Regulatory T cells for Tolerance

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Abbreviations:

aGvHD: acute graft-versus-host disease
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AL: acute leukemia
AL: acute leukemia
arTreg: alloantigen reactive T regulatory cell
arTreg: alloantigen-reactive T regulatory cell
ATG: anti-thymocyte globulin
aTreg: activated T regulatory cell
aTreg: activated Treg
BMT: bone marrow transplantation
CAR: chimeric antigen receptor
cGvHD: chronic graft-versus-host disease
CNI: calcineurin inhibitors
CTLA-4: cytotoxic T-lymphocyte antigen 4
DC: dendritic cell
FACS: fluorescent-activated cell sorting
GMP: good manufacturing practice
GvHD: graft-versus-host disease
HLA: human lymphocyte antigen
HSC: hematopoietic stem cells
IDO: indoleamine 2,3-dioxygenase
IL: interleukin
IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked
iTreg: induced regulatory cell
LDLT: living donor liver transplantation
mAb complex: monoclonal antibody complex
MMF: mycophenolate mofetil
NHP: nonhuman primate
nTreg: natural T regulatory cell
PBL: peripheral blood lymphocyte
PBSCT: peripheral blood stem cell transplant
pTreg: peripheral T regulatory cell
rTreg: resting Treg
SOT: solid organ transplantation
Tconv: T conventional cell
ThRIL: TReg Immunotherapy in the setting of Liver transplantation
Tr1: type 1 regulatory cell
TSDR: T regulatory cell-specific demethylated region
tTreg: thymus-derived T regulatory cell
UCB: umbilical cord blood

Abstract:

Regulatory T cells (Tregs) are critical mediators of immune homeostasis and hold significant promise in the quest for transplantation tolerance. Progress has now reached a critical threshold as techniques for production of clinical therapies are optimised and Phase I/II clinical trials are in full swing. Initial safety and efficacy data are being reported, with trials assessing a number of different strategies for the introduction of Treg therapy. It is now more crucial than ever to elucidate further the function and behaviour of Tregs *in vivo* and ensure safe delivery. This review will discuss the current state of the art and future directions in Treg therapy.

Keyword:

Regulatory T cells; T cell; tolerance; immunosuppression; humanized mouse

Introduction:

The past few decades have marked an era of promising advances in the quest for clinical tolerance, with much of the progress attributed to the growing knowledge of regulatory T cells (Tregs). Findings within that demonstrate the role of Tregs as crucial mediators of immune homeostasis have led many to speculate these cells to be key to tolerance induction. Indeed, from the identification of specific Treg markers to the implementation of Treg cellular therapies in pioneering preliminary clinical studies, the addition of Tregs to the clinical armamentarium is on the horizon. There is still much to learn in the study of Tregs, ranging from optimizing expansion mechanisms to implementing appropriate therapies in clinical trials. This review will focus on the knowledge gained in Treg research thus far, ongoing clinical trials for Treg-based cell therapies, and prospective areas of research.

Regulatory T cells (Tregs): A brief background

Origin*:* Although the concept of T cells that act as suppressors had been proposed as far back as the 1970's and 1980's with contributions from groups Gershon/Kondo [1], Okumura/Tada [2], and Hall/Jelbart/Dorsch [3], research in Tregs only really started to gain traction in the 1990's after the discovery of specific markers that distinguished this suppressor population from other T cells [4]. It was in this period that the $CD4^+CD25^+$ T cell population was identified as having potent regulatory properties in self-tolerance and autoimmune suppression [5]. While a number of other non-CD4 expressing Treg subtypes exist, this review will focus on the extensively studied $CD4^+$ Treg subset. CD25 not only serves as a useful marker for Treg identification, but due to its high affinity for interleukin-2 (IL-2), it is also important for the expansion and maintenance of Tregs [4].

The identification of the FOXP3 gene helped to spearhead investigation into the characterization and function of Tregs due to its role as a 'master regulator' [6, 7]. Foxp3-

mutant scurfy mice and Foxp3-null mice demonstrate $CD4^+CD25^+$ regulatory T cell deficiency that quickly leads to aggressive lymphoproliferative autoimmune syndromes that is reversible with restoration of the Treg compartment. In immunodeficient mice, Tregs can also be generated with the addition of a Foxp3 transgene [8]. In a similar manner, a FOXP3 mutation in humans leads to an X-linked autoimmune lymphoproliferative disorder called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) [9]. Variability in FoxP3 gene expression can lead to differential Treg suppressive function. In particular, the methylation status of the Treg-specific demethylated region (TSDR) within the FoxP3 gene locus in both mice and humans correlates with suppressive function. The demethylation of this evolutionarily conserved region not only serves to regulate FoxP3 gene transcription and the conversion of non-Treg cells to Tregs, but also plays a factor in maintaining suppressive function. However, the mechanisms by which it serves this role is not yet completely understood [10-12].

Tregs may be classified into two main subpopulations, the more conventional naturally-occurring thymus-derived Tregs (nTregs), and induced Tregs (iTregs), which represent a population of suppressive Tregs that are differentiated from $CD4^+$ T cells in the periphery. nTregs and iTregs are also commonly referred to as thymus-derived Tregs (tTregs) and peripherally-induced Tregs (pTregs), respectively [13]. A number of other regulatory $CD4^+$ T cells exist, including Type 1 regulatory T cells (Tr1) and Th3 cells, which exhibit suppressive functions but do not express FOXP3. There have been interesting findings that suggest that transient FOXP3 expression may be necessary for the suppressive function of these cells, but further discussion of non-FOXP3 expressing Tregs is beyond the scope of this review. [14, 15]

***Suppressive Mechanisms*:** Tregs display significant versatility in their suppressive mechanisms, which can depend on the immune environment, the Treg activation state, and target cell type [16]. In transplantation, for example, the suppressive mechanisms can depend on donor-specificity [17]. There are four main processes by which Tregs suppress immune responses: (1) modulation of dendritic cell (DC) function or maturation, (2) inhibitory cytokine release, (3) cytolysis, and (4) metabolic disruption, [18].

(1) Investigation of specific pathways involving cytotoxic T-lymphocyte antigen 4 (CTLA-4) has demonstrated that Tregs can hinder the activation of effector T cells by modulating DCs. Multiple studies have supported the finding that Tregs may downregulate B7 costimulatory molecules CD80 and CD86 on DCs in a CTLA-4 dependent manner to disrupt the activation of effector T cells by APCs. CTLA-4 interacts with CD80/CD86 ligands with high affinity to deprive naïve T cells of CD28-mediated costimulation. [19]. Studies indicate that although CD80/86 is mainly expressed by APCs, naive T cells can also express these molecules, providing a further target for CTLA-4 [20]. Through CTLA-4 ligation, Tregs may also induce DCs to express the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO), which converts tryptophan to kynurerine, a molecule toxic to T cells [16]. Another relevant molecule expressed on Tregs is LAG-3, which binds to MHC-II molecules to suppress DC maturation [18].

(2) TGF-β, IL-10, and IL-35 are considered to be the main regulatory cytokines released by Tregs. These cytokines are involved in tasks such as the direct suppression of effector T cell signaling, regulation of IFN-γ, the induction of Tregs, and the maintenance of FoxP3 expression [16, 21, 22].

(3) Tregs may exert suppression through expression of granzymes that induce programmed cell death. By this mechanism Tregs are able to kill and suppress B cells and possibly hinder effector T cell function through apoptosis [23].

(4) Metabolic disruption is another potent mechanism of suppression. A number of studies have demonstrated that Tregs can induce apoptosis of effector T cells through their high expression of CD25 that allows them to deprive effector T cells of IL-2 [24].

Treg therapy in transplantation: **The overarching goal in Treg therapy in transplantation has been to promote a tolerogenic state, while reducing the dependence on toxic immunosuppressive medication** [25, 26]. Treg therapies could bring advantages in terms of

practicality, such as cost, availability, specificity, and applicability across different organs, due to their potential to be standardized and manufactured industrially [27]. As a select few Treg therapy studies enter Phase II clinical trials, the present stage marks a significant milestone [28]. It is therefore critical that the operational machinery of Tregs is elucidated for safe and effective use.

An important aspect of clinical translation is development of Good Manufacturing Practice (GMP) methods for Treg therapy production [29]. This is complicated by controversies over which subsets of Tregs are best suited for therapy in terms of function and stability.

When isolating Tregs for clinical cell therapy, it is important to be careful of contamination with non-Tregs in the final product. The presence of populations such as effector cells, for example, may be pro-inflammatory [30, 31]. Thus, the isolation of suppressive Treg cell populations is much more complicated than simply the identification of previously discussed markers such as CD4/CD25/FOXP3. Although FOXP3 is constitutively expressed in suppressive Tregs, it is an intracellular marker that cannot be used in the context of isolation. Moreover, CD25, although expressed highly in Tregs, is not exclusive to Tregs and includes other cell types, such as activated effector T cells [6, 15, 32]. Necessarily, other markers must be used to further purify the desired population. CD127, the α-chain of the IL-17 receptor, inversely correlates with FoxP3 expression, and its low expression can be used in conjunction with CD4 and CD25 to isolate a highly suppressive Treg population [33-35].

Isolation of Tregs is also complicated by the need to consider the different subsets within the heterogeneous Treg population that would best function in transplantation. Depending on various environmental and pathological conditions, different Treg populations can express a number of transcription factors, microRNA, chemokine receptors, suppressor molecules, cytokines, and other immune-related proteins [36]. However, the identification of many new markers in recent years has made it possible to dissect this heterogeneity and to hone Treg therapy to identify and select for specific populations that will function optimally in organ

transplantation. CD45RA, for example, distinguishes three Treg subpopulations with functionally different properties: $CD45RA^{+}FoxP3^{lo}$ resting Tregs (rTregs), $CD45RA^{-}FoxP3^{hi}$ activated Tregs (aTregs), and cytokine-secreting $CD45RA^{-}FoxP3^{lo}$ non-suppressive Tregs [12, 37]. However, there are challenges in Treg isolation through CD45RA because naive Treg numbers in the peripheral blood diminish with age [38, 39].

Treg stability is critical in clinical therapy. As a whole, previous studies have demonstrated the durable stability of Tregs, but there remain findings that suggest that at least some subsets of Tregs display a loss of suppressive function or FOXP3 expression. There is evidence that a minor population of Tregs, which have lower expression of CD25, will not only lose their suppressive function but also differentiate into effector T cells in certain conditions, particularly when Tregs are adoptively transferred into lymphopenic recipients [40]. Loss of FoxP3 expression after infusion may be attributed to reduced IL-2 production within the host due to the Tregs not being able to produce IL-2 themselves [41]. Some studies have also indicated that Tregs lose their FoxP3 expression through stress signals elicited by proinflammatory cytokines and liposaccharides, where FoxP3 is ubiquinated by the E3 ligase Stub1 [42]. Some of these “ex-Tregs” may be able to regain their suppressive function, but the exact mechanisms and conditions in which this occurs is not yet clearly defined [40]. A large obstacle in measuring the stability of Tregs post-infusion is the lack of a safe and effective GMP-approved strategy to track the Tregs’ survival in humans. While there have been some studies that label Tregs to provide insight into their behaviour post-infusion [43, 44], an effective method that can be used in the clinical setting would be crucial in guiding treatment post-transplantation.

One marker that has been associated with stability of suppressive function in Tregs is the transcription factor *Helios*. Some have also suggested *Helios* as a marker for distinguishing nTregs from iTregs [45]; however, other recent studies have challenged this notion that the lack of *Helios* expression does not necessarily indicate an iTreg population [46]. The TSDR, which is perhaps the most commonly identified marker for stability of FoxP3 expression, has been demonstrated to be fully demethylated in nTregs, while markedly more methylated in iTregs

[47]. In general, nTregs are thought to be able to maintain FOXP3 expression better than iTregs and have been the focus of many isolation protocols. However, iTreg generation *in vivo* using induction agents may be a useful and perhaps more economically viable strategy in comparison to adoptive cellular therapy [48].

Animal models

Mice*:* In terms of elucidating potential mechanisms and new targets of research, Treg cell therapy in mouse models has offered valuable insights. The pretreatment of mice with infusion of donor alloantigen and anti-CD4 mAb can generate alloantigen-specific CD4+CD25+ T cells that potently regulate rejection of skin allografts in a CTLA-4 and IL-10 dependent manner [49]. In bone-marrow allograft transplantation murine models, transfer of either freshly isolated or *ex vivo* expanded Treg cells promotes tolerance of the allograft while hindering GvHD [50, 51]. In another approach, pretreatment with IL-2/IL-2 monoclonal antibody (mAb complexes) expands Tregs and induces tolerance to pancreatic islets [52]. The adoptive transfer of Tregs in mouse models has also been used to track Treg migration patterns that suggest that Tregs have suppressive functions in both the transplanted graft and secondary lymphoid organs [53].

Humanized mice**:** The majority of studies that have facilitated the translation towards clinical studies and the necessary regulatory body approvals have been performed in humanized mouse models and non-human primates. The differences between mouse and human Tregs necessitate this. For example, human Tregs typically express a memory phenotype, while mouse Tregs are generally antigen-inexperienced due to their housing in controlled environments [54]. Moreover, unlike mouse Tregs that can be clearly recognized with distinct markers such as FoxP3, there is yet to be a more definitive and defining marker for human Tregs. For example, FOXP3 in humans can be expressed by non-Tregs that may also upregulate CD25 while not being suppressive, bringing clear challenges in terms of identification [55].

Humanized mice are created through the engraftment of immunodeficient mice with human mononuclear cells, leukocytes, or hematopoietic stem cells (HSC). Our group has used humanized mice to demonstrate that treatment with *ex-vivo* expanded human Tregs could prevent human transplant arteriosclerosis, skin rejection, and islet rejection [35, 56, 57]. Using this model we have also demonstrated that different Treg populations have distinct tissue-specific homing markers that dictate their migration patterns and regulatory efficacy *in vivo* [58]. Treg populations that were initially assessed in these models have now been taken through to clinical trials in renal transplantation.

***Nonhuman primates (NHP)*:** Nonhuman primate Tregs have provided useful insights into human Tregs and accelerated clinical translation [59]. Bashuda et al. first demonstrated that the adoptive transfer of immunosuppressive anergic T cells that were generated by co-culture with donor alloantigen in the presence of anti-CD80/CD86 mAbs to suppress renal allograft rejection [60]. These cells have now progressed to clinical trials in kidney transplant recipients. In 2011, Ma et al. pretreated NHP cells with anti-thymocyte globulin (ATG) and then performed an adoptive transfer of donor alloantigen-specific Tregs combined with low-dose sirolimus, delaying acute rejection of renal allografts in *Cynomogulous* monkeys [61].

A number of NHP studies have focused on tracking Tregs after infusion, highlighting the challenges in sustaining the suppressive function of Tregs for use in therapy due to their transient nature [62]. Ezzelarab et al. infused multiple doses of *ex vivo*-expanded Tregs into lymphodepleted, MHC-mismatched *cynomogulous* monkey heart graft recipients early post-transplant and treated with tacrolimus, anti-IL-6 mAb, and rapamycin. Here, unlike the previous two NHP trials, the adoptive transfer of Tregs led to a relative decline in graft function along with induction of proinflammatory cytokine expression, antidonor alloantibody production, and an increase in effector memory cells [63]. IFN-γ, IL-6, and IL-15 levels were elevated, which likely led to enhanced Tmem and alloAb responses and destabilization of Tregs. In the study, the authors reason that it is possible that a re-expansion of effector T cells was associated with

the immunosuppressive tapering strategy [64]. The different results between these studies may be attributed to factors such as timing, frequency, and dosage of the injections, as well as the absence of splenectomy and the specific immunosuppressive regimen used.

Clinical trials of Treg cellular therapy

From the first report of human Treg expansion in 2001 and the subsequent first large-scale expansion of human Tregs in 2004 [65, 66], progress has accelerated swiftly towards clinical trials. Research centres worldwide have now begun to translate their Treg cell therapies to the bedside with varying yet promising results [Table 1].

In 2009, Trzonkowski et al. published the first clinical report using *ex vivo* expanded Tregs in two patients with graft-versus-host disease (GvHD) [28]. Peripheral blood was isolated from family donors of patients and cells were flow sorted for $CD4^{+}CD25^{hi}CD127^{-}$ Tregs, and expanded *ex vivo* for adoptive transfer. After the transplantation, the patients were treated with increasing immunosuppression comprised of solumedrol, tacrolimus, mycophenolate (MMF), and ATG, and then were infused with Tregs ($6x10^{7}$ Tregs per infusion) three times (days +75, +82, and +93 post-transplant). In a patient who had developed chronic GvHD (cGvHD) following bone marrow transplantation (BMT) to treat myelodysplastic syndrome, the infusion of *ex vivo* expanded Tregs led to a marked decrease in dependency on suppressive drug treatment as well as alleviation of GvHD complications. The other patient, who had developed acute GvHD (aGvHD) following allogeneic peripheral blood stem cell transplant (PBSCT) for chronic myelocytic leukemia, was treated with three doses of donor expanded Tregs that moderately improved the patient's condition. However, the patient did not survive long after available donor Tregs for infusion were depleted, indicating that more doses may be necessary to sustain suppression. This first clinical trial revealed vital information of the feasibility of adoptive Treg cell therapy in the context of transplantation and also highlighted some areas of required improvement.

In 2011, Di Ianni et al. reported the first clinical trial of adoptive transfer of Tregs to prevent GvHD in the absence of any post-transplantation immunosuppression in 28 patients with acute leukemia (AL) [67]. Their method centred around the early infusion of freshly isolated donor Tregs, followed by conventional T cells (Tconv) at the time of full-haplotype-mismatched HSC, and ultimately demonstrated that this prevented GvHD while also favouring Tconv-mediated post-transplantation immune reconstitution. The same group have continued with a Phase II trial in which they investigated whether the Treg-Tconv adoptive immunotherapy prevents post-transplant leukemia relapse [68]. In this trial with 43 adults with high-risk AL, patients were conditioned with a total-body irradiation-based regimen followed by grafts of $CD34^+$ cells, Tregs (2×10^6/kg), and Tconvs (1×10^6/kg) isolated from donors. The findings were promising in that with this treatment, almost 90% of patients were protected against GvHD, further supporting the safety and efficacy of using freshly isolated Tregs. Notably, in a 46-month follow up of AL patients, the use of Treg-Tcov adoptive therapy favoured post-transplant immunological reconstitution and led to an extremely low cumulative incidence of post-transplant leukemia relapse.

Brunstein et al. have also reported the successful use of *ex-vivo* expanded, partially human leukocyte antigen (HLA)-matched nTregs derived from umbilical cord blood (UCB) in a phase I dose-escalation trial in 23 patients with aGvHD [69]. Interestingly, UCB has become a popular source for Tregs due to its reduced proportion of activated effector T cells and ease of Treg isolation. In this preliminary trial, nTregs were obtained through CD25 isolation, expanded with anti-CD3/anti-CD28-beads and IL-2, and infused at doses of $0.1\text{-}30 \times 10^5$ Tregs/kg. Compared with 108 historical controls, treatment with UCB-derived Tregs reduced the incidence of grade II-IV GvHD with no indication of toxicity. Moreover, in a two-year follow up of the patients, there was no increased incidence of opportunistic infections or relapse in the long-term [70]. Recently, utilizing a novel technique of nTreg expansion, patients were also infused with UCB-derived nTregs expanded in the presence of KT64/86 cells (K562 cells modified to express CD64, a high-affinity Fc receptor, and the CD28 ligand CD86). KT64/86 cells displayed a higher

effectiveness at expanding Tregs, while maintaining FOXP3 expression and suppressive function [71].

Recently, Johnston et al. completed a Phase I trial to treat cGvHD with adoptive transfer of donor Tregs in 10 match-related donor recipients [72]. This 3+3 dose escalation trial (1, 5, $15x10^5$ Treg cells/kg) was the first clinical study with the use of highly purified donor-derived Tregs isolated through high-speed cell sorting, in which cells were enriched for CD25, purified for $CD4^+CD127^{dim}$, and infused without expansion. They reported no toxicity from the infusions with 6 of the patients having stable to improved cGvHD, while the other 4 had unresponsive or progressive disease requiring a new immunosuppressive regimen.

In terms of **solid organ transplantation** (SOT), although met with a separate set of challenges compared to HSCT, implementation of Treg cell therapies have currently demonstrated encouraging results in phase I/II clinical trials and potential for the reduction in the dependency of immunosuppressive drugs. Many trials for Treg therapy in SOT are currently in the initial phases or ongoing, with published data not yet available.

In Japan, 10 end-stage liver failure patients undergoing living donor liver transplantation (LDLT) were treated with a Treg-like cellular therapy [73]. Patients received an adoptive transfer of this *ex-vivo*-generated Treg-like cell product at doses of 0.23-6.37 x 10^6 Tregs/kg, generated through co-culture of recipient lymphocytes with irradiated donor cells in the presence of anti-CD80/86 monoclonal antibodies. The immunosuppressive regimen, which consisted of steroids, MMF, and tacrolimus, was tapered 6 months post-cellular infusion and stopped at 18 months. All patients also underwent splenectomy. Under this treatment, 7 out of the 10 patients achieved complete immunosuppression withdrawal, ranging from 16 to 33 months post-transplantation, and have displayed normal graft function and histology. However, 3 patients presented with signs of acute cellular rejection and had immunosuppression re-started. Interestingly, these 3 patients had an autoimmune cause for their liver disease (primary biliary cirrhosis or primary

sclerosing cholangitis). Unfortunately, due to this, the trial has been suspended and the authors currently cannot extend to the 40 patients as originally planned [73].

In another clinical trial, Bashuda's group in Japan has attempted the adoptive transfer of self-anergic cells in kidney transplant recipients from HLA-mismatched living donors. In the generation of the anergic T cells, both recipients and donors underwent lymphocytapheresis treatment before the transplant and PBMCs from both were co-cultured in the presence of anti-CD80/CD86 mAb, which modulates Treg function, and infused 12 days after the transplant as immunosuppression was also gradually tapered. All 12 recipients who received a splenectomy or rituximab and cyclophosphamide displayed graft survival, but further immunosuppression reduction was withheld due to a high incidence of biopsy-proven acute rejection. Data from the 40 recipients who received rituximab and rATG instead of cyclophosphamide, have yet to be reported. This new approach may be promising for SOT if the optimal balance with immunosuppression can be further clarified [74].

Two groups from the U.S. have recently shared results from their trials of adoptive cellular therapy with Tregs in kidney transplant recipients. The TRACT trial assessed the safety of the infusion of autologous, polyclonally expanded Tregs (0.5, 1, and $5x10^{-9}$ cells) in living donor kidney transplant recipients [75]. Here, patients received alemtuzumab induction for lymphodepletion before being infused with Tregs, which were isolated by $CD25^{+}$ enrichment following $CD8^{+}$ and $CD19^{+}$ depletion by CliniMACS and then expanded with anti-CD3/anti-CD28-coated beads, IL-2, and sirolimus. Thereafter, patients received tacrolimus and mycophenolate-based immunosuppression followed by conversion to sirolimus 30 days post-transplantation, in an effort to aid Treg survival. All 9 patients received this therapy and displayed a 9-20 fold increase in circulating $CD4^{+}CD25^{hi}CD127^{-}FOXP3^{+}$ Tregs in the peripheral blood with no signs of rejection or serious adverse effects.

Similarly, the TASK pilot trial sought to assess the safety and feasibility of injecting $CD4^{+}CD25^{+}127^{-}$ peripheral blood Tregs (320×10^{6} cells) that were FACS-sorted and expanded

polyclonally *ex vivo* with a deuterium label into kidney transplant recipients with subclinical inflammation on a 6-month protocol biopsy [76]. Two of the patients displayed reduced graft inflammation and improved rejection scores with no incidence of acute rejection, while one patient displayed signs of subclinical acute cellular rejection at 6-month post-Treg infusion and was treated with steroids and modified maintenance therapy. The authors mentioned that the third patient possibly had worsened outcomes because he started to develop *de novo* donor-specific antibodies prior to the infusion. The group have also recently initiated a trial to compare the efficacy of infused polyclonal Tregs versus donor alloantigen-reactive Tregs in kidney transplant recipients with subclinical inflammation [NCT02088931].

Our group is currently a partner in the EU-funded ONE Study (Phase I) and MRC-funded TWO Study (Phase II) trials, which aim to investigate the safety and therapeutic efficacy of Tregs in living donor renal transplantation. The central focus of the ONE study, a consortium coordinated in Regensburg and including centres located in the UK, Germany, France, Italy, and the US, is to test and compare a variety of immunoregulatory cell products in the context of a similar patient population, with a reference group of patients [77]. Both the cellular therapy and reference groups received low dose tacrolimus, MMF, and steroids, with the experimental group receiving regulatory cell therapy and the reference group receiving anti-CD25 antibody induction. These immunosuppressive regimens were held consistent throughout all centres. The manufactured cellular products included polyclonal and alloantigen-specific nTregs, Tr1 cells, tolerogenic dendritic cells, and regulatory macrophages. Additionally, Prof Lombardi's group, a ONE Study collaborator, has also initiated an independent clinical trial called ThRIL (TReg Immunotherapy in the setting of Liver transplantation), which similarly tests the safety of Treg therapy in liver transplant recipients [78].

The TWO Study aims to assess the efficacy of Tregs in renal transplant recipients. In this randomized controlled trial, patients in the therapy arm will receive expanded derived nTregs and immunosuppression reduced to a monotherapy. The primary outcome measure is biopsy-

proven acute rejection within 18 months of transplantation and patients will also be intensively monitored for detection of changes in immune phenotype.

Current methods of Treg isolation for cell therapy: Due to the low frequency of nTregs in the peripheral blood, cells must be purified and then expanded *ex vivo* or pooled [Figure 1]. A common GMP-approved method for isolation is CliniMACS (Miltenyi Biotech), in which cells are isolated with the use of magnetic beads. Studies have used this for the depletion of $CD8^+$ effector T cells and the positive selection for $CD25^+$ cells to purify a Treg population. More recently, some have found success in depleting Treg populations from $CD19^+$ and $CD127^+$ cells before Treg enrichment to isolate a more suppressive population with increased purity [34, 79]. However, methods such as CliniMACS that are based on magnetic bead-based isolation are limited. Although purification levels are being improved, there are restrictions in the number of parameters that may be used for isolation and there is little 'fine tuning' in the isolation of cells with intermediate expression levels.

Conversely, fluorescent-activated cell sorting (FACS)-based purification has the potential to yield cell products with higher purity because of the ability to isolate on a greater number of markers than CliniMACS. Not only would this generate populations of higher purity, but the use of different parameters for isolation allow for greater specificity and potency. Indeed, there is specific focus on the development of reliable, high performance clinically-approved FACS-based methods, such as the Cytonome GigaSort system and MACS Quant Tyto. Nonetheless, there remains a gulf to bridge developing the technology to meet GMP requirements, particularly in Europe. Thus, recent research in Treg therapy has focused on optimizing these two main methods.

Clonality (Polyclonal vs. Alloantigen Reactive Tregs): Recent studies have debated the use of polyclonal versus alloantigen-reactive Tregs (**arTregs**) [80]. Polyclonal Tregs can be expanded commonly with the use of anti-CD28/anti-CD3 beads/antibodies and IL-2, with a few groups demonstrating methods for expansion cells in substantial numbers [81]. However, the use of

polyclonal Tregs has its disadvantages in that large populations are necessary for adequate suppression because only a certain number of clones are likely to be donor-reactive. This non-specific reactivity may have detrimental effects through the suppression of other immune responses, leading to vulnerability to opportunistic pathogens and tumours, although in practice experimental studies have not been able to demonstrate this vulnerability [82].

Conversely, arTregs are selected due to their enhanced responsiveness to donor antigens and theoretical reduction in the risk of suppression of third-party immune responses. Thus, studies have found them to be as much as 10 times more suppressive than polyclonal Tregs in preclinical and clinical models [29, 34, 83], with fewer cells necessary to prevent rejection in mice [29, 57]. However, because they are generally expanded using allogeneic APCs, this strategy is difficult to apply to deceased donor SOTs [25]. Additionally, arTregs are generally more expensive and difficult to isolate with great frequency, especially because increased HLA-matching leads to decreased stimulation and expansion of arTregs [29].

Production/Manufacturing:

Cost: Currently, the manufacturing costs of patient-tailored Treg therapies can vary widely depending on isolation procedures, GMP facility costs, incubation period, dosage, and characteristics of Tregs isolated. However, there is potential for such a therapy to be economical in the long-term due to reduced reliance on immunosuppression, complications and hospitalization [25, 84]. In general, the manufacturing costs mean that autologous Treg infusions are more expensive than allogeneic infusions. As the manufacturing of Tregs become more standardized and commercialized, it is anticipated that these treatments will become cheaper. An outstanding question remains whether such therapies will be produced centrally or within each hospital's GMP facility. We believe that the central production of a Treg product has benefits in terms of standardization of production, quality control, enhanced throughput and concentration of expertise.

***Dosage**:* The dosage necessary for Treg therapy is also an area that requires further investigation, for it is dependent on the characteristics of the infused Tregs (stability, purity, and type), as well as the patient's condition and timing of injection. On the one hand, because polyclonal cells are less specifically suppressive than arTregs, they require a higher dosage. Previous mouse studies have demonstrated that a polyclonal Treg to Tconv ratio of 1:1-1:2 is sufficient to prevent allograft rejection [83, 85, 86]. Thus, assuming this proportion is consistent for humans, at least 49 to 79 x 10^9 Tregs will be needed to fulfil this requirement in humans [83]. If lymphodepletion to reduce the Tconv population is performed prior to Treg infusion in the patient, 10% of the total number of Tregs will be necessary to balance the scales. With recently reported strategies that can expand polyclonal Tregs to around 1x10^8 cells/100 mL of peripheral blood, the production of high numbers of polyclonal Tregs is technically feasible [29, 81, 87]. arTregs require a fraction of the polyclonal Treg numbers necessary in order to induce the same suppressive potency, but are more expensive and difficult to expand with high purity under GMP conditions.

Timing: The timing of infusion can impact Tregs' effectiveness in vivo. It is generally thought that Tregs should be infused around the time of transplantation to optimally exert their suppressive effects. Given that the Treg response and infiltration into grafts is typically delayed compared to conventional T cells [88], it can be argued that Tregs must be infused early enough to counteract effector T cell activity before damage has commenced. The delayed migration may be due to differences in their lymphotoxin-dependent migratory patterns [89]. In cases where expansion of donor-specific Tregs is necessary prior to infusion, treatment before or at the time of transplantation is logistically difficult and therefore the therapy can only be given to recipients of living donor transplants where the donor HLA type is known [90]. Moreover, allografts that are normally subject to the trauma of surgery, can promote a proinflammatory microenvironment that destabilizes Tregs through the loss of FoxP3 expression and the induction of an effector phenotype [91, 92]. The timing of infusion also needs to be coordinated with induction therapy that results in lymphodepletion. Given that a high Treg:Tconv ratio plays a vital role in tolerance induction, lymphodepletion may offer an effective therapeutic window

for Tregs to function, particularly when Treg expansion processes are limited [83, 86, 93]. However, induction therapies used at the time of transplantation may also themselves be detrimental to Treg expansion and function [76, 94]. Tregs may therefore need be infused after induction therapy is complete. However, there are insufficient studies focused on varying the timing of Treg therapy in humans, let alone any studies focused on the optimal timing for multiple infusions.

Cryopreservation: Methods for optimizing efficient and effective cryopreservation of Tregs can also open up many possibilities for Treg therapies. Reliable storage can alleviate issues of timing of infusion and depletion of the stock during the treatment protocol. Initial studies have shown that cryopreservation of human Tregs reduces suppressive potency and the CD4+ and FOXP3+ population, most likely due to the fragility of the Tregs [95]. However, using different preservation strategies, some groups, including ours, have found success in the recovery of cryopreserved Tregs, which could be used to prevent GvHD [81] and rejection of vessels [56], skin [35], or islet [96] grafts. Based on recent studies that demonstrate effective strategies for cryopreservation of human Tregs, the safety and efficacy of cryopreserved Tregs is being assessed in clinical trials such as the ONE/TWO Study and ThRIL [78].

Source: The source by which Tregs are isolated from also needs to be taken in consideration. nTregs are commonly isolated from peripheral blood, but they may also be taken from umbilical cord blood. Because cord blood is generally immunologically naïve, the $CD4^+CD25^+$ population lacks the non-suppressive memory and activated T cells that usually coexist within this population in peripheral blood [97]. Thus, unlike in peripheral blood, $CD4^+CD25^+$ expression can reliably be used for isolation of Tregs. Moreover, with less contamination within the isolated population, *ex vivo*-expanded UCB Tregs are generally more potent in suppression than peripheral blood Tregs [98-100]. Pooling of UCB Tregs from multiple sources is a possible strategy for the production of large numbers for therapy. In a comparative study of pooled human UCB versus peripheral blood Tregs, the former was found to be more suppressive *in vivo* [101]. Multiple clinical trials are now using UCB as a source of Tregs.

***Immunosuppression*:** The optimal Treg cell therapy would completely eliminate the use of immunosuppressive drugs due to their adverse effects, but studies have indicated that Tregs cannot prevent rejection without at least some form of adjunctive immunosuppression. Recent findings indicate that some of the commonly used immunosuppressive agents have detrimental effects on human Tregs *in vivo*. For example, basiliximab and calcineurin inhibitors (CNI) such as tacrolimus block IL-2 production, which is necessary for Treg proliferation and function [94, 102]. Addition of IL-2 to the therapeutic regimen may therefore be an option to counteract this effect. Low-dose IL-2 is already being used in clinical trials enhances Treg stability and proliferation through the activation of the STAT5 pathway [103-105].

Thus, the optimal therapy should also consider which drugs can benefit Treg survival or function [106]. This section focuses on immunosuppressive drugs that have shown promise for Treg cell therapy, with more extensive investigations outlining various other drugs' effects on Tregs to be found in other reviews [106, 107].

ATG, a polyclonal rabbit or horse-derived antibody, is a commonly used induction agent in transplantation that has also shown ability to expand $CD4^+CD25^+FOXP3^+$ cells. Lopez et al. found that *ex vivo* expansion of human peripheral blood lymphocytes (PBL) stimulated with rabbit-derived ATG expanded Tregs primarily through the conversion of $CD4^+CD25^-$ cells to $CD4^+CD25^+$ cells [108]. Later findings confirmed these results, suggesting that expansion may be through transcriptional regulation by increased NFAT1, a FOXP3 inducer [109-111]. Clinical studies with ATG induction indicate that both Tconvs and Tregs are depleted, but that Tregs may repopulate faster than Tconvs and be functionally suppressive [102, 112, 113]. Thus, Treg infusion alongside this treatment may be a suitable strategy in shifting the balance towards Tregs over Tconvs.

Rapamycin (sirolimus), has shown much promise to be used concurrently with Treg treatment and has already been implemented in a few clinical trials. Many studies have found that this mechanistic target of rapamycin (mTOR) inhibitor can preferentially promote the survival of Tregs over Tconvs [62, 114-116] and is beneficial in Treg cell therapy in animal models [117]. This effect may be due to its inhibition of the PI3K/Akt/mTOR pathway, which the Tconvs are more dependent on than the Tregs for survival, making Tconvs more prone to apoptosis in the presence of rapamycin [118, 119]. Therapy that is based on targeting this pathway may provide a conducive environment for Treg survival. Rapamycin is commonly used in Treg expansion protocols to reduce effector T cell contamination. There are however findings that rapamycin administration can enhance memory CD8+ T cell responses during viral or bacterial infection [120]. The specific effects of rapamycin in the context of clinical transplantation therefore need further clarification.

It is important to note that many studies indicate contradicting effects of how a certain immunosuppressive drug or regimen affects Treg differentiation and function. This is likely attributed to the differences in timing, dosage, and combination of drugs. These factors need to be carefully considered in future investigations.

Future of Treg cell therapy:

Many questions remain regarding the specificity, stability and function of Tregs, but this has led to an avenue of exciting and novel innovation that can potentially address these concerns. For example, a significant development has been the investigation of chimeric antigen receptor (CAR) Tregs, in which nTregs are transduced with a vector containing a chimeric receptor to produce cells that are genetically modified to target donor antigens with high specificity [121]. Macdonald et al. demonstrated the generation of CAR Tregs generated with HLA-A2, a commonly mismatched antigen in transplantation, that is highly donor antigen-specific and maintains FOXP3 expression while preferentially localizing to the transplanted allograft [122]. This method is therefore able to overcome many of the limitations posed by arTregs, for it is

not dependent on the presence of donor professional APCs [123]. More recently, CAR Tregs have been shown to be efficacious in a human xenograft transplant model [124].

To address the issue of stability of Tregs *in vivo*, there are some recent studies that suggest the use of Treg-derived exosomes. Exosomes are extracellular vesicles that contain proteins and RNA and are known to be secreted by most cells in order to modulate immune response. Unlike human Tregs that may be vulnerable to the loss of suppressive functions under inflammatory conditions, exosomes are unlikely to have their immune functions disrupted. Adoptive transfer of Treg-derived exosomes has been tested in a rat kidney transplant model, where the treatment inhibited T cell proliferation and suppressed acute rejection [125].

After Tregs are infused, tracking their migratory patterns *in vivo* is also crucial to understanding their survival and function within the graft, and different technological strategies have been proposed ranging from the labelling of Tregs during expansion to tomography imaging after infusion [126-128]. Further work must also focus on assessing safety due to the potential toxicities of labelling and imaging.

Conclusion

The evidence for Treg therapy in transplantation is mounting, with clinical studies now progressing through to early phases. The challenges that remain centre of the methodology for introduction of Tregs into clinical therapy and their cost. The latter may be resolved once the former is better understood. If Tregs hold significant benefits to post-transplantation outcomes and facilitate a reduction in immunosuppression, the costs for production may become relatively less significant. A scale up of the therapy may reduce costs, although it is not clear whether this is imminent given that much of the process cannot yet be automated. A greater understanding is required regarding the relative efficacy of Tregs in transplantation and whether their use will completely eliminate or only reduce the need for immunosuppression. There are scant data available to assist in determining dosages, timings and efficacy of the various Treg populations in different transplant types with the majority of

data being in animal models, leading to speculative clinical studies assessing feasibility and only rarely efficacy. We are now truly on the cusp of one of the first breakthroughs in transplantation since the introduction of modern immunosuppression. For the first time the pipeline of novel therapeutics contains not molecules, but cells.

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Figure legend

Figure 1: Treg isolation/expansion methods. (A) Treg isolation/expansion pathways for various methods in Treg cell therapy. (B) Representative methods of Treg expansion or induction used in clinical trials.