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Clinical perspectives for regulatory T cells in transplantation tolerance

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Abstract

Three main types of CD4⁺ regulatory T cells can be distinguished based upon whether they express Foxp3 and differentiate naturally in the thymus (natural Tregs) or are induced in the periphery (inducible Tregs); or whether they are FoxP3 negative but secrete IL-10 in response to antigen (Tregulatory type 1, Tr1 cells). Adoptive transfer of each cell type has proven highly effective in mouse models at preventing graft vs. host disease (GVHD) and autoimmunity. Although clinical application was initially hampered by low Treg frequency and unfavorable ex vivo expansion properties, several phase I trials are now being conducted to assess their effect on GVHD following hematopoietic stem cell transplantation (HSCT) and in type I diabetes. Human Treg trials for HSCT recipients have preceded other indications because GVHD onset is precisely known, time period needed for prevention relatively short, initial efficacy likely to provide life-long protection, and complications of GVHD can be lethal. This review will summarize the clinical trials conducted to date that have employed Tregs to prevent GVHD following HSCT and discuss recent advances in Treg cellular therapy.

Keywords

Regulatory T cell; stem cell transplant; GVHD; cellular therapy

1. Introduction

One of the most significant discoveries in the field of immunology in the last 15 years has been the identification and characterization of specific T cell subsets critical for regulating immune responses [1, 2]. These regulatory T cells (Tregs) are not only required to suppress the activation of self-reactive lymphocytes and autoimmunity [3], they are also required to limit the immune response to chronic pathogens and commensal bacteria in the gut [4]. Adoptive transfer of Tregs (directly ex vivo or after in vitro expansion) can treat or even prevent autoimmune disease in animal models [3, 5]. Further, mouse models demonstrated that adoptive transfer of Treg also prevents graft rejection and GVHD, a frequent and severe

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complicating factor in hematopoietic stem cell transplantation (HSCT) [6]. Graft rejection and GVHD occur when allogeneic immune cells recognize polymorphic major histocompatibility (MHC) and minor histocompatibility antigens expressed on graft or recipient cells as non-self and initiate an immune response against them. Although both the innate and adaptive immune systems contribute to alloresponses, the dominant effects are mediated by allogeneic CD4⁺ and/or CD8⁺ T cells (termed conventional T cells or Tconv), which can directly recognize foreign MHC molecules and associated peptides expressed on allogeneic antigen-presenting cells (APCs) or tissue cells, or indirectly recognize foreign peptides presented by syngeneic APCs [7]. After myeloablative chemoradiotherapy conditioning for HSCT, systemic inflammation is severe and often overwhelms immune regulatory mechanisms, despite the routine use of multi-agent immunosuppressive drug regimens designed to dampen alloreactivity. Despite aggressive early post-HSCT inflammatory response and the purposeful infusion of donor T cells into a lymphopenic environment that maximally supports their expansion, HSCT fosters the development of tolerance as host APCs are replaced with donor APCs and T cells.

Thus, the risk period for adverse donor anti-host alloresponses that culminate in the multi-organ GVHD typically is highest in the first 1–3 months after HSCT until central tolerance occurs. This critical time of need for intense immune regulation for HSCT recipients has a precise onset (the day of HSCT) and effective immune suppression or tolerance induction during this relatively short window of 1–3 months may provide life-long protection against adverse alloresponses since immune tolerance that develops in the absence of GVHD almost invariably allows the host to be taken off all immunosuppressive agents by 5 months post-HSCT. For these reasons, and because Treg cells can be isolated from healthy allogeneic HSCT donors, human Treg cell trials for GVHD prevention have preceded other indications. Clinical trials to test the efficacy of Treg cellular therapy have been slowed because of difficulties in achieving sufficient numbers of highly potent Tregs, without contaminating effector T cells, to be useful for in vivo prevention or treatment of disease. Nonetheless, several clinical studies have been completed to date and others are ongoing [8, 9]. This review will: A) outline the various types of Treg that have been used in clinical trials, B) discuss and compare the clinical trials that have been conducted using adoptive transfer of Treg, C) outline the next phase of clinical trials; and D) discuss how Treg therapy will be improved in the future.

2 Clinical strategies for Treg mediated transplantation tolerance

CD4⁺ regulatory T cells can be divided into three main classes based upon expression of the transcription factor Foxp3 and where they develop. Natural Treg (nTreg) develop in the thymus and express Foxp3 while induced Treg (iTreg) are so-named because they exit the thymus as regular T cells, and Foxp3 expression and suppressive function is induced in the periphery. Like iTreg, Type 1 regulatory T (Tr1) cells arise in the periphery but these cells do not require Foxp3 expression, and IL-10 secretion is the primary mechanism for their suppressive function.

2.1 Natural Tregs

While murine data indicate that nTreg cellular therapy is very promising, a practical problem remained for isolating pure Treg cells for human immunotherapy. In young mice, CD4⁺ CD25⁺ cells are moderately abundant (comprising ~5–10% of secondary lymphoid organs) and the CD25⁺ subset is readily apparent and can be purified to >90% CD4⁺ Foxp3⁺ using magnetic beads. However, a large and overlapping population of CD25^{dim} T effector/memory cells exists in human, and nTreg isolated from PB using magnetic beads under GMP conditions are only 60–70% CD4⁺ CD25⁺ Foxp3⁺, with the majority of the contaminants being CD4⁺ CD25⁺ Foxp3⁻ cells [10–13]. Despite the lack of uniform high level purity of the

target population, the purified cells still suppressed T cell activation in vitro. One drawback to this approach is that only $60\text{--}260 \times 10^6$ Treg can be isolated from Leukapheresis products, and mouse GVHD models indicate a high Treg:Teffector cell ratio (1:1) given at the time of BMT is needed for maximum suppression of GVHD. Thus, the absolute number of Tregs that can be purified using magnetic bead separation may be limiting especially in the context of high numbers of donor T cells as would occur with non-manipulated bone marrow or peripheral blood stem cell grafts. Fresh nTreg are also less effective than in vitro expanded nTreg at suppressing GVHD [14]. Unfortunately, nTregs purified from human blood using magnetic beads do not consistently maintain Foxp3 expression or suppressive function when expanded in vitro with using anti-CD3/CD28 monoclonal antibody (mAb)-coated microbeads and high-dose IL-2 [11, 15]. In contrast, PB nTreg that were sort-purified based on CD25 expression (top 2%) and were >90% CD4+Foxp3+ did maintain Foxp3 expression and suppressive function after expansion, although sorting is not a viable clinical procedure in many centers due to the lack of good manufacturing practice (GMP)-compatible sorters.

In contrast to PB, we found that nTregs were readily purified from umbilical cord blood (UCB) due to the relative paucity of CD25^{dim} non-Tregs in UCB. Similar to PB nTreg, ~70% of the CD4+ cells purified from UCB with CD25 magnetic beads expressed Foxp3+. However, unlike the situation with bead-purified PB nTreg, the cells purified from UCB contained fewer CD4+25^{dim} cells and could be expanded several hundred-fold ex vivo using anti-CD3/28 beads and IL-2 while maintaining Foxp3 expression and suppressive function [11, 15]. These studies allowed us to initiate the first clinical trial to study the safety of ex-vivo expanded nTregs. While ~30-fold more CD25^{high} cells can be isolated from PB than UCB, the CD25^{dim} non-Treg preferentially expand after stimulation, resulting in a product that has lost suppressor activity. The discovery that the immunosuppressant rapamycin selectively expands murine and human CD4+, 25+, Foxp3+ Treg vs. Teffector/memory cells provided an important approach to generate the consistent suppressive function required for the therapeutic use of bead-purified, ex vivo expanded, PB Tregs [16-19]. To determine whether PB nTregs expanded in the presence of rapamycin (Rapa) are capable of suppressing human GVHD, we developed a GMP-compliant purification protocol, and stimulated the purified CD4+25+ cells similar to the protocol used for UCB nTregs (clinical-grade anti-CD3/CD28 mAb-coated beads, 300U/ml IL-2, 17-21 days). While this approach seemed likely to solve the issues of nTreg yield, PB nTreg expansion in rapamycin was 10-20 fold lower than that for UCB nTreg. Decreased expansion was likely due to a combination of rapamycin, which also affects Treg expansion [15], and the fact that, unlike UCB [15], PB contains significant numbers of CD45RA- nTreg [20], which have a lower in vitro proliferative potential than CD45RA+ nTreg (KLH, personal observation). However, like UCB nTreg, bead-purified PB nTreg expanded in the presence of Rapa were suppressive both in vitro and in the xenogeneic model of GVHD [20], and a clinical trial was initiated to assess their ability to suppress GVHD in humans following HSCT using HLA-matched sibling donors.

2.2 Induced Tregs

CD4+ T cells with regulatory activity can be induced in the periphery and are required not only for peripheral tolerance, but also for preventing a general lymphoproliferative disease in response to some types of chronic infections [4]. Murine Treg can also be induced and expanded in vitro by stimulating CD4+25- T cells in the presence of TGF β or all-trans retinoic acid (ATRA) and, like nTregs, adoptive transfer of these iTregs cells suppresses disease [21-23]. TGF β or ATRA also induce Foxp3 expression after stimulation of naïve human T cells (CD4+25-45RA+), but while one study showed these cells to be suppressive [24], other studies observed modest or no suppression [25-27]. However, CD4+25-45RA+ T cells stimulated in the presence of TGF β plus ATRA acquired stable suppressive function

[28]. Similarly, we found that Rapa enhanced TGF β -dependent Foxp3 expression and induced potent suppressor function in naïve (CD4+25-45RA+) T cells [12]. Rapa/TGF β also induced suppressive function in unfractionated (CD4+25-) T cells, which is advantageous therapeutically because it increases yield and decreases cost by avoiding the need to isolate the CD45RA+ cell subset. Rapa/TGF β iTregs express CD25 at levels higher than expanded nTregs, and contain few IL-2, IFN γ or IL-17 secreting cells. Most importantly, Rapa/TGF β iTregs suppress disease in a xenogeneic model of GVHD, opening the door for iTreg cellular therapy for human diseases [12]. Other approaches that have proven useful in rodents have included FoxP3 gene transfer [29, 30], which can be used to suppress murine GVHD in an antigen-specific manner [31].

2.3 Tr1 cells

Tr1 cells are characterized as CD25-, Foxp3-, IL-10+, IL-4- and were first identified over 20 years ago as a suppressive T cell present in the PB of transplanted SCID patients who developed split chimerism that maintained peripheral tolerance by secreting IL-10 [32, 33]. IL-10 secreting Tr1 cells are also found in transplanted β -thalassemic patients, but only those patients with incomplete donor chimerism, suggesting that Tr1 cells are associated with long-term tolerance [34]. Tr1 cells specific for alloantigens can be induced in vitro from naïve T cells by persistent antigen exposure in the presence of IL-10. Although no mechanism exists to uniformly purify out the vast majority of T cells that are not alloantigen-specific and do not adopt the Tr1 phenotype, these cells are anergic and bulk cultures are capable of suppressing disease in a mouse model of GVHD [35]. One potential advantage of Tr1 cellular therapy over nTreg or iTreg is that, since Tr1 cells need to see antigen to secrete IL-10, there is less chance for systemic immunosuppression. A clinical trial is also open to assess the ability of IL-10 induced Tr1 cells to prevent GVHD [36].

3 Treg clinical trials

3.1 Freshly isolated and infused nTregs to prevent GVHD in the clinic

Five years ago, the first human trial employing adoptive transfer of nTreg to suppress GVHD had been initiated by Edinger et. al. who treated 5 HSCT recipients with fresh, bead-purified donor nTreg [13]. nTreg were infused post HSCT followed by delayed lymphocyte infusion (DLI) of an equal number of donor T cells to prevent or treat recurrent hematological malignancies. No infusional toxicity or increase in infection or GVHD was observed.

A similar study using fresh, bead-purified nTreg was initiated in 2008 by Di Lianni, et. al. to assess the effect of adding nTreg pre-HSCT on GVHD prevention and immunologic reconstitution in HSCT [10]. In this trial, nTreg were infused into patients 3 days prior to HLA-haploidentical CD34+ cells supplemented with frozen/thawed mature donor T cells in the absence of any post-transplant immunosuppression. nTreg purification was very consistent, and only 2 of 28 patients enrolled in the study did not receive nTregs due to low purity ($\geq 50\%$ Foxp3+). These studies confirmed the safety of ex vivo purified nTregs, and found they promoted lymphoid reconstitution and did not overtly weaken the graft vs. leukemia effect of the co-transferred mature T cells [10]. However while no GVHD was observed for doses of 0.5- or 1.0×10^6 Tcon/kg plus 2×10^6 nTreg/kg, 2 of 5 patients receiving 2×10^6 Tcon/kg plus 4×10^6 nTreg/kg developed GVHD, indicating 1×10^6 Tcon/kg is the maximum dose unless increased numbers of Treg are given.

3.2 In vitro expanded nTregs

UCB is a valuable alternative for HSCT in patients that lack a suitable related donor, and we recently demonstrated that leukemia free survival following transplantation using two

partially HLA matched UCB units is comparable to matched related or unrelated donors [37]. Because double UCB transplantation has an increased risk of grade II-IV GVHD and because of the favorable properties for UCB nTreg expansion, it is an ideal candidate to test the efficacy of expanded nTreg. Our clinical trial, which started in 2007, included 23 patients who received a double UCB transplant and nTreg expanded from a third UCB unit [38]. The first 5 patients received escalating Treg doses from $0.1 \times 10^6/\text{kg}$ to $3.0 \times 10^6/\text{kg}$. Data from a xenogeneic model of GVHD showed that nTreg efficacy correlated with the number of cells remaining on day 10-14, so the next cohort received on day 14 a second dose of Treg ($3.0 \times 10^6/\text{kg}$), cryopreserved at the time of initial Treg transfer. While thirteen patients in this cohort received the two full doses, variability in nTreg yield resulted in 3 patients receiving only the first dose, 1 patient received 2 doses of 2.1×10^6 Treg and 1 patient received only an initial dose of 0.8×10^6 Treg/kg. Importantly, all Treg cultures passed the release criteria ($\geq 60\%$ CD4+25+) and suppressed in vitro T cell proliferation $>50\%$ at the end of culture at a ratio of 1:2 (PB mononuclear cell:Tregs). Since the primary endpoint of this first-in-human study was the safety and tolerability of expanded UCB nTreg, it is of note that no infusional toxicity was observed; nor was there an increased risk of infection, relapse or early mortality. Even more important, nTreg reduced the incidence of grade II-IV acute GVHD (43% versus 61%, $p=.05$) compared to 108 historical controls treated identically except for Treg.

Another significant goal of this trial, which was possible because the UCB nTregs were from a separate unit, was to track the expanded cells in the recipient and assess key issues like nTreg stability, persistence and the fate of the non-Treg contaminants. Similar to the xenogeneic studies, UCB Treg were only detected in circulation for only ~14 days, with the highest proportion of CD4+CD127-FoxP3+ cells observed on day +2. Furthermore, the % of expanded cells expressing Foxp3 did not change over time, indicating that the expanded nTreg are stable and that the contaminating cells are either anergic or hypoproliferative. While expanded cells could not be detected in the secondary lymphoid organs in the xenogeneic model, we did not determine whether they persist long-term in patients outside of the circulation. Tracking expanded cells also showed that injection of frozen/thawed cells is ineffective, as they achieved $<10\%$ of the circulating numbers produced by fresh cells and persisted only 3 days.

The UCB trial demonstrated that expanded nTreg were safe up to a dose of $3 \times 10^6/\text{kg}$, and although average nTreg yield could support a significant dose escalation, the 100-fold variability in yield makes it impractical. As mentioned in section 2.1, increased Treg yield can be achieved with expansion of PB nTregs in the presence of Rapa. Therefore, we also initiated a clinical trial to assess the ability of PB nTreg to suppress GVHD following HSCT using HLA-matched sibling donors (unpublished data). In this study, as in the fresh nTreg study by Di Ianni, both Treg and HSC came from the same donor. In our case, a non-mobilized apheresis was donated on day -18, and a mobilized apheresis on day 0, whereas in the Di Ianni study they were given on day -4 and day 0, respectively. Accrual on the PB nTreg trial was slow, likely because the 18 days between donations required donors to make two separate trips to the transplant facility. Therefore, the trial was electively discontinued. However, the clinically expanded PB nTreg were as suppressive as their UCB counterparts, and showed no infusional toxicity despite patients receiving 3- and 10-fold higher nTreg doses than the UCB nTreg trial ($10 \times 10^6/\text{kg}$ and $30 \times 10^6/\text{kg}$ vs. $3 \times 10^6/\text{kg}$, respectively).

While sorting is not a viable clinical procedure in many clinical treatment facilities due to the lack of good manufacturing practice (GMP)-compatible sorters, Trzonkowski, et. al. used sort-purified nTreg (CD4+25+127-) expanded in vitro with CD3/28 beads to treat two patients with GVHD, with positive results. However, it is difficult to compare this study

with the other trials, as it used 20-300 fold lower nTreg doses ($0.1 \times 10^6/\text{kg}$ vs. 2- or $30 \times 10^6/\text{kg}$), and the suppressive function of the expanded cells was not shown [39].

3.3 Induced Tr1 cells

Unlike the above trials using polyclonal nTreg, the Tr1 cells used for cellular therapy are donor T cells that have been stimulated ex vivo with purified host monocytes or DC-10 cells in the presence of IL-10 to induce allo-specific Tr1 cells. The first clinical trial employing Tr1 cells is being conducted by Roncarolo, et. al. on patients with high risk malignancies receiving CD34+ selected cells from a haploidentical donor. Since polyclonal stimulation was not used in Tr1 cultures, they contain unprimed T cells capable of responding to nominal and viral antigens in addition to host-specific Tr1 cells [40]. Tr1 cells were infused into patients in the absence of immunosuppression following mononuclear cell engraftment as a DLI. Like nTreg, no infusional toxicity was observed with Tr1 cell transfer and the therapy showed efficacy in that, unlike haploidentical hosts receiving the same dose of unmanipulated DLI [41], acute GVHD was moderate after IL-10-DLI at the therapeutic dose, and no chronic GVHD was observed [36].

4 The next generation of Treg therapy

As outlined above, the major impediment to nTreg cellular therapy has not been safety, but rather generating sufficient numbers of cells to maximize efficacy. The need for large numbers of nTregs was not unexpected, as high doses ($\sim 1:1$ with donor T-cells) are also required to suppress disease in animal models [3, 14, 42]. Whereas lymphopenia can expand nTregs in vivo to a level that can suppress GVHD in rodents if administered several days before the T effector cells, the efficacy of such approaches depends upon sufficient in vivo expansion in patients, some of whom may be receiving immune suppressive drugs. For this reason and due to the fact that most of the targeted patient populations will not be severely lymphopenic at the time of nTreg infusion, a primary focus for the next generation of therapies has been to optimize nTreg expansion, while minimizing loss of suppressive function and contamination with non-Tregs. Another mechanism for increasing Treg yield is to develop a clinical trial using Tregs that have been induced from the considerably more abundant CD4+25- cells which can be purified in much larger numbers than nTregs.

4.1 Improving nTreg stimulation

The initial trials using expanded nTreg utilized anti-CD3/CD28 beads for stimulation because they were the only GMP reagent available and had a safety record in humans. However, stimulation of UCB nTregs with cell-based artificial antigen presenting cells (aAPCs) that express the costimulatory molecule, CD86, and an FcR (CD64) for loading anti-CD3 mAb, increases expansion (~ 4 -fold) over bead-based aAPCs (e.g. anti-CD3/CD28 beads) [15, 43]. A cell-based aAPC has recently been licensed for GMP use that increases nTreg expansion to a similar degree. However, this increase alone is not likely to be sufficient to complete dose escalation trials, so additional approaches still need to be explored to maximize yield.

4.2 Large scale nTreg expansion

Three studies demonstrated that sort-purified nTreg can be expanded $>1,000$ -fold if re-stimulated, but in each case cultures contained high numbers of IL-2- and IFN γ -secreting cells which may exacerbate GVHD [44-46]. In addition, cell sorting is a challenging GMP procedure, and the calculated nTreg yield from PB was not greatly increased over the 2.3×10^9 we obtained with UCB nTreg. Since these experiments were all performed in the absence of rapamycin, which preferentially inhibits the proliferation and differentiation of

non-Treg CD4⁺ cells, we assessed whether re-stimulation in the presence of rapamycin improves nTreg purity and decreases contamination with inflammatory cytokine secreting.

Using GMP-grade reagents and techniques, we found that bead-purified nTregs could be expanded >3,000-fold (yield, $\sim 500 \times 10^9$ cells) with a single re-stimulation in the presence of rapamycin without losing Foxp3 expression or suppressive function [20]. These cultures also contained ~10-fold fewer IL-2 or IFN γ secreting cells than reported for sort-purified cells re-stimulated once in the absence of rapamycin. Furthermore, if PB nTregs were sort-purified, they maintained Foxp3 expression and suppressive function even after four re-stimulations, and expanded up to an amazing 50-million fold [20]. Importantly, both bead-purified and sort-purified nTreg maintained in vivo suppressive function after re-stimulation, and suppressed GVHD in a xenogeneic model of disease. In addition, maximally expanded nTreg showed no genetic abnormalities or TCR V β skewing, and did not adopt a senescent (CD57⁺) phenotype, demonstrating they are not transformed and retain a broad spectrum of reactivities [47]. Highly expanded PB nTregs maintained stimulation-dependent expression of the nTreg marker, TGF-beta complex associated latency activated peptide (LAP), showing re-stimulation represents expansion of natural Treg and not induction of Treg.

4.3 nTreg Banking

While a single re-stimulation greatly increases nTreg yield, it also increases the length of culture. Unfortunately, enrollment on the initial clinical trial using expanded UCB nTreg was already slowed due to the 9-11 day delay in the time to transplant required for nTreg expansion and increasing it even further would likely have a severe effect on subsequent trials. The ability to massively expand nTregs, combined with their ability to suppress third-party responses, will allow the creation of nTreg banks that can be used to treat multiple patients. This will improve nTreg cellular therapy by having a better tested product with known efficacy capable of supporting trials across a broad range of doses. While the optimal situation for banking of cellular products is to inject cells directly post-thaw, we have shown in our xenogeneic model and initial clinical trial that frozen/thawed nTreg have decreased in vivo persistence and efficacy. However, frozen/thawed nTreg can be re-stimulated and further expanded in rapa without losing Foxp3 expression or suppressive function. Thus, a viable strategy to the creation of an off the shelf Treg product could be repetitive stimulations of nTregs followed by freeze/thawing and a brief restimulation of aliquots of frozen nTregs.

4.4 iTregs generated with Rapa + TGF β

As discussed in section 2.2, human CD4⁺25⁻ non-Treg cells stimulated in the presence of TGF β and rapamycin (Rapa/TGF β iTregs) express Foxp3 and are suppressive. Unlike nTregs, which comprise only 1-3% of total CD4⁺ T cells in the periphery, Rapa/TGF β induces suppressive function in bulk CD4⁺ cells, which are 20 to 40-fold more abundant than nTregs. Large scale experiments with cGMP reagents showed that $\sim 240 \times 10^9$ iTreg could be generated from a single apheresis product, >50x more than available in initial nTreg clinical trial. Like nTreg expanded in the presence of Rapa, <4% of cells in Rapa/TGF β iTreg cultures secrete IFN γ or IL-17. A concern in the field has been whether nTregs or iTregs are unstable and can be reprogrammed to become Teffs but, as for nTreg, no evidence for iTreg conversion into Teffs was found in the xenogeneic GVHD model although iTregs did not persist long-term in PB. Importantly, iTreg suppression of GVHD lethality was comparable to nTregs. With these data, a phase I trial of iTregs for GVHD prevention and/or therapy now can be considered.

5 Future of Treg therapies

5.1 Enhancing Treg efficacy by increasing potency

One mechanism to enhance the potency of expanded nTregs is through drug treatment. Because PKC- θ localizes to the proximal immunological synapse in Teffector but not Tregs, pharmacological inhibition of PKC- θ may prove to be able to augment Treg function in vitro and in vivo as has been recently shown [48]. Augmentation of Stat5 signaling, downstream of the IL-2R common gamma chain, and inhibition of stat1 both have been shown to increase Treg expansion in mice and to reduce GVHD lethality [49, 50]. Several Treg subsets have also been identified that display increased potency, including those expressing functional markers that could be used for purification (e.g. HLA-DR+, Lag-3+, CD45RA, CTLA-4)[51-54], although the cGMP purification reagents for these markers are not yet available, and whether they remain more potent after expansion will have to be determined. Alternatively, Shevach's group has recently identified two surface markers specific for nTreg (LAP or CD121) that could be used to re-isolate nTregs after expansion [46]. While the cost of re-isolating billions of nTregs on a per-patient basis would be fairly high, the group also showed that re-isolated nTregs maintained high purity following further stimulation [46].

Although nTregs are fully capable of suppressing third party responses, data from murine models of GVHD and diabetes [5, 55-61] as well as allogeneic human skin grafts [62] show that antigen-specific Treg are significantly more potent. While allo-specific T cells may only represent 2-5% of the repertoire, and expansion of allo-specific nTregs would take a significantly longer time, large numbers of allo-specific Tregs could be generated by re-isolating activated cells (i.e. LAP+) upon allo-re-stimulation of banked Tregs. In addition, T cell receptor gene transfer may be useful in conferring antigen specificity in Tregs that have been polyclonally expanded in vitro [63]. Alternatively, our finding that TGF β /Rapa was able to induce suppressive function in activated memory cells suggests that Tregs could be induced from pathogenic T cells removed from patients experiencing GVHD.

5.2 Enhancing Treg efficacy by increasing stability

The efficacy of Treg therapy can also be improved by increasing the in vitro and in vivo stability of the expanded cells. For example, while retinoic acid alone does not maintain Foxp3 expression and suppressive function in cultures of bead-purified PB nTreg, it does increase the stability of nTreg expanded in the presence of Rapa [25]. IL-2 is required by nTregs to maintain suppressive function and stability in vitro and in vivo [64, 65], and injection of IL-2/anti-IL-2 complexes increases Treg number and suppresses disease in a mouse model of autoimmunity. Since nTreg efficacy in the xenogeneic model of GVHD correlated with in vivo persistence [15] IL-2/anti-IL-2 complexes could be used to enhance nTreg efficacy in humans. A recent study demonstrated that IL-2 also stabilizes Foxp3 expression in murine TGF β -induced Tregs in vivo [66], and even promotes demethylation of the *Foxp3* TSDR. Our experiments showed Foxp3 expression in human CD4+25- T cells cultured with TGF β was also stabilized by Rapa, although demethylation of the *Foxp3* TSDR was not observed even when these cells were expanded in high-dose IL-2 [12]. Interestingly, unlike nTregs, Tregs induced from human CD4+25-45RA+ with TGF β /ATRA were stable in vitro and in vivo even after exposure to IL-1 β and IL-6 [28].

5.3 In vivo induction/expansion of Tregs

Several studies have shown that nTreg present in grafts can be preferentially expanded in vivo or that suppressive function can be induced in donor T cells in vivo. In one such study, decitabine (Dec), a DNA methyltransferase inhibitor capable of inducing Foxp3 expression and suppressive function in murine CD4+25- cells in vitro [67, 68], was shown to suppress

GVHD by inducing suppressor function in vivo [67, 68]. Pharmaceuticals targeting another group of DNA-modifying enzymes, termed histone deacetylases (HDACs), also increase Treg number and function in vivo and suppress graft rejection, and likely GVHD [69]. nTreg stability and Treg induction in vivo are both inhibited by strong pro-inflammatory cytokine responses [48, 70]. We have shown that blocking IL-21 signaling in vivo decreases GVHD-associated Th1 differentiation while increasing the number of Treg and suppressing disease in a Foxp3 dependent manner [71]. Anti-human IL-21 also increased Foxp3+ cell number and suppressed disease in a xenogeneic model of GVHD (unpublished data).

Another powerful mechanism to induce or expand regulatory T cells in vivo are tolerogenic dendritic cells (DC). PD-L1 expressing DC induce murine Treg in vitro, and PD-L1/L2 are required for mouse iTreg development in vivo [72, 73]. The therapeutic potential of PD-L1+ DC immunotherapy was demonstrated in a xenogeneic model of GVHD, where adoptive transfer of human PD-L1 expressing DC suppressed disease [74]. Murine and human Tregs can also be induced by nutrient starvation in vitro, and are particularly sensitive to the conditions of low tryptophan and high tryptophan catabolites created when they are activated by plasmacytoid DC (pDC) expressing the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) [75-77]. IDO expression in colonic APCs is critical for suppressing GVHD-associated gut pathology, and treating mice pre-BMT with a TLR7/8 agonist, which induces IDO expression in gut APC, inhibited GVHD [78, 79]. Murine DC treated ex vivo with HDAC inhibitors also upregulate IDO expression and suppress GVHD [80]. DC can also suppress GVHD by increasing in vivo natural Treg expansion, as occurs when mice are injected with the DC growth factor FLT3 ligand [81]. A recent publication showed that DC-10 cells, the tolerogenic DC subset capable of inducing Tr1 cells in vitro and in vivo, can be purified from human blood by sorting (CD14+11c+83+), or differentiated from anti-CD14 bead purified monocytes [33, 82], opening the door for a potential DC-10 therapy. Lastly, pharmacological inhibition of phosphodiesterase 3 (PDE) resulted in an elevation of cAMP enhancement of murine and human donor-reactive Tregs generated using immature allogeneic DCs [83]. As the in vitro upregulation of intracellular cAMP has been shown to induce alloantigen-specific tolerance leading to GVHD inhibition [84] and PDE inhibition to the generation of murine donor-reactive Tregs capable of suppressing allogeneic skin graft rejection, the in vitro or in vivo use of PDE inhibitors may prove useful in generating iTregs to induce transplantation tolerance.

6 Concluding remarks

Like all therapies, clinical use of ex vivo expanded Tregs is associated with potential risks. Despite early concerns, Treg cellular therapy has not caused any infusional toxicity, and has established a limited safety record with regard to risk of infection, relapse or early mortality. However, efficacy data for Treg at this point are also limited. To achieve maximal efficacy, it is likely to require billions of expanded Tregs, perhaps at multiple timepoints, meaning that in spite of initial successes, significant safety issues likely remain. Perhaps the most troublesome issue is still the possibility expanded Tregs will revert to Tconv cells. The notion of plasticity among the various T cell subsets has gained much attention [85], [86]. While we found no evidence in the xenogeneic model of GVHD or the limited number of patients receiving allelically marked Treg for conversion of human Treg into Teffector cells in PB, this may change with logarithmic increases in Treg dose or under conditions (e.g. including the specific inflammatory environment to which infused Tregs home) that may favor reversion. Indeed, mouse studies show that the majority of adoptively transferred Treg cells maintain their suppressive activity, but the few cells that lose Foxp3 expression and differentiate into Tconv cells can cause severe disease [87]. Understanding why cells lose their Treg function and preventing this de-differentiation in vivo will improve both the safety and efficacy of Treg cell therapy. Because Foxp3 is the master regulator of Treg cell

function [88], alterations in Foxp3 expression or activity are likely involved in converting Tregs to Tconv cells. Foxp3 expression is modulated by DNA methylation via CpG islands in its promoter [89] and by chromatin remodeling [69]. Therefore, administration of selective demethylation agents and/ or HDACs may enhance Treg cell function and fidelity in vivo.

Another area of concern is that excessive Treg activity may blunt the response to infectious agents or lead to higher rates of tumor occurrence or relapse. Although none of the trials conducted thus far have observed any evidence of increased infections due to Treg, these concerns will remain justified until an effective Treg dose is reached. In addition, one study showed that augmenting Treg cell activity via TGF- β administration protected NOD mice from T1D but did not prevent coxsackievirus clearance [90]. Similarly, although murine studies show that excessive Treg impair tumor clearance [91], no effect on relapse rate was observed in human Treg clinical trials, although these studies used retrospective analysis and involved a diverse groups of diseases and without the statistical power to conclude a null effect on disease recurrence. The effect of nTreg on tumor clearance by human cells is critical, and should be addressed in the xenogeneic GVHD model.

Additional experiments are clearly required to examine whether therapeutic levels of adoptively transferred Tregs restrict protective immune responses. However, expansion protocols are now in place for all three types of Tregs, which should allow a direct comparison of suppression of GVHD vs. GVL. If excessive immunosuppression cannot be avoided, the use of suicide vectors may be an attractive way to eliminate introduced Tregs, although this would likely require genetic modification, which requires its own careful assessment of risks and rewards.

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Highlights

- Freshly isolated nTregs have been infused by two groups following HSCT without toxicity.
- Transfer of freshly purified or in vitro expanded Tregs decreases GVHD, although efficacy is limited by nTreg yield.
- Functionally suppressive nTregs can be massively expanded through re-stimulation, allowing 'off-the-shelf' Treg banking.
- Future studies are focused on cell surface markers and ways to increase Treg potency, stability, expansion and induction.