Improving the Efficacy of Regulatory T Cell Therapy

Paulien Baeten1,2 · Lauren Van Zeebroeck2,3 · Markus Kleinewietfeld2,3 · Niels Hellings1,2 · Bieke Broux1,2,4 ©

Accepted: 31 May 2021 / Published online: 5 July 2021 © The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2021

Abstract

Autoimmunity is caused by an unbalanced immune system, giving rise to a variety of organ-specific to system disorders. Patients with autoimmune diseases are commonly treated with broad-acting immunomodulatory drugs, with the risk of severe side effects. Regulatory T cells (Tregs) have the inherent capacity to induce peripheral tolerance as well as tissue regeneration and are therefore a prime candidate to use as cell therapy in patients with autoimmune disorders. (Pre)clinical studies using Treg therapy have already established safety and feasibility, and some show clinical benefits. However, Tregs are known to be functionally impaired in autoimmune diseases. Therefore, ex vivo manipulation to boost and stably maintain their suppressive function is necessary when considering autologous transplantation. Similar to autoimmunity, severe coronavirus disease 2019 (COVID-19) is characterized by an exaggerated immune reaction and altered Treg responses. In light of this, Treg-based therapies are currently under investigation to treat severe COVID-19. This review provides a detailed overview of the current progress and clinical challenges of Treg therapy for autoimmune and hyperinflammatory diseases, with a focus on recent successes of ex vivo Treg manipulation.

Keywords Regulatory T cells · Cell therapy · Autoimmunity · COVID-19 · Gene editing · RNA interference

Introduction

Treatment of autoimmune diseases is mostly based on broad-acting immunomodulatory drugs to restore the balance of the immune system. Since they are not curative, they require lifelong administration and carry a significant risk for side effects. In healthy individuals, regulatory T cells (Tregs) induce peripheral tolerance, thereby preventing an exaggerated immune response of both the adaptive and innate immune system [1]. Already a decade ago, the first-in-man clinical trial using Tregs as a cell therapy was performed. Here, researchers succeeded in controlling the undesired immune response in chronic graft-versus-host disease (GvHD) with high efficacy and limited adverse events [2].

Next to the immunoregulatory properties of Tregs, tissue regenerative functions have been described (as reviewed in [3]). Dombrowski et al. reported that in the damaged central nervous system (CNS), murine Tregs promote oligodendrocyte differentiation and myelin regeneration [4]. In addition, Tregs were found to prevent viral-induced lung damage in mice by producing amphiregulin [5]. Tregs have also been shown to directly induce lung epithelial cell proliferation in both inflammatory injury and non-inflammatory regenerative mouse models [6]. Furthermore, Tregs accumulate in acutely injured skeletal muscle in mice and potentiate muscle repair by amphiregulin production [7]. Tregs have a direct effect on muscle satellite cell expansion and therefore promote muscle regeneration [8]. This newly described Treg function significantly augments the potency when considering cell therapeutic application in autoimmune diseases.

Combined, Tregs are an ideal candidate for cell therapy in autoimmune and hyperinflammatory disorders. Although many steps have been taken toward clinical application, several challenges remain. In this review, we discuss ongoing
(pre)clinical research, the set-backs and opportunities that are intrinsic to Treg therapy.

**Treg phenotype and Plasticity**

CD4^+CD25^{high}CD127^{low} Tregs constitute 5–10% of the total CD4^+ T cell population in the circulation [9, 10]. The majority of Tregs develop in the thymus (tTregs). Their mechanism of suppression has been studied elaborately and entails both cell–cell contact and production of soluble factors (as reviewed in [11]). The transcription factor forkhead box protein 3 (FOXP3) is the master regulator in the development and function of Tregs [12–16]. However, at least in humans, FOXP3 expression is not exclusive for Tregs, as it is also transiently expressed by activated T cells [17–20]. FOXP3 expression and stability of Tregs mainly rely on the methylation status of the gene region. More specifically, the promoter [21] and Treg-specific demethylated region (TSDR) within the 2nd conserved non-coding sequence (CNS2) [22, 23] is completely demethylated in functional Tregs. Determination of the methylation state of the FOXP3 gene region is the most reliable way to identify genuine human Tregs. This discriminates them from recently activated T effector cells (Teff) [21, 23] and in vitro transforming growth factors (G-CSF, IL-6, IFN-γ) induced Treg 

Tregs are known to exhibit functional plasticity, since they can adapt to local inflammatory stimuli and thereby start producing pro-inflammatory cytokines [25–27]. In addition, Tregs are autoreactive by nature, which means that a loss of suppressive capacity (e.g., through unstable FOXP3 expression) could contribute to the development of autoimmunity. Indeed, several studies showed that Tregs collected from inflamed organs in autoimmune disorders have lower FOXP3 levels [28–30]. These “exFOXP3” cells produce pro-inflammatory cytokines and have an activated-memory phenotype [25]. Transfer of exFOXP3 cells into non-obese diabetic (NOD) with recombination activating gene 2 (Rag2) knockout mice leads to the development of autoimmunity [25]. Transfer of myelin-specific exFOXP3 cells into lymphodeficient immunized recipients induced experimental autoimmune encephalomyelitis (EAE) to a similar extent as Teff [31]. In the latter study, researchers found that inflammation in the CNS of EAE mice induced FOXP3 instability and promoted interferon gamma (IFN-γ) production in autoreactive Tregs and that FOXP3 expression stabilized again after resolution of inflammation [31]. Interestingly, various research groups found that in the presence of interleukin 6 (IL-6), FOXP3-expressing mouse Tregs shift toward a T helper 17 (Th17)-like effector phenotype [32–34]. In mice with autoimmune arthritis, IL-17-producing exFOXP3 cells accumulated in inflamed joints, where the conversion to Th17 cells was mediated by fibroblast-derived IL-6 [35]. FOXP3^+IL-17^+ cells are also present in the synovium of patients with active rheumatoid arthritis [35]. In human Tregs, a Th17-like phenotype is induced by IL-1β, IL-23 and IL-21 rather than by IL-6 [36]. In addition, researchers found that only human leukocyte antigen (HLA)-DR^+ Tregs, a Th17-like phenotype is induced by IL-1β, IL-23 and IL-21 rather than by IL-6 [36]. In addition, researchers found that only human leukocyte antigen (HLA)-DR^+ Tregs, is able to secrete IL-17. They only secrete IL-17 upon strong T cell receptor (TCR) stimulation and as a result lose their suppressive capacity [26]. Human memory Tregs were described to constitutively express RAR-related orphan receptor (RORγt) and IL-17, two markers of the Th17 lineage [37]. Tregs from relapsing–remitting multiple sclerosis (RRMS) patients rather show a Th1-like phenotype and have a reduced suppressive function [38]. They produce more IFN-γ than Tregs from healthy controls, while levels of IL-17 secretion remained unaltered. Moreover, expression of T-box transcription factor (Tbet) was increased, and RORγt expression was decreased [38]. This Th1 phenotype can be induced in vitro by addition of IL-12 [38, 39] or in high sodium chloride (NaCl) concentrations [40–42]. Interestingly, type 1 diabetes mellitus (T1DM) patients also display increased proportions of IFN-γ-producing Tregs compared to healthy controls but did not show reduced suppressive activity [43].

The mechanism of FOXP3 instability and subsequent loss of suppressive capacity is not yet fully understood. Some transcriptional regulators of the FOXP3 protein might be involved. For instance, one of those regulators is deleted in breast cancer 1 (DBC1), a component of the FOXP3 complex. DBC1 negatively regulates FOXP3 expression and suppressive function of Tregs, plausibly through the caspase 8-mediated pathway [44]. In addition, Treg-specific overexpression of inhibitor of DNA binding 2 (Id2), an inhibitor of helix-loop-helix DNA-binding transcription factors, increases susceptibility to EAE and spontaneous autoimmunity [45]. In vitro experiments showed that upregulated Id2 expression leads to reduced FOXP3 expression and increased production of Th17-related cytokines in iTregs [45]. Expression of Th subset-related surface markers or transcription factors does not necessarily imply that Tregs are prone to lose their suppressive activity and contribute to disease. For instance, studies showed that expression of interferon regulatory factor 4 (Irf4), a transcription factor essential for Th2 differentiation, in mouse Tregs is essential for suppression of Th2 responses [46]. Tbet expression, a Th1 specifying transcription factor, in Tregs is necessary for suppressing Th1 cells in mice [47]. Moreover, signal transducer and activator of transcription 3 (STAT3) expression in mouse Tregs is indispensable for suppressing Th17 responses in vivo [48]. Treg-specific STAT3 loss results in an enhanced Th17 response, higher mortality and less Treg infiltration [49]. Therefore, it has been hypothesized that Tregs adapt to their environment and can thus exhibit
appropriate suppression of different Th cell subsets. Indeed, upregulation of these transcription factors leads to expression of relevant chemokine receptors and adhesion molecules, enabling Treg migration to their Th counterparts in the target tissue [48].

**Tregs in Autoimmunity and Hyperinflammation**

Tregs control inappropriate immune activity and therefore play a crucial role in preventing autoimmunity and hyperinflammation. Their importance is illustrated by the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome which is caused by a mutation in the FOXP3 gene and results in a complete absence of Tregs and subsequent multi-organ autoimmunity [50–52]. In the mouse model for this condition, called scurfy mouse, diseased mice are rescued by adoptive transfer of Tregs [13, 51, 53, 54]. Indeed, autoimmune and hyperinflammatory diseases are characterized by disturbances in Treg numbers and function.

Treg numbers are decreased in the circulation of untreated RRMS patients [55–59], active systemic lupus erythematosus (SLE) patients [60–63] and patients with active Crohn’s disease [64, 65]. In contrast, the numbers of circulating Tregs is not altered in T1DM patients [66–69]. For coronavirus disease 2019 (COVID-19), conflicting findings on Treg frequency have been reported [70]. COVID-19 is characterized by a hyperinflammatory response directed to the lung and the development of a cytokine storm, where the level of pro-inflammatory cytokines is increased [71, 72]. It was found that severely ill COVID-19 patients have reduced Treg frequencies in the blood [72–77]. In contrast, Rendeira et al. found that the Tregs were increased in COVID-19 patients [78–81]. When looking for Tregs in the target tissue, they were shown to be increased in the inflamed intestinal mucosa of Crohn’s disease patients [64, 65, 82, 83] and in the cerebrospinal fluid (CSF) of RRMS patients [59, 84, 85].

In contrast, Treg numbers seem to be reduced at the site of inflammation in patients with T1DM [86].

Apart from changes in Treg frequency, there is also a reduction in the suppressive activity of these cells in several autoimmune diseases. Circulating Tregs are found to be defective in MS, specifically in untreated RRMS patients [59, 85, 87–90]. Tregs of T1DM patients have an impaired suppressive capacity as well [66, 67, 86] although this could not be confirmed by Putnam et al. [68]. Furthermore, there are conflicting reports about the functionality of Tregs in active SLE [61, 63, 91–93], which still warrants further investigation. In patients with Crohn’s disease, circulating Tregs were found to remain functional [64, 65]. Since these reports all investigated the function of circulating Tregs, it could be argued that the most functional Tregs leave the circulation to suppress Teff in the inflamed tissue, but only very limited data are available. One report shows that Tregs isolated from lymph nodes at the inflamed intestinal mucosa of patients with Crohn’s disease are still suppressive [65]. In contrast, in the EAE mouse model, it has been found that CNS-derived Tregs are unable to suppress Teff isolated from CNS at peak of disease [94].

Altogether, it is clear that disease severity and progression in autoimmune and hyperinflammatory diseases is partly caused by loss of Treg functionality. Therefore, administration of Tregs is an interesting intervention to restore the immune balance. However, since Tregs of patients are dysfunctional in many autoimmune diseases, infusion of autologous Tregs might not be sufficient. Therefore, allogeneic Treg transplantation can be considered but includes several risks, such as GvHD and transplant rejection [95]. In that point of view, isolation and ex vivo manipulation of autologous Tregs in order to induce and stabilize their suppressive activity might be safer and more efficient.

**Current Status of Treg therapy for Autoimmunity and Hyperinflammation**

Researchers worldwide have started to investigate the potential of Treg therapy, with the first clinical trial being reported in 2009 [2]. Preclinical studies in animal models have provided indications about the efficacy, safety and feasibility of Treg therapy. Several phase I and some phase II clinical studies have now been reported and concluded that Treg therapy is well-tolerated, with some indications of efficacy. These studies are further discussed below, with a focus on the use of iTregs to treat autoimmunity and hyperinflammation.

**Preclinical Studies**

First evidence on the potential of Treg therapy has been provided by preclinical experiments using animal models of autoimmune diseases (listed in Table 1). Adoptive transfer of polyclonal CD4+CD25+ cells reduced disease severity and immune cell infiltration into the spinal cord of EAE animals [96–98] and delayed the development of diabetes in NOD mice [99]. Similarly, transfer of CD4+CD25+ Tregs delayed the natural disease development in SLE-prone animals and decreased renal damage [100]. A second injection with Tregs into these SLE-prone mice delayed progression and further decreased mortality [100]. In already established disease, administration of polyclonal CD4+CD25+ cells into mice with inflammatory bowel disease gradually improved clinical signs and restored colonic architecture [101]. In an attempt to further improve efficiency of Treg therapy, it was shown that self-antigen-specific Tregs are even more potent in protecting against development of EAE [98, 102, 103] and diabetes [104–106].

To investigate the function of human Tregs in vivo, humanized mouse models have been developed. Here, human
Table 1 An overview of preclinical studies using Treg therapy in animal models for autoimmune diseases

| Disease | Source Tregs | Timing and dose | Effect | Reference |
|---------|--------------|----------------|--------|-----------|
| EAE CD4⁺CD25⁺ | Naïve mice LN | 3 days before EAE induction 2 × 10⁶ cells | Protection against induction and progression Less immune cell infiltration in spinal cord | [96] |
| EAE CD4⁺CD25⁺ | Naïve mice Spleen and LN | 2 days before EAE induction 2.5 × 10⁶ cells | Decreased severity disease | [97] |
| EAE CD4⁺CD25⁺CD62Lʰ⁻ | Polyclonal/antigen-specific LN | 1 day before and 18 days after EAE induction 2 × 10⁵ cells (polyclonal) 1–3 × 10⁶ cells (antigen-specific) | Polyclonal before induction: little protection Antigen-specific before: complete protection Antigen-specific after: reduced disease severity | [98] |
| EAE CD4⁺CD25⁺ | CNS of EAE mice during remission/ LN of naïve mice | 1 day before and after EAE induction 2 × 10⁴ cells | CNS-derived Treg: protection LN-derived Treg: no protection | [103] |
| EAE CD4⁺CD25⁺ | MBP89-101-IA5-ζ Tg mice Spleen and LN | At time induction and 11 days after induction 1 × 10⁶ cells | At induction: protection After induction: reduced severity disease | [102] |
| T1DM CD4⁺CD25⁺CD62Lʰ⁻/ʰ⁻ | Prediabetic animals Spleen | Co-transfer activated T cells and Tregs 5 × 10⁵ Tregs | CD62Lʰ⁻: no delay CD62Lʰ⁻: delay | [99] |
| T1DM CD4⁺CD25⁺ | Diabetic mice Antigen-specific Pancreas | Co-transfer Tregs and activated T cells 0.5–2 × 10⁵ Tregs | 5 × 10²: no protection 1 × 10³: complete protection | [104] |
| T1DM Retroviral FOXP3-transduced CD4⁺ T cells | Antigen-specific/polyclonal Naïve animals Spleen | Transfer after onset 1 × 10⁵ cells | Antigen-specific: stabilization disease Polyclonal: no effect | [105] |
| T1DM In vitro expanded CD4⁺CD25⁺ | Antigen-specific Spleen and LN | Co-transfer Tregs and activated T cells 2–5 × 10⁶ Tregs | Protection against disease induction | [106] |
| SLE In vitro expanded CD4⁺CD25⁺CD62Lʰ⁻ | Polyclonal Healthy SLE-prone animals Spleen and LN | Transfer before and during (2nd injection) development 6 × 10⁶ cells | Before: delayed development, decreased renal damage During: delayed progression, decreased mortality | [100] |
| IBD CD4⁺CD25⁺ | Spleen | 4 weeks after disease induction 1 × 10⁶ cells | Gradual disappearance symptoms Restore colonic architecture and less infiltrates | [101] |

CD62L L-selectin, CNS central nervous system, EAE experimental autoimmune encephalomyelitis, FOXP3 forkhead box protein 3, IBD inflammatory bowel disease, LN lymph nodes, MBP myelin basic protein, T1DM type 1 diabetes mellitus, Tg transgenic, SLE systemic lupus erythematosus.

Immune cells are injected, either intravenously or intraperitoneally, into immunodeficient mice [107–111]. Transfer of human CD34⁺ hematopoietic stem cells (HSCs) is most commonly used [111, 112] and has originally been developed for preclinical testing of gene therapies [113]. Human T cells drive xenograft versus host disease (xGVHD) in these mice, which can be inhibited by co-transfer of Tregs [114, 115], making this an ideal model for studying Treg function [116]. Adoptive transfer of CD49d⁺ [117] or CD39ʰ⁻ [118] Tregs completely prevents xGVHD. Infusion of expanded human Treg prevented rejection of a human pancreatic islet allograft in a humanized mouse model [119]. Lastly, infused CD45RA⁺ Tregs home to human small intestines in a severe combined immune deficiency (SCID) xenotransplant model [120] indicated that systemically administered Tregs find their way to the place of action.
Clinical Trials

Although other sources of Tregs (e.g. iTreg, induced pluripotent stem cells [iPSC], type 1 regulatory T cell [Tr1]) are being explored in (pre)clinical studies [105, 121–128], this review focuses on Treg therapy in autoimmunity and hyperinflammation (listed in Table 2), since this is the most commonly used source of Tregs in clinical trials. It is worth mentioning that early Treg clinical trials have mostly focused on graft rejection and GvHD (reviewed in [129]). Briefly, Treg therapy lowered the incidence of acute GvHD [130, 131], relieved or stabilized symptoms of chronic GvHD [2, 132] and reduced the need for immunosuppressive treatment in chronic GvHD patients [2, 132] and after organ transplantation [133] with a 2-year graft survival [134].

Infusion of in vitro expanded autologous, polyclonal CD4⁺CD25⁺⁺CD127⁻ Tregs has been evaluated in different clinical trials for T1DM [135–138] and SLE [139] and was found to be well-tolerated. In T1DM patients receiving Treg therapy, insulin use and connecting peptide

| Disease | Phase | Product | Expansion | Dose and infusion | Effect | Reference study ID |
|---------|-------|---------|-----------|-------------------|--------|-------------------|
| T1DM I  | Polyclonal | Polyvalent Autologous | Anti-CD3/CD28 beads | 10–20×10⁶ cells/kg bodyweight | Well-tolerated | [135] |
|         |       | MACS + FACS | IL-2 | Single infusion | Increased C-peptide | |
|         |       | CD4⁺CD25⁺⁺CD127⁻ | 14 days | | Decreased insulin use | |
|         |       | Autologous | | | Cells stay present up to 4 months | |
| T1DM I  | Polyclonal | Polyvalent Autologous | Anti-CD3/CD28 beads | 10–30×10⁶ cells/kg bodyweight | Well-tolerated | [136, 137] |
|         |       | FACS | IL-2 | Single or double infusion | Increased C-peptide | |
|         |       | CD4⁺CD25⁺⁺CD127⁻ | | | Decreased insulin use | |
|         |       | Autologous | | | Decreased HbA1c levels | |
|         |       | FACS | | | Prolonged remission but still progression | |
|         |       | CD4⁺CD25⁺⁺CD127⁻ | | | Cells stay present up to 1 year before returning to baseline after 2 years | |
|         |       | | | | 2nd dose beneficial | |
| T1DM I  | Polyvalent | Autologous | Anti-CD3/CD28 beads | 0.05–26×10⁸ cells | Well-tolerated | [138] |
|         |       | Autologous | IL-2 | Single infusion i.v. | No opportunistic infections | |
|         |       | FACS | 14 days | | Indications for improved metabolic activity | |
|         |       | CD4⁺CD25⁺⁺CD127⁻ | | | Cells stay present up to 1 year | |
| T1DM II | Polyclonal | Autologous | Yes | Low or high dose Single infusion | Completed | NCT02691247 |
| T1DM I/II | UC blood | Yes | | 1–5×10⁹ Tregs/kg bodyweight Combined with insulin | Ongoing | NCT02932826 |
| MS I    | Polyclonal | Autologous | Yes | | Ongoing | EudraCT 2014–004,320-22 |
| SLE I   | Polyclonal | Autologous | Yes | Anti-CD3/CD28 beads | Safe | [139] |
|         |       | FACS | IL-2 | 1×10⁸ cells/kg bodyweight Single infusion | Rapid peripheral loss | |
|         |       | CD4⁺CD25⁺⁺CD127⁻ | | | Stable disease for 48 weeks | |
| SLE I   | Polyclonal | Autologous | Yes | 0.5–10×10⁹ Tregs/kg bodyweight Single infusion | Ongoing | NCT03185000 |
| SLE I   | Polyclonal | Autologous | Yes | | Ongoing | NCT04468971 |
| SLE I   | Polyclonal | Autologous | Yes | | Ongoing | NCT04468971 |

C-peptide connecting peptide, COVID-19 coronavirus disease 2019, FACS fluorescence activated cell sorting, HbA1c haemoglobin A1c, IL interleukin, i.v. intravenous, MACS magnetic activated cell sorting, MS multiple sclerosis, SLE systemic lupus erythematosus, T1DM type 1 diabetes mellitus, UC umbilical cord
(C-peptide) levels remained stable; however, these patients still progressed [136–138]. Still, clinical trials in T1DM are progressing into phase II studies. In SLE, infused Tregs were found in skin biopsies of a patient with active skin disease, resulting in a transient disease stabilization [139]. In MS and Crohn’s disease, the first clinical trials with Treg therapy are being executed, and the results have not been reported yet (at the moment of writing).

Recently, Gladstone et al. treated 2 patients with severe hyperinflammatory COVID-19 with 2–3 doses of allogeneic, cryopreserved Tregs derived from umbilical cord blood [140]. The infusion was well-tolerated and led to a decrease of inflammation and recovery of both cases. A phase I clinical trial (“RESOLVE”) has now been initiated to evaluate safety and efficacy of Treg therapy in severe COVID-19.

Challenges and Opportunities of Treg Therapy in the Clinic

Overall, clinical studies already demonstrate the successful manufacturing of Treg for infusions, provide evidence that Treg therapy is well-tolerated and show some clinical benefit [135–140]. Four important steps need to be taken when applying Treg cell therapy: (1) isolation, (2) expansion, (3) re-infusion of pure and stable cells and (4) in vivo follow-up of the treatment. However, all of these steps come with challenges, but also opportunities, as discussed below. In the next chapter, we will provide novel insights from fundamental studies that could tackle these challenges.

The first step is Treg isolation. There are many potential sources: autologous or donor blood, umbilical cord blood [130], fresh or cryopreserved samples [141]. Each of them has their own advantages and disadvantages. Fresh, autologous blood is the best option to avoid rejection and is independent of donor availability. However, patients with autoimmune diseases may not fully benefit from their own Tregs as they are shown to have reduced functioning and circulate in lower numbers. Off-the-shelf products, like cryopreserved cells, cannot be directly injected since a round of in vitro reactivation is still required after thawing to obtain viable and suppressive Tregs [141].

Treg isolation can be done using magnetic (MACS) or fluorescence-activated cell sorting (FACS), and both are being used in clinical studies [2, 135–139]. MACS is performed in a closed, sterile system; uses good manufacturing practice (GMP) consumables and can quickly process high numbers of untouched cells. However, the purity of the cellular product is better with FACS since manual gating and careful selection of the purest and most precise population is possible, but it is very time-consuming. The second disadvantage of FACS is the droplet formation which makes it harder to work in GMP conditions. However, new custom-made FACS systems are being manufactured to enable researchers to work under GMP restrictions [142, 143]. Currently, samples are usually pre-enriched with MACS and further purified with FACS, although this increases the cost [2, 135, 142]. FOXP3 is the best marker to isolate pure Tregs but cannot be used for viable cell sorting and subsequent culturing and infusion. Isolation of CD4+CD25highCD127low cells, as currently used in clinical trials, results in a functional but heterogeneous Treg population.

Including more surface markers (e.g. CD45RA, CD121, CD49d, CD39, CD154, latency-associated peptide [LAP]) can result in a better defined and purer Treg population (Table 3). CD45RA is already being implemented in a

| Table 3: Additional surface markers to identify stable and potent Tregs |
|-----------------------|-----------------|-----------------|
| Marker | Result | Reference |
| CD45RA+ | No switch to a Th17-like phenotype | [120] |
| | Completely demethylated TSDR | |
| | Retain suppressive activity in vitro | [144] |
| | Maintain stable Tregs phenotype after ex vivo expansion | |
| LAP+ | 90% FOXP3+ | Better suppression in vitro compared to CD4+CD25highCD127low | [161] |
| | No cytokine production | |
| CD121+ | 90% FOXP3+ | Better suppression in vitro compared to CD4+CD25high | [161] |
| | No cytokine production | |
| CD49d− | 90% FOXP3+ | Highly suppressive in vitro | [117, 145] |
| | No cytokine production | Stable FOXP3 expression during expansion | |
| CD39high | Highly suppressive in vitro | Stable FOXP3 expression during IL-1β and IL-6 challenge | [118, 162] |
| CD154− | Highly demethylated TSDR | |
| | Highly suppressive in vitro | [163] |

FOXP3 forkhead box protein 3, IL interleukin, LAP latency-associated peptide, Th T helper, TSDR Treg-specific demethylated region
running clinical trial for Crohn’s disease [120]. These markers and their combinations create new opportunities to increase efficacy, since the most potent Tregs can be selectively isolated.

When a sterile-operating FACS system is available, any combination of markers is possible. However, when using MACS, there is a limit to the number of markers that can be included. We suggest to add CD45RA+, CD49d− and CD39high as additional markers, since these have been shown to identify highly potent human Treg subsets that are effective in xenogeneic models, remain stable during in vitro expansion and are not affected by an inflammatory environment [117, 118, 120, 144, 145].

Preclinical studies have revealed that self-antigen-specificity of Tregs correlates with therapeutic potency. Some studies found that polyclonal Tregs are effective in delaying induction and progression of disease [96, 97], while others could not confirm these findings [98, 103, 105]. However, there is consensus that lower numbers of cells are needed when using self-antigen-specific Tregs, since suppression of the immune response is very targeted. In one study, as little as 1 × 10⁶ self-antigen-specific Tregs were shown to be sufficient to protect against the development of diabetes in mice [104]. However, this is difficult to translate into human clinical studies, since causative self-antigens have not been identified for most autoimmune diseases and can vary between patients. Moreover, self-antigen-specific Tregs are only present in low numbers in peripheral blood which makes expansion in vitro challenging. Polyclonal Tregs furthermore provide bystander suppression by production of immunomodulatory cytokines, making this modality effective in diseases with an unknown causative antigen [146–149]. However, with new developments in the field to induce selective antigen-specificity and the discovery of causative antigens, antigen-specific Tregs could hold great potential for future treatments. Indeed, in preclinical studies, antigen specificity is introduced in Tregs by overexpression of a recombinant TCR or a chimeric antigen receptor (CAR). CAR proteins are a synthetic fusion of co-receptors and recognize unprocessed proteins on lins and the TCR signalling domains. They are independantly isolated.

Use of antigen-specific Tregs has recently been extensively reviewed in [154, 155]. Here, we propose a different approach to increase efficiency of Treg therapy. Because of the low number of Tregs in blood, in vitro expansion of the isolated Tregs is the inevitable second step of Treg therapy. During expansion, it is important to maintain the suppressive nature of the cells and to prevent outgrowth of non-Tregs. As Tregs are anergic in vitro [156], efficient protocols for cell expansion under GMP conditions are essential, and they have been reported over the years. In addition, fully closed [157] and even automated [158] expansion systems have been developed. Advantages of these systems are improved biosafety, lower risk of product contamination, standardization, lower costs and less variation. Up to now, all clinical trials have used anti-CD3/CD28 beads and IL-2 to successfully expand Tregs of autoimmune patients [135–139]. During a 2-week expansion, the expansion rates ranged between 29.8 and 1366.8 with significant donor variation [138, 159].

There are however some considerable disadvantages related to expansion. First, use of anti-CD3/CD28 beads leads to a 10% loss of cells, since beads need to be removed before re-infusion [142]. Second, there is a risk of contamination with Teff or microorganisms during the in vitro culture. Third, a loss of suppressive function after Treg expansion has been reported. Researchers found that in vitro culturing of CD4⁺CD25highCD127low Tregs resulted in loss of Treg phenotype (FOXP3 levels and TSDR methylation status), loss of suppressive function and induction of inflammatory cytokine production, specifically in the CD45RA⁻ subpopulation [120, 144, 160]. In contrast, other research groups were successful in expanding functional Tregs. Several studies report that expanded Tregs are still suppressive, produce no pro-inflammatory cytokines and have a 100% demethylated TSDR after 2 weeks of culturing, without Teff contamination [2, 135, 136, 138]. Therefore, consensus was reached to limit the in vitro expansion time to a maximum of 2 weeks [160]. In addition, careful Treg isolation is of crucial importance; additional selection of CD45RA⁺ significantly enhances Treg stability over the culturing period [120, 144].

Many research groups have attempted to optimize the in vitro Treg expansion protocol. First, the mTOR kinase inhibitor rapamycin was found to selectively expand highly suppressive FOXP3⁺ Tregs [24, 157, 164–166]. Production of inflammatory cytokines is prevented as well [157, 165, 166], and cells retain a demethylated TSDR [24]. Rapamycin should be included in the protocol when Tregs are isolated using MACS, in which CD25⁺ (potentially containing activated Teff), and not exclusively CD25high cells, are selected. The addition of a vitamin A derivate, all trans retinoic acid, was additionally found to augment the effects of rapamycin [166]. Rapamycin, all trans retinoic acid and calcineurin inhibitor tacrolimus are also shown to influence the chemokine receptor homing profile of Tregs [166, 167]. Rapamycin induces expression of skin-homing C–C chemokine receptor 4 (CCR4) and cutaneous lymphocyte-associated antigen (CLA), while all trans retinoic acid and tacrolimus result in gut-homing α4β7 integrin expression.
Depending on the disease, using these supplements during in vitro culture might therefore result in a more suited Treg phenotype. In addition, culturing cells in mild hypothermic conditions prevents loss of FOXP3 and demethylated TSDR status, while augmenting cell expansion. Interestingly, mildly hypothermia enables Treg expansions that were refractive to in vitro expansion under regular conditions [168]. Finally, the type of medium and serum is also crucial for optimal Treg expansion. Golab et al. determined that X-VIVO™ medium combined with human serum induces the highest expansion rates while maintaining Treg phenotype [169].

Altogether, it is of crucial importance to monitor the phenotype and potency of Tregs after in vitro expansion. This is important for the third step of Treg therapy, which is reinfusion of Tregs into the patient. Before administration of Tregs can be considered, the cellular product must meet all release criteria defined by Food and Drug Administration (FDA) (Table 4). These criteria are sterility, purity, identity and potency [142]. Several research groups take along extra criteria, including the in vitro suppressive capacity and the methylation status of TSDR [138, 142]. However, for both criteria, several days are needed to obtain results, during which the cells could have changed again. Therefore, the European Medicines Agency (EMA) and its advanced therapy medicinal product (ATMP) guidelines require stability of the final product for a longer time period. This allows the cellular product to be stored before infusion, while more time-consuming quality control measurements are completed [158]. Tests to measure these release criteria also need to be accurate, precise, specific, suitable and robust. Once the cells are sufficient in number and quality, infusion into the patient is possible. Determination of the dose depends on the potency of the cells (polyclonal or antigen-specific), the disease and disease activity. Doses used in clinical trials range from 0.1 up to 100 million cells/kg bodyweight (Table 2). Although most clinical trials use a single infusion, a 2nd dose is found to be beneficial in T1DM, since the metabolic outcome after 1 year was found to be better in these patients compared to patients receiving 1 dose [136].

Once Tregs are administered, the final step of the therapy is careful follow-up of the patient. First, in vivo monitoring of the infused cells should be performed. Using different labelling methods, it was shown that infused Tregs peak during the first 2 weeks in the blood and slowly decay afterward but remain detectable for 1 year after infusion [130, 136–139, 170]. Since patient sampling is mostly restricted to blood, it is unclear whether the infused cells die or alternatively reach the target tissue. In one study, infused Tregs were shown to be present in skin biopsies of an SLE patient receiving the cellular product [139]. Therefore, more methods that investigate tissue infiltration are needed, and new, non-invasive whole-body imaging techniques are in development. One research group transfected mouse Tregs with a human sodium iodide symporter (NIS), making them detectable with single-photon emission computed tomography (SPECT/CT) after injecting a radiolabel, without affecting Treg phenotype or function [171]. In another study, infused 111Indium tropolonate-labelled Tregs were detectable in the liver up to 72 h after infusion, using SPECT/CT [172]. Here, labelling did not affect Treg function or phenotype and decayed after 72 h. These methods have so far only been performed in mice, but they have great potential to be used in humans, to track infused cells into the target organ. Finally, efficacy of treatment is monitored using disease-specific clinical methods. For instance, disease-specific parameters like C-peptide levels are measured in T1DM patients [135–137]; presence of inflammatory cytokines is determined in skin biopsies of SLE patients; follow-up on brain lesions using magnetic resonance imaging (MRI) of MS patients and colonoscopy of patients with Crohn’s disease to calculate the disease activity score are performed.

The use of Treg therapy could possibly induce a global immune suppression in the patients, especially when polyclonal Tregs are used, with a risk of developing side effects. In general, Treg therapy is found to be well-tolerated. However, opportunistic infections have been reported in some studies [132, 173]. In addition, contamination of the cellular product with Teff or unstable Tregs could even exacerbate disease. This risk is avoided by sufficient control of the in vitro culture conditions, and monitoring Treg phenotype/function before administration, as described above. Finally, malignancies have been proposed as a possible adverse event of Treg therapy, since Tregs suppress anti-tumour immunity. Although a direct correlation was not found, skin cancers did occur in patients receiving Treg therapy in one study [132]. Specifically in autoimmunity, additional challenges arise. The inflammatory environment in the target tissue could cause Treg instability, leading to their differentiation

Table 4 Release criteria defined by FDA and EMA (ATMP) before administration of cells as a therapy into patients

| Release criteria          | Minimum criteria                  |
|--------------------------|-----------------------------------|
| Sterility                | Mycoplasma Absent                 |
|                          | Anaerobic and aerobic bacterial growth Absent |
|                          | Fungal growth <5 EU/kg            |
|                          | Endotoxin                         |
| Purity                   | % CD4+ cells >90%                 |
|                          | % CD8+ cells <5%                  |
|                          | Residual beads <100 beads per 3×10⁶ cells |
| Identity                 | % FOXP3+ cells >60%               |
| Potency                  | % viability >75%                  |
| Stability of final product | Several hours (overnight)         |

*FOX3* forkhead box protein 3

Springer
into unpredictable, autoreactive Teff, as discussed before. In addition, the causative self-antigen is not always known, and therefore, incorrect antigen-specificity or incomplete coverage leads to an ineffective intervention [163].

**Ex Vivo Manipulation of Tregs**

As mentioned, maintaining functional stability of Tregs is crucial since they will be exposed to a highly inflammatory environment in autoimmunity or hyperinflammation. Also, when considering autologous transplantation, the isolated Treg population may be less functional. As discussed earlier, additional markers to isolate a highly functional and stable Treg population could be considered. However, isolating specific subpopulations of Tregs will lead to a low yield, requiring an extensive in vitro expansion step which ideally should be limited to 2 weeks. To overcome these issues, we propose ex vivo manipulation to ensure proper and stable Treg functioning in vivo [174]. Several strategies are discussed below (Fig. 1), based on recent progress made within this field.

Stable FOXP3 gene expression is ensured by viral vector-induced ectopic overexpression of FOXP3 and Helios or the use of dCas9 to direct a transcriptional activator VPR to the FOXP3 gene. Demethylation of the TSDR region of the FOXP3 gene and histone acetylation can be enhanced using dCas9 fused to enzymes TET and p300 or by targeting DNMT, resulting in stable FOXP3 expression. Control of ubiquitination of the FOXP3 protein by enhancing its acetylation (HDAC inhibition, p300 induction), inhibiting cytokine-induced Stub1 levels and enhancing USP7 expression, prevents FOXP3 degradation. Enhancing RNF31 expression induces atypical ubiquitination resulting in FOXP3 stability. Inhibiting kinase PIM1 or DBC1 preserves FOXP3 levels. Functionality of Tregs is boosted by inhibiting TNF-α-recruited PKCθ. Introducing engineered IL-2–IL-2Rβ and CAR into the cells specifically expands transfected Tregs. Targeting JNK1 leads to IL-2 independent survival of Tregs. Ac acetylation, CAR chimeric antigen receptor, DBC1 deleted in breast cancer 1, dCas9 catalytically inactive Cas9, DNMT DNA-methyltransferases, FOXP3 forkhead box protein 3, HDAC histone deacetylase, Helios zinc-finger protein 2 (IKZF2), JNK1 c-Jun N-terminal kinase 1, P phosphorylation, p300 histone acetyltransferases, PIM1 kinase, PKCθ protein kinase, RNF31 ring finger protein 31, shRNA short hairpin RNA, Stub1 E3 ubiquitin ligase, TCR T cell receptor, TET ten-eleven translocation, TNF-α tumour necrosis factor alpha, TSDR Treg-specific demethylated region, Ub ubiquitination, USP7 deubiquitinase. Created with BioRender.com.
Stable Gene Expression

To obtain a stable Treg phenotype and function, investigation initially focused on stabilizing FOXP3 gene expression. At the beginning of the century, researchers successfully induced FOXP3 gene expression in human naïve CD4+ T cells using viral vectors. This resulted in typical FOXP3+ Tregs with in vitro suppressive function [175–178]. In addition, these converted Tregs remained stable in both in vitro and in vivo inflammatory conditions [50]. More recently, FOXP3 gene expression was induced in Jurkat cells using a catalytically inactive Cas9 protein (dCas9) bound to an active domain of the transcriptional activator VPR and single-guide RNAs (sgRNAs) targeting important FOXP3 gene regions [179]. Another important transcription factor that regulates the function of Tregs is Helios (zinc-finger protein 2 [IKZF2]). Helios is present on the FOXP3 promotor as a transcription factor [180] and correlates with the suppressive capacity of Tregs [181]. Helios+ Tregs are highly suppressive, while Helios− Tregs exclusively produce inflammatory cytokines [182]. Forced overexpression of Helios using a retroviral vector in naïve mouse T cells undergoing in vitro Treg differentiation had no additive effect on FOXP3 levels but improved the suppressive capacity of the cells [183]. Furthermore, ectopic expression of both FOXP3 and Helios in human CD4+ T cells results in highly suppressive Tregs that delay disease in the xenogeneic GvHD model [184].

Epigenetic Editing

As discussed previously, the epigenetic status of the FOXP3 gene is crucial for its expression in Tregs, and consequently, for stable Treg function. In mouse and human iTregs, the TSDR region of the FOXP3 gene is completely demethylated by the ten-eleven-translocation (TET) enzyme, and this is counterbalanced by DNA-methyltransferases (DNMTs) [21, 22]. In addition, histone acetylation has been described as an important mechanism in mouse Tregs, involving histone acetyltransferases (p300) and histone deacetylase (HDAC). Using dCas9 fused to the active parts of TET enzyme or p300, the enzymes can be guided to the FOXP3 gene to demethylate the CNS2 region or induce histone acetylation at the promotor, respectively, in mouse iTregs [185]. The p300-iTregs were very stable in in vitro inflammatory conditions and retained FOXP3 levels, in contrast to partial stabilizing of FOXP3 in the TET-iTregs [185]. In addition, TET enzyme overexpression induced with retroviral vectors enhanced mouse iTreg stability in both in vitro and in vivo inflammatory conditions by promoting CNS2-specific demethylation [186]. Furthermore, the use of RNA interference to reduce DNMT expression resulted in an increase in FOXP3 levels in naïve CD4+ mouse cells [187].

Next to gene editing, the culture conditions were also found to affect the methylation state of the FOXP3 gene. Lowering oxygen levels [186] and adding vitamin C [186, 188] to mouse and human iTregs were shown to induce TET enzyme activity, leading to suppressive iTregs with stable FOXP3 levels in inflammatory conditions. In addition, the methylation inhibitor 5-azacytidine [187, 189] and the acetylation enhancer Trichostatin A (TSA) [189] both increase FOXP3 levels in naïve CD4+ mouse cells. Next, TSA maintained FOXP3 expression in human Tregs and prevented cytokine-induced IL-17 production [36]. In addition, inhibition of HDAC through pharmacological inhibitors nicotinamide (NAM) [190, 191], which is already in clinical use [192, 193], or Ex-527 [194] increases FOXP3 levels and its transcriptional targets.

FOXP3 Protein Stability

To ensure stability of the FOXP3 protein, it has to be protected from polyubiquitination, which leads to degradation. Modulating the acetylation of the FOXP3 protein using p300 and HDAC prevents ubiquitination and induces stable FOXP3 expression in mouse and human Tregs [190]. In addition, it was reported that recruitment of inflammatory cytokine-induced E3 ubiquitin ligase Stub1 to FOXP3 increases polyubiquitination of FOXP3. Therefore, researchers used RNA interference to inhibit Stub1 in both human and mouse Tregs. This prevented degradation of FOXP3, even in in vitro inflammatory conditions, thereby protecting mice from colitis induction [195].

In another report, ectopic expression of both deubiquitnase USP7 and FOXP3 in human embryonic kidney (HEK) 293 T cells reduced FOXP3 polyubiquitination [196]. In contrast, atypical ubiquitination seems to stabilize FOXP3 protein expression in human Tregs, since lentiviral overexpression of Ring finger protein 31 (RNF31) ubiquitinitates FOXP3 and leads to an enhanced suppressive capacity in vitro [197].

Phosphorylation of the FOXP3 protein also influences its functionality. In human Tregs, PIM1 kinase phosphorylates FOXP3, resulting in decreased chromatin binding activity of the transcription factor [198]. Therefore, knockdown of PIM1 in human Tregs using short hairpin RNA (shRNA) results in enhanced gene expression of FOXP3 target genes and increased suppression in vitro [198].

Finally, DBC1 is a protein which is part of the FOXP3 complex and negatively regulates FOXP3 expression [44]. Loss of DBC1 in both human and mouse cells using shRNA results in stable FOXP3 levels, even in in vitro inflammatory conditions, and an enhanced suppressive function in vivo [44].
Treg Function

Treg function is in many cases directly correlated to (stable) FOXP3 expression, as described above. However, several strategies have been successfully reported that enhance Treg function without (directly) targeting FOXP3 gene expression. For instance, RNA interference to target protein kinase PKCθ in human Tregs was shown to enhance their suppressive capacity [199]. PKCθ is recruited to the immunological synapse after stimulation with tumour necrosis factor alpha (TNF-α), leading to nuclear factor-κB (NF-κB) activation, thereby reducing Treg function and enhancing Teff function [200]. This TNF-α-induced loss of suppressive capacity was thus prevented by knockdown of PKCθ.

Cell Survival and Expansion

For cell therapy products, the survival of the infused cells in vivo is of crucial importance for the efficacy of the treatment. Tregs are highly dependent on IL-2 for their survival and expansion [201]. However, to avoid challenges related to toxicity and off-target effects of systemic IL-2 infusion, an orthogonal IL-2–IL-2 receptor β chain (IL-2Rβ) pair was engineered, which can be used to selectively expand transfected cells expressing the orthogonal IL-2Rβ after infusion with orthogonal IL-2 in mice [202]. Another strategy is to add part of the cytoplasmic domain of IL-2Rβ to a CAR cassette, to induce antigen-dependent survival and proliferation of human cells [203]. However, this would only be feasible in autoimmune diseases with known auto-antigenic triggers. Finally, an IL-2 independent mechanism was explored by performing knockdown of c-Jun N-terminal kinase 1 (JNK1) in mouse Tregs, which leads to apoptosis-resistance and enhanced suppressive function in vitro. Consequently, in the context of islet transplantation, this resulted in prolonged in vivo islet allograft survival [204].

Conclusions

Altogether, it is evident that while Treg therapy in autoimmunity and hyperinflammation has provided encouraging results, many challenges remain. Experts agree on the need

Table 5 Recommendations for future Treg therapy

| Step | Recommendation | Advantages | Challenges | Reference |
|------|----------------|------------|------------|-----------|
| Isolation | GMP-compatible FACS | Sterility, Purity | Time-consuming | [142, 143] |
| Newly-defined Treg surface markers | Purity, Less heterogeneous, Validated | Limited number of combinations possible, FACS required | [117, 118, 120, 144, 145, 161, 163] |
| TCR and CAR induced antigen-specificity | Safety, Effectiveness, Validated | Antigen unknown, Patient variability | [98, 102, 146, 150–152] |
| Expansion | Closed and automated manufacturing systems | Safety, Sterility, Decreases costs, Less variation | Know-how, Facility required | [158] |
| Addition of growth factors | Purity, Stability, Desired migratory phenotype | | | [24, 157, 164–167] |
| Follow-up | New labelling methods for in vivo monitoring | Cells remain unaffected, Safety, Tracking possible | Time-limited effects, Research limited to small animals | [171, 172] |
| Disease-specific monitoring | Monitor efficacy | Require high sensitivity and specificity biomarkers | [135–137] |
| Ex vivo manipulation | Stable expression of functional molecules | Stability, Functionality, Long term effects | Low transfection rate, Increased culturing time | [44, 175–179, 183–187, 190, 195–199] |
| Boosts survival and expansion | Long term effect | Low transfection rate, Increased culturing time | [202–204] |

CAR chimeric antigen receptor, FACS fluorescence activated cell sorting, GMP good manufacturing practice, TCR T cell receptor, Tregs regulatory T cell
for cell expansion before infusion, and we and others propose to use this window of opportunity to manipulate Tregs in vitro to enhance their suppressive capacity and ensure their stability after administration [174]. One drawback to this strategy is the prolonged manipulation and expansion time needed in vitro. However, following this procedure ensures that infused Tregs remain stable and suppressive in vivo thereby limiting severe side effects. General recommendations for future use of Tregs as a therapy are listed in Table 5.

Most strategies use genetic editing methods on cultured cells, which is a very targeted approach. Especially retroviral and lentiviral vector delivery of RNA interference are applied. They have already been used in clinical trials in several diseases and syndromes, and a gene therapy for SCID patients using retroviral vectors is already FDA- and EMA-approved (Strimvelis®). Both types of viral vectors were found to be highly efficient and long-lasting. However, lentiviral vectors have superior safety profiles [205, 206], since retroviral vectors were found to integrate near proto-oncogenes, causing leukaemia [207, 208]. For cancer, CAR-T cell therapy is FDA and EMA approved (Yescarta® and Kymriah®). The main advantage of a gene editing approach is that it ensures long-lasting effects, in contrast to adapting culturing conditions (e.g. rapamycin supplementation), which is only transiently effective. Furthermore, new and advanced techniques, like dCas9 or redesigned CARs, are being explored. Another advantage of gene editing is that inducible suicide genes can be included as well and can be used as a rescue strategy in the case of adverse events.

In conclusion, ex vivo adaptation of Tregs during expansion and prior to administration ensures the survival, stability and functionality in vivo. Prioritizing future research towards this strategy is predicted to lead to significant progress in the field of Treg therapy in autoimmunity and hyperinflammation.

**Author Contribution** PB and LV performed the literature survey and wrote the first draft and following revisions of the manuscript. MK, NH and BB participated in the manuscript writing and reviewed the manuscript. PB and BB conceptualized the topic of the review.

**Funding** The authors are funded by Fonds voor Wetenschappelijk Onderzoek (FWO), the Belgian Charcot Stichting, Stichting MS Research, MS International Foundation, and MoveS.

**Declarations**

**Conflict of Interest** The authors declare no competing interests.

---

**References**

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 55(3):1151–64

2. Trzonkowski P, Bieniaszewska M, Juscińska J, Dobyszuk A, Krzysztyniak A, Marek N et al (2009) First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. Clin Immunol 133(1):22–26. https://doi.org/10.1016/j.clim.2009.06.001

3. Li J, Tan J, Martino MM, Lui KO (2018) Regulatory T-cells: potential regulator of tissue repair and regeneration. Front Immunol 9:585. https://doi.org/10.3389/fimmu.2018.00585

4. Dombrowski Y, O’Hagan T, Dittmer M, Penalva R, Mayoral SR, Bankhead P et al (2017) Regulatory T cells promote myelin regeneration in the central nervous system. Nat Neurosci 20(5):674–680. https://doi.org/10.1038/nm.4528

5. Arpaia N, Green JA, Molledo B, Arvey A, Hemmers S, Yuan S et al (2015) A distinct function of regulatory T cells in tissue protection. Cell 162(5):1078–1089. https://doi.org/10.1016/j.cell.2015.08.021

6. Mock JR, Garibaldi BT, Aggarwal NR, Jenkins J, Limjunyawong N, Singer BD et al (2014) Foxp3+ regulatory T cells promote lung epithelial proliferation. Mucosal Immunol 7(6):1440–1451. https://doi.org/10.1038/mi.2014.33

7. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cefletti M, Jang Y et al (2013) A special population of regulatory T cells potentiates muscle repair. Cell 155(6):1282–1295. https://doi.org/10.1016/j.cell.2013.10.054

8. Castiglioni A, Corna G, Rigamonti E, Basso V, Vezzoli M, Monno A et al (2015) FOXP3+ T cells recruited to sites of sterile skeletal muscle injury regulate the fate of satellite cells and guide effective tissue regeneration. PLoS ONE 10(6):e0128094. https://doi.org/10.1371/journal.pone.0128094

9. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA (2001) CD4+CD25+CD127- regulatory cells in human peripheral blood. J Immunol 167(3):1245–1253. https://doi.org/10.4049/jimmunol.167.3.1245

10. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S et al (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 203(7):1701–1711. https://doi.org/10.1084/jem.20060772

11. Sambucci M, Gargano F, Guerrera G, Battistini L, Borsellino G (2019) One, no one, and one hundred thousand: T regulatory cells’ multiple identities in neuroimmunity. Front Immunol 10:2947. https://doi.org/10.3389/fimmu.2019.02947

12. Horii S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299(5609):1057–1061. https://doi.org/10.1126/science.1079490

13. Fontenot JD, Gavin MA, Rudensky AY (2003) FOXP3+ regulatory T cells. Nat Immunol 4(4):330–336. https://doi.org/10.1038/nn904

14. Walker MR, Kasprowicz DJ, Gersuk V, Benard A, Van Landeghen M, Buckner JH et al (2003) Induction of Foxp3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 112(9):1437–1443. https://doi.org/10.1172/JCI19441
Clinical Reviews in Allergy & Immunology (2022) 62:363–381

15. Cosmi L, Liotta F, Lazzeri E, Francalanci M, Angeli R, Mazzinghi B et al (2003) Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. Blood 102(12):4107–4114. https://doi.org/10.1182/blood-2003-04-1320

16. Williams LM, Rudensky AY (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 8(3):277–284. https://doi.org/10.1038/nri1437

17. Morgan ME, van Bilsen JH, Bakker AK, Heemskerk B, Schilham MW, Hargreaves FC et al (2005) Expression of Foxp3 mRNA is not confined to CD4+CD25+ regulatory T cells in humans. Hum Immunol 66(1):13–20. https://doi.org/10.1016/j.humimm.2004.05.016

18. Gavin MA, Torgerson TR, Houston E, DeRoos P, Ho WY, Stray-Pedersen A et al (2006) Single-cell analysis of normal T cell-specific demethylated region. J Immunol 194(1):113–124.

19. Stray-Pedersen A et al (2006) Single-cell analysis of normal FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc Natl Acad Sci U S A 103(17):6659–6664. https://doi.org/10.1073/pnas.0509481003

20. Zhou X, Bailey Rosse WA, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong S10875-1182/1002/11182/journ

21. Janson PC, Winardal ME, Marits P, Thor M, Ohlsson R, Winquist O (2008) FOXP3 promoter demethylation reveals the committed Treg population in humans. PLoS ONE 3(2):e1612. https://doi.org/10.1371/journal.pone.0001612

22. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J et al (2007) Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 5(2):e38. https://doi.org/10.1371/journal.pbio.0050038

23. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J et al (2007) DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3+ conventional T cells. Eur J Immunol 37(9):2376–2389. https://doi.org/10.1002/eji.200737594

24. Rossetti M, Spreafico R, Saidin S, Chua C, Moshref M, Leong JY et al (2015) Ex vivo-expanded but not in vitro-induced human regulatory T cells are candidates for cell therapy in autoimmune diseases thanks to stable demethylation of the FOXP3 regulatory T cell-specific demethylated region. J Immunol 194(1):113–124. https://doi.org/10.4049/jimmunol.1401145

25. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M et al (2009) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol 10(9):1000–1007. https://doi.org/10.1038/ni.1774

26. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C et al (2009) IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood 113(18):4240–4249. https://doi.org/10.1182/blood-2008-10-183251

27. Kleinewietfeld M, Hafer DA (2013) The plasticity of human Treg and Th17 cells and its role in autoimmunity. Semin Immunol 25(4):305–312. https://doi.org/10.1016/j.smim.2013.10.009

28. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Spouros E et al (2008) Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. Immunity 28(5):687–697. https://doi.org/10.1016/j.immuni.2008.03.016

29. Bluestone JA, Tang Q, Sedwick CE (2008) T regulatory cells in autoimmune diabetes: past challenges, future prospects. J Clin Immunol 28(6):677–684. https://doi.org/10.1007/s10875-008-9242-z

30. Wan YY, Flavell RA (2007) Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature 445(7129):766–770. https://doi.org/10.1038/nature05479

31. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H et al (2013) Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. Immunity 39(5):949–962. https://doi.org/10.1016/j.immuni.2013.10.016

32. Zhu J, Yamane H, Paul WE (2010) Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol 28:445–489. https://doi.org/10.1146/annurev-immunol-030409-101212

33. Xu L, Kitan and Fuss I, Strober W (2007) Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. J Immunol 178(11):6725–6729. https://doi.org/10.4049/jimmunol.178.11.6725

34. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP et al (2008) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity 29(1):44–56. https://doi.org/10.1016/j.immuni.2008.05.007

35. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T et al (2014) Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med 20(1):62–68. https://doi.org/10.1038/nm.3432

36. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I (2008) Human CD25highFoxp3low regulatory T cells differentiate into CD17-producing cells. Blood 112(6):2340–2352. https://doi.org/10.1182/blood-2008-01-133967

37. Ayyoub M, Deknyftd F, Raimbaut I, Dousset C, Lefueve L, Bioley G et al (2009) Human memory FOXP3+ Tregs secrete IL-17 ex vivo and constitutively express the T(H)17 lineage-specific transcription factor RORgamma t. Proc Natl Acad Sci U S A 106(21):8635–8640. https://doi.org/10.1073/pnas.0900621106

38. Dominguez-Villar M, Baecher-Allan CM, Hafer DA (2011) Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease. Nat Med 17(6):673–675. https://doi.org/10.1038/nm.2389

39. Feng T, Cao AT, Weaver CT, Elson CO,Cong Y (2011) Interleukin-12 converts Foxp3+ regulatory T cells to interferon-gamma-producing Foxp3+ T cells that inhibit colitis. Gastroenterology 140(7):2031–2043. https://doi.org/10.1053/j.gastro.2011.03.009

40. Hernandez AL, Kitz A, Wu C, Lowther DE, Rodriguez DM, Vudattu N et al (2015) Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells. J Clin Invest 125(11):4212–4222. https://doi.org/10.1172/JCI81151

41. Sumida T, Lincoln MR, Ukeje CM, Rodriguez DM, Akazawa H, Noda T et al (2018) Activated beta-catenin in Foxp3+ regulatory T cells links inflammatory environments to autoimmunity. Nat Immunol 19(12):1391–1402. https://doi.org/10.1038/s41590-018-0236-6

42. Muller DN, Wilck N, Haase S, Kleinewietfeld M, Linker RA (2019) Sodium in the microenvironment regulates immune responses and tissue homeostasis. Nat Rev Immunol 19(4):243–254. https://doi.org/10.1038/s41577-018-0113-4

43. McClymont SA, Putnam AL, Lee MR, Eisenh JH, Liu W, Hulme MA et al (2011) Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. J Immunol 187(7):3918–3926. https://doi.org/10.4049/jimmunol.1003099

44. Gao Y, Tan J, Chen W, Li Q, Nie J, Lin F et al (2015) Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. Proc Natl Acad Sci U S A 112(25):E3246–E3254. https://doi.org/10.1073/pnas.1421463112

45. Hwang SM, Sharma G, Verma R, Byun S, Rudra D, Im SH (2018) Inflammation-induced Id2 promotes plasticity in...
multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level. Immunology 123(1):79–89. https://doi.org/10.1111/j.1365-2567.2007.02690.x

60. Crispin JC, Martinez A, Alcocer-Varela J (2003) Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 21(3):273–276. https://doi.org/10.1016/s0896-8411(03)00121-5

61. Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C et al (2006) Regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 27(2):110–118. https://doi.org/10.1016/j.jaut.2006.06.005

62. Liu MF, Wang CR, Fung LL, Wu CR (2004) Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. Scand J Immunol 59(2):198–202. https://doi.org/10.1111/j.1365-2567.2004.01370.x

63. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgam K, Trad S et al (2005) Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 175(12):8392–8400. https://doi.org/10.4049/jimmunol.175.12.8392

64. Maud J, Loddenkemper C, Mundt P, Berg E, Giese T, Stullmack A et al (2005) Peripheral and intestinal regulatory CD4+CD25(high) T cells in inflammatory bowel disease. Gastroenterology 128(7):1868–1878. https://doi.org/10.1053/gast.2005.03.043

65. Saruta M, Yu QT, Fleschner PR, Manley PJ, Schmidt-Weber CB, Banham AH et al (2007) Characterization of FOXP3+CD4+ regulatory T cells in Crohn’s disease. Clin Immunol 125(3):281–290. https://doi.org/10.1016/j.clim.2007.08.003

66. Lindley S, Dayan CM, Bishop A, Roeo BO, Peakman M, Tree TI (2005) Defective suppressor function in CD4+CD25(high) regulatory T-cells from patients with type 1 diabetes. Diabetes 54(1):92–99. https://doi.org/10.2337/diabetes.54.1.92

67. Brusko TM, Wassfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA (2005) Functional defects and the influence of age on the frequency of CD4+CD25+ T-cells in type 1 diabetes. Diabetes 54(5):1407–1414. https://doi.org/10.2337/diabetes.54.5.1407

68. Putnam AL, Vendrame F, Dotta F, Gottlieb PA (2005) CD4+CD25(high) regulatory T cells in human autoimmune diabetes. J Autoimmun 24(1):55–62. https://doi.org/10.1016/j.jaut.2004.11.004

69. Brusko T, Wassfall C, McGrail K, Schatz R, Viener HL, Schatz D et al (2007) No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 56(3):604–612. https://doi.org/10.2337/db06-1248

70. Rahimzadeh M, Naderi N (2021) Towards understanding of regulatory T cells in COVID-19: a systematic review. J Med Virol. https://doi.org/10.1002/jmv.26891

71. Ragab D, Salah Eldin H, Taeimah M, Khatib R, Salem R (2020) The COVID-19 cytokine storm: what we know so far. Front Immunol 11:1446. https://doi.org/10.3389/fimmu.2020.01446

72. Qin C, Zhou L, Hu Z, Zhang S, Yang S, Tao Y et al (2020) Dysregulation of immune response in patients with coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis. 71(5):762–768. https://doi.org/10.1093/cid/ciaa248

73. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H et al (2020) Clinical and immunological features of severe and moderate coronavirus disease 2019 (COVID-19) in Wuhan, China. Clin Infect Dis. 71(5):762–768. https://doi.org/10.1093/cid/ciaa248

74. Meckiff BJ, Ramirez-Suastegui C, Fajardo V, Chee SJ, Kusnadi AM, Yu QT, Fleshner PR, Mantel PY, Schmidt-Weber CB, Bennett CL, Oc Curran K, Hellings N, Thewissen M, Somers V, Hensen K, Venken K, Hellings N, Thewissen M, Somers V, Hensen K, Rummons JL et al (2008) Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting COVID-19 patients

75. Springer
with different severity of illness. JCI Insight 5(10). https://doi.org/10.1172/jci.insight.137799
76. Sadeghi A, Tahaemsebi S, Mahmood A, Kuznetsova M, Valizadeh H, Taghizadeh A et al (2021) Th17 and Treg cells function in SARS-CoV2 patients compared with healthy controls. J Cell Physiol 236(4):2829–2839. https://doi.org/10.1002/jcp.30047
77. Jia R, Wang X, Liu P, Liang X, Ge Y, Tian H et al (2020) Mild cytokine elevation, moderate CD4(+) T cell response and abundant antibody production in children with COVID-19. Virol Sin. 35(6):734–743. https://doi.org/10.1007/s12250-020-00265-8
78. Rendeiro AF, Casano J, Vorkas CK, Singh H, Morales A, DeSimone RA et al (2021) Profiling of immune dysfunction in COVID-19 patients allows early prediction of disease progression. Life Sci Alliance 4(2). https://doi.org/10.26508/lsa.202000955
79. Chen X, Huang J, Huang Y, Chen J, Huang Y, Jiang X et al (2020) Characteristics of immune cells and cytokines in patients with coronavirus disease 2019 in Guangzhou, China. Hum Immunol. 81(12):702–708. https://doi.org/10.1016/j.humimm.2020.08.006
80. De Biasi S, Meschiari M, Giebellini L, Bellinazzi C, Borella R, Fidanza L et al (2020) Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with COVID-19 pneumonia. Nat Commun 11(1):3434. https://doi.org/10.1038/s41467-020-17292-4
81. Tan M, Liu Y, Zhou R, Deng X, Li F, Liang K et al (2020) Immunopathological characteristics of coronavirus disease 2019 cases in Guangzhou, China. Immunology. 160(3):261–268. https://doi.org/10.1111/imm.13223
82. Eastaff-Leung N, Maharrack N, Barbou B, Cummins A, Barry S (2010) Foxp3+ regulatory T cells. Th17 effector cells, and cytokine environment in inflammatory bowel disease. J Clin Immunol 30(1):80–89. https://doi.org/10.1007/s10875-009-9345-1
83. Wang Y, Liu XP, Zhao ZB, Chen JH, Yu CG (2011) Expression of CD4+ forhake box P3 (Foxp3)+ regulatory T cells in inflammatory bowel disease. J Dig Dis 12(4):286–294. https://doi.org/10.1111/j.1751-2980.2011.00505.x
84. Fritzsching B, Haas J, Konig F, Kunz P, Fritzsching E, Poschl J et al (2011) Intracerebral human regulatory T cells: analysis of CD4+ CD25+ FOXP3+ T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients. PLoS ONE 6(3):e17988. https://doi.org/10.1371/journal.pone.0017988
85. Feger U, Luther C, Poeschel S, Melms A, Toloza E, Wiendl H (2007) Increased frequency of CD4+ CD25+ regulatory T cells in the cerebrospinal fluid but not in the blood of multiple sclerosis patients. Clin Exp Immunol 147(3):412–418. https://doi.org/10.1111/j.1365-2249.2006.03271.x
86. Ferraro A, Socci C, Stabilini A, Valle A, Monti P, Piemonti L et al (2011) Expansion of Th17 cells and functional defects in T regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes. Diabetes 60(11):2903–2913. https://doi.org/10.2337/db11-0090
87. Viglietta V, Baecher-Allan C, Weiner HL, Haller DA (2004) Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 199(7):971–979. https://doi.org/10.1084/jem.20031579
88. Dhaeze T, Peelen E, Hombrout A, Peeters L, Van Wijmeersch B, Lemkens N et al (2015) Circulating follicular regulatory T cells are defective in multiple sclerosis. J Immunol 195(3):832–840. https://doi.org/10.4049/jimmunol.1500759
89. Haas J, Hug A, Viehaver A, Fritzsching B, Falk CS, Filser A et al (2005) Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur J Immunol 35(11):3343–3352. https://doi.org/10.1002/eji.200526065
90. Venken K, Hellings N, Hensen K, Rummens JL, Medaer R, D’Hooghe MB et al (2006) Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. J Neurosci Res 83(8):1432–1446. https://doi.org/10.1002/jnr.20852
91. Iikuni N, Lourencou EV, Hahn BH, La Cava A (2009) Cutting edge: regulatory T cells directly suppress B cells in systemic lupus erythematosus. J Immunol 183(3):1518–1522. https://doi.org/10.4049/jimmunol.0901163
92. Valencia X, Yarboro C, Illie G, Lipsky PE (2007) Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. J Immunol 178(4):2579–2588. https://doi.org/10.4049/jimmunol.178.4.2579
93. Yan B, Ye S, Chen G, Kuang M, Shen N, Chen S (2008) Dysfunctional CD4+, CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arthritis Rheum 58(3):801–812. https://doi.org/10.1002/art.23268
94. Korn T, Reddy J, Gao W, Bettelli E, Awasthi A, Petersen TR et al (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. Nat Med 13(4):423–431. https://doi.org/10.1038/nm1564
95. Mancusi A, Piccinelli S, Velardi A, Pierini A (2019) CD4(+) FOXP3(+) Regulatory T cell therapies in HLA haploidentical hematopoietic transplantation. Front Immunol 10:2901. https://doi.org/10.3389/fimmu.2019.02901
96. Kohan AP, Carpenter PA, Anger HA, Miller SD (2002) Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. J Immunol 169(9):4712–4716. https://doi.org/10.4049/jimmunol.169.9.4712
97. Zhang X, Koldicz DN, Izikson L, Reddy J, Nazareno RF, Sakaguchi S et al (2004) IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. Int Immunol 16(2):249–256. https://doi.org/10.1093/intimm/dxh029
98. Stephens LA, Malpass KH, Anderton SM (2009) Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg. Eur J Immunol 39(4):1108–1117. https://doi.org/10.1002/eji.200839073
99. Szanya V, Ermann J, Taylor C, Holness C, Fathman CG (2002) The subpopulation of CD4+CXCR5+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CC Chemokine Receptor 7. J Immunol 169(5):2461–2465. https://doi.org/10.4049/jimmunol.169.5.2461
100. Scalapino KI, Tang Q, Bluestone JA, Bonyhadi ML, Daikh DI (2006) Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol 177(3):1451–1459. https://doi.org/10.4049/jimmunol.177.3.1451
101. Mottet C, Uhlig HH, Powrie F (2003) Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 170(8):3939–3943. https://doi.org/10.4049/jimmunol.170.8.3939
102. Mekala DJ, Geiger TL (2005) Immunotherapy of autoimmune encephalomyelitis with redirected CD4+CD25+ T lymphocytes. Blood 105(5):2090–2092. https://doi.org/10.1182/blood-2004-09-3579
103. McGauchy MJ, Stephens LA, Anderton SM (2005) Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. J Immunol 175(5):3025–3032. https://doi.org/10.4049/jimmunol.175.5.3025
104. Herman AE, Freeman GI, Mathis D, Benoist C (2004) CD4+CD25+ regulatory T cells depend on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med 199(11):1479–1489. https://doi.org/10.1084/jem.20040179
CD4+CD25highCD127- regulatory T cells preserves beta-cell function in type 1 diabetes in children. Diabetes Care 35(9):1817–1820. https://doi.org/10.2373/dcc12-0038

136. Marek-Trzonkowska N, Mysiowiec M, Dobyszuk A, Grabowska M, Derkowska I, Jusincska J et al (2014) Therapy of type 1 diabetes with CD4(+)CD25(high)CD127-regulatory T cells prolongs survival of pancreatic islets - results of one year follow-up. Clin Immunol 153(1):23–30. https://doi.org/10.1016/j.clim.2014.03.016

137. Marek-Trzonkowska N, Mysiowiec M, Iwaszkiewicz-Grzes D, Gliwinski M, Derkowska I, Zalinska M et al (2016) Factors affecting long-term efficacy of T regulatory cell-based therapy in type 1 diabetes. J Transl Med 14(1):332. https://doi.org/10.1186/s12967-016-1090-7

138. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK et al (2015) Type 1 diabetes immunotherapy using polyclonal regulatory T cells. Sci Transl Med 7(315):315ra189. https://doi.org/10.1126/scitranslmed.aad4134

139. Dall’Era M, Pauli ML, Remedios K, Taravati K, Sondova PM, Putnam AL et al (2019) Adoptive Treg cell therapy in a patient with systemic lupus erythematosus. Arthritis Rheumatol 71(3):431–440. https://doi.org/10.1002/art.40737

140. Gladstone DE, Kim BS, Mooney K, Karaba AH, D’Alessio FR (2020) Regulatory T cells for treating patients with COVID-19 and acute respiratory distress syndrome: two case reports. Ann Intern Med. https://doi.org/10.7326/L20-0681

141. Golab K, Grose R, Placencia V, Winn H, Poulikos I, Marzi M et al (2017) T cell based therapy: strategies to overcome the impact of cryopreservation on the Treg viability and phenotype. Oncotarget 9(11):9728–9740. https://doi.org/10.18632/oncotarget.23887

142. Golab K, Grose R, Trzonkowski P, Wickrema A, Tibudan M, Marek-Trzonkowska N et al (2016) Utilization of leukapheresis and CD4 positive selection in Treg isolation and the ex vivo expansion for a clinical application in transplantation and autoimmune disorders. Oncotarget 7(48):79474–84. https://doi.org/10.18632/oncotarget.13101

143. Hulsps R, Villa-Komaroff L, Koksal E, Etienne K, Rogers P, Tuttle M et al (2014) Purification of regulatory T cells with the use of a fully enclosed high-speed microfluidic system. Cytotherapy 16(10):1384–1389. https://doi.org/10.1016/j.cytj.2014.05.016

144. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek M et al (2012) Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. Eur J Immunol 39(4):1088–1097. https://doi.org/10.1002/eji.200838904

145. Haase D, Puan KJ, Starke M, Lai TS, Soh MY, Karunanithi I et al (2015) Large-scale isolation of highly pure “untouched” regulatory T cells in a GMP environment for adoptive cell therapy. J Immunother 38(6):250–258. https://doi.org/10.1007/JCI.000000000000083

146. Kim YC, Zhang AH, Yoon J, Culp WE, Lees JR, Wucherpfennig KW et al (2018) Engineered MBP-specific human Treg ameliorate MOG-induced EAE through IL-2-triggered inhibition of effector T cells. J Autoimmun 92:77–86. https://doi.org/10.1016/j.jaut.2018.05.003

147. Prowse F, Carlin J, Leach MW, Maurie S, Coffman RL (1996) A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med 183(6):2669–2674. https://doi.org/10.1084/jem.183.6.2669

148. Asseman C, Maurie S, Leach MW, Coffman RL, Prowse F (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 190(7):995–1004. https://doi.org/10.1084/jem.190.7.995

149. Collisson LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM et al (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 450(7169):566–569. https://doi.org/10.1038/nature06306

150. Wright GP, Notley CA, Xue SA, Bendle GM, Holler A, Schumacher TN et al (2009) Adoptive therapy with redirected primary regulatory T cells results in antigen-specific suppression of arthritis. Proc Natl Acad Sci U S A 106(45):19078–19083. https://doi.org/10.1073/pnas.0907396106

151. Elinav E, Adam N, Waks T, Eshhar Z (2009) Amelioration of colitis by genetically engineered murine regulatory T cells redirected by antigen-specific chimeric receptor. Gastroenterology 136(5):1721–1731. https://doi.org/10.1053/j.gastro.2009.01.049

152. De Paula PA, Schmidt A, Zhang AH, Maldonado T, Konigs C, Scott DW (2020) Engineered regulatory T cells expressing myelin-specific chimeric antigen receptors suppress EAE progression. Cell Immunol 358:104222. https://doi.org/10.1016/j.cellimm.2020.104222

153. Elinav E, Waks T, Eshhar Z (2008) Redirection of regulatory T cells with predetermined specificity for the treatment of experimental colitis in mice. Gastroenterology 134(7):2014–2024. https://doi.org/10.1053/j.gastro.2008.02.060

154. Janssens I, Cools N (2020) Regulating the regulators: is introduction of an antigen-specific approach in regulatory T cells the next step to take autoimmunity? Cell Immunol 358:104236. https://doi.org/10.1016/j.cellimm.2020.104236

155. Ferreira LMR, Muller YD, Bluestone JA, Tang Q (2019) Next-generation regulatory T cell therapy. Nat Rev Drug Discov 18(10):749–769. https://doi.org/10.1038/s41573-019-0041-4

156. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN (2001) Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol. 31(4):1122–31. https://pubmed.ncbi.nlm.nih.gov/11298337/

157. Fraser H, Safinia N, Grageda N, Thirkell S, Lowe K, Fry LJ et al (2018) A rapamycin-based GMP-compatible process for the isolation and expansion of regulatory T cells for clinical trials. Mol Ther Methods Clin Dev. 8:198–209. https://doi.org/10.1016/j.omtm.2018.01.006

158. Marin Morales JM, Munch N, Peter K, Freund D, Oelschlagel U, Holg K et al (2019) Automated clinical grade expansion of regulatory T cells in a fully closed system. Front Immunol 10:38. https://doi.org/10.3389/fimmu.2019.00038

159. Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T et al (2009) Expansion of human regulatory T-cells from patients with type 1 diabetes. Diabetes 58(3):652–662. https://doi.org/10.2337/db08-1168

160. Marek N, Bieniaszewska M, Krzyzaniak A, Jusincska J, Mysliwska J, Witkowski P et al (2011) The time is crucial for ex vivo expansion of T regulatory cells for therapy. Cell Transplant 20(11–12):1747–1758. https://doi.org/10.3727/s0090736911x566217

161. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM (2009) Selective expression of latency-associated peptide (LAP) and IL-1 receptor type II (CD11a/CD11b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. Blood 113(21):5125–5133. https://doi.org/10.1182/blood-2009-01-199950

162. Bonsellino G, Kleinsvietfeld M, Di Miti D, Sternjak A, Diamantini A, Giometto R et al (2007) Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolsis of extracellular ATP and immune suppression. Blood 110(4):1225–1232. https://doi.org/10.1182/blood-2006-06-064527

163. Noyan F, Lee YS, Zimmermann K, Hardtke-Wolenski M, Taubert R, Warnecke G et al (2014) Isolation of human antigen-specific
regulatory T cells with high suppressive function. Eur J Immunol 44(9):2592–2602. https://10.1002/eji.201344381

164. Battaglia M, Stabilini A, Migliavacca B, Horejs-Hoeck J, Kaipper T, Roncarolo MG (2006) Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients. J Immunol 177(12):8338–8347. https://10.4049/jimmunol.177.12.8338

165. Safinia N, Vaikunthanathan T, Fraser H, Thirkell S, Lowe K, Blackmore L et al. (2016) Successful expansion of functional and stable regulatory T cells for immunotherapy in liver transplantation. Oncotarget 7(7):7563–77. https://10.18632/oncotarget.6927

166. Scotta C, Esposito M, Fazekasova H, Fanelli G, Edozie FC, Ali N et al. (2013) Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4(+)CD25(+)/FOXP3(+) T regulatory cell subpopulations. Haematologica 98(8):1291–1299. https://10.3324/haematol.2012.074088

167. Scotta C, Fanelli G, Hoong SJ, Romano M, Lamperti EN, Sukthankar M et al. (2016) Impact of immunosuppressive drugs on the therapeutic efficacy of ex vivo expanded human regulatory T cells. Haematologica 101(1):91–100. https://10.3324/haematol.2015.128934

168. Marek-Trzonkowska N, Frekarska K, Filipowicz N, Piotrowski A, Guca M, Vogt K et al. (2017) Mild hypothermia provides Treg stability. Sci Rep 7(1):11915. https://10.1038/s41598-017-10151-1

169. Golab K, Krzysztofik A, Marek-Trzonkowska N, Misawa R, Wang LI, Wang X et al. (2013) Impact of culture medium on CD4(+)CD25(high)CD127(low)/neg Treg expansion for the purpose of clinical application. Int Immunopharmacol 16(3):358–363. https://10.1016/j.immuno.2013.02.016

170. Chandran S, Tang Q, Sarwal M, Lasszik ZG, Putnam AL, Lee K et al. (2017) Polyclonal regulatory T cell therapy for control of inflammation in kidney transplants. Am J Transplant 17(11):2945–2954. https://10.1111/ajt.14415

171. Sharif-Pagahale E, Sunasse K, Tavare R, Ratnasothy K, Koers A, Ali N et al. (2011) In vivo SPECT reporter gene imaging of regulatory T cells. PLoS ONE 6(10):e25857. https://10.1371/journal.pone.0025857

172. Oo YH, Ackrill S, Cole R, Jenkins L, Anderson P, Jeffery HC et al. (2019) Liver homing of clinical grade Tregs after therapeutic infusion in patients with autoimmune hepatitis. JHEP Rep 1(4):286–296. https://10.1016/j.jheprep.2019.08.001

173. Brunstein CG, Blazar BR, Miller JS, Cao Q, Hippen KL, McKenna DH et al. (2013) Adoptive transfer of umbilical cord blood-derived regulatory T cells and early viral reactivation. Biol Blood Marrow Transplant 19(8):1271–1273. https://10.1016/j.bmt.2013.06.004

174. Baeten P, Hellings N, Broux B (2020) In vitro tailoring of regulatory T cells prior to cell therapy. Trends Mol Med 26(11):1059–1060. https://10.1016/j.molmed.2020.08.008

175. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S et al. (2004) Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 16(11):1643–1656. https://10.1093/intimm/dxh165

176. Oswald-Richter K, Grill SM, Shariat N, Leelawong M, Sundrud MS, Haas DW et al. (2004) HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. PLoS Biol 2(7):E198. https://10.1371/journal.pbio.0020198

177. Allan SE, Alstad AN, Merinold N, Crellin NK, Amendola M, Bacchetta R et al. (2008) Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. Mol Ther 16(1):194–202. https://10.1038/sj.mt.6300341

178. Aarts-Riemens T, Emmelot ME, Verdonck LF, Matis T (2008) Forced overexpression of either of the two common human FOXP3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. Eur J Immunol 38(5):1381–1390. https://10.1002/eji.200737590

179. Forstneric V, Ozen I, Ogorevc J, Lainscek D, Przakin A, Lebar T et al. (2019) CRISPRa-mediated FOXP3 gene upregulation in mammalian cells. Cell Biosci 9:3. https://10.1186/s13578-019-0357-0

180. Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen HR, Bruno TC et al. (2010) A role for the transcription factor Helios in human CD4(+)CD25(+) regulatory T cells. Mol Immunol 47(7–8):1595–1600. https://10.1016/j.molimm.2010.02.001

181. Baine J, Basu S, Arnes S, Sellers RS, Macian F (2013) Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. J Immunol 190(3):1008–1016. https://10.4049/jimmunol.1200792

182. Bin Dhurban K, d’Hennezel E, Nashi E, Bar-Or A, Rieder S, Shevach EM et al. (2015) Coexpression of TIGIT and FCRL3 identifies Helios+ human memory regulatory T cells. J Immunol 194(8):3687–3696. https://10.4049.jimmunol.1401803

183. Takatori H, Kawashima H, Matsuki A, Meguro K, Tanaka S, Iwamoto T et al. (2015) Helios enhances Treg cell function in cooperation with Foxp3. Arthritis Rheumatol 67(6):1491–1502. https://10.1002/art.39091

184. Seng A, Krausz KL, Pei D, Koesters DC, Fischer RT, Yankee TM et al. (2020) Coexpression of FOXP3 and a Helios isoform enhances the effectiveness of human engineered regulatory T cells. Blood Adv 4(7):1325–1339. https://10.1182/bloodadvances.201900965

185. Okada M, Kanamori M, Someya K, Nakatsukasa H, Yoshimura A (2017) Stabilization of Foxp3 expression by CRISPR-dCas9-based epigenome editing in mouse primary T cells. Epigenetics Chromatin 10:24. https://10.1186/s13324-017-0129-1

186. Someya K, Nakatsukasa H, Ito M, Kondo T, Tateda K, Akamura T et al. (2017) Improvement of Foxp3 stability through CNS2 demethylation by TET enzyme induction and activation. Int Immunol 29(8):365–375. https://10.1093/intimm/dxx049

187. Kim HP, Leonard WJ (2007) CREB/ATF-dependent T cell receptor-induced Foxp3 gene expression: a role for DNA methylation. J Exp Med 204(7):1543–1551. https://10.1084/jem.200701009

188. Yue X, Trifari S, Ajio T, Tsagarakou A, Pastor WA, Zepeda-Martinez JA et al. (2016) Control of Foxp3 stability through modulation of TET activity. J Exp Med 213(3):377–397. https://doi.org/10.1084/jem.20151438

189. Moon C, Kim SH, Park KS, Choi BK, Lee HS, Park JB et al. (2009) Use of epigenetic modification to induce FOXP3 expression in naive T cells. Transplant Proc 41(5):1848–1854. https://10.1016/j.transproced.2009.02.101

190. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O et al. (2010) Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood 115(5):965–974. https://10.1182/blood-2009-02-207118

191. van Loosdregt J, Brunen D, Fleskens V, Pals CE, Lam EW, Coffier PJ (2011) Rapid temporal control of Foxp3 protein degradation by sirtuin-1. PLoS ONE 6(4):e19047. https://10.1371/journal.pone.0019047

192. Greenbaum C, Kahn SE, Palmer JP (1996) Nicotinamide’s effects on glucose metabolism in subjects at risk for IDDM. Diabetes 45(11):1631–1634. https://doi.org/10.2337/diab.45.11.1631

193. Lamper EF, Klinghammer A, Scherbaum WA, Heinze E, Haaster B, Giani G et al. (1998) The Deutsche Nicotinamide Intervention
Study: an attempt to prevent type 1 diabetes. DENIS Group. Diabetes. 47(6):980–984. https://doi.org/10.2337/diabetes.47.6.980

194. Kwon HS, Lim HW, Wu J, Schonzer M, Verdin E, Ott M (2012) Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. J Immunol 188(6):2712–2721. https://doi.org/10.4049/jimmunol.1100903

195. Chen Z, Barbi J, Bu S, Yang HY, Li Z, Gao Y et al (2013) The ubiquitin ligase Stub1 negatively modulates regulatory T cell suppressive activity by promoting degradation of the transcription factor Foxp3. Immunity 39(2):272–285. https://doi.org/10.1016/j.immuni.2013.08.006

196. van Loosdregt J, Fleskens V, Fu J, Brenkman AB, Bekker CP, Pals CE et al (2013) Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. Immunity 39(2):259–271. https://doi.org/10.1016/j.immuni.2013.05.018

197. Zhu F, Yi G, Liu X, Zhu F, Zhao A, Wang A et al (2018) Ribosomal protein 31-mediated atypical ubiquitination stabilizes forkhead box P3 and thereby stimulates regulatory T-cell function. J Biol Chem 289(32):20099–20111. https://doi.org/10.1074/jbc.RA118.005802

198. Li Z, Lin F, Zhuo C, Deng G, Chen Z, Yin S et al (2014) PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. J Biol Chem 289(39):26872–26881. https://doi.org/10.1074/jbc.M114.586651

199. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M et al (2010) Protein kinase C-theta mediates negative feedback on regulatory T cell function. Science 328(5976):372–376. https://doi.org/10.1126/science.1186068

200. Rawlings DJ, Sommer K, Moreno-Garcia ME (2006) The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes. Nat Rev Immunol 6(11):799–812. https://doi.org/10.1038/nri1944

201. Humblet-Baron S, Franckaert D, Dooley J, Bornschein S, Cauwe B, Schonefeldt S et al (2016) IL-2 consumption by highly activated CD8 T cells induces regulatory T-cell dysfunction in patients with hemophagocytic lymphohistiocytosis. J Allergy Clin Immunol 138(1):200–9 e8. https://doi.org/10.1016/j.jaci.2015.12.1314

202. Sokolosky JT, Trotta E, Parisi G, Picton L, Su LL, Le AC et al (2018) Selective targeting of engineered T cells using orthogonal IL-2 cytokine-receptor complexes. Science 359(6379):1037–1042. https://doi.org/10.1126/science.aar3246

203. Kagoya Y, Tanaka S, Guo T, Anczurowski M, Wang CH, Saso K et al (2018) A novel chimeric antigen receptor containing a JAK-STAT signaling domain mediates superior antitumor effects. Nat Med 24(3):352–359. https://doi.org/10.1038/nm.4478

204. Tripathi D, Cheekatlja SS, Paidipally P, Radhakrishnan RK, Welch E, Thandi RS et al (2018) c-Jun N-terminal kinase 1 defective CD4+CD25+FoxP3+ cells prolong islet allograft survival in diabetic mice. Sci Rep 8(1):3310. https://doi.org/10.1038/s41598-018-21477-9

205. Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C et al (2013) Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 341(6148):1233151. https://doi.org/10.1126/science.1233151

206. Biffi A, Montini E, Liorioli L, Cesani M, Fumagalli F, Plati T et al (2013) Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341(6148):1233158. https://doi.org/10.1126/science.1233158

207. Hacein-Bey-Ahina S, Hauer J, Lim A, Picard C, Wang GP, Berry CC et al (2010) Efficacy of gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 363(4):355–364. https://doi.org/10.1056/NEJMoa1000164

208. Howe SI, Mansour MR, Schwarzwelder K, Bartholomae C, Hubank M, Kempski H et al (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J Clin Invest 118(9):3143–3150. https://doi.org/10.1172/JCI35798

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.