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Improving the efficacy of regulatory T cell therapy

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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 a cell therapy in patients with autoimmune disorders. (Pre)clinical studies using Treg therapy have already established safety, 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

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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 towards 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 relies on the methylation status of the gene region. More specifically, the promotor [21] and Treg-specific demethylated region (TSDR) within the 2^{nd} conserved non-coding sequence (CNS2) [22, 23] are 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 factor beta (TGF-β)-induced Tregs (iTregs) [22-24], as they exhibit a partially demethylated TSDR, resulting in an unstable *FOXP3* expression [21].

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 autoimmune diabetes [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 towards 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]. $FOXP3^{+}IL\text{-}17^{+}$ 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^{-}, but not DR^{+} Tregs are 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 tTregs 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 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 $CD39^{high}$ [118] Tregs completely prevent 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 tTreg 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 $CD4^{+}CD25^{high}CD127^{low}$ 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 (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 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 1 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 have 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 $CD4^{+}CD25^{high}CD127^{low}$ cells, as currently used in clinical trials, results in a functional but heterogeneous Treg population.

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 $2x10^6$ 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 $2.5x10^6$ cells	Decreased severity disease	[97]
EAE	$CD4^+CD25^+CD62L^{high}$ Naïve mice Polyclonal / antigen-specific LN	1 day before and 18 days after EAE induction $2x10^5$ cells (polyclonal) $1\text{-}3x10^5$ 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 $2x10^4$ cells	CNS-derived Treg: protection LN-derived Treg: no protection	[103]
EAE	$CD4^+CD25^+$ MBP89-101-IA^S-ζ Tg mice Spleen and LN	At time induction and 11 days after induction $1x10^6$ cells	At induction: protection After induction: reduced severity disease	[102]
T1DM	$CD4^+CD25^+CD62L^{low/high}$ Prediabetic animals Spleen	Co-transfer activated T cells and Tregs $5x10^5$ Tregs	$CD62L^{low}$: no delay $CD62L^{high}$: delay	[99]
T1DM	$CD4^+CD25^+$ Diabetic mice Antigen-specific Pancreas	Co-transfer Tregs and activated T cells $0.5\text{-}2x10^4$ Tregs	$5x10^3$: no protection $1x10^4$: complete protection	[104]
T1DM	Retroviral FOXP3-transduced $CD4^+$ T cells Antigen-specific / polyclonal Naïve animals Spleen	Transfer after onset $1x10^5$ 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 $2\text{-}5x10^6$ Tregs	Protection against disease induction	[106]
SLE	*In vitro* expanded $CD4^+CD25^+$ $CD62L^{high}$ Polyclonal Healthy SLE-prone animals Spleen and LN	Transfer before and during (2^{nd} injection) development $6x10^6$ cells	Before: delayed development, decreased renal damage During: delayed progression, decreased mortality	[100]
IBD	$CD4^+CD25^+$ Spleen	4 weeks after disease induction $1x10^6$ 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

Table 2: Published and running trials using Tregs as a cell therapy in autoimmunity and hyperinflammation.

Disease	Phase	Product	Expansion	Dose and Infusion	Effect	Reference Study ID
T1DM	I	Polyclonal Autologous MACS+FACS $CD4^+CD25^{hi}CD127^{lo}$	Anti-CD3/CD28 beads IL-2 14 days	10-20x10^6 cells / kg bodyweight Single infusion	Well-tolerated Increased C-peptide Decreased insulin use Cells stay present up to 4 months	[135]
T1DM	I	Polyclonal Autologous FACS $CD4^+CD25^{hi}CD127^{lo}$	Anti-CD3/CD28 beads IL-2	10-30x10^6 cells / kg bodyweight Single or double infusion	Well-tolerated Increased C-peptide Decreased insulin use Decreased HbA1c levels Prolonged remission but still progression Cells stay present up to 1 year back return to baseline after 2 years 2^{nd} dose beneficial	[136, 137]
T1DM	I	Polyclonal Autologous FACS $CD4^+CD25^{hi}CD127^{lo}$	Anti-CD3/CD28 beads IL-2 14 days	0.05-26x10^8 cells Single infusion i.v.	Well-tolerated No opportunistic infections Indications for improved metabolic activity Cells stay present up to 1 year	[138]
T1DM	II	Polyclonal Autologous	Yes	Low or high dose Single infusion	Completed	NCT02691247
T1DM	I/II	UC blood	Yes	1-5x10^6 Tregs / kg bodyweight Combined with insulin	Ongoing	NCT02932826
MS	I	Polyclonal Autologous	Yes		Ongoing	EudraCT 2014-004320-22
SLE	I	Polyclonal Autologous FACS $CD4^+CD25^{hi}CD127^{lo}$	Anti-CD3/CD28 beads IL-2	1x10^8 cells / kg bodyweight Single infusion	Safe Rapid peripheral loss Stable disease for 48 weeks	[139]
Crohn's disease	I/II	Polyclonal Autologous $CD4^+CD25^{hi}$ $CD127^{lo}CD45RA^+$	Yes	0.5-10x10^6 Tregs / kg bodyweight Single infusion	Ongoing	NCT03185000
COVID-19	I	Allogeneic UC blood	Yes	1-3x10^8 cells	Ongoing	NCT04468971

C-peptide: connecting peptide; COVID-19: coronavirus disease 2019; FACS: fluorescence activated cell sorting; HbA1c: hemoglobin 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

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 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 $CD39^{high}$ 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 $1x10^4$ 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 between the antigen recognition domain of immunoglobulins and the TCR signalling domains. They are independent of co-receptors and recognize unprocessed proteins on the cell surface. Introduction of transgenic TCR or CAR does not influence Treg properties [146, 150-152], and has been shown to decrease inflammation and ameliorate disease in autoimmune animal models [98, 102, 146, 150-153]. 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 micro-organisms during the *in vitro* culture. Third, a loss of suppressive function after Treg expansion has been reported. Researchers found that *in vitro* culturing of $CD4^{+}CD25^{high}CD127^{low}$ 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].

Table 3: Additional surface markers to identify stable and potent Tregs.

Marker	Result	Reference
$CD45RA^+$	No switch to a Th17-like phenotype Completely demethylated TSDR Retain suppressive activity *in vitro* Maintain stable Tregs phenotype after *ex vivo* expansion	[120] [144]
LAP^+	90% $FOXP3^+$ Better suppression *in vitro* compared to $CD4^+CD25^{high}CD127^{low}$ No cytokine production	[161]
$CD121^+$	90% $FOXP3^+$ Better suppression *in vitro* compared to $CD4^+CD25^{high}$ No cytokine production	[161]
$CD49d^-$	90% $FOXP3^+$ Highly suppressive *in vitro* No cytokine production Stable FOXP3 expression during expansion	[117, 145]
$CD39^{high}$	Highly suppressive *in vitro* Stable FOXP3 expression during IL-1β and IL-6 challenge	{Gu, 2017 #387;Borsellino, 2007 #208}
$CD154^-$	Highly demethylated TSDR Highly suppressive *in vitro*	[162]

FOXP3: forkhead box protein 3; IL: interleukin; LAP: latency-associated peptide; Th: T helper; TSDR: Treg-specific demethylated region

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, 163-165]. Production of inflammatory cytokines is prevented as well [157, 164, 165] 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 $CD25^{high}$ cells are selected. The addition of a vitamin A derivate, all *trans* retinoic acid, was additionally found to augment the effects of rapamycin [165]. Rapamycin, all *trans* retinoic acid and calcineurin inhibitor tacrolimus are also shown to influence the chemokine receptor homing profile of Tregs [165, 166]. 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, mild hypothermia enables Treg expansions that were refractive to *in vitro* expansion under regular conditions [167]. 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 [168].

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 **re-infusion** 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].

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	< 0.8
Anaerobic and aerobic bacterial growth	Absent
Fungal growth	Absent
Endotoxin	< 5 EU/kg
Purity	
% $CD4^+$ cells	> 90%
% $CD8^+$ cells	< 5%
Residual beads	< 100 beads per $3x10^6$ cells
Identity	
% $FOXP3^+$ cells	> 60%
Potency	
% viability	> 75%
Stability of final product	Several hours (overnight)

FOXP3: forkhead box protein 3

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 afterwards but remain detectable for 1 year after infusion [130, 136-139, 169]. 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 [170]. In another study, infused ^{111}Indium tropolonate-labelled Tregs were detectable in the liver up to 72h after infusion, using SPECT/CT [171]. Here, labelling did not affect Treg function or phenotype and decayed after 72h. 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 are 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, 172]. 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 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 [162].

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* [173]. Several strategies are discussed below (Fig. 1), based on recent progress made within this field.

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 [174-177]. 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 [178]. 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 [179] and correlates with the suppressive capacity of Tregs [180]. $Helios^+$ Tregs are highly suppressive, while $Helios^-$ Tregs exclusively produce inflammatory cytokines [181]. 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 [182]. 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 [183].

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 tTregs, the TSDR region of the *FOXP3* gene is completely demethylated by the ten-eleven-translocation (Tet) enzyme, and this is counterbalanced by DNA-methyltransferases (DNMT) [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 [184]. The p300-iTregs were very stable in *in vitro* inflammatory conditions and retained FOXP3 levels, in contrast to partial stabilization of FOXP3 in the Tet-iTregs [184]. 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 [185]. Furthermore, the use of RNA interference to reduce DNMT expression resulted in an increase in FOXP3 levels in naïve $CD4^+$ mouse cells [186].

Next to gene editing, the culture conditions were also found to affect the methylation state of the *FOXP3* gene. Lowering oxygen levels [185] and adding vitamin C [185, 187] 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 [186, 188], and the acetylation enhancer Trichostatin A (TSA) [188] 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) [189, 190], which is already in clinical use [191, 192], or Ex-527 [193] 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 [189]. 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 [194].

In another report, ectopic expression of both deubiquitinase USP7 and FOXP3 in human embryonic kidney (HEK) 293T cells reduced FOXP3 polyubiquitination [195]. In contrast, atypical ubiquitination seems to stabilize FOXP3 protein expression in human Tregs, since lentiviral overexpression of Ring finger protein 31 (RNF31) ubiquitinates FOXP3 and leads to an enhanced suppressive capacity *in vitro* [196].

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 [197]. Therefore, knockdown of PIM1 in human Tregs using short hairpin RNA (shRNA) results in enhanced gene expression of FOXP3 targets genes and increased suppression *in vitro* [197].

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 [198]. 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 [199]. 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 [200]. 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 [201]. 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 [202]. 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 [203].

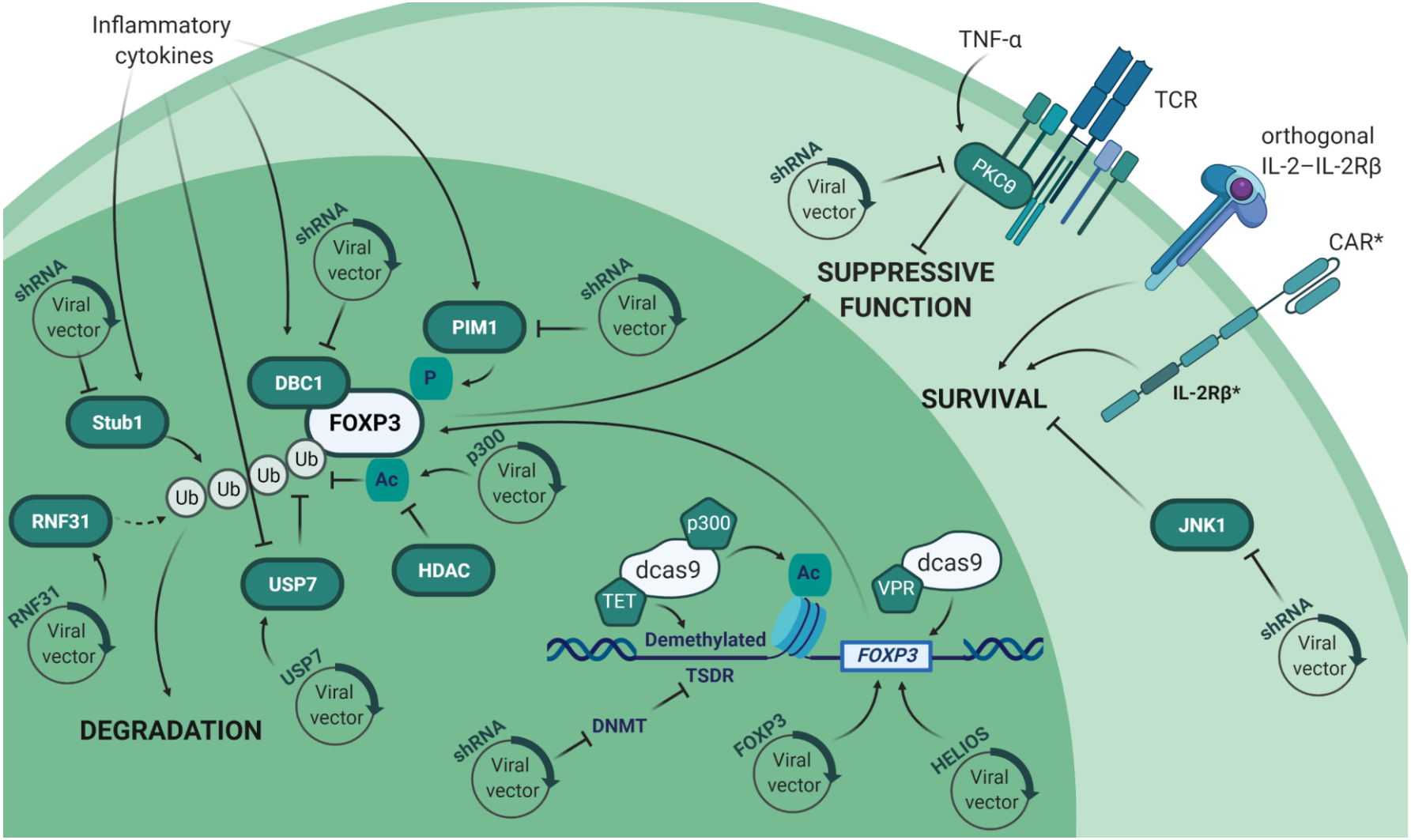

Fig. 1: Strategies to boost Treg function and survival. Stable *FOXP3* gene expression is ensured by viral vector-induced ectopic overexpression of FOXP3 and Helios or the use of dCas9 to direct a transcriptional activator VPR to the *FOXP3* gene. Demethylation of the TSDR region of the *FOXP3* gene 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.
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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, 162]
	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, 163-166]
Follow-up	New labelling methods for *in vivo* monitoring	Cells remain unaffected Safety Tracking possible	Time-limited effects Research limited to small animals	[170, 171]
	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, 174-178, 182-186, 189, 194-198]
	Boosts survival and expansion	Long term effect	Low transfection rate Increased culturing time	[201-203]

CAR: chimeric antigen receptor; FACS: fluorescence activated cell sorting; GMP: good manufacturing practice; TCR: T cell receptor; Tregs: regulatory T cell

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 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 [173]. 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 (Strimveli®). Both types of viral vectors were found to be highly efficient and long-lasting. However, lentiviral vectors have superior safety profiles [204, 205], since retroviral vectors were found to integrate near proto-oncogenes, causing leukaemia [206, 207]. 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.

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