

### Regulatory T cells: Genetic modifications and impact on disease treatment

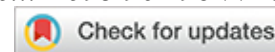
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### ABSTRACT

Regulatory T cells, also known as Tregs, play a significant role in the immune system since they control cells such as mast cells, basophils, and eosinophils. For this reason, it is necessary to understand the activation mechanisms of Tregs, such as inhibitory cytosines, cytolysis, metabolic alteration, and dendritic cells. Due to Tregs's functions and scientific advances in genetic engineering, the manipulation of these cells has been achieved. In this sense, gene transfer using Lenti-/Retro Virus has been using transposases and reprogrammable nuclease systems. However, it is crucial to consider that genetic engineering has helped generate Tregs and increase their specificity, which contributes considerably to treating diseases, thanks to using chimeric antigens and transgenic T cells (CAR and TCR, respectively). Therefore, the classification of regulatory T cells is given according to their origin, as is the case of shy Tregs, which develop in the thymus, and conventional T cells, which are induced *in vivo*. Finally, it is essential to consider that these Tregs are being used in stage I/II clinical trials to obtain precise results on their safety, viability, and efficacy.

**Keywords:** Regulatory T cells; Tregs; genetically engineered.

### INTRODUCTION

In the 1970s, the proposal of subsets of T cells capable of eliminating an immune response was first put forward, leading researchers to investigate T cells' existence.<sup>1</sup> Subsequently, it was possible to define regulatory T cells (Tregs) that encompass several specialized cell populations to perform cellular extrinsic immunosuppression. They can regulate the protective immune response by limiting collateral tissue damage and autoimmunity. Many subsets of human Treg, such as CD8 post-Treg,<sup>2</sup> IL10-producing Tr1 cells<sup>3</sup> and Th3 cells, are eliminated through secretion of TGF $\beta$ .<sup>4</sup> The therapeutic potential of mimics was certified with evidence supporting that Treg can prevent all immunity, thus becoming an ideal candidate for executing tolerance-promoting protocols. Thanks to scientific advances, solid plans for manufacturing Treg have been developed to isolate and expand a functional and stable product. In this research we will learn about the different regulatory mechanisms, subtypes, applications and technical challenges of T cells.

#### Generation mechanism of Regulatory T cells

Lack of regulatory T cells can result in autoimmunity, immunopathology, allergy, or metabolic disease<sup>5</sup> since these cells play an essential role when the immune system response fails.<sup>6</sup> In addition, regulatory T cells work together with effector T cells to mediate the immune system's reaction upon arriving at the site of inflammation,<sup>6</sup> such as mast cells, basophils, and eosinophils.<sup>7</sup> Therefore, it is necessary to comprehend the regulatory T-cell mechanisms [Fig. 1] to understand the phases of the disease.<sup>8</sup>

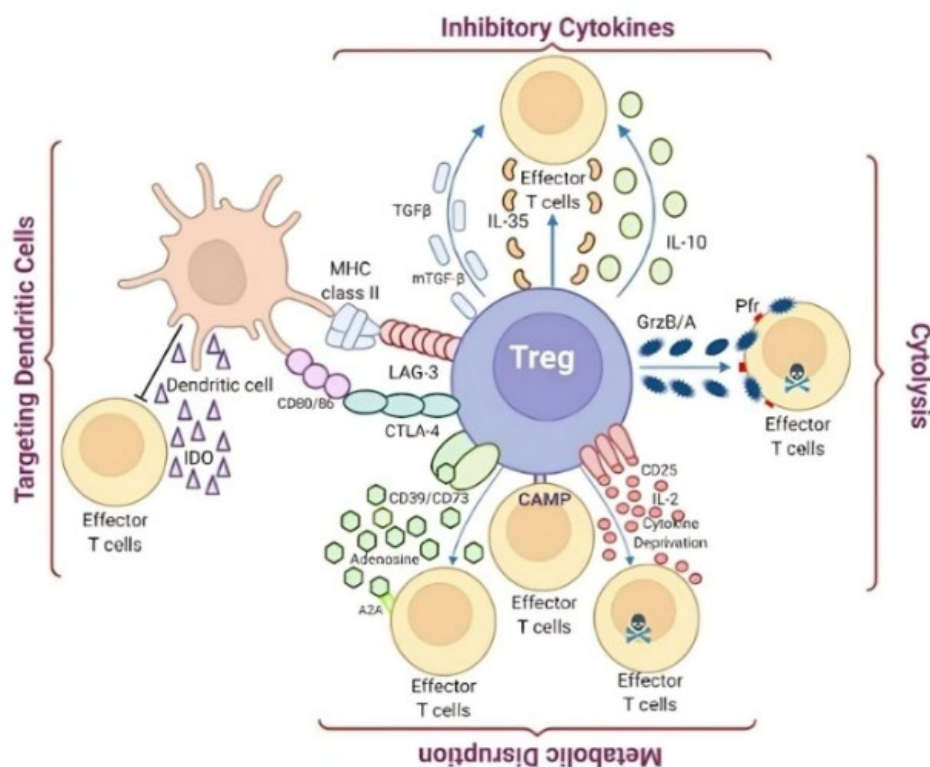


Figure 1. Mechanism of T cell regulation

### Inhibitory Cytokines

The appearance of inhibitory cytokines is the first pathway of suppressive action.<sup>9</sup> For instance, inhibitory cytokines, TGF $\beta$ , interleukin-10, and interleukin-35,<sup>10</sup> are a fundamental part of Treg cell-mediated suppression. However, how they contribute to the natural Treg cells that originate in the thymus is unclear.<sup>11</sup> Because the degree of suppression in which Treg cells are involved is directly related to the number of CD25+ CD4+ T cells and their suppressive activity, which depends on the concentration of the antigens to which they respond.<sup>12</sup>

### Cytolysis

The second mechanism of Treg-mediated suppression is cytolysis, which depends on the cell contact of target cells via granzymes A and B13 in a perforin-dependent and -independent manner.<sup>14</sup> Recent studies have shown that activated Treg stimulates apoptosis of effector T cells via a death-inducing ligand-receptor five pathways linked directly to tumor necrosis factor (TRAIL-DR5). Blockade of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) suppresses cytolysis and suppression.<sup>15</sup>

In addition, galectin-1 has been shown in several studies to inhibit effector functions by triggering growth arrest and apoptosis of activated T cells or blocking the secretion of proinflammatory cytokines; this may result in Treg suppressor function.<sup>16</sup>

### Metabolic Disruption

Regulatory T cells have perfected their specific metabolic characteristics to suppress effector T cells. Thus, when these cells come into contact with tumor cells, they express CD39 and CD73 (ectonucleotidases) and COX-2 (cyclooxygenase 2).<sup>17</sup> In this way, it is possible that prostaglandin E2 (PGE2) and adenosine, a product of COX-2 and the ectonucleotidases CD38 and CD73, are generated.<sup>17</sup> Consequently, cAMP (cyclic adenosine

monophosphate) levels increase as they bind to A2aR<sup>18</sup> and EP2R receptors; consequently, a robust proinflammatory signal is given. Likewise, high levels of cyclic adenosine monophosphate can hinder the metabolism of effector T cells.<sup>17</sup>

### Targeting Dendritic Cells

It is essential to consider that dendritic cells primarily present antigens and determine T-cell responses. These antigens are captured and processed to present in MHC products, and the subsequent differentiation of T cells is controlled.<sup>19</sup> Thus, regulatory T cells may be precursors of maturation or function of dendritic cells (DCs) necessary to activate effector T cells.<sup>11</sup> Thus, cytotoxic T lymphocyte antigen four expressed by regulatory T cells may alter the proper maturation of antigen-presenting cells, such as DCs, by binding to CD80/86.<sup>18</sup> In addition, it is necessary to consider that stimulation by immature DC antigen or low CD80/86 expression may cause T cells to reach an anergic state characterized by hypoproduction and hyperproliferation of cytokines after antigen stimulation.<sup>18</sup> On the other hand, several studies have shown that lymphocyte activation gene three can suppress the maturation of dendritic cells.<sup>12</sup>

### T cell generation by genetic commerce

Advances in genetic engineering have allowed the manipulation of regulatory T cells to increase.<sup>20</sup>

Generation of T cells by genetic engineering	
Gene Transfer Using Lenti-/Retro-Virus	Several studies have shown that regulatory T cells from healthy donors in vitro can be efficiently transcribed because this transfer system corrects defective replication by placing gene expression cassettes into the genome.
Gene transfer using transposases	This transfer system allows the random insertion of different cargo sizes into T cells. However, this cargo insertion must be monitored over long periods.
Programmable nuclease systems	The CRISPR-Cas system has made nuclease design more efficient. This allows double-stranded DNA breaks to be solved by establishing a DNA template to eliminate the mutation. Thus, this principle is applied to CD4+ regulatory T cells in which GFP insertion is approximately 40%.

Table 1. Characteristics of Generation of T cells.

### Genetic engineering and its contribution to the specificity of regulatory T cells

A fascinating approach to regulatory T cells is adoptive transfer, which aims to explore physiological characteristics in particular autoregulation important for the treatment of immune-mediated diseases such as GVHD (graft-versus-host disease).<sup>21 22 23</sup> Likewise, according to several studies, it has been shown that the transfer of polyclonal regulatory T cells tends to be effective in preventing graft-versus-host disease.<sup>24</sup>

Thus, thanks to the advances generated by genetic engineering, it has been possible to use the antigenic specificity of regulatory T cells using CAR or TCR receptors (chimeric antigens and transgenic T cells).<sup>25</sup>

However, chimeric antigen technology has additional benefits over transgenic T cells since the CAR technology prevents the restriction given by the human leukocyte antigen system known as HLA from occurring after the cells are activated.<sup>26</sup> Also, CARs have the advantage of directly targeting specific antigens without the restriction provided by the Major Histocompatibility Complex.<sup>26</sup>

## Subtypes and applications

Regulatory T cells (Tregs) are T lymphocytes that regulate or suppress other immune system cells. Furthermore, Treg maintains immune homeostasis.<sup>27</sup> Tregs are classified according to their origin as thymic Tregs (Tregs) or natural Tregs (nTregs) developed in the thymus. On the other hand, conventional T cells (Tcons) are induced in vivo in peripheral tissues that suffer from non-inflammatory or inflammatory conditions. Furthermore, peripheral Tregs (pTregs) are typically present on mucosal surfaces and induced Tregs (iTregs) are those induced from Tcons in vitro.<sup>28</sup> Tregs can be classified, considering the functional and phenotypic distinctions. T cell receptors (TCRs) of tTregs generally recognize self-antigens, whereas iTregs express TCRs specific to foreign antigens.<sup>28</sup> Treg cells are characterized by the T cell co-receptor CD4 expression and the IL-2 receptor chain CD25. Therefore, its phenotype is CD4+CD25+. In addition, the specific expression of the transcription factor Forkhead box P3 (FoxP3) allows the development and function of these cells.<sup>29</sup>

### CD4 tTregs

CD4 tTregs are a specialized subpopulation of T lymphocytes that suppress the activation of the immune system. Thus, the homeostasis of this system is maintained, and tolerance towards autoantigens is favored.<sup>30</sup> Most current studies isolate human Tregs from peripheral blood. However, third-party umbilical cord blood (UCB) derived Tregs have been successfully used in clinical trials.<sup>28</sup> tTreg has relatively strong lineage fidelity. However, confident, but not all, studies indicate that under definite conditions in an inflammatory environment, tTreg can lose Foxp3 identity and expression, causing loss of immunosuppressive capacity.<sup>31</sup> Furthermore, decreased Foxp3 expression can cause immunopathology by undermining the suppressor function of tTregs, resulting in Th2 cell formation.<sup>28</sup> Therefore, finding an approach that supports the steadiness and function of tTregs in the inflammatory state is necessary.

### CD4 iTregs

iTregs have been developed; as a result, the relative scarcity of tTregs in the peripheral blood and the time and cost of in vitro Treg expansion protocols since they significantly limit their clinical usefulness. Therefore, to generate iTreg, scientific induce Tcons in vitro.<sup>28</sup> The advantages of the approach of the iTregs can be generated in relatively high numbers and overcome the limitations of the small starting population of tTregs and in vitro expansion. Moreover, iTregs can be highly effective in tolerance induction colitis, which is further augmented by combining them with tTregs.<sup>28</sup> In addition, adoptively transferred CD4 iTregs have a potent capacity to inhibit GVHD, but, in the process, develop impaired graft-versus-leukemia (GVL) capacity.<sup>32</sup>

### CD8 iTregs

CD8 iTregs or "adaptive or induced" Tregs differentiate from activated T cells in the periphery or cell culture. CD8 iTregs defining markers are still under study and necessary to reach an accord.<sup>33</sup> CD8 iTregs have been studied in diverse cell-dependent and independent mechanisms to moderate immune suppression and homeostasis.<sup>34</sup> Future research will focus on developing unique markers to identify iTreg subsets for use in the clinic.

### Type 1 Regulatory (Tr1) Cells

Type 1 regulatory (Tr1) cells también se encuentran en la periferia. They are a type of Treg that expresses CD4+CD49β+Lag3+ and has transitory and lower level FoxP3 with activation.<sup>35</sup> Tr1 cells support maintaining immunological homeostasis and development tolerance.<sup>36</sup> Tr1 cells are being studied to determine their

therapeutic potential; in this case, the groups manipulate multiple strategies, including in vitro gene editing, to increase Tr1 numbers in vivo.

### Follicular Regulatory T (TFR) Cells

Follicular regulatory T (TFR) cells are cells T CD4<sup>+</sup> with specialized roles in regulating humoral immunity.<sup>37</sup> This cell type shares phenotypic characteristics with T follicular helper cells (TFH).<sup>28</sup> T cell inducible costimulatory (ICOS), CD40L, and PD-1 restrictive TFR function.<sup>37</sup> These specialized regulatory cells prevent TFH cells from supporting B cell progress. As a result, TFR plays an essential role in antibody production and regulation of autoimmunity.

### nTreg

Natural regulatory T cells (nTreg) are characterized by the constitutive expression of the IL-2 receptor alpha chain (CD25).<sup>38</sup> In more recent studies, the factor of FoxP3 (forkhead box protein 3) transcription is the primary regulator of the development and function of nTreg cells;<sup>39</sup> simultaneously, it became the essential phenotypic marker.

Subset	Specific marker	Secretory products	Actions	Location
tTreg	Foxp3		Activation of the immune system.	Peripheral blood, UCB
iTreg	CD4, Foxp3	IL-10, TGF- $\beta$	Similar to nTreg	Periphery
Tr1	CD4, CD25	IL-10	Suppress effector Th cell migration and functions	Generated from non-Treg cell precursors and draining lymph nodes
TFR	PD-1, CXCR5		Regulating humoral immunity	Germinal centers
nTreg	CD4, CD25, Foxp3	IL-10, TGF- $\beta$	Block T cell proliferation,	Thymus

Table 2. Characteristics of subsets of regulatory T cell (Treg)

### Genetic engineering techniques to modify regulatory T cells (Tregs)

#### CRISPR/Cas9

Several preliminary investigations have utilized CRISPR-Cas9 to disrupt genes within human T cells, as evidenced by published preclinical studies. These endeavors include the knockout of the gene responsible for encoding C-C chemokine receptor type 5 (CCR5) in CD4<sup>+</sup> T cells, resulting in T cells resistant to HIV infection.<sup>40</sup> Additionally, the knockout of the gene encoding CD7 in CD7 CAR T cells has been conducted to prevent fratricide, given that T cells express CD7. Clinical trials involving CRISPR technology have recently commenced in the United States. The inaugural CRISPR clinical trial in the country, initiated by CRISPR Therapeutics and Vertex, focuses on treating patients with the blood disorder  $\beta$ -thalassaemia.<sup>41</sup> These studies have progressed despite recent reports indicating that CRISPR-Cas9 editing may induce significant genomic deletions and rearrangements distant from the target site.<sup>42</sup> Efforts are underway to develop high-fidelity versions of Cas9 and alternative CRISPR-Cas systems. Notably, CRISPR-mediated editing extends beyond altering genomic sequences; novel CRISPR-Cas systems and their variants have been engineered for targeted DNA methylation, gene activation, and direct RNA editing. CRISPR-Cas9 genome editing holds the potential to insert antigen receptors into precise genomic locations while simultaneously editing multiple genes that regulate Treg cell function.<sup>40</sup>

#### Chimeric Antigen Receptors (CARs)

In the study cited in MacDonald KN 2022<sup>40</sup>, CAR utilization to redirect human Treg cells has also involved CEA. However, instead of preventing or mitigating disease in the intestine or lung, the suppression mediated by human CEA CAR Treg cells *in vivo* was demonstrated through partial protection of a CEA-expressing tumor from CEA CAR T cell-induced death in immunodeficient mice. One immediate limitation of this scenario was that CAR Treg and CAR T cells, administered at a 1:1 ratio, targeted the same antigen. Therefore, the observed partial protection could result from competition between these cell types for antigen rather than genuine suppression. Subsequently, another research team generated human CD19 CAR Treg cells capable of suppressing the proliferation and cytotoxic activity of CD19 CAR Teff cells *in vitro*.<sup>40</sup> These cells migrated to CD19-expressing B cell-derived tumors in mice, preventing CD19 CAR Teff cell-mediated tumor destruction *in vivo* at a ratio as low as one CAR Treg cell per 16 CAR Teff cells.

### **Polyclonal Treg Therapy**

Polyclonal Treg therapy involves using autologous Tregs that are expanded *ex vivo* to reinstate tolerance in autoimmune disorders. Several clinical trials employing this therapy for autoimmune disease treatment have been concluded or are ongoing.<sup>42</sup> The initial clinical trial, conducted in children with T1D, demonstrated that the adoptive transfer of autologous polyclonal Tregs extended the survival of pancreatic islets. Some patients exhibited signs of clinical remission and maintained insulin independence for up to a year without encountering adverse effects.<sup>43</sup> Another clinical trial involving adult T1D patients using *ex vivo*-expanded autologous polyclonal Tregs demonstrated the safety and tolerability of the treatment.<sup>30</sup> Ongoing clinical trials in T1D and other autoimmune conditions investigate optimal treatment regimens, including the number of cells transferred, required doses, and intervals between doses.<sup>43</sup>

### **Completed and Ongoing Clinical Trials**

Several phase I or phase I/II clinical trials have been completed or started. The clinical trials aim to test Treg infusion's safety, feasibility, and efficacy in patients. The following describes results from published studies in autoimmune diseases and transplantation and gives an overview of the ongoing clinical trials.

### **Tregs in Autoimmunity**

Clinical trials have potentiated the confirmation of the therapeutic potential of Tregs in several animal studies. Treg therapy has been applied through the medications abatacept and belatacept. They are fusion proteins containing the moiety of receptor CTLA-4, the receptor responsible for Tregs' considerable suppressive powers; it has been used to introduce Treg therapy. These medications have already been shown to help maintain immunosuppression following solid organ transplantation and treating autoimmune disorders.<sup>44</sup> In a study, a research group from the United Kingdom demonstrates that good manufacturing practice GMP-grade Tregs show preferential solid homing to the liver and spleen with minimal localization to other organs after intravenous infusion. Also, Tregs showed that they have the metabolic capacity to survive within inflamed tissues. Thus, patients with autoimmune liver diseases could be excellent candidates for Treg therapy.<sup>45</sup> In 2019, Izumi et al. published results from several trials investigating novel therapeutic targets, demonstrating that translational research in pemphigus and pemphigoid is fast-growing. Thus, the researchers expect several novel treatments to be available shortly to treat pemphigus and pemphigoid patients.<sup>46</sup> The primary issue is the limited abundance of nTregs, comprising only 5–10% of CD4+ T cells in peripheral blood. Regardless of their source, it is necessary to purify the material to isolate a homogeneous population of Tregs. This purification process typically involves immunomagnetic or fluorescence-activated cell sorting (FACS). Immunomagnetic sorting,

while more practical due to its closed vessel setup, suffers from the drawback of yielding Treg preparations with relatively low purity. Although this is acceptable for applications involving fresh cells, *in vitro* expansion procedures often result in the overgrowth of impurities alongside Tregs, as Tregs have a low proliferation rate. Consequently, the final purity of the product tends to be poorer than the initial material.<sup>45</sup>

### **Tregs in Solid Organ Transplantation**

Tregs play a role in developing and maintaining tolerance to solid organ allotransplants. Tregs produced from patients are being studied to prevent organ rejection and reduce immunosuppression after kidney or liver transplants. In addition, expanded polyclonal nTregs and antigen-specific nTregs are being studied.<sup>46</sup> A group of researchers from London has recently provided results from the successful expansion under GMP conditions of polyclonal Tregs isolated from end-stage liver disease patients awaiting liver transplantation and stable liver transplant recipients under maintenance immunosuppression. Treg infusion was safe and correctly tolerated and can improve donor-specific immune responses.<sup>46</sup> The Northwestern University in the USA developed a protocol for the large-scale isolation and expansion of human Tregs and conducted a phase I clinical trial. Tregs would be infused in adult patients after kidney transplantation. It also lays the groundwork for a phase II clinical trial testing the efficacy of Treg infusion for tolerance induction or drug minimization.<sup>46</sup> Preclinical studies have demonstrated Treg's ability to treat autoimmune diseases and prevent graft rejection, but clinical researchers are still far from these ultimate goals. However, early clinical trials have demonstrated the safety and feasibility of Treg infusions, and new Phase II trials are currently being initiated or planned.<sup>47</sup> Moreover, using Tregs in solid organ transplantation has been shown to mitigate adverse outcomes and promote graft tolerance. The authors discuss the multiple mechanisms by which Tregs suppress immune responses, including the secretion of suppressive cytokines like IL-10 and TGF- $\beta$  and the inhibition of IL-2 mRNA induction in target T cells. These immunosuppressive capabilities are harnessed to develop treatments that minimize rejection and protect transplanted organs. The review also highlights the promising results from several studies where Tregs were administered to transplant recipients, indicating their potential to revolutionize transplantation medicine.<sup>29</sup>

### **Tregs in Clinical Trials for Chronic Inflammatory Diseases**

The use of regulatory T cells (Tregs) in treating chronic inflammatory diseases has been extensively investigated in recent clinical trials, showing their potential to modulate the immune response and reduce chronic inflammation. Adoptive transfer of Tregs, where the cells are cultured and expanded *ex vivo* before being infused into patients, has shown promising results in reducing inflammation and improving symptoms in inflammatory bowel diseases. For instance, in a recent study, patients with ulcerative colitis treated with an infusion of autologous *ex vivo* expanded Tregs demonstrated a 50% reduction in inflammatory markers and significant improvements in quality of life, with a maximum tolerated dose of  $10 \times 10^6$  Tregs/kg.<sup>48</sup> However, these trials face significant limitations, including variability in Treg expansion and the need for standardized protocols to ensure consistency in results. Additionally, some patients experienced mild side effects, such as fever and fatigue, necessitating careful monitoring.<sup>49</sup>

Advances in Treg-based therapy have also involved the genetic optimization of these cells to improve their specificity and functionality. Initial phase clinical trials have demonstrated that these therapies are feasible and well-tolerated, with significant potential for treating inflammatory and autoimmune disorders. The authors highlighted the importance of Tregs as a multifaceted and adaptable therapy. However, they noted the critical challenge of maintaining the stability and sustained function of genetically modified Tregs in chronic

inflammatory environments, as 20% of patients in the trials experienced a loss of Treg function after six months.<sup>27</sup> Furthermore, long-term monitoring is crucial to evaluate the safety of these therapies, as genetic modifications could introduce additional risks, such as inappropriate immune activation.

Finally, studies have demonstrated that Tregs can maintain functionality in established systemic inflammation and reverse fatal autoimmune diseases. For example, authors found that Tregs could effectively treat chronic inflammatory diseases, presenting a new therapeutic paradigm. In their study, 70% of patients showed a reduction in disease symptoms and a 40% decrease in inflammatory markers.<sup>50</sup> However, maintaining the immunosuppressive function of Tregs in highly inflammatory environments and the heterogeneity of patient responses present significant challenges. The authors emphasized the importance of developing methods to improve Treg stability and function and identifying specific biomarkers to predict treatment efficacy and personalized therapies. For example, they proposed using DNA methylation analysis of the FOXP3 promoter region to identify trustworthy and stable Tregs.<sup>51</sup>

### Safety Concerns in Treg Cell Immunotherapy

The risk of off-target effects or uncontrolled cell proliferation in regulatory T cells (Tregs) is a significant concern in immunotherapy. One study highlights that Tregs, which express the transcription factor FOXP3, are critical for maintaining immune homeostasis but pose challenges in cancer therapy due to their immunosuppressive functions. These cells can inadvertently suppress antitumor responses, complicating their therapeutic targeting. Regulatory T cells are pivotal in sustaining peripheral tolerance and warding off autoimmune diseases, yet they impede the efficacy of antitumor immune responses and therapies.<sup>53</sup>

Off-target effects are particularly problematic in the context of Tregs due to their ubiquitous role in immune regulation. The use of Tregs in tumor environments must be carefully managed to avoid systemic autoimmune reactions. Strategies such as targeting effector Tregs rather than all FOXP3+ T cells have been proposed to mitigate these risks. While the complete removal of Treg cells can lead to harmful autoimmune responses, a viable approach for stimulating robust tumor immunity without triggering autoimmunity involves specifically targeting the terminally differentiated effector Treg cells.<sup>54</sup>

Tregs' suppression of antitumor immunity is also facilitated by their interaction with other immune cells, such as dendritic cells (DCs). A study on the suppression mechanisms of Tregs found that regulatory T cells influence the release of chemokines from dendritic cells, which is independent of their role in controlling T cell proliferation.<sup>55</sup> This interaction highlights the complexity of the immune environment and the potential for off-target effects when modifying Tregs.

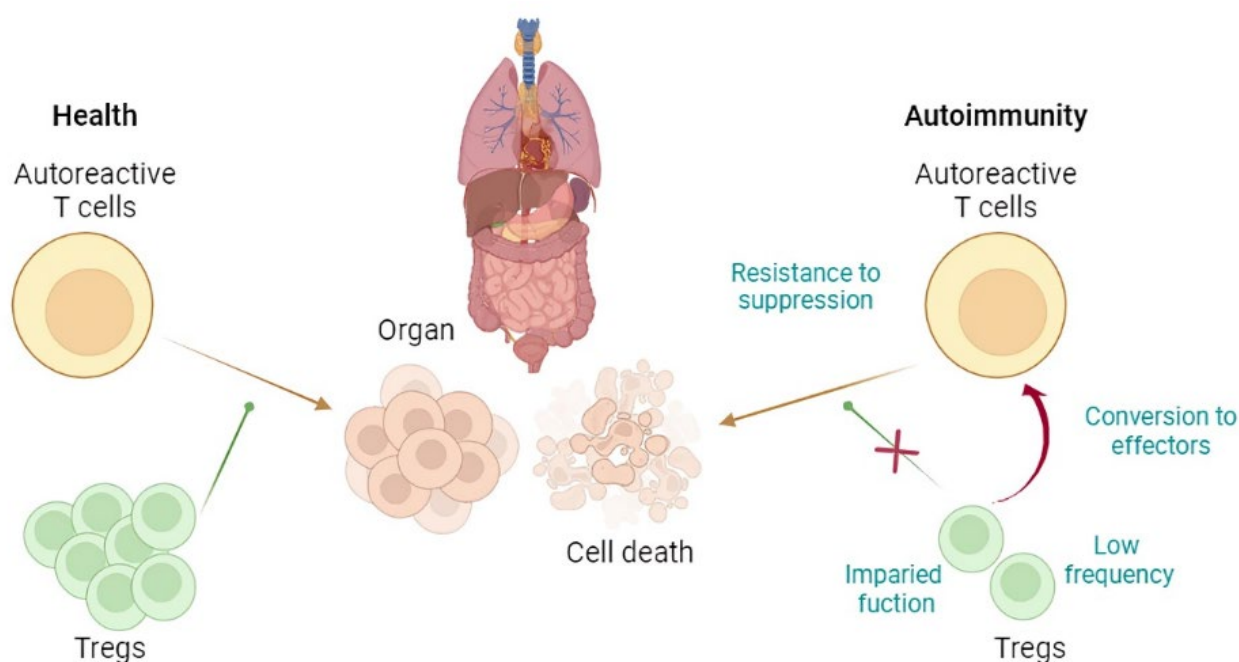
Uncontrolled cell proliferation is another critical issue with Treg therapies. If Tregs proliferate uncontrollably, they can exacerbate immune suppression rather than enhance immune response. This is particularly concerning in cancer therapy, where an overabundance of Tregs can impede antitumor responses. Manipulating regulatory T cells in clinical settings offers a hopeful method for managing undesirable immune reactions, although the interaction of various factors is not yet fully understood.<sup>56</sup> This underscores the necessity of developing precise control mechanisms for Treg proliferation.

To mitigate these risks, various strategies are being explored. One approach involves the use of drugs like pentoxifylline (PTXF), which can impair the generation and maintenance of activated Tregs. Administering the FDA-approved medication pentoxifylline (PTXF) daily significantly slowed tumor growth in a mouse model of melanoma transplantation by modifying the identity and functionality of Treg cells.<sup>57</sup> This highlights the potential of pharmacological interventions in controlling Treg activity.

## Technical challenges and future perspectives in studying human Tregs

One of the challenges for the researchers is to identify a homogeneous Treg population since many defining markers - CD25,<sup>1</sup> FOXP3<sup>2,3</sup> and CTLA-4<sup>4</sup> are increased upon effector T-cell activation. This is why researchers choose a combination of different Treg markers based on standard protocols and their experience with experimentation. To evaluate the functions of Treg, it is necessary to consider the ability of these cells to eliminate proliferation in target cells.<sup>5</sup> Thornton and Shevach 1998 performed the "proliferation assay",<sup>6</sup> which consists of isolating putative regulatory and responder cell populations by immunomagnetic or fluorescence-activated cell sorting (FACS). There are two types of assays based on carboxyfluorescein succinimidyl ester (CFSE), and 3H-thymidine, where the former has an advantage in that cytofluorometry is used simultaneously to label the responder cell population exclusively. At the same time, the latter is applied to measure and evaluate the proliferation of regulatory and responder cells for a short period while remaining in the co-culture.<sup>7</sup>

It is essential to consider one factor in particular, which is the isolation method. Compared to FACS-based protocols, immunomagnetic isolation obtains higher purity and helps analyze many Treg markers during the same period, better defining the Treg population. Investigations of Tregs in phenotype and frequency assessment are one of the current challenges scientists face, as there are limitations when performing assays due to their ability to reproduce inflammatory characteristics in vitro. Several investigations of Tregs applied in autoimmune diseases have shown that Tregs maintain tolerance when effector T cells are eliminated [Fig 2].



**Figure 2. Deficiency of regulatory T cells in autoimmunity. In health, Tregs maintain tolerance by suppressing effector T cells. Tregs fail to suppress autoreactive effector T cells in organ-specific autoimmune diseases, implying that target cells die. Reported reasons include insufficient numbers of Treg, impaired suppressive capacity, transformation of Treg into effector cells, and resistance of effector T cells to Treg-mediated suppression.**

Clinical trials of Treg immunotherapy mostly use adoptive transfer of CD4+CD25+ or CD4+CD25+CD127- cells.<sup>8</sup> However, there are more precisely defined subsets of human Treg. The use of these Treg subsets may

benefit certain disease states. Treg immunotherapy has a solid preclinical evidence base and emerging data supporting the safety and efficacy of Treg immunotherapy regimens in patients with a clinical condition. Clinical tolerance is required for ex vivo expansion with applied transport and in vivo manipulation to extend and enhance endogenous Treg function, which represents promising strategies for treating autoimmune and allo-immune diseases. For clinically applicable Treg immunotherapy protocols to be successful, researchers must overcome significant barriers, including Treg stability, isolation and storage of subpopulations. Given that immune regulation disorders underlie many clinical disorders, the design of safe and effective immunotherapies using Treg cells could greatly benefit.

Building upon these foundational challenges, future research must strategically target enhancing Treg stability and refining the specificity of Treg markers. These focal areas are crucial for advancing our basic scientific understanding of Tregs and vital for developing more effective and safe therapies.

Recent research into manipulating the expression of key markers such as CD25 and FOXP3 through genetic editing techniques provides a promising basis for more effective therapies. Enhanced stability of Tregs could allow for more durable interventions that are less susceptible to variability in inflammatory environments and after in vitro expansion, potentially revolutionizing treatments for autoimmune diseases and preventing transplant rejection.<sup>58</sup>

Furthermore, developing precise quantification techniques, such as DNA methylation analysis of the FOXP3 promoter region, could offer a more reliable and specific method for identifying actual Treg cells. This is crucial for implementing Treg-based therapies, ensuring that only cells with genuine regulatory capabilities are selected for clinical expansion and application.<sup>59</sup>

Developing strategies for the selective depletion of Treg subpopulations contributing to specific disease pathologies is another critical frontier in research. Recent studies indicate that strategies targeting specific markers predominantly expressed on Tregs, such as CD25 in tumor contexts, could selectively modulate these cells in pathological settings, offering a more targeted and potentially more effective therapeutic approach.<sup>60</sup>

Addressing these specific areas in future research will significantly advance the use of Treg cells for treating various clinical conditions. Such advancements promise not only to overcome current limitations in treating autoimmune diseases and transplant management but also have the potential to fundamentally transform therapeutic strategies in other medical fields where immune regulation is critical.

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## CONCLUSIONS

Taken together, Tregs have been demonstrated to have a unique role in regulating or suppressing immune system cells, maintaining immune homeostasis, and controlling inflammation induced by pathogens and environmental toxins. As a result, Treg has recently emerged as a pivotal player in promoting the repair and regeneration of various organ systems. The basic mechanisms by which Treg cells function are Inhibitory Cytokines, Cytolysis, Metabolic Disruption, and Targeting Dendritic Cells.

Preclinical and clinical research has established the potential of Treg-based therapies as a promising alternative in hematopoietic stem cell transplantation, induction and maintenance of resistance to large organ allogeneic transplants, and treatment of autoimmune diseases. Preclinical studies have confirmed the feasibility and tolerability of Treg therapy. However, little data is available on the efficacy and reproducibility of this approach in clinical trials. Future studies include research on specific markers, recognizing Treg subsets with relevant properties, and recognizing gold standard techniques for deriving, stabilizing, and manufacturing larger Tregs used in the clinic, preserving its suppressor function.

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