Published in final edited form as:

Trends Mol Med. 2004 January ; 10(1): 3–9.

TGF- β , T-cell tolerance and anti-CD3 therapy

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Abstract

Recent studies by Chatenoud and co-workers suggest that non-mitogenic F(ab')₂ fragments of anti-CD3 antibodies, which cannot bind the Fc receptor, induce a prolonged period of tolerance and prevent diabetes in nonobese diabetic (NOD) mice. Tolerance is established by regulatory T cells through the production of transforming growth factor- β 1 (TGF- β 1), but the mechanism by which TGF- β 1 confers this activity is unclear. Analysis of mice deficient in TGF- β 1 suggests that TGF- β 1 raises the threshold at which intracellular calcium activates T cells to a level that prevents an autoimmune response.

Differential response to self and non-self is the hallmark of the adaptive immune system. T cells activated by self must be either destroyed or tolerized to prevent autoimmune diseases such as diabetes mellitus, arthritis and multiple sclerosis. The challenge to developing treatments is to avoid the side-effects associated with immunosuppression, such as nephrotoxicity (caused by FK506 or cyclosporin A treatments) and increased frequency of infection (Table 1). Recent studies in which non-stimulating anti-CD3 antibody therapy has been used to treat nonobese diabetic (NOD) mice and diabetic individuals show promise. The study by Chatenoud and co-workers [1] suggests that treating NOD mice with anti-CD3 F(ab')₂ fragments, which cannot cross-link surface CD3 molecules on T cells and are therefore non-mitogenic, induces tolerance by generating regulatory T (T_{reg}) cells. In turn, the T_{reg} cells inhibit T cells reactive towards pancreatic islet cells (autoimmune T cells) through the production of transforming growth factor- β 1 (TGF- β 1). This anti-CD3 therapy has the important advantage that long-term tolerance has been shown to occur without adverse effects on the immune system.

Loss of T-cell activity can lead to immunodeficiency, whereas T-cell activation from the aberrant recognition of self-antigens can lead to autoimmunity. Self-tolerance is achieved primarily by the negative selection of immature thymocytes [2]; thus, when autoreactive T cells escape negative selection and travel to the periphery, autoimmune responses can occur [3]. T_{reg} cells then come into play by preventing T-cell-mediated autoimmune responses through the induction of peripheral tolerance [4,5].

Most experimentally induced and spontaneous autoimmune diseases arise from a deregulation of T-cell homeostasis [6,7]. Immune regulation is achieved by tightly regulated interactions among cytokines, surface receptors and transcription regulators [8]. A deficiency in some of these factors, such as TGF- β 1, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or FoxP3, leads to severe autoimmune disease, and overexpression of either TGF- β 1 or FoxP3 is protective [9–11]. Many autoimmune diseases have been attributed to a loss of T_{reg}-cell function, which involves cell–cell contact and the production of TGF- β 1 and interleukin

(IL)-10 [12]. However, the role of IL-10 in T_{reg} suppressor function in mice is restricted to the mucosal immune system and seems not to be involved in autoimmune disease [13]. TGF- β 1, by contrast, prevents autoimmunity in numerous tissues [14,¹⁵].

NOD mice develop spontaneous diabetes, and females are more prone to disease than males. Both CD4⁺ and CD8⁺ T cells are found in the pancreas of NOD mice; these cells produce pro-inflammatory cytokines such as inter-feron- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), resulting in the destruction of islet cells and the development of type 1 diabetes mellitus by 5 months of age [16]. The disease progresses slowly owing to the inhibitory role of T_{reg} cells. But when NOD mice are genetically combined with CD28- or B7-deficient mice, which have no T_{reg} cells, diabetes develops more rapidly.

T_{reg} cells constitutively express CTLA-4, a negative regulatory molecule that binds with higher affinity to B7.1 and B7.2 than CD28. Activation of CTLA-4 by cross-linking enhances the production of TGF- β 1 by T_{reg} cells and its accumulation on their surface [12,¹⁷]. Blocking co-stimulation by CTLA-4-immunoglobulin (CTLA-4-Ig) results in a depletion of T_{reg} cells and severe diabetes in both male and female NOD mice, confirming that co-stimulation is essential for the development of T_{reg} cells [12]. These results are consistent with the recent study by Chatenoud and co-workers [1], in which tolerance in diabetic NOD mice was established by TGF- β 1-producing T_{reg} cells upon treatment with F(ab')₂ fragments of anti-CD3 antibodies that cannot bind the Fc receptor. Hints on how TGF- β 1 carries out this function are now becoming evident.

Regulatory T cells in tolerance

T_{reg} cells develop during thymic education, are positively selected for self-antigens, and home to peripheral tissues where they prevent T-cell activation in a contact-dependent manner. *in vitro* studies have shown that T_{reg} cells inhibit the proliferation of T helper (Th) cells in response to stimulation of the T-cell receptor (TCR) by anti-CD3, and that T_{reg}-depleted CD4⁺ Th cells proliferate more vigorously than undepleted cells [18]. T_{reg} cells inhibit the production of IL-2 and IFN- γ by Th cells [19] (Figure 1), and they also inhibit graft-versus-host disease (GVHD) [20]. T_{reg} functions have been observed in both CD4⁺CD25⁺ and CD4⁺CD25⁻ subsets of CD4⁺ T cells [21]. These studies strongly suggest that T_{reg} cells, whether they express CD25 or not, are suppressor cells that show immunoregulatory function.

T_{reg} cells constitutively express both CTLA-4 and the conventional co-receptor CD28. Co-stimulatory signals through CD28 are important to prevent activation-induced cell death (AICD) of CD4⁺ Th cells, whereas they are essential for T_{reg}-cell development [22,²³]. T_{reg} cells produce TGF- β 1, and blockade of TGF- β 1 with neutralizing antibodies prevents the inhibitory action of T_{reg} cells on T cells [12]. Cross-linking of CTLA-4 induces TGF- β 1 production by CD4⁺ T cells, and antibodies specific for CTLA-4 block T_{reg}-cell function *in vivo* [1,^{12,17}], suggesting that TGF- β 1 production is tightly regulated and important for the function of CD4⁺CD25⁺CTLA-4⁺ T_{reg} cells *in vivo* (Figure 2).

The murine *Foxp3* gene (encoding a forkhead/winged helix transcription factor also known as Scurfin) is essential for the development and maintenance of T_{reg} cells in the periphery, as shown by conditional gene ablation and overexpression studies [24,²⁵], and a defect in the human *FOXP3* gene leads to IPEX syndrome – a systemic autoimmune disease [11,²⁶]. Retroviral transfection of naïve T cells with *Foxp3* converts them to T_{reg} cells [11]. Although overexpression of *Foxp3* in T cells induces *Il10*, rendering them T_{reg} cells, it is not clear whether *Foxp3* mutant T cells can produce TGF- β 1 upon cross-linking of CTLA-4. Overexpression of *Foxp3* delays the development of autoimmune disease in *Ctla4*^{-/-} mice, suggesting that there could be a link between these two signaling pathways [24]. These studies indicate that co-

stimulatory signals through CD28 and CTLA-4 might induce the expression of *Foxp3*, which is essential for the development and function of T_{reg} cells.

The induction and function of T_{reg} cells have been studied extensively in NOD mice, which develop spontaneous autoimmune type I diabetes (Figure 1). NOD mice show a Th1 autoimmune response due to enhanced macrophage production of IL-12, which is controlled by the gene locus *Idd4* [27]. Blocking co-stimulatory signals inhibits experimentally induced autoimmune diseases ([28,²⁹]; and see references in Ref. [16]). These results seem to contrast with observations made in NOD mice; however, it is possible that effector T cells do not require co-stimulation in the spontaneous autoimmune disease of NOD mice, but they do in experimentally induced autoimmune disease. The accelerated progression of disease in CD28-deficient NOD mice could result from a blockade of T_{reg}-cell development caused by a lack of co-stimulation (see references in Ref. [16]). Therapeutic induction of tolerance by non-depleting antibodies specific for CD4 and CD8, anti-CD40L and CTLA-4-Ig have all shown promise in transplantation models, and their effects seem to be mediated through the generation of T_{reg} cells (see Ref. [30] and references therein).

Other spontaneous autoimmune mouse models, such as *Ctla4*^{-/-} and *Tgfb1*^{-/-} mice, succumb to severe multifocal autoimmune inflammatory disease around weaning age, suggesting that both CTLA-4 and TGF-β1 are essential for T_{reg} function. Treatment of day-2 *Tgfb1*^{-/-} mice with CTLA-4-Ig, which binds with high affinity to B7.1 and B7.2 and blocks co-stimulation through both CD28 and CTLA-4, does not reverse the severe autoimmune disease (T. Doetschman, D.A. Rowley, J.A. Bluestone, unpublished). This is consistent with the idea that co-stimulation of CD28 is dispensable for spontaneous T-cell-mediated autoimmune disease. There is no exacerbation of autoimmune disease in *Tgfb1*^{-/-} mice treated with CTLA-4-Ig, suggesting that there are no functional T_{reg} cells to be affected, consistent with the idea that TGF-β1 is required for the development and/or activity of T_{reg} cells.

Is TGF-β1 important for T_{reg} function and tolerance?

There is confusion regarding the role of TGF-β1 in the function of CD4⁺CD25⁺ T_{reg} cells, because there are several subsets of T cells that show regulatory function *in vitro*. Both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells can be effective as T_{reg} cells [31], but the mechanism by which they suppress T-cell response is still unclear. Both IL-10 and TGF-β1 can be produced by T_{reg} cells, and the classification of T_{reg} cells has been based on the cytokines that they secrete. For example, type 1 T_{reg} cells (T_{r1} cells) secrete IL-10, and Th3 cells secrete both IL-10 and TGF-β1 [30,³²].

A recent study by Kemper *et al.* [33] using human peripheral blood lymphocytes has shown that T_{r1} cells produce both IL-10 and TGF-β1 upon stimulation with anti-CD3 and anti-CD46 or complement factor C3b (CD46 binds to C3b). IL-10 was found to mediate the suppression of Th cells but it was not clear whether TGF-β1 was responsible for the induction of IL-10 [33]. Another study has indicated that T_{reg}-cell function is dependent on TGF-β1 secretion and cell–cell contact [12], although Piccirillo *et al.* [18] have shown that *Tgfb1*-deficient T_{reg} cells have suppressor function, albeit at higher cell numbers than the usual 1:1 cell ratio, and that T cells deficient in TGF-β signaling (by expression of a dominant-negative TGF-β type II receptor) are also inhibited by T_{reg} cells. This latter study clearly demonstrated the existence of TGF-β1-independent CD4⁺CD25⁺ T_{reg} cells *in vivo*; however, it did not clarify whether these cells were CD25^{hi}, in which case they could be sequestering IL-2 (the CD25 ligand), thereby preventing T-cell proliferation by IL-2 starvation rather than by active suppression.

Because mouse models in which TGF-β signaling is disrupted develop cell-autonomous autoimmune disease [34] (Table 2), the physiological relevance of TGF-β1-independent T_{reg} cells is unclear. Belghith *et al.* [1] explain the confusion surrounding the role of TGF-β1 in

T_{reg} function by demonstrating that NOD mouse $CD4^+CD25^{hi}$ T_{reg} cells, but not $CD4^+CD25^+$ cells, show regulatory function, and that the absence of T_{reg} function in $CD4^+CD25^+$ cells is due to a lack of TGF- β 1 production. Another study has shown that T cells deficient in TGF- β signaling are not inhibited by T_{reg} cells *in vivo*, suggesting that in mice TGF- β -dependent T_{reg} cells are the main factors that induce tolerance *in vivo* [35]. This is also supported by the fact that *Tgfb1* knockout, *Tgfb2* conditional knockout and dominant-negative *Tgfb2* transgenic mice show similar autoimmune phenotypes. Taken together, these *in vitro* and *in vitro* studies suggest that T_{reg} cells that do not produce TGF- β 1 are not as effective *in vitro* (Table 2).

Recently, it has been shown that TGF- β 1 induces IL-10 in T_{reg} cells [36], suggesting that it might mediate some of its suppressor function through the induction of *Il10*, at least in the gut mucosa where IL-10 seems to have its chief regulatory effect [13]. There is also evidence that TGF- β 1 can induce both deletion (apoptosis) and anergy of activated T cells [37,38]. It is worth mentioning that only *Tgfb1* knockout mice develop autoimmune disease, whereas both *Il-10* and *Tgfb1* knockout mice develop inflammatory colitis [13-15,39] (Table 2).

TGF- β 1 is required for tolerance *in vitro*

As demonstrated by Chatenoud and co-workers [1], TGF- β 1 is central to the function of T_{reg} cells. Accordingly, overexpression of TGF- β 1 in pancreatic islet cells protects NOD mice from islet-cell destruction and autoimmune diabetes [10,40]. T cells expressing a dominant-negative TGF- β type II receptor are spontaneously activated and are not inhibited by T_{reg} cells *in vitro*, suggesting that TGF- β signaling in Th cells is essential for the ability of T_{reg} cells to inhibit Th-cell activation [35]. Consistent with the tolerogenic role of TGF- β 1-producing T cells, *Tgfb1*^{-/-} mice die from a multifocal autoimmune disease at weaning age [14,15], and they can be rescued by T-cell elimination [41]. These results clearly indicate that TGF- β 1 has an essential role in T-cell regulation and autoimmune disease, but they do not elucidate the mechanisms of TGF- β 1 function.

We and our co-workers [42] have found that TGF- β 1 prevents autoimmune disease by increasing the threshold of activation through the Ca^{2+} -calcineurin signaling pathway. *Tgfb1*^{-/-} thymocytes have an increased concentration of intracellular calcium ($[Ca^{2+}]_i$) and show an activated phenotype after suboptimal stimulation. Consistent with this, *Tgfb1*^{-/-} thymocytes are more resistant to inhibition of activation mediated by FK506, which is dependent on Ca^{2+} -calcineurin signaling. In the periphery, *Tgfb1*^{-/-} T cells show an anergic response, AICD and TCR downmodulation, suggesting a previously activated state [43]. Addition of TGF- β 1 to $CD4^+$ T cells during activation by anti-CD3 plus anti-CD28 prevents Ca^{2+} influx, activation of the transcription factor NFATc and nuclear translocation; this is in addition to the known effects of TGF- β 1 on cytokine production and the proliferation of naïve T cells [44].

Taken together, these studies suggest that TGF- β 1 produced by Th ($CD4^+CD25^-$) and T_{reg} cells raises the threshold for activation by Ca^{2+} in T cytotoxic and Th cells such that the recognition of self-antigens is not sufficient for activation. At this time we do not know the relative contributions of autocrine TGF- β 1 (produced by Th cells) and paracrine or juxtacrine TGF- β 1 (produced by T_{reg} cells) to Ca^{2+} homeostasis in T cells.

Immunotherapy for autoimmune diseases

Antibodies against the CD3 complex have been useful in treating graft rejection, GVHD and autoimmune disease [45]. However, whole Ig molecules that can bind to the Fc receptor are mitogenic, cause an increase in cytokine secretion and lead to generalized immunosuppression owing to a depletion of T cells. The study by Chatenoud and co-workers [1] suggests that F

(ab⁺)₂ fragments of anti-CD3 that cannot bind to the Fc receptor can induce prolonged tolerance and prevent diabetes in NOD mice. F(ab⁺)₂ fragments of anti-CD3 do not activate naïve T cells but induce the apoptosis of activated T cells [45]. In addition, the treatment of diabetic patients with humanized non-activating anti-CD3 antibodies improved insulin production for over a year (see Ref. [46] and references therein).

The mechanism by which this treatment induces tolerance involves the generation of TGF- β 1-producing T_{reg} cells from pre-existing CD4⁺CD25⁺ T cells. Because overexpression of TGF- β 1 in pancreatic islet cells protects NOD mice from islet-cell destruction and autoimmune diabetes [10, 40], it might be worthwhile considering combinatorial treatments with TGF- β 1 ligand and anti-CD3 F(ab⁺)₂ fragments for preventive treatment. Because systemic treatment with TGF- β 1 could have multiple detrimental effects, delivery would have to be targeted specifically to the pancreas. Given recent concerns regarding gene therapy, other methods for the targeted delivery of TGF- β 1 need to be developed. Alternatively, treatment with autologous TGF- β 1-producing T_{reg} cells generated *in vitro* by using anti-CD3, anti-CD46 or anti-CTLA-4 antibodies could inhibit the progression of autoimmune diabetes.

Concluding remarks

Important advances in our understanding of the cellular and molecular basis of T-cell regulation are being made. The contribution by Chatenoud's group [1] suggests that treatment with F(ab⁺)₂ anti-CD3 fragments provides long-term remission of overt diabetes in NOD mice by inducing the differentiation of CD4⁺CD25⁺ T_{reg} cells from preexisting CD4⁺CD25⁺ T cells, and also by inducing CD4⁺CD25⁺ T_{reg} cells to produce TGF- β 1. Their study also helps to clarify some of the confusion surrounding the requirement of TGF- β 1 for T_{reg}-cell function. It is now clear that CD4⁺CD25^{hi} cells can function as T_{reg} cells independent of TGF- β 1, and that CD4⁺CD25⁺ T cells have regulatory functions only when they produce TGF- β 1.

Coupled with recent studies on the molecular mechanism by which TGF- β 1 protects against autoimmune disease in mice [42, 43], a picture is evolving in which TGF- β 1-mediated regulation of Ca²⁺ homeostasis in T cells can increase the intracellular Ca²⁺ threshold for activation such that autoimmune responses are inhibited. Future efforts should include developing therapies involving anti-CD3 as well as targeted induction or delivery of TGF- β 1 ligand.

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(a) Normal mice

(b) NOD mice

(c) NOD mice

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Figure 1.

Regulatory T (T_{reg}) cells prevent diabetes in nonobese diabetic (NOD) mice. **(a)** CD4⁺CD25⁺ T cells develop in the thymus and prevent peripheral T-cell activation and autoimmune disease. T_{reg} cells inhibit proliferation and cytokine production by T helper (Th) cells on stimulation *in vitro* and *in vivo*. On stimulation, T_{reg} cells produce transforming growth factor-β1 (TGF-β1), which in turn inhibits the activation of Th cells. CD4⁺CD25^{hi} T_{reg} cells also inhibit Th-cell responses in a TGF-β1-independent manner, although their role *in vivo* is still unclear. **(b)** Deficiency in functional CD4⁺CD25⁺ T_{reg} cells causes diabetes in NOD mice. In these mice, Th cells are spontaneously activated and T_{reg} cells do not produce TGF-β1, thereby resulting in autoimmune type 1 diabetes. **(c)** Induction of functional T_{reg} cells prevents

diabetes in NOD mice through TGF- β 1-mediated inhibition of Th-cell activation. Treatment of NOD mice with anti-CD3 generates functional CD4⁺CD25⁺ T_{reg} cells from CD4⁺ CD25⁻ T cells. Blockade of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)-mediated co-stimulation inhibits the production of TGF- β 1 by T_{reg} cells and thus reverses the protection mediated by anti-CD3 treatment. Consequently, blockade by anti-TGF- β 1 also reverses the protection mediated by T_{reg} cells.

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Figure 2.

Production of transforming growth factor-β1 (TGF-β1) is essential for the function of regulatory T (T_{reg}) cells. On activation of CD4⁺CD25⁻ T cells by anti-CD3, T helper (Th) cells produce pro-inflammatory cytokines such as interleukin (IL)-2, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). The anti-inflammatory cytokines TGF-β1 and IL-10 (not shown) are also induced to a lesser extent. Treatment of CD4⁺CD25⁻ T cells with the non-mitogenic anti-CD3 F(ab')₂ fragment induces expression of CD25, resulting in CD4⁺CD25⁺ T_{reg} cells. Activated T_{reg} cells produce mainly TGF-β1 and IL-10, but do not produce IL-2, IFN-γ or TNF-α. Co-stimulation through CD28 is essential for the development of T_{reg} cells and their survival, whereas co-stimulation through cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is essential for their functional response. Expression of the transcription factor gene *Foxp3* is essential for the development and function of T_{reg} cells, which inhibit Th-cell activation through the juxtacrine activity of TGF-β1. Blue cylinder, CD25; orange cylinder, CD28; pink cylinder, CD4; yellow cylinder, CTLA-4; green cylinder, T-cell receptor; red cylinder, CD3.

Table 1Factors involved in the induction of tolerance^a

Treatment	Mechanism of action	Target cells or molecules	Disadvantages
Anti-CD3	Activation and depletion of T cells	T cells	Generalized immunosuppression
Anti-CD40L	Depletion of activated T cells and generation of T _{reg} cells	Activated T cells	None
Anti-CD4	Blockade of co-stimulation	CD4 ⁺ T cells	None
Tacrolimus (FK506)	Inhibition of T-cell activation	Calcineurin	Generalized immunosuppression and nephrotoxicity
Cyclosporin A	Inhibition of T-cell activation	Calcineurin	Generalized immunosuppression and nephrotoxicity
CTLA-4-Ig	Blockade of co-stimulation	T cells	Accelerates diabetes in NOD mice because it blocks T _{reg} cell generation
Anti-LFA-1 ^b	Blockade of co-stimulation and extravasation of T cells	T cells	Does not induce long term tolerance; requires continuous treatment
T _{reg} cells (CD4 ⁺ CD25 ⁺)	Inhibition of effector T-cell activation	T cells	None
Anti-CD3 F(ab') ₂	Activates T _{reg} cells and TGF-β1 production	CD4 ⁺ T cells	None

^a Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; Ig, immunoglobulin; LFA, leukocyte function-associated antigen-1; NOD, nonobese diabetic; TGF-β1, transforming growth factor-β1; T_{reg} cell, regulatory T cell.

^b Although anti-LFA-1 treatment has been shown to induce long-term tolerance in transplantation models, it does not do so in *Tgfb1*^{-/-} mice because these mice succumb within a week of terminating the antibody treatment.

Table 2TGF- β 1 is essential for inducing self tolerance *in vivo*^a

Knockout mice	Phenotype	Comments	Refs
<i>Tgfb1</i>	Multifocal inflammatory autoimmune disease mediated by IFN- γ and T-cell dependent; mice die within 3 weeks of birth	TGF- β 1 is essential for preventing T-cell activation; TCR downmodulation is an indication of self-antigen recognition <i>in vivo</i>	[14,43]
<i>Tgfb2</i> transgenic dominant-negative mice	Mice develop autoimmune inflammatory disease roughly 5 months after birth	TGF- β signaling in T cells is essential for inducing tolerance; transgenic receptor expression might be leaky, hence the delay	[47,48]
Inducible knockout of <i>Tgfb2</i> in hematopoietic cells	Mice develop autoimmune inflammatory disease after injecting poly(I)-poly(C) or IFN- α or IFN- β to delete TGF- β -RII through the Cre-flox targeting system	TGF- β signaling in T cells is essential for inducing tolerance; these T cells are not inhibited by any other T _{reg} cells	[49]
<i>Ctla4</i>	Massive lymphoproliferative inflammatory autoimmune disease mediated by Th2 cells; mice die roughly 3 weeks after birth	CTLA-4 is essential for T _{reg} -cell development and function; overexpression of <i>Foxp3</i> rescues these mice from the lethal autoimmune disease	[24,50]
<i>Foxp3</i>	Similar to <i>Ctla4</i> knockout mice	<i>Foxp3</i> is essential for T _{reg} development and function and upregulates IL-10; retroviral transfection of CD4 ⁺ T cells programs them to become T _{reg} cells	[11,24,25]
<i>Il10</i>	Enterocolitis; no autoimmune diseases	Inflammation in the colon is due to activation of T cells against gut bacteria	[13]
<i>Smad3</i>	Multifocal formation of pyogenic abscesses in the wall of the stomach and the intestine; involuted thymus, smaller spleen and enlarged lymph nodes	No apparent autoimmune diseases but impaired mucosal immunity; T cells show activated phenotype	[51]

^a Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IFN, interferon; IL, interleukin; TGF- β , transforming growth factor- β ; Th, T helper; T_{reg} cell, regulatory T cell.