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Regulatory T cell therapy: Current and future design perspectives

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Abstract

Regulatory T cells (Tregs) maintain immune equilibrium by suppressing immune responses through various multistep contact dependent and independent mechanisms. Cellular therapy using polyclonal Tregs in transplantation and autoimmune diseases has shown promise in preclinical models and clinical trials. Although novel approaches have been developed to improve specificity and efficacy of antigen specific Treg based therapies, widespread application is currently restricted. To date, design-based approaches to improve the potency and persistence of engineered chimeric antigen receptor (CAR) Tregs are limited. Here, we describe currently available Treg based therapies, their advantages and limitations for implementation in clinical studies. We also examine various strategies for improving CAR T cell design that can potentially be applied to CAR Tregs, such as identifying co-stimulatory signalling domains that enhance suppressive ability, determining optimal scFv affinity/avidity, and co-expression of accessory molecules. Finally, we discuss the importance of tailoring CAR Treg design to suit the individual disease.

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Highlights:

- The tolerogenic effect of Tregs can be effectively harnessed for cellular therapy
- Limitations of polyclonal Treg therapy led to the generation of engineered Tregs
- CAR Tregs confer antigen specificity without requirement for MHC restriction
- CAR Treg design is currently based on 2nd generation CAR T cells for cancer therapy
- Developments to improve potency of CAR T cells for cancer may be applied to Tregs

1. Introduction

1.1 Phenotype

Regulatory T cells (Tregs) are a dynamic and specialized subset of $CD4^+$ T cells which play an indispensable role in the suppression of exacerbated immune responses, maintenance of peripheral tolerance and tissue integrity [1]. Tregs are characterized by the expression of high levels of the IL-2 receptor α-chain CD25, and the lineage specific transcription factor forkhead box protein 3 (FoxP3). The suppressive function of Tregs relies heavily on high and stable expression of FoxP3, which together with other transcription factors, determines the functional program of Tregs by inducing expression of specific genes and epigenetic signature during their development [2-4]. Constitutive stable expression of FoxP3 is considered indispensable for lineage maintenance as the ablation of Foxp3 from mature Tregs leads to loss of function and conversion to other T helper (Th) cell types [5].

1.2 Classification

Two broad categories of Tregs have been described according to the site of origin: central, naturally occurring or thymus derived Tregs (tTreg) and peripheral Tregs (pTreg) [6]. In the thymus, tTregs are selected positively through MHC-II dependent T cell receptor (TCR) interactions, resulting in a relatively high avidity selection [7]. On the contrary, pTregs originate from conventional $CD4^+$ T cells (T_{conv}), usually in the presence of TGF-β and IL-2 [7]. Together, both these types of Tregs play specialized roles in controlling both innate and acquired immune responses to self and foreign antigen. After development, maintenance of functional stability and homeostatic proliferation requires continuous signaling in Tregs [8, 9]. The interaction of cognate antigen with TCR initiates activation of Tregs. However, complete activation requires a secondary signal which is provided by co-stimulatory molecules like CD28, ICOS and/or CD40 [10-12]. In the presence of the TCR signal alone, both T_{conv} and Tregs undergo a state of anergy and unresponsiveness. A broad spectrum of co-stimulatory and co-inhibitory receptors and their ligands are engaged in activation during TCR dependent Treg activation [13, 14].

1.3 Immune suppressive function

In a healthy individual, Tregs accumulate in non-lymphoid organs and barrier tissues such as skin, lung and the gastrointestinal tract [15-18]. During an inflammatory response, Tregs migrate from the inflamed tissue to draining lymph nodes and exert immune suppression not only at the site of inflammation but also in local secondary lymphoid tissues [19-21]. Tregs exert their immunosuppressive function by direct cell-contact dependent or independent mechanisms (**Figure 1**). Some of these mechanisms for modulation of the immune response involve the secretion of inhibitory cytokines like TGF-β, IL-10 and IL-35 [22-26], consumption of IL-2 [27-30], production of lytic proteins such as granzyme and perforin [31, 32], and modification of APCs by down-regulation or trogocytosis of peptide-MHC II, CD80 and CD86 [33-35]. Besides these mechanisms, antigen specificity also plays an important role in Treg mediated suppression through physical co-clustering of TCR stimulated Tregs with IL-2 producing auto-reactive T cells in lymph nodes to suppress autoimmunity in a negative feedback manner [36].

Over the years, research on Treg biology has undergone significant advances. Accumulated evidence demonstrates that Treg play an essential role in the control of a variety of physiological and pathological immune responses, including anti-microbial and anti-tumor responses and transplant immunity [37-39]. In this review, we examine the different types of Treg based therapies currently being tested, as well as introduce various strategies for improving CAR T cell design that can potentially be applied to CAR Tregs to improve function, persistence, and efficacy.

2. Role of Tregs

2.1 Sentinel for T cells that escape thymic selection

In general, Tregs constitute only 1 to 2% of peripheral blood lymphocytes (PBL). Perturbations in Treg numbers often results in the pathology of many common autoimmune diseases [40], whereas loss of function gene mutations in the Foxp3 gene leads to the development of a range of autoimmune and inflammatory disorders known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [41, 42]. Several studies in different autoimmune disease models have indicated an association of disease phenotype mainly with Treg function rather than defective Treg numbers in patients. In multiple sclerosis [43], type I diabetes [44], psoriasis [45] and

myasthenia gravis [46], peripheral blood Tregs were found to have reduced capacity to suppress T cell proliferation and interferon gamma (IFN-γ) production. Recent evidence in patients with allergic or asthmatic disease suggested decreased frequency of IL-10 secreting Tregs in comparison to healthy individuals [47, 48].

2.2 Dampen and curtail inflammatory responses

Although Tregs can effectively regulate T_{conv} cells under normal circumstances, certain conditions like infection require robust effector function. Tregs play an important role in controlling the balance between induction of a proinflammatory anti-pathogen response and an anti-inflammatory response to prevent damage to host cells. For example, in virus induced encephalitis, and in lung inflammation following influenza A infection, Tregs were found to reduce disease severity by suppressing the over-activation of the immune response [49, 50]. This balance can be destabilized following the sensing of specific local proinflammatory signals like IL-6 and IFN-γ, which causes Tregs to lose their suppressive phenotype [51, 52]. The role of Tregs in chronic viral infections is not completely understood to date. In patients with hepatitis B and hepatitis C virus infection, an increase in the number of peripheral Tregs have been reported which prevents effective antiviral immunity [53-55].

2.3 Tregs in the tumor microenvironment

In contrast to infection, within the tumor microenvironment, the dominant suppression of Tregs over T_{conv} becomes exaggerated and pathological. Increased activity of Tregs protects tumor tissues from immune surveillance and hence recognition. In patients with different cancer types e.g. lung, pancreatic, breast, liver and skin, an increased proportion of Tregs have been reported, which inhibits proliferation and IFNγ production by T_{conv} and NK cell mediated cytotoxicity [56-60]. Studies performed in murine tumor models have demonstrated that ablation of Tregs triggers a rapid, spontaneous immune response against the tumor tissue and improves the effectiveness of anti-cancer immunotherapy [61-63].

3. Sources of Tregs for cell therapy

The importance of Tregs in inducing both *in vivo* and *ex vivo* tolerance underscores their immense potential as a therapeutic tool. Of these, the use of cellular Treg based therapies has shown promising outcomes in both pre-clinical studies as well as in the clinic.

3.1 *In vivo* induction of antigen specific Tregs

$CD4^+$ T_{conv} cells can develop into Tregs depending on a mixture of contact dependent and cytokine signals present during antigen presentation by professional APCs. For example, repetitive stimulation of naive T cells with antigen presenting immature DCs leads to the induction of IL-10 producing Tregs [64]. Animal models of autoimmune and allergic diseases have provided evidence for the induction of IL-10 producing Tregs following peptide administration [65-67]. However, in the non-obese diabetic (NOD) mouse model and in multiple sclerosis patients, administration of self or altered peptides resulted in severe inflammatory or anaphylactic side effects [68, 69].

For induction of Tregs, use of monoclonal antibodies (mAbs) has shown significant outcomes. Studies in the NOD mouse model reported that anti-CD3 treatment can induce immunoregulatory mechanism by selectively depleting pathogenic cells and inducing TGF-β secreting Tregs [70, 71]. In patients with early onset of type I diabetes, anti-CD3 monoclonal antibody treatment resulted in maintenance of residual beta cell function and required administration of lower insulin doses [72, 73]. A phase 2 clinical trial of teplizumab (an Fc receptor-nonbinding anti-CD3 monoclonal antibody) administered to relatives of diabetes I patients showed delayed progression of the disease [74]. This therapy has also been tested in other animal models of autoimmunity, multiple sclerosis [75, 76], colitis [77], rheumatoid arthritis [78] and transplantation [79]. Another candidate for mAb based approach to induce Treg is anti-CD45RO/RB which was shown to induce anergic and suppressive human antigen specific $CD4^+$ and $CD8^+$ T cells upon stimulation *in vitro* [80]. A recent preclinical study has demonstrated the potential of anti-CD45RC mAb administration in the prevention of allograft rejection and graft versus host disease (GvHD) inhibition [81].

Other approaches to induce and expand Tregs *in vivo* involve the use of the immunosuppressive drug rapamycin [82], administration of low dose IL-2 [83, 84], IL-2/IL-2 antibody complex [85-87], use of Tumor necrosis factor receptor 2 (TNFR2) blockers [88, 89]. All these strategies have been used in several clinical trials either individually or in combination with polyclonal Tregs.

3.2 *Ex vivo* expanded polyclonal Tregs

The clinical application of polyclonal Tregs is considered a next generation cellular therapy for several autoimmune diseases and inflammatory immune disorders. Tregs

isolated from peripheral blood are stimulated and expanded *in vitro* using anti-CD3/CD28 antibody coated beads and high dose IL-2 [90, 91]. Expanded Tregs retain expression of cell specific genes and are reportedly more efficient in suppressive function [92]. Another approach for the preferential expansion of Tregs over other T cell subsets involves the use of anti CD28 superagonists, with reportedly high Treg stability [93].

3.2.1 Thymic derived, natural Tregs

The first preclinical proof of concept for use of polyclonal Tregs was demonstrated in 1995 by Sakaguchi and co-workers, who demonstrated that $CD4^+CD25^+$ T cells could be used to transfer tolerance in athymic nude mice by suppressing self-reactive lymphocytes [94]. Since then, approaches that involve boosting Treg to T_{conv} ratios have been tested in several disease settings. Tregs with a polyclonal specificity have demonstrated potential in various preclinical models of GvHD [95-97], solid organ transplantation [98, 99] and autoimmune diseases [96, 100, 101]. Several clinical trials examining the safety and feasibility of polyclonal Tregs for type I diabetes [102, 103], transplantation [104] and GvHD [105-107] have been carried out, demonstrating their efficacy in immunotherapy (Table 1). Some other clinical trials for polyclonal treg therapy in autoimmune hepatitis (NCT02704338), Crohn's disease (NCT03185000), Pemphigus (NCT03239470) and Alzheimer's disease (NCT03865017) are also under investigation. Beside autoimmune diseases, the use of polyclonal Tregs in other disease models have also shown significant therapeutic potential. In non-immune diseases like cardiovascular disease, obesity, type 2 diabetes and degenerative diseases, administration of polyclonal Tregs are reported to reduce the inflammation and morbidity rate by contributing to tissue homeostasis and repair [108, 109]. In genetic disorders, characterized by mutations or defect in production of an essential protein, such as for clotting factor VIII or IX in hemophilia, lack of tolerance to protein replacement therapy is often observed. In this case, adoptive transfer of polyclonal Tregs effectively suppressed immune responses to therapeutic proteins in preclinical animal studies [110, 111]. Polyclonal Treg cell therapy is therefore generally considered safe and efficacious, although obtaining sufficient cell numbers can be challenging in many disease scenarios [112, 113].

While results from preclinical and Phase I/II clinical studies demonstrating the safety and feasibility of Treg infusion therapy are encouraging, outcomes from Phase III

studies will truly indicate whether polyclonal Treg therapy will become a common standard of care in the treatment of different autoimmune diseases, transplant rejection and GvHD.

3.2.2 FoxP3 transduced T cells

There are a few approaches for circumventing the requirement of large polyclonal Treg cell numbers for therapy. Tregs constitutively express the transcription factor FoxP3, which is critical for their immunosuppressive function. Several groups have shown that ectopic expression of FoxP3 can confer a suppressive phenotype to naive or memory $CD4^+$ T cells [4, 114]. In a mouse model with recent onset of type I diabetes, a single injection of 10^5 FoxP3 transduced islet specific T cells was reported to stabilize and reverse the disease condition [115]. Lentiviral delivery of FoxP3 gene into IPEX patient-derived $CD4^+$ T cells mirrored Treg population from healthy donors, with characteristic features like decreased proliferation, hyporesponsiveness, reduced cytokine release and suppressive activity [116]. These induced Tregs were demonstrated to be stable in inflammatory conditions not only *in vitro* but also *in vivo* in a xenograft mouse model of GvHD [116]. Forced expression of FoxP3 in $CD4^+$ T cells isolated from FVIII immunized mice generated antigen specific suppressor Treg like cells, that conferred long lasting prevention of inhibitory immune response against FVIII replacement therapy [117]. Several other studies have shown the efficiency of FoxP3 transduced Tregs in combating autoimmune diseases like allergy [118], renal injury [119] and collagen induced arthritis [120]. Recently, Honaker and co-authors demonstrated the use of the CRISPR/Cas9 system for stable and high-level expression of FoxP3 in T_{conv} cells. These edited Treg like cells were able to suppress the immune response in a xeno-GvHD mouse model [121]. Further, CRISPR based gene correction for regulated expression of FoxP3 demonstrated that gene editing in IPEX can preserve HSPC differentiation potential and edited regulatory and effector T cells restored their regulatory phenotype and function [122]. These studies demonstrate applicability of gene correction in the treatment of autoimmune diseases.

Another approach which has been used to enforce Treg differentiation involves use of the cell permeable form of FoxP3, linked to the protein transduction domain (PTD) from the HIV transactivator of transcription, which allows FoxP3 to be delivered to the cytoplasm and nucleus. This protein form has been shown to induce a Treg phenotype in both human and mouse T cells [123, 124]. Repeated infusion resulted in the

amelioration of the scurfy phenotype, inflammatory bowel disease or rheumatoid arthritis in preclinical animal models [125, 126]. However, a major limitation of this approach involves the high cost for human patients and also a requirement for further exploration in terms of immunosuppressive specificity and stability.

3.3 TCR enrichment

The major risk with polyclonal Treg therapy is off-target suppression of immune responses, which might lead to an increased susceptibility to opportunistic infections or suppression of anti-tumor activity [127]. Developing antigen specific Treg therapy therefore provides a more effective and safer approach. Several preclinical studies using *ex vivo* or *in vivo* expanded antigen (Ag) specific Tregs have shown improved potency and lower risk of pan-immunosuppression [128-133]. *In vitro* priming of Tregs with alloantigen can generate tailor made Tregs with appropriate antigen specificity. Jiang *et al* reported induction of human Tregs specific for human leukocyte antigen A2 (HLA A2) peptide (138-170 aa) through peptide pulsing of immature DCs. These Tregs efficiently suppressed T_{conv} cells in a cell contact dependent manner [134]. Studies performed in a type I diabetes mouse model demonstrated that pancreatic lymph node Tregs pulsed with islet antigen were significantly better in prevention and treatment of disease as compared to polyclonal Tregs [131-133]. Similar results were obtained in a skin allograft and GvHD mouse model, suggesting the improved efficacy of antigen specific Tregs [128-130]. Further, in a humanized mouse model for transplantation, antigen specific Tregs demonstrated better efficacy when used in much lower numbers as compared to polyclonal Tregs [135, 136]. In a recent study, Tregs isolated from FVIII sensitized mice, expanded *in vitro* with FVIII, antigen presenting cells and IL2 were found to suppress anti-FVIII antibody response and induce long term tolerance to FVIII [137]. Overall, these studies demonstrate the potential of antigen specific Tregs in transplant rejection, autoimmunity and recombinant protein therapies.

3.4 Antigen specific engineered Tregs

Regardless of the improved outcome from TCR enriched antigen specific Tregs, the main limitation of this approach are complex cell culture requirements, and a low starting population of antigen specific Tregs, especially in genetic disorders with large mutations that result in a lack of protein expression like hemophilia, Pompe and Fabry's disease [138-141]. To overcome these limitations, engineered Tregs

expressing antigen specific transgenic TCRs or synthetic chimeric antigen receptors are an alternative approach to induce targeted immunosuppression.

3.4.1 TCR transgenic Tregs

The first proof of concept for the use of T cells over-expressing the alpha (α) and beta (β) chains of antigen specific TCRs was obtained in the field of cancer immunotherapy [142]. This approach was later applied to redirect Treg specificity towards target antigens involved in autoimmune diseases. Several preclinical studies in mouse models have shown that TCR engineered Tregs are more efficient in suppression of effector responses against specific antigens in colitis, multiple sclerosis, arthritis and autoimmune diseases [143-146]. Further, *in vitro* expanded Tregs with direct alloantigen specificity conferred by transgenic TCR were more efficient in tolerance induction to MHC mismatched heart grafts [147].

The success achieved in mouse Tregs encouraged the development of human TCR transgenic Tregs. Kim and co-workers showed that TCR transduced Tregs recognizing a HLA class II restricted peptide to the C2 domain of FVIII were able to suppress both T and B cell responses against FVIII in HLA transgenic hemophilia A mice [148]. Hull and colleagues demonstrated the efficacy of lentiviral mediated islet antigen specific TCR transfer in human Tregs in the prevention of diabetes [149]. In a recent study, single cell TCR analyses of islet Tregs revealed their specificity for insulin and other islet derived antigen and these antigen specific Tregs were reported to be efficient in protecting NOD mice from diabetes [150].

There are some limitations of this approach such as the requirement for MHC restriction and risk of mispairing with endogenous TCR, although this can be addressed by introducing disulphide links or knocking out the endogenous αβ TCR. A major concern is that the majority of these transgenic Tregs were generated using TCRs isolated from T_{conv} cells and it is highly likely that the intrinsic affinity and specificity of TCRs isolated from Tregs are distinct from T_{conv}, which can affect the stability of engineered Tregs, avidity and migration to specific niches.

3.4.2 Chimeric antigen receptor (CAR) Tregs

Engineering antigen specific T cells through the incorporation of chimeric antigen receptors (CARs) has found unprecedented success in the treatment of hematologic malignancies [151, 152]. The synthetic CAR molecule, comprised of an extracellular

antigen binding domain from a monoclonal antibody and intracellular T cell signaling domains [153] can identify the target antigen in an MHC independent manner without the requirement for antigen presentation, thus overcoming the limitations posed on TCR transgenic Tregs.

Building on the success of 2nd generation CARs in cancer treatment, to date, all CAR Treg studies have used an identical design that includes a single co-stimulatory domain linked to the primary CD3ζ signaling domain. Almost a decade ago, initial preclinical studies with second generation CAR Tregs were performed in mouse models of colitis and xeno-transplantation [154, 155] and the first human CAR Tregs were generated [156]. Since then several studies on CAR Tregs have showed improved efficacy, enhanced persistence and stability in different disease models like colitis [143, 155, 157], GvHD [158-161] and skin rejection [162]. The possible mechanisms by which CAR Tregs may induce immunosuppression are represented in **Figure 2.**

In recent years, CAR Treg technology has been applied to induce immune suppression against soluble antigens. In a hemophilia A model, human Tregs expressing FVIII specific CAR Tregs were able to suppress the proliferation of FVIII specific T_{conv} cells [148, 163]. Tregs possess a unique feature of bystander suppression which enables rational design to target Treg cells to the inflamed tissue, without necessarily targeting cell surface antigens. Taking advantage of this property, a CAR molecule was developed targeting citrullinated vimentin (CV), which is present abundantly and exclusively in extracellular matrix of inflamed joints in rheumatoid arthritis (RA) patients [164]. These cells were able to proliferate in the presence of synovial fluid from RA patients, suggesting that presence of CV in inflamed joints is sufficient to activate these CAR Tregs. This approach can prove beneficial in certain inflammatory settings as direct targeting of antigen expressing cells may be detrimental due to the reported cytolytic activity of CAR Tregs in certain cases [165]. In a recent study, CAR Tregs designed against insulin were found to be functionally stable and suppressive in *in vitro* experiments and persisted *in vivo*, but were unable to prevent spontaneous diabetes in NOD/Ltj female mice model [166].

B cell targeting antibody receptor (BAR) Tregs, which comprises an extracellular antigen domain (rather than the scFv of a CAR), complexed to primary and co-

stimulatory signaling molecules, is another strategy which has recently been used to demonstrate suppression of FVIII specific B cells both *in vitro* and in a hemophilia A mouse model [167, 168]. This approach provides promising results in these initial studies and require further studies using different disease models.

4. Considerations for CAR Treg design

CARs have opened up avenues to engineer Tregs against a wide variety of antigens. However, there are several aspects of CAR design which can be improved upon with context to Treg engineering. One of the major issues in the Treg based cellular therapy is the stability and plasticity of their phenotype. Tregs demonstrate plastic differentiation depending on the TCR signal strength which determines the binding of FoxP3 to a set of regulatory factors [169, 170], and the microenvironment which can affect the post-translational modification of FoxP3 [171]. In an inflammatory environment or due to the strong signals, Tregs can exhibit features of Th cells, such as the secretion of pro-inflammatory cytokines and the expression of Th specific transcription factors, but also still maintain the expression of Foxp3 [172, 173]. Thus, tailoring CAR Treg design according on the disease model is critical. Another important factor in adoptive cellular therapy is the potential for a transient suppressive effect due to short lived persistence. Natural Tregs exhibit the phenomenon of Infectious tolerance i.e. conversion of T_{conv} cells into Tregs by a small number of antigen specific Tregs, thus generating long-lived antigen specific tolerance [174, 175]. The exact molecular mechanism of this phenomenon is not known yet but studies have shown that secretion of cytokines like TGF-β [176, 177], catabolism of tryptophan [176, 178] and interaction with DCs through co-inhibitory molecules like CTLA4 and PD1 [179] play an important role. With CAR Tregs, it is not known if these cells can induce infectious tolerance, more importantly in case of soluble antigens where contact dependent suppression might not occur. In these circumstances, it might be important to consider multiple infusions in order to extend tolerance. These different aspects of Treg behaviour should be taken into consideration while designing antigen specific Tregs for therapeutic purposes. In this section, we describe some of the modular design approaches which can be utilized to achieve the full potential of these therapies.

4.1 Costimulatory molecules

Extensive research has been carried out on the function of different co-stimulatory molecules to optimize the efficacy of T_{conv} cell-based CAR therapies for oncology. Currently, the most commonly used costimulatory molecules in CAR T_{conv} therapy are CD28 and 4-1BB [180] (**Figure 3A**). Although both molecules have proved to be remarkably effective in enhancing CAR therapy, they have exhibited very dissimilar kinetics, persistence and toxicity profile in patients [181-183]. Other groups have studied the functional effect of CARs expressing other co-stimulatory domains like ICOS or OX-40. The use of ICOS based CARs resulted in a greater propensity towards TH1/TH17 polarization and increased secretion of Th17 associated cytokines, with enhanced persistence in a xenograft tumor model [184, 185]. Addition of the OX40 endodomain led to reduced secretion of IL-10 by T_{conv} cells without altering the expression of other proinflammatory cytokines [186]. In a different approach, inhibitory CARs expressing PD1 or CTLA4 when used together with CD28 or 4-1BB CARs, limited toxicity by restricting off-target T cell stimulation [187].

Like T_{conv} cells, Tregs express a number of co-signaling molecules, which can both positively and negatively control Treg differentiation and function. But unlike T_{conv} CARs, fewer studies have been undertaken to investigate the impact of different co-signaling molecules on CAR Tregs. CD28 costimulation is essential for optimal Treg activation and function [188, 189] and hence used in the majority of CAR Tregs. Tregs expressing CD28 CAR were able to induce immune tolerance but did not persist for more than 3 weeks in the NOD/SCID gamma (NSG) mouse model [158]. 4-1BB TNF receptor expressing 2^{nd} generation CAR T_{conv} cells have shown improved persistence and reduced exhaustion in tumors [190]. In a mouse model for preventing transplant rejection, a comparison of CAR Tregs expressing either CD28 or 4-1BB costimulatory signaling domains indicated that incorporation of the CD28 costimulatory molecule effectively inhibited graft rejection, while 4-1BB did not [191]. The role of 4-1BB in Treg activation and function is not completely understood. A few reports have shown involvement of 4-1BB in the improvement of Treg expansion and suppression [192-194] while others have reported inhibition of suppressive function [195-197], thus meriting further study. However, understanding the importance of other co-signaling molecules in maintaining CAR Treg suppressive functions will be critical for improving their use in immunotherapy.

For T_{conv} CARs, a small subset of studies has been performed using 3rd generation CARs, in which a combination of co-signaling domains are used to further tailor T cell functionality. For example, combining CD28 and 4-1BB co-stimulatory domains resulted in increased expression of type I interferon, greater expansion and improved B-cell acute lymphoblastic leukemia regression in xenografts as compared to 2nd generation CARs [198]. Similarly, in a phase I dose escalation study, Ramos and co-authors demonstrated the effectiveness of 3rd generation CARs in the eradication of minimal residual disease and more durable remissions [199]. In addition, incorporation of the OX-40 domain resulted in reduced secretion of IFN-gamma and IL-2 with reduced antitumor activity *in vivo* [200]. This strategy has not yet been tested in CAR Tregs.

From a clinical perspective, it is critical to develop a CAR that maximizes the suppressive property of Tregs while simultaneously inducing poor cytotoxicity or proinflammatory cytokine responses if accidentally incorporated into a T_{conv} cell. Therefore, there is a need to identify appropriate co-signaling domains that can redirect CAR Tregs, while maintaining phenotype stability, cytokine production, survival and persistence.

4.2 Affinity and avidity tuning

In comparison to the TCR, the antibody based CAR scFv has a much higher affinity and avidity to the cognate antigen [201]. The impact of receptor affinity on determining Treg signaling and suppressive function has been postulated, but not conclusively reported. High levels of repeated signaling can lead to destabilization of the Treg phenotype and hence a resultant loss of suppressive activity [52, 202]. In contrast, signaling mediated by low amounts of a strong TCR agonist has been demonstrated to increase the persistence of Tregs *in vivo* [203]. Depending on the disease model, affinity of CAR molecules towards the target antigen can result in different outcomes.

In tumor therapy, the affinity of CAR molecule to the target antigen has been shown to play an important role in the efficacy and persistence of these cells. Lowering the scFv affinity towards target antigen has been shown to limit on-target, off-tumor toxicities [204]. In a recent study, lower affinity CD19 CAR T cells showed increased proliferation and cytotoxicity *in vitro* and enhanced anti-tumor activity and longer persistence with decreased toxicities *in vivo* [205]. It is therefore expected that the use

of moderate or low affinity CARs could have an impact on CAR Treg suppressive function *in vivo*. Recently, Sprouse and co-workers conducted an extensive study to demonstrate the role of high or low affinity TCR Tregs on the development of diabetes in NOD TCR knockout mice. The results from this study reported that both low and high affinity TCR Tregs use distinct non-redundant suppressive mechanisms for combined effective control of tissue specific autoimmune response [206].

Besides affinity, overall receptor avidity may affect therapeutic outcome. Studies performed with T_{conv} cells suggest that antigen specificity is not the only factor that influences functional efficacy [207, 208]. In adoptive T cell based therapies for the treatment of cancer or viral diseases, T cells expressing low avidity TCR showed a reduced ability to respond to limited antigen concentrations together with an incapacity to eliminate viral infections and tumors [207, 209, 210]. Further a skin allograft rejection model demonstrated that TCR avidity is important for the optimal function of Tregs [211]. Promoter usage and lentiviral transduction efficiency of T cells results in heterogenous expression of the CAR molecule, making it hard to ensure consistent behaviour among individual CAR T cells as avidities may vary. The integration of the CAR construct into the endogenous TCR locus using CRISPR/Cas9 system limited CAR expression in T_{conv} cells and has shown reduced tonic signaling and exhaustion. The CRISPR edited CAR T cells demonstrated enhanced T cell potency as compared to conventionally generated CAR T cells in a mouse model [212, 213]. Therefore, finetuning CAR affinity and avidity is an attractive strategy that can be applied to Tregs in order to modulate the outcome of CAR therapy.

4.3 Multi-antigen targeting

In cancer, tumor relapse is the one of the major issues of CAR T cell therapy. To overcome this problem, CAR T cells with specificity towards multiple tumor antigens are being tested in preclinical models [214]. Approaches used to generate multi-antigen CARs either involve co-administering CAR T cells with different antigen specificities (pooled CARs), incorporating a single CAR molecule expressing two distinct antigen binding domains in tandem (tandem CARs), or using a bi-directional vector to co-transduce 2 CAR molecules with different specificities into a single cell (**Figure 3B**). Pooled CARs, tandem CARs and dual antigen specific CARs has been investigated in glioblastoma using human epidermal growth factor receptor-2 (HER-2)/IL-13Rα2, B cell- Acute lymphoblastic leukaemia (B-ALL) using CD19/CD123

specific CAR T cells [215, 216] and in other models [217-219]. The CARs were able to generate a distinct response to each antigen, prevent antigen escape and improve antitumor efficacy [219].

To date, multi-antigen specific CARs have not been used for Tregs. However, a study on islet Tregs with multiple specificity for insulin as well as other islet derived antigens protected against diabetes in NOD mice [150]. This suggests that a similar approach can be used to generate CAR Tregs with multiple specificities, to improve targeting.

4.4 Logic-gated CARs

With the reported improvement in efficacy of dual antigen specific CAR T cells in tumor models, several groups have now applied Boolean logics to further modulate the outcome of these therapies (**Figure 3C**). Lanitis and co-authors proposed an AND gate strategy to physically isolate the CD3ζ signal from the co-stimulatory CD28 signal, assembled into separate CAR moieties individually targeting mesothelin and a folate receptor. These CAR T cells only transmit a signal upon encountering both antigens, which results in highly selective antitumor efficacy [220]. AND gated CARs also exhibit the natural biological properties of T cells, such as optimized proliferation, cytokine secretion, cytotoxicity, tumor-specific homing and off-tumor toxicity reduction [220]. Recently, the AND logic gate strategy was combined with the Notch receptor system where the binding of antigen to the first CAR triggers the SynNotch receptor to release a transcriptional regulator, which regulates the expression of a second CAR molecule [221]. Using this approach, it is possible to accomplish highly controlled signaling in Tregs, which can overcome issues related to overstimulation and hence loss of function.

Another interesting approach which has recently been utilized to overcome immunosuppression in the tumor microenvironment of a pancreatic cancer model is the generation of trivalent CAR T cells that respond only to tumor specific expression patterns. Engineered T cells redirected to recognize the tumor specific prostate stem cell antigen (PSCA) and cytokines TGF-β and IL-4 transmitted individual signals including antigen recognition, co-stimulation and cytokine secretion [222]. These three signals led to activation, amplification and persistence of T cells, which resulted in safe and selective lysis at tumor sites by CAR T cells. Generation of CAR Treg with a similar strategy can improve the potency and long-term effectiveness of the therapy. For

example, CAR Tregs recognising a specific antigen and immune enhancing cytokines such as IL6 and IFN-γ could suppress inflammation driven by antigen specific T_{conv} cells.

4.5 Co-expression and armored CARs

The impact of accessory gene co-expression with CAR molecules in T cells is being analyzed for tumor therapy. A tumor specific CD30 CAR in conjugation with chemokine receptor CCR4 resulted into enhanced tumor targeting by modulating the trafficking and homing to the tumor microenvironment [223]. Using a similar approach, in a B cell leukemia mouse model, CD28ζ 2^{nd} generation CAR expressed with 4-1BB in trans induced more potent antitumor responses than 3^{rd} generation CAR and showed reduced exhaustion and increased persistence of CAR T_{conv} cells *in vivo* [198]. Besides these studies, several other groups have generated CAR T cells in association with molecules like CD80, CD40L or IL-15/inducible suicide gene (iCasp9) which has enhanced their functionality through multiple mechanisms [224-226]. Other modifications, such as armored CARs carrying a cytokine payload, are currently being tested [227-230]. In a study performed by Markley and Sadelain, use of CD19 CAR T cells constitutively expressing IL-2, IL-7, IL-15 or IL-21 showed improved anti-lymphoma activity *in vivo* than CARs without cytokines [231].

Translation of this approach for Tregs could involve constitutive co-expression of inhibitory molecules like CTLA4 or PD1 that can help to improve the suppressive activity of Tregs in a contact dependent manner. CAR Tregs expressing immunosuppressive cytokines such as, IL-10, IL-35 or TGF-β either constitutively or induced upon activation, have the potential to improve contact independent suppression. Importantly, these approaches will likely require optimization to tailor the function of Treg therapies to different diseases.

5. Concluding remarks

In the past decade, multiple studies have addressed the efficacy and potential of Treg based therapies. There are several formats of Tregs, from polyclonal to antigen specific, which are being used in clinical trials. CAR Tregs are a very promising approach but designs based on CAR T_{conv} cells for cancer may not be well suited for preserving the immune suppressive function of Tregs. There are key differences in the biology of Tregs and T_{conv} with respect to TCR stimulation, co-receptor ligation or

cytokine production. Overall, further studies on the modular design of CARs and its impact on Tregs in different disease models are required to establish a new generation of cellular therapies.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any conflict of interest.

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Figure Legends

Figure 1: Mechanism of Treg suppression. Tregs are able to suppress immune responses by direct and indirect mechanisms. Indirect mechanisms include the production of anti-inflammatory cytokines like IL-10, IL-35 and TGF-β, which can suppress T_{conv} and NK cells. Release of perforin and granzyme can damage the target cell membrane leading to apoptosis. Expression of CD39 and CD73 on Tregs mediate conversion of ATP to adenosine and AMP causing reduced proliferation of T_{conv} cells. Tregs have been observed to mediate a direct effect on T_{conv} cells through receptor-ligand interactions like PD1-PDL1, ICOS-ICOSL, TRAIL-DR5. By depriving IL2 from the microenvironment, these cells reduce the proliferation of T_{conv} and NK cells. Interaction of Tregs with antigen presenting cells (APC) via CTLA4, PD1 and other co-inhibitory molecules leads to direct APC suppression and indirect suppression of T_{conv} cells.

Figure 2: Mechanism of CAR Treg mediated suppression. CAR Tregs can identify either cell-surface or soluble antigen. Interaction of CAR Tregs with cells expressing the target antigen on the cell surface activates the CAR Treg, which can secrete anti-inflammatory cytokines, perforin and/or granzyme, or upregulate co-inhibitory receptors like CTLA-4. CAR Tregs also upregulate CD25 which can indirectly suppress T_{conv} cells by consumption of IL2. In the case of soluble antigen, CAR Tregs can identify antigen bound non-specifically to the surface of APCs or to the antigen specific B cells receptor (BCR) on B cells, thus leading to initiation of both contact dependent and independent suppression, although exact mechanisms remain to be defined.

Figure 3: Next generation modifications of CAR constructs. A) Structure of 2nd and 3rd generation CAR molecules. B) Different formats of multi-antigen specific CARs. C) Representation of different forms of AND logic gated CAR cells.

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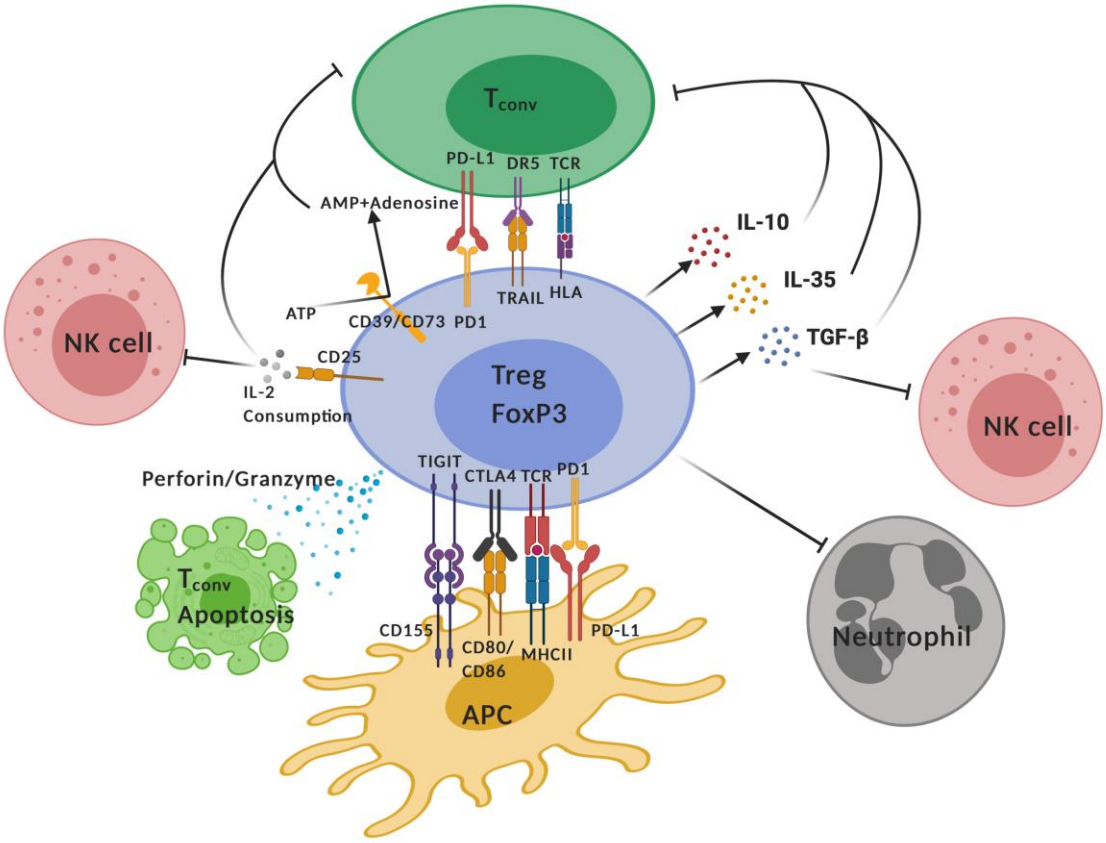
Figure 1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NK cell
CD25
IL-2
Consumption
Treg
FoxP3
NK cell
TIGIT
CTLA4
TCR
PD1
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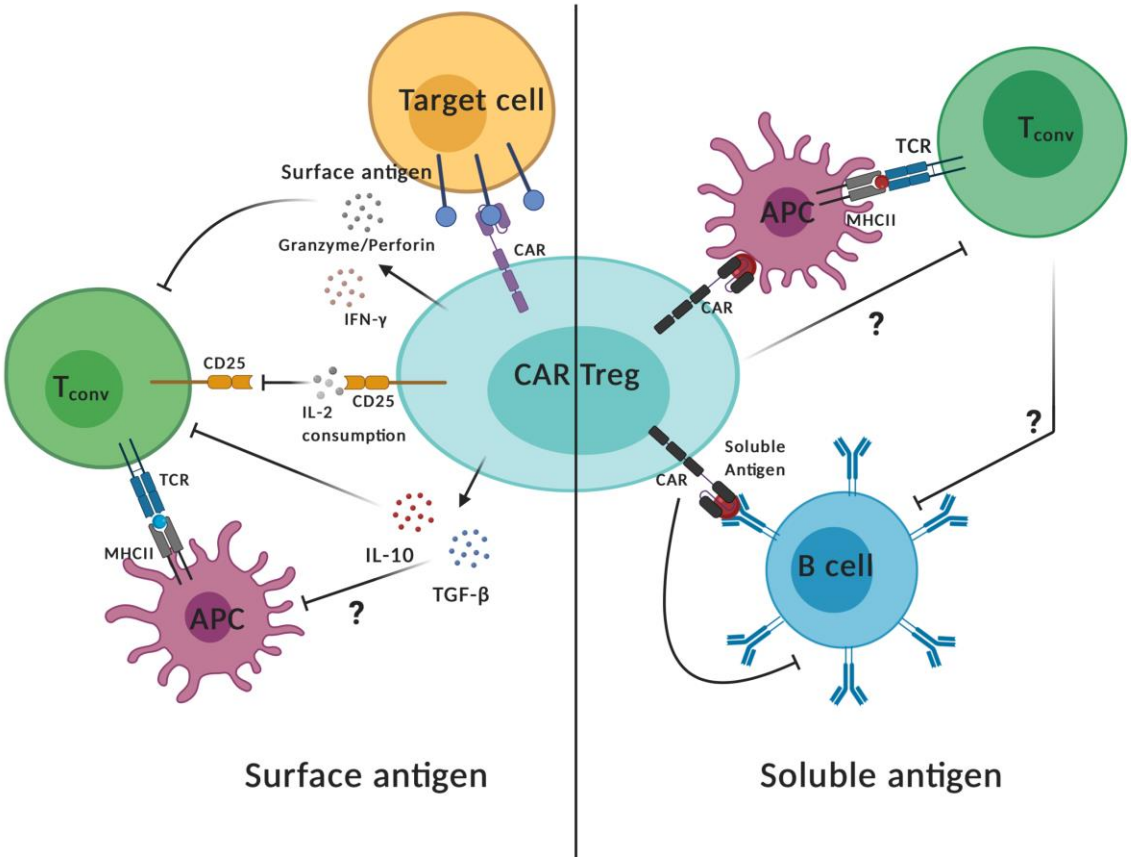
CD86
MHCII
PD-L1
Neutrophil
APC

Figure 2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CAR
CAR Treg
CD25
IL-2
consumption
CD25
Tconv
TCR
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APC
IL-10
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Soluble
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CAR
B cell
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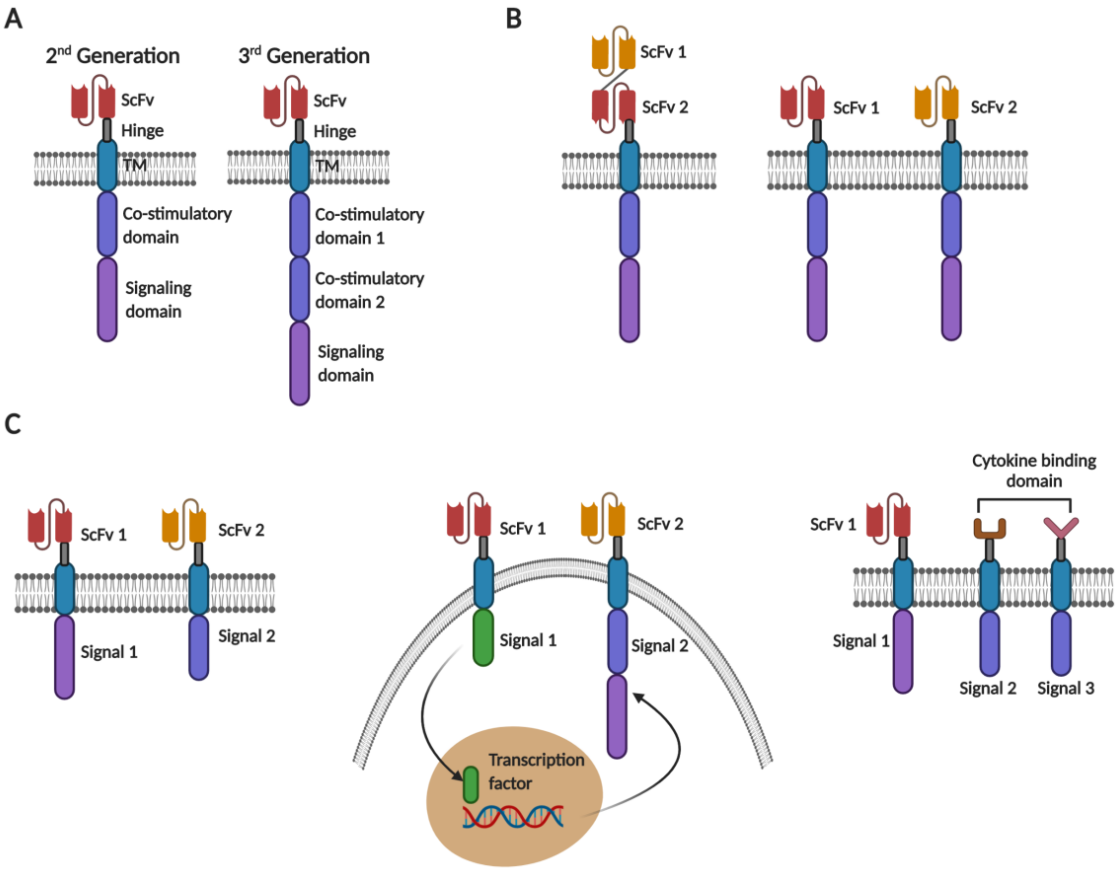
Surface antigen
Soluble antigen

Figure 3


A
2nd Generation
3rd Generation
ScFv
Hinge
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Co-stimulatory domain
Signaling domain
Co-stimulatory domain 1
Co-stimulatory domain 2
B
ScFv 1
ScFv 2
C
Signal 1
Signal 2
Transcription factor
Cytokine binding domain
Signal 3

Table 1: Completed and on-going clinical trials with polyclonal Treg infusions in different diseases

Study ID	Phase	Enrollment	Age (in years)	Product	Dose	Status
Type I diabetes						
NCT01210664	I	14	18-45	Autologous polyclonally expanded Tregs	0.05, 0.4, 3.2 and 26 x 10e8	Completed
ISRCTN06128462	I	12	5-28	Autologous polyclonally expanded Tregs	10 and 30 x 10e6/Kg	Completed
NCT02691247	II	113	8-17	Autologous polyclonally expanded Tregs	2.5 and 20 x 10e6/Kg	Active, not recruiting
NCT02772679	I	16	18-45	Autologous polyclonally expanded Tregs	3 and 20 x 10e6/Kg	Recruiting
NCT03011201	I/II	40	>18	UCB Polyclonally expanded Treg	2 x 10e6/Kg	Recruiting
NCT02932826	I/II	40	6-16	UCB Polyclonally expanded Treg	2 x 10e6/Kg	Recruiting
NCT03444064	I	18	18-68	Autologous polyclonally expanded Tregs	4-16 x 10e8	Recruiting
GvHD						
NCT02385019	I/II	22	>18	Donor Tregs	0.5, 1 and 2-3 x 10e6/kg	Unknown
NCT03683498	I	16	Child, adult and older	Donor Tregs	0.5, 1 and 2 x 10e6/kg	Recruiting
NCT01795573	I	38	18-70	Ex-vivo Expanded Donor Regulatory T Cells	NA	Active, not recruiting
NCT02749084	I/II	20	>18	Multiple infusion of Donor Tregs	0.5, 1 and 2 x 10e6/kg	Recruiting
NCT01911039	I	20	>18	Donor T Regulatory Cells	0.1, 0.5 and 1.5 x 10e6/kg	Unknown
Kidney Transplant						
NCT02145325	I	10	18-65	Autologous polyclonally expanded Tregs	0.5, 1, 5 x 10e9	Active, not recruiting
NCT02129881	I/II	12	>18	Autologous polyclonally expanded Tregs	1, 3 and 6 x 10e6/Kg	Completed
NCT02371434	I/II	9	18-65	Autologous polyclonally expanded Tregs	0.5, 1, 5 x 10e6/Kg	Unknown
NCT02244801	I/II	16	18-70	Donor alloantigen reactive Tregs	3 and 9 x 10e8	Completed
NCT02091232	I/II	8	>8	Belatacept conditioned Tregs	4 and 9 x 10e8	Active, not recruiting
NCT02088931	I	3	18-50	Autologous polyclonally expanded Tregs	3.2 x 10e8	Unknown
NCT02711826	I	40	>18	Donor alloantigen reactive Tregs	1 x 10e6/Kg	Recruiting
ISRCTN11038572	IIb	136	>18	Autologous polyclonally expanded Tregs	5-10 x 10e6/Kg	Active, Not recruiting
NCT01446484	I	30	1-18	Autologous polyclonally expanded Tregs	2 x 10e8	Unknown

NCT03284242	NA	12	18-65	Autologous polyclonally expanded Tregs	NA	Not yet recruiting
Liver Transplant						
NCT02166177	I	9	18-70	Autologous polyclonally expanded Tregs	0.5-1 and 3-4.5 x 10e6/Kg	Completed
NCT02188719	I	24	21-70	Donor alloantigen reactive Tregs	0.5, 2 and 8 x 10e8	Recruiting
NCT02474199	I	18	18-70	Donor alloantigen reactive Tregs	4 x 10e8	Recruiting
NCT01624077	I	1	10-65	Autologous induced Tregs	1 x 10e6/Kg	Unknown
NCT03577431	I/II	9	17-70	Belatacept conditioned Tregs	2.5-500 x 10e6	Not yet recruiting

1081

1082 UCB: Umbilical cord blood