**REVIEW ARTICLE**

# Umbilical Cord Expanded Regulatory T Cells as a Therapeutic Target for Treatment of Autoimmune Diseases

**[1]Ahmad G. Elsayed*, [1]Talaat Othman, [2]Amal F. Moustafa**
[1]Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt
[2]Department of Thoracic Medicine, Faculty of Medicine, Mansoura University

## INTRODUCTION

Umbilical cord blood (UCB) is a valued tissue in the umbilical cord, this can be easily collected and stored for clinical uses following delivery. Up to now, growing evidence demonstrates that cells extracted from UCB have significant potential for treating multiple diseases, with also the advantage of lack of risk to the donors, minimal ethical problems, a low occurrence of viral infections, a low incidence of graft-versus-host disease (GVHD), and high accessibility. The first successful UCB transplantation was done in 1988 for treating a 5-year-old boy with from Fanconi anemia. Since this date, more than 30000 UCB transplants have been achieved with great success for a variety of diseases, including hematological disorders, cancer, neurological disorders, and autoimmune diseases.

Umbilical cord blood cells comprise a large population of naïve highly-functioning Treg cells and numerous types of stem cells with potent ability to suppress immune response (Fig.1). These cells have the ability to suppress the activity of effector T cells and restore immune tolerance via the release of immunosuppressive cytokines and cell-to-cell contact. [1]

***Keywords:*** autoimmunity, cord blood, graft-versus-host disease, hematopoietic stem cell transplantation, regulatory T cell, type 1 diabetes.

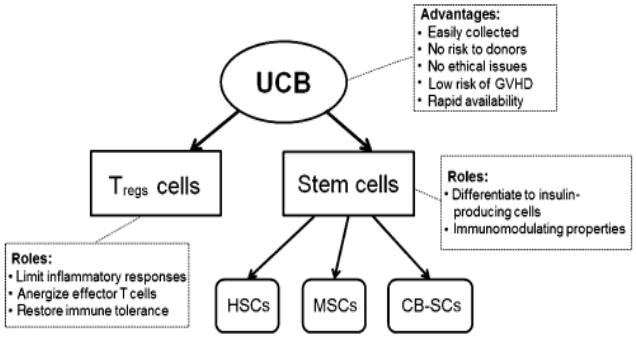


**Fig 1:** Components of umbilical cord blood (UCB)[1]

***Corresponding author:***
Ahmad G. Elsayed
Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt
Tel.: 01001326700
E-mail: ahmedgomaa@mans.edu.eg

UCB cells acts as a promising treatment for type 1 diabetes mellitus as an example of an autoimmune disease. The UCB contains various stem cell types, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and cord blood-derived multipotent stem cells (CB-SC), as well as a large population of immature unprimed highly functional regulatory T (Treg) cells. There are several advantages of UCB over other tissue sources of stem cells, including ease of collection, no risk to the donors, no ethical issues, a low risk of graft-versus-host disease (GVHD), and rapid availability. The UCB extracted stem cells not only differentiate into insulin-producing beta cells, but show immunomodulating effect and protect the regenerated insulin-producing cells against re-attack as well. These high-functioning Treg cells could bound the inflammatory responses and anergize effector T cells or reestablish the immune tolerance by their regulatory functions on multiple cell types.

**Collection of umbilical cord blood:**

Umbilical cord blood (UCB) is a very rich source of transplantable stem cells, which is considered a useful treatment for plenty of genetic diseases, immune deficiencies and blood malignancies in pediatric and adult patients lacking a suitable related or unrelated donor. UCB Banking is a method of preserving potentially life-saving cells that are usually wasted after the interruption of the blood flow from the umbilical cord to the newborn infant. UCB harvesting after vaginal delivery or Caesarean section is a simple, risk-free and quick procedure that does not change the normal course of the birth. Since UCB is a graft source and UCB transplantation provides good results, the UCB collection rate for banking should be optimized. [2]

**Regulatory T cells:**

***The origin and Development of CD4+CD25+Foxp3+ regulatory T cells***

Regulatory T cells of the CD4+CD25+Foxp3+ phenotype have been, till now, the most frequently studied type of regulatory T cells. They are critically important in the immune response regulation in both pathological and physiological conditions. Thymus-derived regulatory T cells are the main mediators of central immune tolerance, while peripherally derived regulatory T cells are highly involved in the regulation of peripheral immune tolerance at the sites of inflammation.

The development of thymus-derived Tregs needs two signals: one derived from the high affinity TCRs and the other delivered by other factors such as Interleukin-2 (IL-2) or CD28 ligands (CD80/CD86). Alteration of thymocytes with intermediate TCR affinity into tTregs is not restricted by IL-2 and CD80/CD86 molecules. To the contrary, the conversion of thymocytes with high TCR affinity into tTregs needs the presence of IL-2 and CD80/CD86 molecules. The interaction between CD28 and CD80/CD86 molecules helps tTreg survival probably by affecting the production of IL-2 and induction of anti-apoptotic protein Bcl-2. Co-stimulation through the CD28 molecule is crucial for thymus-derived Tregs development and maintains their stability in the peripheral lymphoid organs. It is unclear if these signals must be provided at the same time, though, they are necessary for the induction of the expression of the Foxp3 transcription factor. The results of recent studies propose another pathway of tTreg development starting from the double-negative (DN) CD4−CD8− thymocytes stage when the rearrangement of TCR encoding genes is not finalized (Fig. 2b). This suggests that tTreg selection may be to some extent independent of the TCR signal. [3]

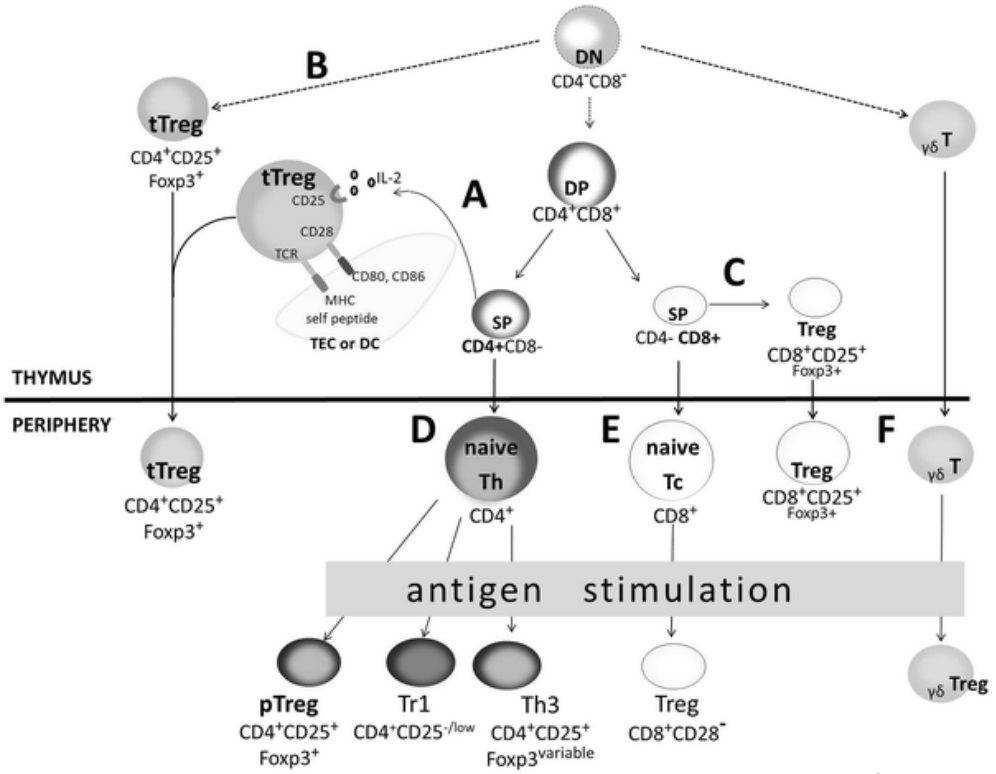


**Fig. 2**: This diagram explains how the regulatory T cell are developed. [4]

Thymus-derived regulatory T cells (tTregs) are originating from SP CD4+CD8− thymocytes (a), an alternative pathway of their development from DN is also assumed (b); CD8+CD25+Foxp3+ Tregs can also develop in the thymus from SP CD8+CD4− thymocytes (c). Both types of thymic Tregs migrate towards peripheral lymphoid organs as mature T cells exhibiting their suppressive potential. Peripherally induced Tregs are differentiating from antigen-activated Th CD4+ cells into peripheral T regs (pTregs) of CD4+CD25+Foxp3+ phenotype. Moreover, Tr1 and Th3 are developed (d); naive T CD8+ cells can differentiate into CD8+CD28− Tregs (e); γδ Tregs can arise from antigen-activated γδ T cells (f). DP double-positive, DN double-negative, SP single-positive, TEC thymic epithelial cells, and DC dendritic cells

***Transcription Factors and Surface Markers***

Treg-cell lineage commitment is mainly dependent on the expression of transcription factor forkhead box P3 (Foxp3). Various mutations in the human FOXP3 gene will result in a syndrome of multi-organ autoimmunity (immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. [5]

Foxp3 as a crucial factor for the function and the stability of regulatory T cells, its transcription is

activated by signal transducer and activator of transcription (STAT) 5, another transcription factor found to affect Treg survival and differentiation.[6]

Foxp3 is one of the most precise markers to identify Tregs; still, Foxp3+ cells without any suppressive function are also existing in humans. Another remarkable limitation of this marker is the fact, that cells must be permeabilized in order to stain Foxp3 intracellularly. As a result, the permeabilized cells are not viable anymore and cannot be used for more functional testing. For this reason, reliable markers expressed on the surface of Tregs are required to isolate and identify this cell population for further functional experiments.[7]

Primarily, Treg characterization was mainly dependant on their higher expression of surface IL-2 receptor α-chain (CD25) until it was clearly understood that CD25 can also be elevated in activated T-cells without any suppressive function. For that, additional surface-markers were anticipated to define Tregs including cytotoxic T-lymphocyte associated Ag-4 (CTLA-4)[8] glucocorticoid-induced tumor necrosis factor receptor (GITR), programmed cell death-1 (PD-1), adhesion molecule CD62L and many others, whereas CD127, CD49d, CD6 and CD26 were proposed to be negative markers. For all mentioned reasons above, the use of a combination of several markers is now recommended for a reliable identification of Tregs because none of these molecules is Treg-specific.[9]

**Regulatory T cells cytokines:**

One prominent feature accredited to Tregs is the secretion of cytokines applying suppressive function on various immune cells. The major Treg-cytokines include interleukin (IL)-10 and Transforming growth factor beta (TGF-β), the TGF-β is so important for the maintenance of immunological tolerance by interfering with the differentiation, proliferation and survival of lymphocytes and other immune cells**.** [10]

IL-10, to the contrary, seems to be principally essential for the control of inflammation at environmental interfaces. Moreover, IL-10 does not only induce suppression of pathogenic Th17-cell response, it is also required to maintain Treg suppressive activity and expression of Foxp3 as well. In addition, IL-10 was stated to interfere with Th1-cell migration to intestinal inflammatory sites.[11] On the other hand, nTregs were described to be a natural source of IL-35, thereby triggering differentiation of naïve T-cells into a distinct iTreg-subset exerting its suppressive function exclusively via production of IL-35 (iTr35-cells). Particularly, iTr35-cells differ from Foxp3+ Treg-subsets because they lack the expression of Foxp3. Probably, IL-35 is needed for optimal suppressive function of Foxp3+ Tregs and has been proposed to contribute to the maintenance of immune tolerance in the gut.[12] But still the exact physiological role of IL-35 in vivo is under debate and needs further research.

***Modes of regulatory T Cells-Mediated Suppression***

A key question of current research on Tregs is to understand the mechanism of Treg-mediated suppression. It is well known that Foxp3+CD25+CD4+ natural Tregs suppress the proliferation of naive T cells and their differentiation to effector T cells in vivo. They also have the ability to suppress the function of natural killer (NK) cells, also the effector functions of differentiated CD4+ and CD8+ T cells, and natural killer T cells, B cells, macrophages, osteoclasts, and dendritic cells.[13] In vitro, Tregs can suppress the proliferation and cytokine production (especially IL-2) of responder T cells when the two populations are cocultured and after antigenic stimulation in the existence of antigen-presenting cells (APC). Once activated, Tregs gain the ability to suppress responder T cells even they share antigen specificity with the Treg.[14]

Numerous mechanisms of Treg-mediated suppression have been proposed, and these include

- Secretion of immunosuppressive cytokines by the Treg.
- Direct cell to cell contact dependent suppression.
- Functional modification or killing of APC (Figure 3).[15]

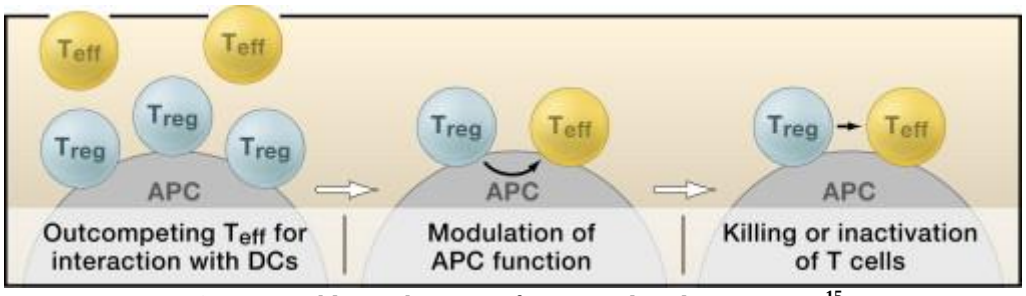


**Fig. 3:** Possible Mechanisms of Treg-Mediated Suppression[15]

More than one mechanism of Treg-mediated suppression may act to manipulate a particular immune response in a synergistic and consecutive manner. Activated Tregs by an antigen are recruited to antigen-presenting cells (APCs), particularly dendritic cells (DCs), and compete with antigen-specific naive T cells in interactions with dendritic cells mainly because of the expression of adhesion molecules (such as Lymphocyte function-associated antigen 1 (LFA-1)) by Tregs in a higher rate. Following this step, Tregs then modulate dendritic cell function. For example, Tregs downregulate the expression of dendritic cell CD80 and CD86 by a CTLA-4-dependent mechanism. Some Tregs may additionally differentiate to inactivate or kill responder T cells by granzyme and perforin or immunosuppressive cytokines (such as IL-10). [16]

***Induction of Tregs by TGF-β***

since the discovery that TGF- β could induce Foxp3 in naïve T cells, the importance of TGF- β for iTreg induction has been well recognized. Although TGF- β 1-deficient mice exhibited normal tTreg development in the thymus, but peripherally induced Tregs were significantly decreased in number. [17] The phosphorylation and activation of Smad transcription factors is a major signaling pathway induced by TGF- β. In T cells, Smad2 and Smad3 are activated by TGF- β, and afterward form a trimer with Smad4. [18]

Foxp3 gene locus is identified by three intronic enhancers, designated 'conserved noncoding sequences' (CNSs) 1, 2, and 3, in addition to a promoter, the enhancers and the promotor were found to play central roles in Treg cell differentiation (fig. 4). Notably, CNS1 contains two consecutive Smad-binding sites, this was confirmed by chromatin immunoprecipitation (ChIP) assays which reveal the recruitment of Smad3 into CNS1. It was found that the mice with conditioned deletion of CNS1 region exhibit spontaneous allergic Th2-type inflammation in the intestine and lung, and defects in peripherally induced Treg but not in tTreg. hence came the importance of CNS1 for iTreg/pTreg generation. [19]

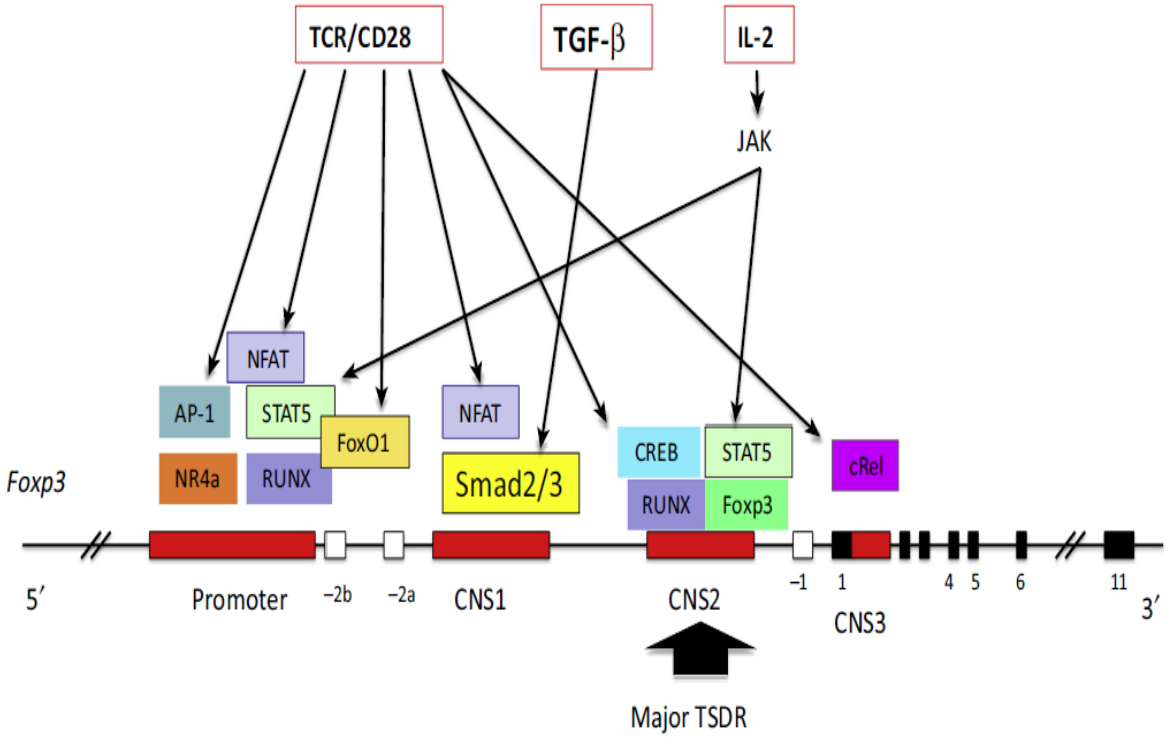


**Fig. 4:** Signals and Transcription Factors Involved in Foxp3 Induction and also the expression of Induced Tregs. [20]
***Abbreviations:*** AP-1, activator protein-1; JAK, Janus kinase; NFAT, nuclear factor of activated T cells; RUNX, Runt-related transcription factor; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TGF, transforming growth factor.

For Foxp3 induction in induced Tregs (iTregs), activation of the transcription factors Smad2 and Smad3 is critical and Smads are recruited to the (CNS1) region. The CNS2 region acts as an enhancer for Foxp3 transcription and is bound by transcription factors such as Foxp3, cAMP response element-binding protein (CREB), and STAT5. CpG islands of this region are found to be hypomethylated in thymic Tregs and establish a Treg-specific demethylated region (TSDR). This region is highly methylated in freshly generated iTregs, therefore Foxp3 expression is unstable.

**Regulatory T cell frequencies in autoimmune diseases**

***Circulating regulatory T cell frequencies***

In Systemic lupus erythematosus (SLE), multiple sclerosis (MS) and rheumatoid arthritis (RA) there are reports documenting decreased, or normal or even increased circulating Treg frequencies relative to health. Reports are also conflicting in autoimmune thyroid disease and psoriasis, in which decreased and normal Treg frequencies have been observed. Also, although the majority of studies show numerical decrease of Treg in inflammatory bowel disease (IBD)**,** there was a study that found increased frequencies**.** On the other hand, while most studies have settled that T1D patients have a normal frequency of circulating Tregs**,** decreased frequencies were found in one recent study**.** Supporting the point of view of presence of Tregs defect in the development of autoimmunity, CD4+ CD25+ FOXP3+ Tregs are also reduced in number in patients with undifferentiated connective tissue disease, which is considered as an early-stage connective tissue or systemic autoimmune disease**.**

Lack of standardization between studies is one proposed explanation for the observed discrepancies. This is a because of the absence of a uniformly recognized marker able to reliably define a homogenous human Treg population. The expected result is the use of distinct combinations of Treg markers, which define the real Treg population with variable degrees of accuracy. For example, In MS, studies defining Tregs as CD4+ CD25+ high CD4+ CD25+ FOXP3+ or CD4+ CD45RO- CD25 high cells have reported increased or equivalent Treg levels relative to health, whereas those using CD39 positivity and/or CD127 negativity as substitute or additional markers have documented decreased Treg frequencies. Even in one study examining the frequency of CD4+ CD25+ cells in RA, the frequency was increased, while studies using more complicated gating strategies, encompassing high CD25 expression, FOXP3 positivity or CD127 negativity, report normal or even decreased Treg frequencies.[21]

It is clear, however, that the use of different Treg markers may not fully explain the inconsistencies observed in these autoimmune conditions. In SLE, for example, the same Treg markers (CD4 positive CD25 positive high FOXP3 positive) have, in different studies, gave results consistent with increased [22] and decreased [23] Treg frequencies.

Considering this, it should be noted that flow cytometry gating is, to some degree, subjective. Even with standardized staining procedures, the use of appropriate controls and sophisticated analysis software, placing of gates still depends partly on the individual selection. Studies using the same defining Treg markers are, hence, not necessarily comparable. A good example of this is the placing of CD25 gates; there is currently no agreement about what constitutes ‘high’ or ‘bright’. The interpretation of Treg research is more complicated in the absence of detailed information about the methods of gating. [24]

It is also important to show out that regulatory T cell frequencies in autoimmune disease are affected by disease stage and treatment regimen. In SLE, as an example, Tregs have been reported to be less frequent in patients with active disease in comparison to those with inactive disease. [25] In MS, disease duration is linked with the frequency of memory Tregs, well-defined by the expression of CD45RA. Patients with short disease duration have a lower frequency of both naive and memory Treg subsets, while the frequency of memory Tregs in age-matched subjects with chronic disease is equal to that seen in health[26]. In the setting of RA, a lower frequency was found in patients with early/active disease, while a normal frequency of Tregs was found in patients with well managed disease. [27] Furthermore, although Ehrenstein et al.[27] did not find numerical Treg defects in RA, significantly the frequency of Tregs increased over time in patients responding to anti-TNFα therapy. The same happens in psoriasis.[28] Tregs are found at lower frequencies in untreated patients in comparison to healthy controls. Additionally, Treg frequencies increase significantly during treatment, in patients responding to antitumor necrosis factor alpha (anti-TNFα) therapy, while in non-responders the Treg frequency remains the same or even decreases during therapy.[29] Correspondingly, in inflammatory bowel disease (IBD), Treg frequencies are lower in patients with active disease compared to those with inactive disease. So, treatment with the anti-TNFα monoclonal antibody Infliximab treatment increases Treg frequency in IBD, predominantly in patients responding to therapy.[30] These findings demonstrate that Treg frequencies in autoimmune diseases are deeply influenced by disease stage and treatment regimen, highlighting the necessity to carefully consider these variables when interpreting published researches.

**Regulatory T Cell Therapy:**

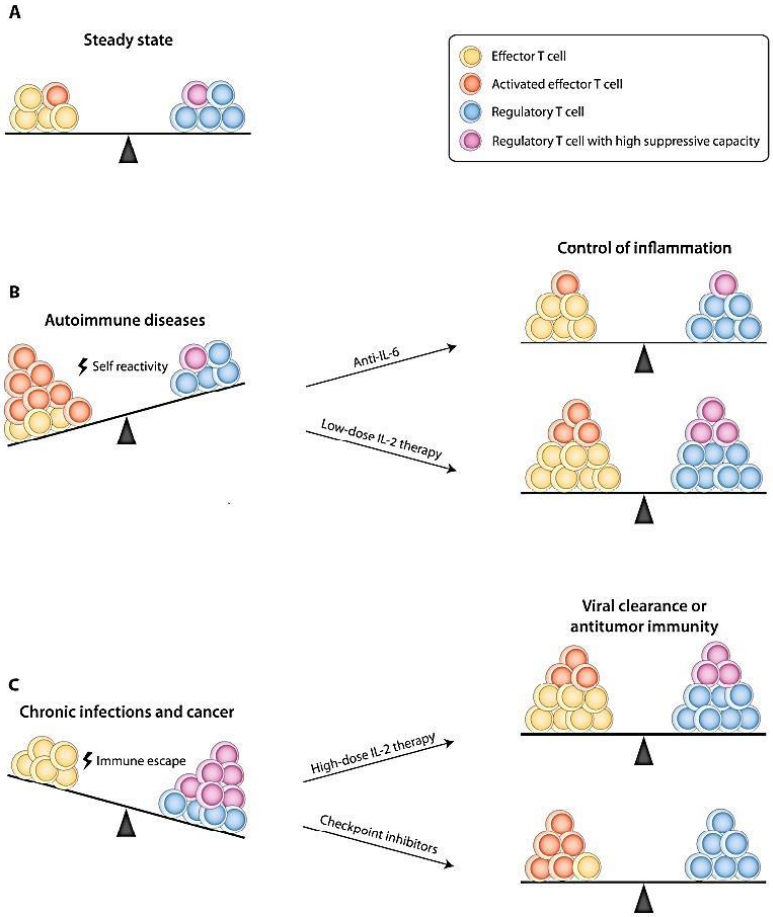


**Fig. 5:** Therapeutics directing the effector T cell–Treg balance.[31]

These are approaches that can reestablish a dysfunctional ratio of effector T cells and Tregs in different diseases to the healthy state (A). (B) In autoimmune diseases, self-tolerance is lost, and the bias goes towards the autoreactive effector T cells. In Th17-driven autoimmune diseases, blocking of IL-6 signalling makes bias towards Treg and inhibits Th17 differentiation. With adding low-dose of IL-2, there is more expansion of Tregs and therefore reestablishes a healthy effector T cell–Treg balance, this represents a broad approach for treatment of autoimmunity. (C) On the contrary, introduction of high-dose IL-2 results in expansion of effector T cells and induction of antitumor immune effects in cancer patients. Checkpoint inhibitors block signalling through inhibitory receptors such as PD-1 or CTLA-4 and can so inverse T cell exhaustion, which enhances immune evasion of the tumour or pathogens. Blockade of these pathways can also promote the function of effector T cells by reducing the suppressive activity of Tregs.[31]

Direct Treg infusion to patients is another way to increase the count of Tregs to treat T1D. In animal models, one infusion of Tregs prevents and reverses diabetes by suppressing priming of effector cells in the lymph node and hinders the effector T cell function in the inflamed tissue**.** [32] By using in vitro Expansion of a highly pure population of Tregs which can be isolated from patients with T1D using fluorescence-activated cell sorting based on cell surface phenotype of CD4 positive CD25 positive CD127 low/negative with short-term polyclonal stimulations, Billions of Tregs can be produced from one unit of blood (~450 ml) under good manufacturing process, making it possible to evaluate this therapy in clinical settings. [33]

**Restoring Regulatory T cell and Effector T Cell Imbalance to Treat T1D as an example of autoimmune diseases**

***Low-Dose IL-2***

The critical importance of IL-2 in Treg homeostasis and apparent Treg survival defects in non-obese diabetic (NOD) mice and patients with T1D provide a basis for using IL-2 therapy to augment Tregs. IL-2 has pleotropic effects on a variety of cell types, and sharp sensitivity of Tregs to IL-2 supports the use of low-dose IL-2 to selectively target Tregs**.** [34]

Amended clinical outcomes for patients using low-dose IL-2 therapy have been detected in chronic graft-versus-host disease and some other autoimmune diseases. [35] In spite of reported IL-2 signaling defects, Tregs from patients with T1D treated with low-dose IL-2 therapy can still respond to IL-2, augmenting the use of this therapy for enhancing Tregs in T1D patients.[36] In a T1D IL-2 and rapamycin combination trial, Tregs increased, but also CD56 high NK cells and CD4+ memory T cells, with an associated decrease in β cell function.[37] These studies made a spotlight on the importance of dosing as well as potential side effects of this treatment. Accordingly, defining the optimal dose is critical for the efficiency of IL-2 therapy. Clinical trials designed to address IL-2 dosing in T1D are ongoing.[38]

***CTLA-4 Ig***

CTLA-4 Ig binds to costimulatory ligands CD80 and CD86, preventing CD28-mediated costimulation. Treg development and homeostasis also require CD28 signaling. However, there is a measurable difference between the amount of costimulation needed for Treg homeostasis versus the amount needed for effector T cell differentiation. Partial blockade of costimulation using CTLA-4 Ig can efficiently prevent effector cell activation while slightly affecting Tregs in mice and human patients.[39] A trial of abatacept, a fusion protein of human CTLA-4 extracellular domain and human IgG1, in participants with newly diagnosed T1D, revealed a decrease in the rate of β cell loss. This helpful effect in the β cell function and haemoglobin A1c (HbA1c) lasted for at least 1 year after abatacept treatment ended.[40]

## CONCLUSIONS

Immunotherapy is an endless research process which needs a lot of effort to expand its horizon. From this review we can conclude that while UCB is a very rich source for stem cells, it is rich in Tregs as well and therefore has the potential to prevent or delay the onset of autoimmune diseases. Moreover, expansion of UCB T regs carries a very promising potential for treating of autoimmune diseases. From our research we have already found that UCB is superior to adult peripheral blood (APB) regarding regulatory T cells expression of suppressor genes and the avoidance of ethical issues.

**Abbreviations:**

**APB:** adult peripheral blood, **APC:** antigen-presenting cells, **anti-TNFα:** antitumor necrosis factor alpha, **AP-1:** activator protein-1, **CREB:** cAMP response element-binding protein, **ChIP:** chromatin immunoprecipitation, **CNSs:** conserved noncoding sequences,**CB-SC:** cord blood-derived multipotent stem cells, **CTLA-4:** cytotoxic T-lymphocyte associated Ag-4, **DN:** double-negative, **Foxp3:** forkhead box P3, **GITR:** glucocorticoid-induced tumor necrosis factor receptor, **GVHD:** graft-versus-host disease, **HbA1c:** haemoglobin A1c, **HSCs:** hematopoietic stem cells, **IPEX:** immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, **iTregs:** induced regulatory T cells, **IBD:** inflammatory bowel disease, **(IL)-10:** interleukin, **(IL-2):** Interleukin-2, **JAK:** Janus kinase**, MSCs:** mesenchymal stem cells, **MS:** multiple sclerosis, **NK:** natural killer cells, **nTregs:** natural regulatory T cells, **NFAT:** nuclear factor of activated T cells, **NOD:** non-obese diabetic, **PD-1:** programmed cell death-1, **Tregs:** regulatory T cells, **RA:** rheumatoid arthritis, **RUNX:** Runt-related transcription factor,**STAT-5:** signal transducer and activator of transcription, **SP:** single-positive, **SLE:** Systemic lupus erythematosus, **TCR:** T cell receptor, **Th3:** T helper 3 cell, **TGF-β:** Transforming growth factor beta, **TSDR:** Treg-specific demethylated region, **T1D:** type 1 diabetes, **Tr1:** Type 1 regulatory T cells, **UCB:** Umbilical cord blood

**Acknowledgement:**

To all members in the 'Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt'.

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