suppressive function. The research carried out during the past few years has contributed considerably to our knowledge of regulatory T cells, but many questions remain unanswered. We shall have to await future studies in order to discover the true significance of the nature and function of T_{reg} cells.

Note added in proof

After submission of the present manuscript three review articles focusing on the genetics, the development and the functions of T_{reg} cells supporting our present conclusions were published (43, 44, 45).

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