Regulatory T cells as immunotherapy

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Regulatory T cells (Tregs) suppress exuberant immune system activation and promote immunologic tolerance. Because Tregs modulate both innate and adaptive immunity, the biomedical community has developed an intense interest in using Tregs for immunotherapy. Conditions that require clinical tolerance to improve outcomes – autoimmune disease, solid organ transplantation, and hematopoietic stem cell transplantation – may benefit from Treg immunotherapy. Investigators have designed *ex vivo* strategies to isolate, preserve, expand, and infuse Tregs. Protocols to manipulate Treg populations *in vivo* have also been considered. Barriers to clinically feasible Treg immunotherapy include Treg stability, off-cell effects, and demonstration of cell preparation purity and potency. Clinical trials involving Treg adoptive transfer to treat graft versus host disease preliminarily demonstrated the safety and efficacy of Treg immunotherapy in humans. Future work will need to confirm the safety of Treg immunotherapy and establish the efficacy of specific Treg subsets for the treatment of immune-mediated disease.

Keywords: regulatory T cells, immunotherapeutics, inflammation, tolerance, adoptive transfer, expansion

INTRODUCTION

Autoimmunity and alloimmunity protect the host against malignancy and infection; however, unrestrained immune system activation leads to clinical disorders. Induction of immunologic tolerance is essential to improving outcomes in diseases typified by immune system activation: autoimmune disease (1), solid organ transplantation (SOT) (2), and hematopoietic stem cell transplantation (HSCT) (3, 4). In these states, conventional T cells coordinate adaptive immunity and underlie the pathogenesis of autoimmune disease, allograft rejection, and graft versus host disease (GVHD). Current strategies to induce tolerance include immunosuppressive pharmacotherapies that cause functional deletion or anergy of reactive conventional T cells. Toxicity limits use of these drugs, leading investigators to design immunotherapies based on the immune regulatory system. This review focuses on immunotherapy using regulatory T cells (Tregs).

Induction of peripheral immunologic tolerance requires Tregs, which suppress autoimmunity and promote allograft survival (5). Thymic deletion of self-reactive T cells provides a mechanism of *central* tolerance; Tregs represent a *peripheral* system to maintain self-tolerance and prevent over-exuberant immune responses. Mice with mutations in a critical Treg gene (*Foxp3*) develop scurfy, a fatal lymphoproliferative syndrome characterized by multi-organ inflammation (6). IPEX (immunodysregulation, polyendocrinopathy, and enteropathy, X-linked) occurs in humans with loss-of-function *FOXP3* mutations (7). Constitutive expression of the forkhead box protein 3 transcription factor (Foxp3 in mice and FOXP3 in humans) is necessary for Tregs to regulate self-tolerance (8, 9). Polymorphisms of cytotoxic T-lymphocyte antigen 4 (CTLA-4) – a co-signaling molecule with vital importance to Treg function (10) – are also linked to autoimmunity (11). **Table 1** lists Treg markers relevant to their use in immunotherapy.

Immunologically, Tregs comprise a subset of $CD4^+$ lymphocytes that suppresses activation, proliferation, and effector responses of both innate and adaptive immune cells (17). Functional Tregs also express the interleukin-2 (IL-2) receptor α-chain (CD25), although activated conventional T cells also transiently express CD25. Like conventional T cells, Tregs require T cell receptor (TCR) stimulation and costimulation for activation. *Natural Tregs* (nTregs) are derived centrally in the thymus (12); *induced Tregs* (iTregs) upregulate FOXP3 in the periphery following antigen exposure and, for example, stimulation from transforming growth factor β (TGF-β) (24). nTregs comprise 5–10% of the circulating $CD4^+$ population. Circulating and tissue iTreg numbers depend on anatomic location as well as specific inflammatory environmental conditions. Abbas et al. recently published recommendations for Treg nomenclature (25); in this review, we will use nomenclature used by cited authors.

Gershon proposed using Tregs for immunotherapy decades ago (26); however, clinical implementation of protocols employing Treg immunotherapy has proved challenging. In this review, we discuss strategies for using Tregs as immunotherapy, address barriers to the use of Tregs, provide promising examples of Treg immunotherapy in animal models and clinical trials, and conclude with future directions for the field.

PRACTICAL USE OF Tregs FOR IMMUNOTHERAPY

Adoptive transfer of autologous or donor-derived Tregs represents an exciting immunotherapeutic strategy (27). Broadly, protocols for adoptive transfer call for Treg isolation from the host or a donor, enrichment, expansion, and re-infusion. **Figure 1** diagrams such a protocol. Advantages of an *ex vivo* expansion strategy include the ability to perform careful cellular phenotyping and govern the dose of administered cells (28). As the contribution of reduced Treg *number* versus reduced Treg *function* remains unclear in autoimmune pathogenesis (29, 30), it is advantageous from an experimental perspective to maintain control over the phenotype and number of infused Tregs.

Table 1 | Treg markers relevant to their use as immunotherapy with selected references.

Marker	Alterative name or identifier	Function	Relevance to Treg immunotherapy
Foxp3	Forkhead box protein 3	Transcription factor, master regulator of Treg development and function	Identifies Treg lineage in mice; expressed in human CD4+ Tregs (12)
CTLA-4	Cytotoxic T-lymphocyte antigen 4, CD152	Transmits inhibitory signal to APCs	Important mechanism of Treg suppressive function (10)
LAP	Latency-associated peptide	Component of TGF-β latent complex	Identifies Treg subset with TGF-β-mediated function (13)
GITR	Tumor necrosis factor receptor superfamily member 18 (TNFRS18), activation-inducible TNFR family receptor (AITR)	Cell signaling	Important mechanism of Treg suppressive function (14)
ICOS	Inducible T cell costimulator, CD278	Costimulator on T cells	Involved in Treg expansion and IL-10 production, particularly during Th2 inflammation (15)
LAG-3	Lymphocyte activation gene 3, CD223	CD4 homolog with MHC class II binding properties	Expressed on Tregs (16)
CD3	TCR co-receptor complex	TCR signal transduction	Stimulation required for Treg expansion
CD4		Interacts with MHC class II molecules on APCs and amplifies TCR signals	Identifies CD4+ lymphocyte subset
CD25	IL-2 receptor α-chain	IL-2 receptor component	Expressed by CD4+Foxp3+ Tregs but also other T cells (17)
CD28		Costimulator required for T cell activation	Stimulation required for Treg expansion (18)
CD44		Hyaluronic acid receptor	Marker of activated Tregs (19)
CD45RO	Leukocyte common antigen (RO isoform)	Protein tyrosine phosphatase, receptor type, C	Positive Treg marker, also identifies memory T cells
CD45RA	Leukocyte common antigen (RA isoform)	Protein tyrosine phosphatase, receptor type, C	Minor Treg marker, also identifies naïve T cells
CD49b	Integrin VLA-4 α4β1 α-chain	Cell adhesion and signaling	Expressed on Tregs (16)
CD62L	L-selectin	Lymphocyte cell adhesion molecule	May be marker of effective disease-modulating Treg subset (20, 21)
CD69	Transmembrane C-Type lectin	Cell signaling	Marker of activated Tregs that suppress via membrane-bound TGF-β1 (22)
CD127	IL-7 receptor α-chain	IL-7 receptor	Negative Treg marker (23)

Peripheral or banked umbilical cord blood (UCB) may serve as a Treg source. A frozen UCB unit yields approximately $5–7.5 \times 10^6$ Tregs; an adult peripheral blood apheresis unit can yield on the order of 10^8 Tregs (28). Successful isolation requires labeling cell surface markers with a tagged antibody and sorting via fluorescence-activated cell sorting (FACS) or magnetic bead separation. Unfortunately, no cell surface markers uniquely identify Tregs. Although Foxp3 expression specifies the Treg lineage in mice (31), T cells promiscuously express FOXP3 in humans (32). Regardless, FOXP3 detection requires cell permeabilization, which renders cells unusable for adoptive transfer. Because activated CD4+ conventional T cells may also transiently express CD25, patterns of CD127 (the IL-7 receptor α-chain) (23), CD49b (the integrin VLA-4 α4β1 α-chain) (16), lymphocyte activation gene 3 (LAG-3) (16), CD45RA, CD45RO, and latency-associated peptide (LAP) (13) can identify Tregs and facilitate their isolation. Although Tregs express CTLA-4, glucocorticoid-induced TNFR family related gene (GITR) (14), CD69 (22), and CD44 (19), activated non-Tregs may also express these markers.

Ex vivo stimulation with anti-CD3/CD28 microbeads in the presence of recombinant human (rh) IL-2 expands Tregs for subsequent manipulation (33, 34). The resultant Tregs have polyclonal reactivity due to non-specific TCR stimulation. However, other protocols generate donor alloantigen-specific Tregs for establishment of allograft tolerance. In one method, Tregs are expanded in the presence of donor antigen-presenting cells (APCs). These Tregs have more potency than polyclonally reactive Tregs and demonstrate a more favorable safety profile *in vivo* (35, 36). Retroviral

FIGURE 1 | Schematic of a strategy to isolate, expand, and infuse Tregs.
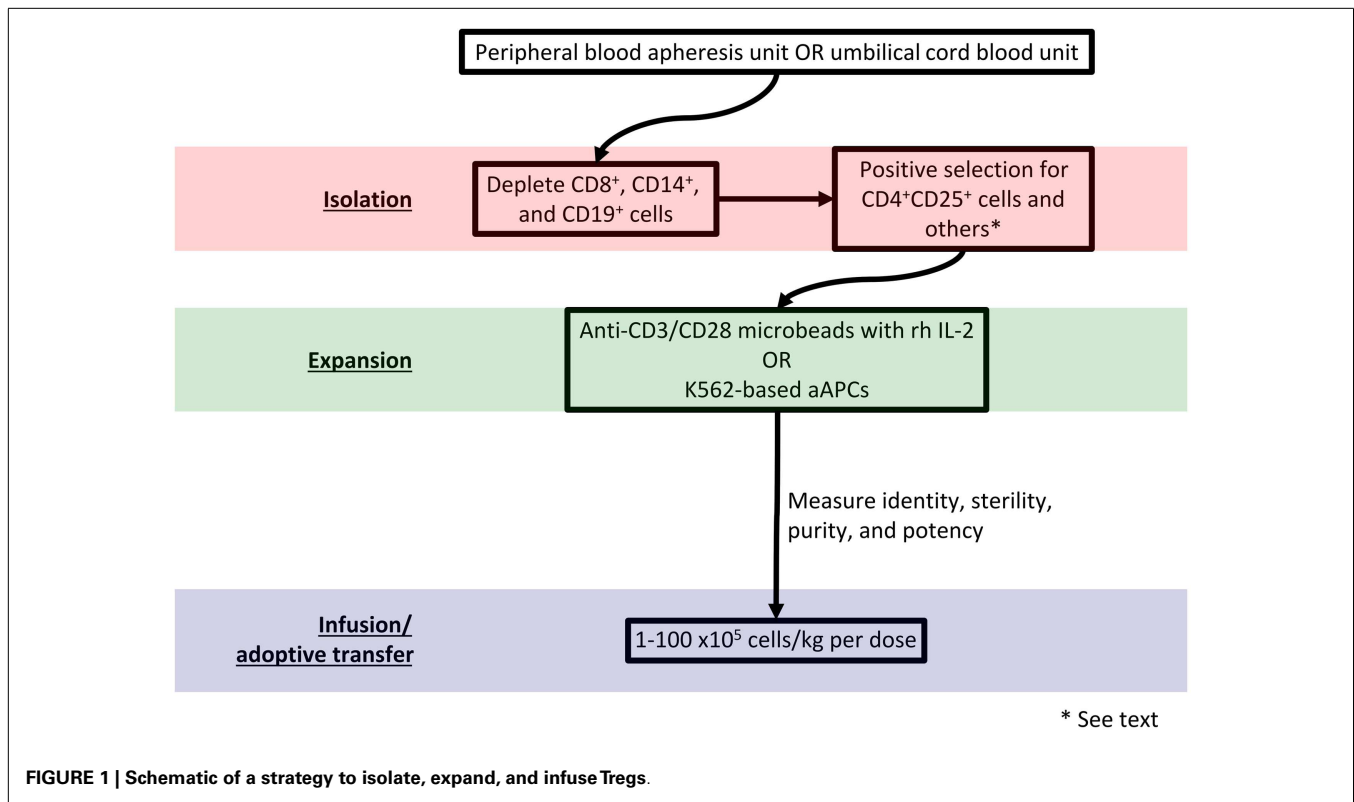
vector transduction of genes encoding TCRs with known antigen specificities also produces alloantigen-reactive Tregs (37). Anti-CD3 antibody-loaded K562-based artificial antigen-presenting cells (aAPCs) may efficiently expand Tregs with a high level of purity and potency (38, 39). Genetic modification that adds cell surface molecules and secreted factors to K562-based aAPCs could further refine the expanded Treg population (40).

It remains unclear what constitutes a therapeutic dose of Tregs. The therapeutic dose in a given application will depend on Treg potency, disease state and activity, and whether protocols employ polyclonal or antigen-specific Tregs (41). In a phase I dose-escalation trial of Tregs for prevention of acute GVHD, Blazar's group used Treg dosages between 1×10^5 and 30×10^5/kg (42). Di Ianni et al. used 40×10^5/kg of Treg in a similar trial (43). Based on animal studies, effective immunosuppression and tolerance induction may require up to 1×10^9 Tregs per infusion (44). To that end, Hoffmann et al. reported a protocol capable of a 4×10^4-fold Treg expansion in 3–4 weeks (45); however, the purity and phenotype of these cells was difficult to ascertain. Using the aAPC method, a 1×10^3-fold expansion of human peripheral blood Tregs can be performed in approximately 3 weeks (39).

Ex vivo conversion of CD4$^+$CD25$^-$ naïve T cells into iTregs with suppressor function represents an alternative strategy to *ex vivo* nTreg isolation and expansion (46). Exposure of naïve CD4$^+$CD25$^-$ or CD4$^+$CD45RO$^-$ T cells to TGF-β (47) with the addition of IL-2, IL-10, or vitamin D3 (48), indoleamine 2,3-dioxygenase (49), all-trans retinoic acid (50), *Foxp3*-expressing retroviruses (12), or epigenetic modifiers (DNA methyltransferase inhibitors or histone deacetylase inhibitors) (51) accomplishes

such a conversion. Lan et al. have suggested that iTregs have more potency than nTregs on a cell-by-cell basis (52), making strategies that expand iTregs attractive for Treg immunotherapy. Future work will need to validate methods of identifying nTregs versus iTregs in humans and assessing their stability and plasticity (53).

A variety of strategies induce Treg number or potency *in vivo* including expansion of nTregs and conversion of non-Tregs to iTregs (54). For example, treating mice prior to allografting with a donor alloantigen and a non-depleting anti-CD4 antibody achieves Treg expansion. Tregs generated by this method prevent allograft rejection (55–58). Moreover, adoptive transfer of Tregs isolated from treated animals abrogates rejection (59). nTregs isolated from naïve animals may also prevent rejection, although long-term allograft survival requires 10-fold more Tregs compared with Tregs isolated from tolerant mice treated with antigen exposure alone (60).

Injection of IL-2/IL-2 monoclonal antibody (mAb) complexes into mice results in a 10-fold *in vivo* Treg expansion (61). Mice treated with this protocol display immunologic tolerance and resistance to experimental autoimmune encephalomyelitis and islet allograft rejection. Simultaneous injection of IL-2/IL-2 mAb complexes and recombinant granulocyte-colony stimulating factor (G-CSF), which causes expansion of myeloid-derived suppressor cells (MDSCs), augments induction of immunologic tolerance. Expansion of MDSCs in addition to Tregs supports MHC class II-mismatched skin allograft survival (62). In a phase 1 dose-escalation trial of subcutaneous IL-2 to treat active chronic GVHD, daily low-dose IL-2 was well-tolerated and led to sustained Treg expansion with improvement in GVHD manifestations (63).

Other pharmacotherapies target particular facets of Treg biology. IL-2-dependent STAT-5 activates Tregs (64), whereas effector T cells employ the phosphoinositide 3-kinase/Akt/mTOR pathway (65). The mTOR inhibitor rapamycin exploits the latter pathway to preferentially expand Tregs (66–68). Clinically, rapamycin increases the number of $CD62L^{high}$ Tregs in the peripheral blood of lung transplant recipients (20) and expands the Treg population in renal transplant patients (69). Anti-thymocyte globulin (ATG), a T cell-depleting polyclonal antibody that promotes Treg generation in mice (70), supports allograft survival when combined with CTLA-4-Ig and rapamycin in a MHC-mismatched skin allograft model (71). In that model, memory T cell-Treg balance shifted in favor of Tregs. Glucocorticoids have broad effects on T cells; however, glucocorticoids may interact with Langerhans cells to promote Treg expansion in contact dermatitis (72). The lymphocyte depleting mAb alemtuzumab (anti-CD52 mAb) may have favorable effects on Treg survival when combined with rapamycin (73). Standard dosages of calcineurin inhibitors such as cyclosporine A and tacrolimus impair Tregs (74). However, treatment with low-dose cyclosporine may increase Treg numbers in the skin of atopic dermatitis patients (75). Compared to conventional doses, low doses of calcineurin inhibitors may allow patients to continue the production of IL-2, which Tregs require for expansion and survival (76, 77). Weng et al. published that the proteasome inhibitor bortezomib reduced acute GVHD severity and prolonged survival time by triggering generation of Tregs (78). A recently reported high-throughput screening assay may increase the number of known compounds with positive effects on Tregs (79).

BARRIERS TO USE OF Tregs FOR IMMUNOTHERAPY

Regulatory T cell functional stability represents a challenge for using Tregs for immunotherapy. A minor population of $Foxp3^+$ cells loses Foxp3 expression over time; these "ex-Foxp3" cells may display an activated conventional T cell phenotype and become pathogenic in vivo (80). Loss of Foxp3 expression has been associated with a pro-inflammatory microenvironment and switching to an effector T cell phenotype characterized by IL-17 and interferon-γ secretion (81–83). While Tregs delivered to a normal host tend to retain their suppressive function, a proportion of Tregs adoptively transferred into a lymphopenic environment may differentiate into pathogenic T cells (84, 85). Exploiting the epigenetic control of the Foxp3 gene could maintain Foxp3 expression and Treg stability (85–87). Both DNA methylation at the Foxp3 upstream control regions (88) and chromatin remodeling (89) help determine Treg plasticity. Pharmacologic DNA methyltransferase inhibitors or histone deacetylase inhibitors could maintain Treg fidelity following adoptive transfer (51). IL-2 therapy might also promote Treg stability after infusion (63).

Despite the fact that some costimulatory pathways differentially affect conventional T cells versus Tregs, no single pathway completely selects for a specific T cell subset (90). Therefore, administration of pharmaceuticals that stimulate Tregs may also activate conventional T cells (off-cell effect). Indeed, a phase I clinical trial of TGN1412 – a super-agonist anti-CD28 antibody – caused massive cytokine storm and multi-organ dysfunction in six healthy adults who required intensive care following

administration of the drug (91). The misadventure with TGN1412 highlights the risks of drugs designed to modulate T cell activity without selectively targeting specific T cell subsets. As above, drugs that modify T cell epigenetic signatures may add specificity to T cell pharmacotherapy (92–94).

Memory T cells provide a significant barrier to the induction of clinical tolerance (95), and depleting donor-reactive T cells permits Tregs to control allograft rejection (96). Therefore, investigators desire drug protocols that functionally deplete memory T cells while maintaining immunoregulation. Alefacept, an LFA-3-Ig fusion protein that polymerizes CD2, leads to selective memory T cell elimination. When administered with CTLA-4-Ig, alefacept prevents acute rejection and promotes kidney transplant engraftment in a non-human primate model (97). Efalizumab, an anti-LFA-1 antibody, showed efficacy in islet-cell transplantation (98) but was withdrawn from the market after four patients with psoriasis developed progressive multifocal leukoencephalopathy (99). Functional Tregs themselves also potently suppress memory T cell proliferation in humans and may not require additional pharmacotherapy to overcome the effect of memory T cells if administered in sufficient dosages (100).

The United States Food and Drug Administration mandates documentation of sterility, identity, purity, and potency of a cell therapy product before administration to patients (21 CFR §1271). Sterility and identity are relatively facile to demonstrate; purity and potency are more problematic. Investigators will need to empirically determine the acceptable level of non-Treg contamination in cell preparations. CpG demethylation of the Foxp3 conserved non-coding sequence 2 (CNS2) identifies committed suppressive Tregs (85, 86); therefore, methylation status of the FOXP3 CNS2 region may indicate Treg purity and stability in cells destined for clinical use. As Tregs have many mechanisms of action, difficulty exists in elucidating which mechanisms regulate a specific disease in an inflammatory environment (101, 102). Therefore, in vitro assays – such as the ability of Tregs to inhibit conventional T cell proliferation – may inadequately describe the potency of cell preparations. For example, Golovina et al. reported that $CD4^+$ T cells expanded in the presence of rapamycin were effective in an in vitro suppression assay, but these cells failed to function in an in vivo xeno-GVHD model (18). These findings imply that investigators may need to develop disease-specific Treg potency testing systems prior to use in humans. Non-human primates have been used to validate SOT protocols (103), but even these models may lead to erroneous conclusions (104).

Cryopreservation of Treg cell preparations presents technical challenges (105), although investigators have developed feasible cryopreservation protocols. One popular method involves liquid nitrogen cryopreservation with 20% human pooled serum and 15% DMSO. After 1 year, Tregs showed 70–80% viability; stimulation and subsequent expansion restored Treg function to pre-cryopreservation levels (106). Strategies to freeze already-expanded Tregs also exist. In their seminal clinical trial of Treg immunotherapy for GVHD (reviewed below), Brunstein et al. cryopreserved ex vivo-expanded Tregs that were not initially infused (42). Their protocol used a freezing medium containing Plasma-Lyte A™ (Baxter, Deerfield, IL, USA), 10% DMSO, and human serum albumin. The thawed cells had an immediate post-thaw

viability exceeding 50%. However, an increase in peripheral blood Tregs following infusion was not observed, whereas the authors had observed a significant increase following the first infusion of non-cryopreserved Tregs. Other authors using a mouse GVDH model detected preserved *in vivo* suppressive function after thawing aAPC-expanded Tregs (39). Further refinement of cryopreservation strategies could facilitate an "on demand" treatment for acute inflammatory disease or acute allograft rejection without the time delay required for *ex vivo* isolation and expansion.

Potential adverse effects of Treg infusion or expansion include those associated with immunosuppression, including infection and malignancy. Interestingly, Di Ianni et al. observed improved immunity to opportunistic pathogens in their trial of Treg infusion for GVHD prevention following HSCT (43). Brunstein et al. similarly reported no increased risk of infection following Treg infusion for acute GVHD (42). Numerous studies implicate Tregs in suppressing anti-tumor immunity [reviewed in (107)]. Future study will need to carefully examine the effect of Treg manipulation on infectious risk and neoplasia.

EXAMPLES OF Treg IMMUNOTHERAPY
ALLOGRAFT TOLERANCE
Graft versus host disease results from donor T cell-mediated systemic inflammation that overwhelms immune regulatory mechanisms following allogeneic HSCT (108). Clinical disease results when donor (i.e., graft) cells recognize host cells as foreign and incite an inflammatory reaction. Inflammation often causes tissue damage despite routine post-HSCT immunosuppressive pharmacotherapy designed to dampen T cell alloreactivity. In contrast to SOT, HSCT eventually fosters the development of tolerance, as donor APCs and T cells replace host leukocytes. Therefore, risk of alloreactive immunity peaks in the first few months following HSCT, highlighting GVHD as an ideal application for Treg immunotherapy. The availability of Tregs from HSCT donors makes Treg immunotherapy protocols particularly feasible.

Strong pre-clinical work supports the use of CD4$^+$CD25$^+$ Tregs to suppress acute GVHD (109). Trzonkowski et al. reported the first two cases of *ex vivo*-expanded donor-derived Tregs to successfully treat post-HSCT GVHD (33). A phase I dose-escalation trial demonstrated the safety profile and efficacy of human UCB-derived partially HLA-matched *ex vivo*-expanded Tregs in reducing the incidence of grades II–IV GVHD in 23 patients compared with 108 controls (42). These investigators isolated Tregs with anti-CD25 magnetic beads, expanded them with anti-CD3/CD28 microbeads and rh IL-2, and infused the expanded Tregs at the time of HSCT. Di Ianni et al. used adult expanded Tregs isolated from the same HLA-haploidentical donor to assess safety and efficacy in prevention of chronic GVHD in 28 patients undergoing HLA-haploidentical HSCT for high-risk acute leukemia (43). These patients also received donor conventional T cells to enhance immune reconstitution and to promote the graft versus leukemia effect. Chronic GVHD developed in only 2 of 28 patients. Other trials of Treg adoptive transfer are ongoing (110, 111).

In 1995, Sakaguchi et al. published their watershed observation that Tregs from naïve mice prevented rejection of allogeneic skin grafts in nude mice given CD25$^-$ T cells (17). Their work laid the foundation for the use of Treg immunotherapy to promote tolerance following SOT. Indeed, induction of tolerance to alloantigen via costimulatory blockade requires Tregs (112). In a MHC-mismatched mouse orthotopic lung transplant model, blockade of CD154 increased Tregs and was associated with attenuation of acute cellular rejection (113). In a chimeric humanized mouse system, *ex vivo*-expanded Tregs prevented transplant arteriosclerosis *in vivo* by limiting effector cell function and allograft infiltration (114). Clinical trials of Treg adoptive transfer to promote SOT tolerance have not been published; however, this review highlights pre-clinical work that could inform the design of post-SOT Treg immunotherapy protocols. Results from The ONE Study (115) should shed light on Treg immunotherapy for induction of tolerance following SOT.

ATOPIC DISEASE
Atopy is a complex immune phenomenon characterized by Th2-predominant inflammation, production of allergen-specific immunoglobulin E (IgE), attraction of pro-inflammatory cells, and the degranulation of effector cells (e.g., mast cells) (116). Literature supports a functional role for Tregs in maintaining allergen tolerance in normal individuals. Indeed, an imbalance between Tregs and Th2 cells leads to an atopic phenotype (117). The E3 ligase Itch has recently been identified as a critical protein controlling the Treg response to Th2 inflammation (118) and may be a therapeutic target in atopic disease states. Allergen-specific immunotherapy decreases allergen-specific T-cell proliferation, Th2-type cytokine production, and inflammatory cell activity (119). Generation of IL-10-producing Tregs may be a prominent mechanism underlying these findings (15). The antidepressant drug desipramine appears to alleviate allergic rhinitis by regulating Tregs and Th17 cells (120). Although clinical trials have not attempted adoptive transfer of Tregs for allergic disease, protocols to expand allergen-specific Tregs may potentially benefit atopic patients.

AUTOIMMUNE DISEASE
Numerous studies have demonstrated diminished numbers of peripheral blood Tregs in patients with autoimmune conditions and that a Treg deficit associates with disease development (121). Redistribution of the Treg population to the tissue compartment does not fully explain the association between peripheral blood Treg deficiency and disease development (122). Moreover, some autoimmune conditions alter the functional activity of Tregs. Such a functional alteration exists in rheumatoid arthritis (123) and multiple sclerosis (124).

Failure to control islet-specific conventional T cells results in type 1 diabetes mellitus (DM1). Risk of DM1 increases with the loss of FOXP3-expressing Tregs (125), and Treg adoptive transfer to non-obese diabetic (NOD) mice can prevent the development of DM1 (41, 126). Interestingly, 80% of IPEX patients develop DM1 in infancy (127). Marek-Trzonkowska et al. recently published a study demonstrating that a donor-derived CD4$^+$CD25^{high}CD127$^-$ Treg infusion preserves β-cell function and may delay DM1 onset in children (128).

Despite positive results in DM1 and other animal models of autoimmune disease including myasthenia gravis (129), adoptive transfer of nTregs has not met with universal success. Adoptive

transfer of nTregs had only a nominal effect on controlling disease progression in a collagen-induced arthritis model (130) and failed to suppress glomerulonephritis and sialadenitis in mice with established lupus (131). nTregs have had variable achievement in controlling other Th17-mediated autoimmune diseases (132). The inability of nTregs to treat many autoimmune disorders may relate to pro-inflammatory cytokines that suppress their function (123, 133) or convert them to pathogenic T cells upon adoptive transfer. Additionally, activated Th17 cells may resist many suppressive mechanisms employed by nTregs. iTregs might be a more appropriate Treg subset for use in autoimmune immunotherapy, as data suggest that iTregs more effectively suppress autoimmune activation compared with nTregs possibly due to differential stability in inflammatory environments (52).

ACUTE INFLAMMATORY DISEASE

Our group established that resolution of experimental murine acute lung injury requires Tregs (134). Mice lacking all mature lymphocytes ($Rag-1^{-/-}$) do not resolve their injury by day 10 following an intratracheal injection of $E. coli$ lipopolysaccharide (LPS), whereas wild-type mice normalize. Adoptive transfer of 1×10^6 congenic $CD4^+CD25^+$ cells up to 48 h after receiving LPS restores resolution in $Rag-1^{-/-}$ mice to that of wild-type mice. Moreover, Treg adoptive transfer limits fibroproliferation following acute lung inflammation (135). Tregs also promote repair from ischemic acute kidney injury (136) and have protective immunomodulatory effects following acute stroke (137). These findings not only demonstrate the importance of Tregs in tissue injury repair but also open the door to studying Treg immunotherapy for other acute inflammatory conditions.

FUTURE DIRECTIONS AND CONCLUSION

Most clinical trials of Treg immunotherapy employed adoptive transfer of $CD4^+CD25^+$ or $CD4^+CD25^+CD127^-$ cells. However, more precisely defined human Treg subsets exist; exploiting these Treg subsets may benefit certain disease states. For example, inducible costimulator-expressing ($ICOS^+$) Tregs secrete more IL-10 than $ICOS^-$ Tregs and could improve conditions characterized by relative IL-10 deficiency, such as atopic disease (15). $ICOS^+$ Tregs may also play an important role in dendritic cell function (138). Another example is the $CD62L^+$ subpopulation of $CD4^+CD25^+$ Tregs, which appears to most effectively treat acute GVHD (21). In rheumatoid arthritis, abnormal Treg function may stem from defective CTLA-4 (139); therefore, augmentation of functional $CTLA-4^+$ Tregs may be advantageous in rheumatoid arthritis. Understanding Treg subset trafficking and survival via chemokine and integrin signals will be key to selecting appropriate Treg subsets for a given application (140).

Genetic reprograming of Tregs, possibly using clinical-grade lentiviral vectors, represents an attractive strategy to fine tune Treg subpopulations (141). Induction of a chimeric immune receptor into Tregs prevented mouse models of experimental autoimmune encephalomyelitis (142) and colitis (143). Engineered TCRs that redirect Treg specificity could also improve Treg potency (144), as Varela-Rohena et al. demonstrated in conventional T cells (145).

Use of Tregs for immunotherapy has a solid pre-clinical database, and emerging data support the safety and efficacy of Treg immunotherapy protocols in patients whose clinical scenario requires induction of clinical tolerance. Both *ex vivo* expansion with adoptive transfer and *in vivo* manipulation to expand and augment the function of endogenous Tregs represent promising strategies to treat autoimmune and alloimmune conditions. In order for clinically feasible Treg immunotherapy protocols to succeed, investigators will need to surmount significant barriers including Treg stability, isolation and storage of Treg subpopulations, and off-target effects of *in vivo* Treg strategies. Because immune dysregulation underlies myriad clinical disorders, designing safe and effective immunotherapies that utilize Tregs could be of great benefit.

AUTHOR CONTRIBUTIONS

Benjamin D. Singer and Franco R. D'Alessio made substantial contributions to the conception of the work, drafting the work, and critically revising it for important intellectual content. Landon S. King provided substantial contributions to the design of the work, and has critically revised the manuscript. All authors share final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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