The promise of CD4+FoxP3+ regulatory T-cell manipulation *in vivo*: applications for allogeneic hematopoietic stem cell transplantation

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ABSTRACT

CD4+FoxP3+ regulatory T cells (Tregs) are a non-redundant population critical for the maintenance of self-tolerance. Over the past decade, the use of these cells for therapeutic purposes in transplantation and autoimmune disease has emerged based on their capacity to inhibit immune activation. Basic science discoveries have led to identifying key receptors on Tregs that can regulate their proliferation and function. Notably, the understanding that IL-2 signaling is crucial for Treg homeostasis promoted the hypothesis that *in vivo* IL-2 treatment could provide a strategy to control the compartment. The use of low-dose IL-2 *in vivo* was shown to selectively expand Tregs *versus* other immune cells. Interestingly, a number of other Treg cell surface proteins, including CD28, CD45, IL-33R and TNFRSF members, have been identified which can also induce activation and proliferation of this population. Pre-clinical studies have exploited these observations to prevent and treat mice developing autoimmune diseases and graft-*versus*-host disease post-allogeneic hematopoietic stem cell transplantation. These findings support the development of translational strategies to expand Tregs in patients. Excitingly, the use of low-dose IL-2 for patients suffering from graft-*versus*-host disease and autoimmune disease has demonstrated increased Treg levels together with beneficial outcomes. To date, promising pre-clinical and clinical studies have directly targeted Tregs and clearly established the ability to increase their levels and augment their function *in vivo*. Here we review the evolving field of *in vivo* Treg manipulation and its application to allogeneic hematopoietic stem cell transplantation.

Introduction

The identification of CD4+FoxP3+ regulatory T cells (Tregs) as a non-redundant cell population essential for the maintenance of peripheral self-tolerance has stimulated strong interest in their potential therapeutic application to promote allograft acceptance and ameliorate autoimmune diseases.[1-5] The finding that Tregs are often present at tumor sites has also raised the prospect of augmenting antitumor immunity by diminishing their numbers or function.[1,6-11] Accordingly, the fields of transplantation, autoimmunity and oncology have converged on a common objective to selectively manipulate the Treg compartment to inhibit or promote conventional T-cell (Tconv) antigen-specific adaptive immune responses.

Clinical procedures developed to harvest Tregs for study and therapeutic application have been primarily based on cell surface expression of CD4, CD25 and CD127.[12-14] Employing magnetic bead or flow cytometric isolation methodology, viable and enriched preparations of Tregs have been generated for subsequent *ex vivo* expansion and translational use in patients.[15-18] Inherent in such *in vitro* manipu-

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lations is the absence of the precise *in situ* microenvironment wherein individual cell populations differentiate, undergo expansion and mediate effector function. Several established strategies have incorporated the use of microbead and antigen-presenting cell (APC)-based technologies to expand Tregs incorporating anti-CD3, CD28, and anti-TNFR family mAbs together with cytokines (e.g. IL-2, TGFβ, and retinoic acid).[19] Successful expansion ranging from approximately 100-1300x was reported from starting populations of peripheral blood ($CD4^+CD127^{lo/-}$) and umbilical cord ($CD25^+$) cells.[15,20] Notably, employing these Tregs in phase I studies reported no apparent toxicities or adverse effects.[15,21]

Although Tregs can be induced to expand *in vitro*, size and purity of the initiating culture, the maintenance of FoxP3 expression and functional activity during culture vary and depend both on starting cell populations and culture conditions. Consistency of reagents from batch to batch also poses challenges for ultimate clinical application. Generation of a GMP product containing high cell numbers for adoptive therapy requires infrastructure and significant economic investment.[22-24] Accordingly, routinely generating adequate numbers of functional Tregs *in vitro* as a readily available adoptive therapy remains translationally challenging.[25] Several excellent articles which include discussion of *in vitro* expansion methods have recently been published and we refer readers to these thorough reviews.[26-30] Strategies to manipulate Tregs *in vivo* have and continue to be examined to circumvent the practical and economic considerations that limit the feasibility of *in vitro* approaches. The provocative finding that low-dose IL-2 more efficiently stimulates Tregs *versus* Tconv populations has fostered optimism that selective manipulation of the FoxP3 compartment *in situ* can be exploited for clinical benefit. Because the production and expansion of effector *versus* Tregs is associated with the development of chronic graft-*versus*-host disease (cGvHD), correction of a Treg:Tconv cell imbalance would have therapeutic benefit.[31] This review presents a historical overview and survey of experience with *in vivo* Treg expansion and associated changes in their functional capacity. Pre-clinical and clinical studies designed to augment Treg levels and function *in vivo* examining therapeutic benefit in the setting of GvHD prevention and therapy will be discussed.

Targeting cell surface receptors for *in vivo* Treg expansion, function and therapeutic application

Experimentally, a number of molecules expressed on Tregs have been shown to expand natural Tregs and/or augment their functional activity *in vivo*, including IL-2, several members of the TNFR family (TNFR2/TNF; TNFRSF25/TL1A; OX-40/OX-40L; 4-1BB/4-1BB-L) as well as CD28 and IL-33 to suppress autoimmune responses, allograft rejection and GvHD (Table 1 and Figure 1). While other molecules have been found to modulate Tregs *in vivo* (e.g. CD45, GITR/GITRL), these are not discussed here because they have not been assessed in GvHD.[32-34]

IL-2 / CD25

Over the past decade, IL-2 treatment has been the approach most extensively utilized in pre-clinical and

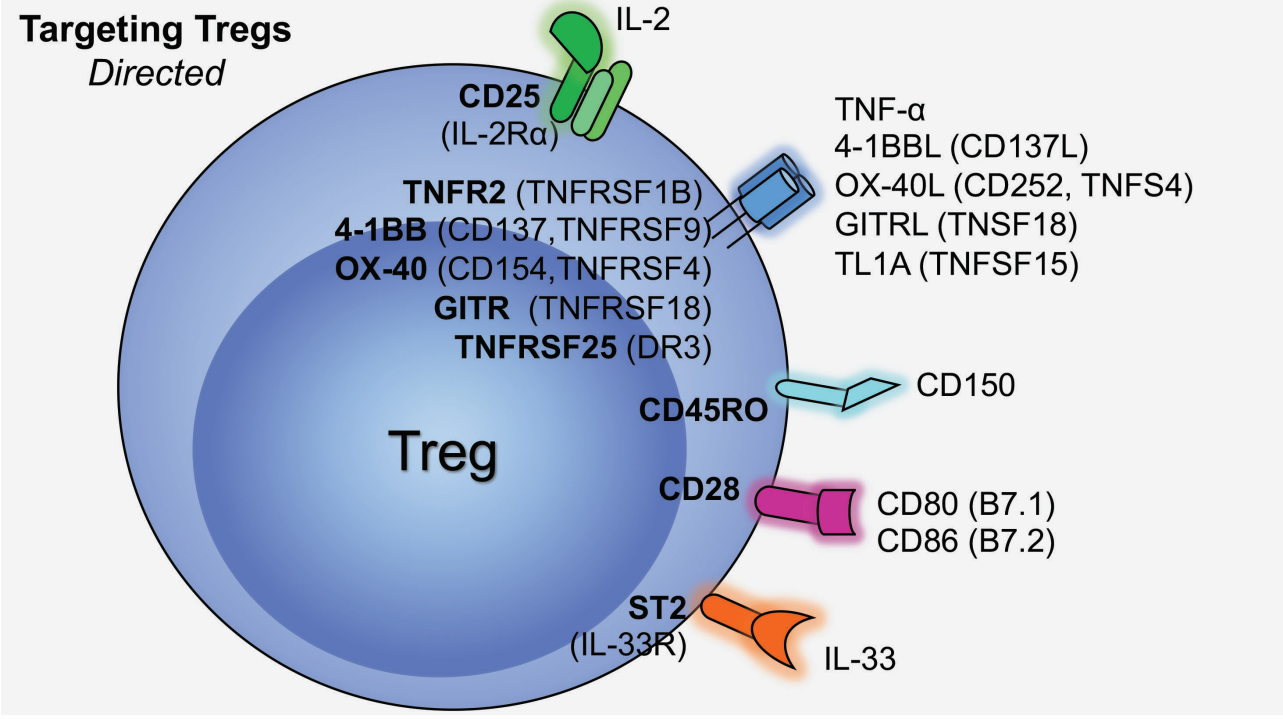

Figure 1. Receptors reported to stimulate Treg expansion *in vivo*. Targeting CD25, members of the TNFR superfamily, CD45RO, CD28 and ST2 was shown to be an effective approach to increase Treg frequency and/or functionality for their pre-clinical / clinical therapeutic application in autoimmune diseases, solid organ transplantation and graft-*versus*-host disease.

clinical settings to expand Tregs *in vivo* to ameliorate GvHD. Therapeutic strategies have varied the reagents, timing of administration and targeting donor/recipient populations (Table 1).

IL-2/CD25 targeting to manipulate Tregs in vivo. IL-2 is a pleiotropic cytokine which plays a dual role in maintaining tolerance and contributing to immunity *in vivo*.[35] Tregs but not Tconv cells constitutively express high CD25 levels, although Tconv up-regulate this receptor after activation. Importantly, downstream signaling in Tregs *versus* Tconv is more sensitive to IL-2 stimulation.[36] Accordingly, high doses (HD) of IL-2 can target $CD4^+$ effector cells and stimulate immunity whereas low-dose (LD; 100-fold lower) IL-2 selectively activates Tregs, promoting tolerance.[36] Human recombinant IL-2 was first approved by the US Food and Drug Administration (FDA) in 1998 for use at HD to stimulate immunity toward metastatic cancers (renal cell carcinoma and melanoma).[37] LD IL-2 has minimal side effects[38] and, together with its effects on *in vivo* Treg expansion, is of interest for tolerance induction.

Multiple studies demonstrated that free LD IL-2 treatment results in Treg expansion leading to efficient reversion of autoimmune type 1 diabetes (T1D),[39] amelioration of experimental autoimmune encephalomyelitis (EAE)[40]

Table 1. Summary of reagents and properties discussed in this review with regard to *in vivo* Treg manipulation.

Study	Target	Reagent	Clone	Property	Combinations	References
Animal	CD25 (IL-2Rα)	Free LD IL-2	n/a	agonistic	----------	39-41, 79
		Free LD IL-2	n/a	agonistic	Sirolimus	42, 43, 80
		Free LD IL-2	n/a	agnostic	Dexamethasone	44, 83
		Free LD IL-2	n/a	agnostic	IL-33	56
		IL-2 Complex (IL-2/anti-IL2)	JES-1A12	agonistic	----------	45-50, 77,78
		IL-2 Complex	JES-1A12	agonistic	Sirolimus	51
		AAV-IL-2	n/a	agonistic	----------	52, 53
		CD25-IL-2 FP	n/a	agonistic	----------	54
		IgG-IL-2 FP	n/a	agonistic	----------	55
		IL-233		agonistic	----------	56
	TNFRSF1B (TNFR2)	TNFR-/- mice	n/a	n/a	----------	88, 89, 92, 93, 97
		TNFR2-Fc	n/a	agonistic		90
		α-TNF-α mAb	XT3.11	blocking	----------	91
		TNC-scTNF80	n/a	agonistic	----------	94, 95
		TNF-α	n/a	agonistic	----------	96
		α-TNFR2 mAb	TR75-54.7	blocking	----------	97
		STAR2	TNFR2	agonistic	----------	98
	TNFRSF4 (OX-40)	α-OX-40 mAb	OX-86	agonistic	----------	100-107, 109
		OX-40-L overexpression	n/a	agonistic	----------	100-104
		α-OX-40-L mAb	RM134L	blocking	----------	108
		α-OX-40-L mAb	KY1005	blocking	----------	110
		α-OX-40-L mAb	KY1005	blocking	Sirolimus	110
	TNFRSF9 (4-1BB)	4-1BB-L-Fc	n/a	agonistic	----------	113
		α-4-1BB mAb	3H3	agonistic	----------	114-116, 120, 121
		α-4-1BB mAb	158321	agonistic	----------	117
		α-4-1BB mAb	1AH2	agonistic	----------	118
		α-4-1BB-L mAb	TKS1	blocking	----------	119
	TNFRSF25 (DR3)	α-TNFRSF25 mAb	4C12	agonistic	----------	126, 127, 130, 131, 133, 135
		α-TNFRSF25 mAb	4C12	agonistic	α-OX-40 (OX-86)	128, 129
		TL1A-Ig	n/a	agonistic	----------	127,130,133
		TL1A-Ig	n/a	agonistic	IL-2	130, 132, 136,137
	CD28	α-CD28SA mAb	D665 (ms)	agonistic	----------	139, 142, 146, 148, 153
		α-CD28SA mAb	JJ316 (rat)	agonistic	----------	140, 141, 143-145,147, 149,150-152
	ST2 (IL-33R)	IL-33	n/a	agonistic	----------	158, 159, 161-165
		IL-33	n/a	agonistic	IL-2	56
		IL-233	n/a	agonistic	----------	56
		ST2-/- donor cells	n/a	n/a	----------	167
		ST2-Fc	n/a	n/a	----------	167
		IL-33-/- recipients	n/a	n/a	----------	167
Human	CD25 (IL-2Rα)	LD IL-2	n/a	agonistic	----------	70-73, 75, 76
		LD IL-2	n/a	agonistic	Tacrolimus	75
		LD IL-2	n/a	agonistic	Sirolimus + Tacrolimus	81, 82
		Ultra LD IL-2	n/a	agonistic	----------	74, 84
	CD28	α-CD28 mAb	TGN1412	agonistic	----------	154

LD: low dose; HD: high dose: n/a: not applicable.

and improved long-term allograft survival in a corneal transplant model.[41] These findings led to combination therapy with synergistic effects on Treg expansion using free LD IL-2 with sirolimus in transplant models, i.e. cornea[42] and skin.[43] Similar results in combination with dexamethasone (Dex) were observed in EAE.[44]

To increase circulating IL-2 half-life and decrease the required dose, antibody/cytokine (α-IL-2/IL-2) complexes (IL-2C) are under development. Notably, *in vivo* utilization of different IL-2C resulted in targeting specific cell subsets, i.e. mAbJES-1A12/IL-2 and S4B6/IL-2 preferentially expanded Tregs and Tconv, respectively.[45] The IL-2C (mAbJES-1A12+IL-2) caused selective Treg expansion and suppression of allergic airway inflammation,[46] contact hypersensitivity,[47] and experimental myasthenia gravis.[48] Furthermore, in renal and cardiac ischemia reperfusion models, IL-2C expanded Tregs attenuated acute renal damage improving renal and myocardial recovery.[49,50] Using a combination therapy of IL-2C, sirolimus and islet Ag peptide, protection against spontaneous and induced T1D in NOD mice occurred following increased Treg levels and function.[51] Recombinant adeno-associated viral vector that continuously releases IL-2 achieved persistent and sufficient levels of LD IL-2 while avoiding toxic effects.[52,53] Importantly, this viral vector controlled diabetes after sustained Treg expansion without impairing immune responses to infection, vaccination and cancer. Recently, an IL-2 modification creating a fusion protein between murine IL-2 and CD25[54] achieved markedly extended half-life [16 hours *vs*. free IL-2 (<15 minutes)] and selective Treg expansion controlling T1D. Other examples of long-lived IL-2 fusion protein (FP) include: IgG-IL2 FP[55] and IL-2+IL-33 (IL-233),[56] discussed below.

IL-2/CD25 Treg manipulation in pre-clinical and clinical allogeneic hematopoietic stem cell transplantation. Adoptive transfer of Tregs is a promising therapy to diminish GvHD. In humans, development of cGvHD is associated with poor Treg reconstitution post HSCT.[57,58] We and others have been examining the application of donor Tregs as a prophylactic strategy to prevent the development of GvHD in experimental models.[59-65] Experiments demonstrated that donor Tregs inhibit lethal acute GvHD (aGvHD) only at high ratios, i.e. 1:1 (Treg:Tconv).[59] Because circulating Tregs account for only approximately 5-10% of $CD4^+$ T cells, a practical obstacle is collecting sufficient numbers of Tregs from donors or recipients for use in allogeneic hematopoietic stem cell transplantation (aHSCT) to suppress activation of anti-host reactive Tconv in T-cell-replete grafts, thus *in vivo* expansion is attractive.

A. Treatment of recipients: pre-clinical and clinical models. In the early 1990s, using a fully MHC-mismatched bone marrow transplant (BMT) model, studies demonstrated that short-term human recombinant IL-2 administration (50,000 U twice daily) starting the day of the BMT significantly reduced GvHD mortality.[66] Moreover, IL-2 treatment did not prevent allogeneic engraftment and preserved graft-*versus*-leukemia (GvL) effect. The IL-2 effect on GvHD but not on GvL was explained by selective inhibition of CD4-mediated activity.[66] Clinical and immunological effects of IL-2 administration in patients following TCD allogeneic and autologous BMT were evaluated. Strikingly, patients who received TCD-BM and LD IL-2 ($2\text{-}6\text{x}10^5$ U/m^2 daily for 90 days) to enhance GvL exhibited high circulating NK cells.[67,68] Since $CD25^+$ Tregs had not yet been identified, the success of these studies was not immediately attributed to Treg expansion. Later, discovery of this suppressive population[69] and the recognition that their infusion inhibited immune responses opened a new era of GvHD prophylaxis and treatment.[59-62,64,65] Indeed, the addition of IL-2 ($6\text{x}10^5$ IU/m^2 daily) with donor $CD4^+$ T cells resulted in expansion of Tregs in patients post transplant.[70] A seminal phase I dose-escalation study demonstrated that therapy of LD IL-2 daily for eight weeks in patients with steroid refractory active cGvHD was well tolerated and induced significant Treg expansion (*Online Supplementary Table S1*).[71] This treatment diminished cGvHD manifestations, including decreased cutaneous sclerosis in a considerable number of patients.[71] Notably, this IL-2 regimen induced selective activation of pStat5 in Tregs *versus* Tconv, which was associated with increased thymopoiesis and production of Tregs.[72] These observations demonstrated that more nTreg homeostasis appeared to be restored following IL-2 therapy in patients with cGvHD.[73]

Ultra LD IL-2 ($0.1\text{-}0.2\text{x}10^6$ IU/m^2 thrice weekly) was administered as aGvHD prophylaxis in pediatric patients after aHSCT starting <day 30 and continuing 6-12 weeks. Treg levels increased in recipients of matched related donor and the Treg functional suppressor activity was restored. IL-2 treated recipients had diminished humoral but not virus-specific cellular immune responses. Importantly, treated patients showed GvHD inhibition with no increase in leukemia/lymphoma relapse rates *versus* controls, suggesting that ultra LD IL-2 increased Tregs without impairing GvL.[74] In contrast, when IL-2 ($1\text{x}10^6$ IU/m^2 daily) was administered in a patient seven years post-GvHD onset, therapeutic effects were decreased.[75] The patient experienced partial improvement of GvHD symptoms, Treg levels were increased after one week but declined despite continual IL-2 administration for approximately two months. A clinical trial randomized 90 subjects to determine whether LD IL-2 administration post transplant could reduce the incidence of both leukemia relapse and cGvHD. Patients were treated with LD IL-2 post-HSCT (day 60) for two weeks (followed by a 2-week hiatus) and compared with untreated controls. The treated group had a lower incidence of cGvHD, accompanied by a significant increase in GvHD-free and GvHD progression-free survival at three years, but administration did not decrease the incidence of leukemia relapse. Correlative studies demonstrated that circulating Treg and natural killer (NK) cells were increased in the IL-2 cohort during the treatment periods.[76]

In pre-clinical studies (Figure 2), treatment with LD IL-2C in MHC-matched HSCT recipients transplanted following reduced-intensity conditioning (RIC) regiments, administered either prior or following aHSCT, promoted expansion and activation of host Tregs within the first 7-10 days post transplant. Notably, host-*versus*-graft responses (HvG) were inhibited, resulting in enhanced donor chimerism and long-term hematopoietic cell engraftment.[77] This work indicated for the first time that *in vivo* administration of IL-2C following BMT was a viable approach to expand host Tregs and consequently regulate alloimmunity following transplantation.[78] In contrast, using an MHC-haploidentical aHSCT model, IL-2 administration post transplant for ten days did not prevent GvHD nor improve survival; comparable results were

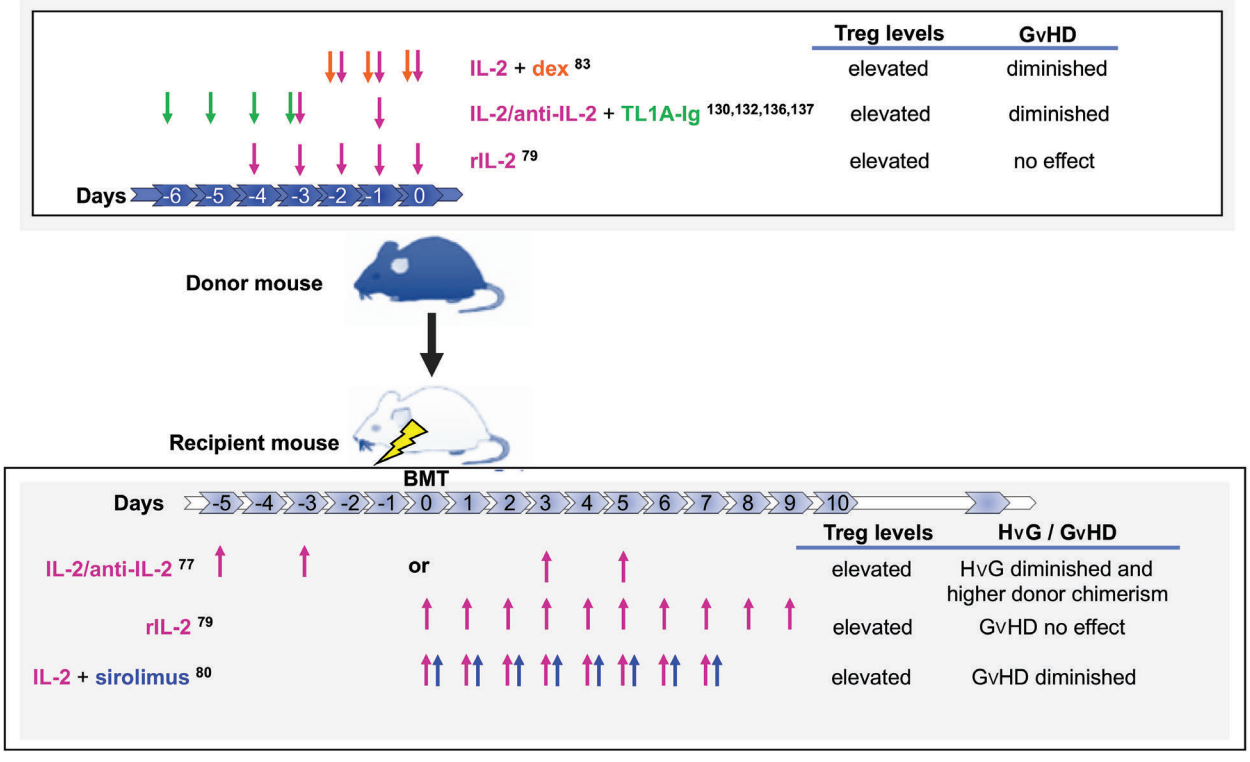

Figure 2. Low-dose IL-2-based treatment of donors / recipients in pre-clinical graft-*versus*-host disease (GvHD) models. IL-2 administration alone or in combination has been extensively utilized in several mouse models to ameliorate GvHD. The success of this strategy relies on the importance of the IL-2/IL-2R signaling pathway on Treg survival, proliferation and suppressive function *in vivo*. HvG: host-*versus*-graft.

observed in a xenogeneic GvHD model.[79] In a murine major-MHC mismatched aHSCT, no effect occurred when treating recipients with LD IL-2 (twice daily) alone. However, the concomitant administration of the mTOR inhibitor sirolimus (0.5 kg/mg daily) in the first week post transplant improved survival and diminished aGvHD. Importantly, *in vivo* treatment with IL-2 plus sirolimus resulted in higher donor Treg expansion *versus* either IL-2 or sirolimus alone. These donor-derived expanded Tregs comprised both expanded natural Tregs and increased conversion of induced Tregs.[80] Initially in a human trial, superior Treg reconstitution and aGvHD prevention was observed in patients treated with the combination of sirolimus and tacrolimus *versus* peri-transplant methotrexate and tacrolimus.[81] In order to improve Treg recovery and based on previous reports, LD IL-2 administration (3 times/wk for 90 days) post transplant was added to the sirolimus/tacrolimus treatment regimen in 20 patients (*clinicaltrials.gov identifier: 01927120*) (*Online Supplementary Table S1*). This phase II trial demonstrated augmented peripheral blood Treg reconstitution in the first month post HSCT in the LD IL-2 (200,000 IU/m^2) plus sirolimus/tacrolimus treated group compared to sirolimus/tacrolimus alone. However, this early Treg expansion was not maintained and did not ameliorate acute or cGvHD.[82] National Institutes of Health clinical trials involving IL-2 and aHSCT are summarized (*Online Supplementary Table S1*).

B. Treatment of donors: pre-clinical and clinical models. Using an MHC-haploidentical aHSCT model, LD IL-2 treatment in donor mice was inadequate as a GvHD prevention strategy. Although IL-2 administration over five days elevated Treg levels in the graft, no improvement in recipient weight loss or survival was observed.[79] These results were attributed to a <1:1 ratio of Tregs:Tconv using a LD IL-2 only Treg expansion strategy. Notably, when donor mice were treated with Dex (5 mg/kg daily) and LD IL-2 for three days, higher Treg levels were observed in comparison to treatment with either reagent alone. In a fully-MHC mismatched aHSCT, mice receiving Dex+IL-2 pre-treated donor cells exhibited improvement in survival and diminution of aGvHD[83] (Figure 2). We are currently not aware of any studies reporting the use of T cells from IL-2 treated HSCT human donors. A recent report noted that treatment of healthy individuals with ultra LD rhIL-2 (50,000-200,000 U/m^2/day) for five days elevated peripheral Tregs with augmented suppressive activity without detection of acute or long-term on/off-target effects.[84]

TNFR super family

The TNF family currently comprises 29 receptors and 19 ligands. In this section, we discuss those members reported to be capable of regulating the Treg compartment *in vivo* including TNFR2, OX-40, 4-1BB and TNFRSF25.[85]

TNFRSF1B (TNFR2)/TNF-α

TNFRSF1B (TNFR2)/TNF-α targeting to manipulate Tregs in vivo. TNFR2 is highly and constitutively expressed on murine Tregs and other immune cells and further up-regulated after activation. It is also found on some endothelial and cells of the nervous system whereas TNFR1 is ubiqui-

tously expressed. TNF-α, the natural ligand, is a pleiotropic cytokine with dual function (pro- and anti-inflammatory) which exists in both membrane-bound and soluble forms. Membrane bound TNF-α preferentially interacts with TNFR2 and results in a suppressive function due to the lack of a cytoplasmic death domain. TNF-α/TNFR1 interactions lead to pro-inflammatory responses.[86,87] TNF-α/TNFR2 interactions reportedly promoted activation and expansion of murine Tregs *in vivo*.[88] This interaction correlates with suppressive function and phenotypic stability of those Tregs.[89] Furthermore, TNF-α expanded Tregs were protective against T1D[90] as well as in the setting of infections[91] and septic shock.[88] Additionally, TNFR2-deficient Tregs lost their capacity to control colitis[92] and EAE.[93] Using a selective agonist to increase binding specificity and target only mouse TNFR2 (TNC-scTNF80) Treg expansion, reduced inflammation in a model of chronic inflammation[94] and established arthritis were reported.[95]

TNFRSF1B (TNFR2)/TNF-α Treg manipulation in pre-clinical allogeneic hematopoietic stem cell transplantation. Serum TNF-α levels are elevated in individuals with GvHD. Due to its pleiotropic nature, this cytokine and the balance between its corresponding receptors modulate both GvHD and GvL. TNF-α *in vivo* selectively enhanced proliferation and activation of Tregs *versus* Tconv and *in vitro* increased Treg CTLA-4 and TGF-β levels.[96] Notably, "unfavorable" (low) numbers of donor TNF-α-primed Tregs (1:10 Treg:Tconv), decreased aGvHD and augmented survival in recipients of a fully MHC-mismatched aHSCT.[96] This diminution of aGvHD promoted by TNF-α primed Tregs was further explored by other investigators demonstrating that this cytokine is produced by Tconv and the effect is dependent on TNFR2 expressed by Tregs.[97] *In vivo* treatment of recipient mice before aHSCT with STAR2, a selective mouse TNFR2 agonist, resulted in expansion of radiation-resistant host Tregs concomitant with a reduction of aGvHD and increased survival. Importantly, STAR2 treatment did not interfere with the transplanted T-cell-mediated GvL or the immune response against infectious opportunistic (cytomegalocirus) pathogens.[98]

TNFRSF4 (OX-40)/TNFSF4 (OX-40-L)

TNFRSF4 (OX-40)/TNFSF4 (OX-40-L) targeting to manipulate Tregs in vivo. OX-40 (CD134) is constitutively and highly expressed on murine Tregs and is up-regulated upon activation on $CD4^+/CD8^+$ Tconv cells and to a lesser extent on NK, NKT cells and neutrophils. OX40 ligand, (OX-40-L, CD252), is expressed on a number of different cell types including activated professional APCs, activated T cells, NK cells, mast cells and endothelial cells.[99] A role for OX-40 in the development, homeostasis and suppressive activity of Tregs has been implicated from studies using young knockout (KO) $OX\text{-}40^{-/-}$ mice.[100,101]

The role of OX-40 on Treg expansion and function remains controversial. The use of agonistic anti-OX-40 mAb (OX-86) or APCs over-expressing OX-40L resulted in clear but weak proliferation and expansion of mouse Tregs *in vivo* and enhanced suppressive capacity in models of colitis and EAE.[100-104] Addition of IL-2 together with anti-OX-40 mAb further amplified Treg expansion as well as suppressive activity in a heart transplant model.[105] However, other studies where clone OX-86 was administered failed to induce Treg expansion and found inhibition of Treg function in models of skin grafts and cancer.[106,107]

TNFRSF4 (OX-40)/TNFSF4 (OX-40-L) manipulation in pre-clinical allogeneic hematopoietic stem cell transplantation. Two decades ago, inhibition of the OX-40/OX-40-L axis was found to diminish murine lethal aGvHD in recipients post aHSCT.[108] Indeed, infusion of pre-treated Tregs with OX-86 or intraperitoneal (i.p.) injection of OX-86 resulted in the inhibition of Treg suppressive activity on GvHD development.[109] Additionally, when an OX-40L blocking antibody (KY1005) was combined with sirolimus in a non-human primate GvHD model, synergistic inhibition of T-cell activation while preserving post-HSCT Treg reconstitution was observed.[110] Importantly, KY1005/sirolimus GvHD prophylaxis resulted in long-term GvHD-free survival and significant control of aGvHD.[110] Interfering with the OX-40L/OX-40 pathway in the setting of aHSCT did not reduce Treg reconstitution although impairment of this pathway resulted in impaired Treg development in young mice.[100,101]

TNFRSF9 (4-1BB)/TNFSF9 (4-1BB-L)

TNFRSF9 (4-1BB)/TNFSF9 (4-1BB-L) targeting to manipulate Tregs in vivo. Analogous to OX-40, 4-1BB (CD137) is constitutively expressed on Tregs and is up-regulated upon activation on CD4 and CD8, B, NK and myeloid cells. CD137-L is the only known intercellular ligand for CD137 and is expressed on APC after activation, although the extracellular domain of 4-1BB binds to fibronectin and galectin-9. 4-1BB co-stimulatory activity is well appreciated to promote proliferation and survival of CD8 T cells.[111,112] However, this pathway can also induce Treg proliferation *in vivo*, as shown by experiments wherein Tregs were coated with 4-1BBL-Fc prior to infusion.[113] Accordingly, agonistic anti-4-1BB mAb was shown to enhance the numbers of Tregs and ameliorate or inhibit disease in several experimental models of autoimmunity including, T1D,[114] colitis,[115] EAE,[116] and psoriasis.[117]

TNFRSF9 (4-1BB)/TNFSF9 (4-1BB-L) manipulation in pre-clinical allogeneic hematopoietic stem cell transplantation. Several laboratories have investigated the implication of 4-1BB/4-1BBL interactions in GvHD and GvL. 4-1BB/4-1BB-L interactions increased GvHD-induced lethality and allogeneic bone marrow rejection mediated by either $CD4^+$ or $CD8^+$ donor T cells. Moreover, treatment with agonistic anti-4-1BB mAb augmented GvL effects of delayed lymphocyte infusion in an acute myeloid leukemia (AML) model by stimulating an allogeneic anti-tumor response.[118] Blockade of 4-1BB-L in F1 mice ameliorated aGvHD, but aggravated cGvHD with high levels of IgE and anti-dsDNA IgG autoantibody.[119] Furthermore, stimulation of 4-1BB using an agonistic mAb prevented cGvHD by inhibition of autoantibody production through activation induced donor $CD4^+$ T-cell death accompanied by diminished host B-cell activation and decreased autoantibody production.[120] As noted above, it has been demonstrated that stimulation of 4-1BB promotes *in vivo* Treg proliferation and suppressive activity.[113] Following anti-4-1BB mAb (3H3) *in vivo* treatment, IL-2 production by memory T cells was increased resulting in the induction of Treg expansion in an Ag-independent manner.[121] Notably, in this haploidentical (parent-into-F1) aGvHD model, host Tregs survived long term. Furthermore, pre-conditioning with anti-4-1BB mAb ameliorated GvHD by increasing Treg suppressive activity against alloreactive donor T cells. When anti-4-1BB

mAb-primed host Tregs were adoptively transferred into BDF1 mice, less severe disease was observed.[121]

TNFRSF25 (DR3)/TNFSF15 (TL1A)

TNFRSF25 (DR3)/TNFSF15 (TL1A) targeting to manipulate Tregs in vivo. TNFRSF25 is constitutively expressed on Tregs and up-regulated upon activation on $CD4^+$ and $CD8^+$ T cells. Low levels are also present on NK, NKT cells and ILC2/3 subsets. Its natural ligand, TL1A, is primarily expressed on endothelial cells and upon activation on APC and T cells, including Tregs.[122,123] TNFRSF25 co-stimulation promotes proliferation, effector function and survival, as well as apoptosis depending on signal strength.[124,125]

Recent studies, using either agonistic mAb (clone 4C12) or a TL1A-Ig fusion protein, have shown that triggering the TNFRSF25 pathway in the absence of antigen leads to a significant expansion of Tregs *in vivo* which is dependent on the TCR and IL-2.[126,127] Notably, TNFRSF25 stimulation expands Tregs to a greater degree than CD25 stimulation (*via* IL-2) alone. Moreover, TNFRSF25 stimulation alone has the strongest *in vivo* effect on Treg expansion compared to other TNFRSF members including GITR, OX-40 or 4-1BB.[126] Stimulating multiple receptors on Tregs can have synergistic effects, though the levels of expansion achieved depend on the molecular targets. Thus, targeting two TNF family members, TNFRSF25 (4C12) and OX-40 (OX-86)[128,129] in the context of co-stimulation or vaccination led to increased frequency of Tregs *versus* either alone. However, when both TNFRSF25 and CD25 are stimulated,[130] there is a marked elevation of the compartment, much greater than administration of TL1A-Ig or IL-2 alone or 4C12 together with OX-86. It is likely that the former is observed because sufficient IL-2 is provided to maintain the elevated Treg levels.

TNFRSF25-induced expansion, either alone or in combination with IL-2, leads to upregulation of activation markers on Tregs and enhanced suppressive activity.[131,132] Our own studies have demonstrated that ICOS-1, Nrp-1, PD-1 and other molecules are more highly expressed after combined TNFRSF25 and CD25 *in vivo* stimulation compared with targeting the individual receptors.[132] Tregs expanded *in vivo via* the TNFRSF25 are protective against allergic lung inflammation and EAE.[126,127,133] Furthermore, they also prolonged graft survival in a mouse model of heterotopic allogeneic heart transplantation.[134]

TNFRSF25 (DR3)/TNFSF15 (TL1A) manipulation in preclinical allogeneic hematopoietic stem cell transplantation. As noted above, TNFRSF25 stimulation using 4C12 or TL1A-Ig selectively and dramatically promotes *in vivo* Treg expansion. Single administration of 4C12 resulted in a significant increase in splenic and lymph node Treg numbers with enhanced suppressive function.[135] In a major MHC-mismatched GvHD model, recipients of 4C12-treated donor T cells showed a significant increase in overall survival and diminished GvHD.[130,135] Importantly, T cells from 4C12-treated donors exhibited preserved graft-*versus*-tumor (GvT) activity.[135] Expansion of Tregs with 4C12 treatment induced phenotypic changes in Tregs, including higher expression of activation and maturation molecules.[131] Mice treated with purified 4C12-Tregs ($CD4^+CD25^+$) showed diminished GvHD with improved survival, demonstrating that these Tregs possessed higher *in vivo* suppressive activity.[131] Notably, administration of 4C12 into recipients with ongoing GvHD augmented mortality and worsened the disease because upon alloantigen exposure TNFRSF25 stimulation induced effector T-cell proliferation and activation.[131] However, prophylaxis of recipients with 4C12 induced host-derived Treg expansion with a reduction in GvHD severity.[131] Critically, the success of this approach to prevent GvHD is dependent on the timing of TNFRSF25 stimulation and the status of donor T-cell activation.

Because of the success of IL-2 in pre-clinical and clinical GvHD studies and the expression of TNFRSF25 in the Treg population, we developed a strategy to transiently manipulate Tregs *in vivo* combining LD IL-2C with TL1A-Ig. This "two-pathway" approach markedly expands (5-7-fold) and selectively activates Tregs. Transplantation of TL1A-Ig/IL-2C Treg expanded donor spleen into recipients resulted in amelioration of GvHD severity in both fully MHC-mismatched and MHC-matched aHSCT settings[130,132] (Figure 2). *In vivo* Treg expansion was superior with TL1A-Ig/IL-2C treatment compared to 4C12 mAb administration and GvHD was more completely ameliorated after transplant of two-pathway expanded donor Tregs. Furthermore, recipients of transplants using spleen cells from TL1A-Ig/IL-2 expanded donors demonstrated preserved GvL while GvHD was effectively diminished.[130] In fact, because TL1A-Ig/IL-2 expanded Tregs expressed higher activation and functional molecules, very low numbers of these expanded cells (corresponding to a ratio of 1:6 expanded Tregs/Tconv) very effectively suppress GvHD post aHSCT.[132] Furthermore, TL1A-Ig/IL-2 expanded Treg therapy was shown to be as effective as post-transplant cyclophosphamide for GvHD prophylaxis while more rapid thymic reconstitution providing earlier recovery of recipient immune function.[136] GvHD is promoted by alloreactive donor T cells and inflammation; therefore, we proposed to regulate both pathways. Donor Treg expansion was combined with EP11313 (a bromodomain and extra-terminal bromodomain inhibitor, BETi) using short-term treatment in the recipient in a fully MHC-mismatched aHSCT. This strategy was found to significantly reduce early GvHD clinical scores including decreased ocular and skin involvement. Importantly, utilizing highly purified TL1A-Ig/IL-2 expanded donor Tregs *in vivo*, this second assessment of the combinatorial strategy further supported the notion that selective BETi can be used for GvHD treatment in combination with Treg adoptive therapy.[137]

CD28/CD80/86 (B7.1/2)

CD28/CD80/86 (B7.1/2) targeting to manipulate Tregs in vivo. CD28, a key co-stimulatory molecule, is expressed on all T cells and provides signals for activation and survival. Its ligands, CD80 and CD86, are expressed on APC. CD28 super-agonists (CD28SA) are mAbs which induce polyclonal T-cell proliferation in the absence of TCR ligation.[138,139] In rodents, *in vivo* application of CD28SA at low doses efficiently expands Tregs and partially inhibits expansion of autoreactive T cells. In addition, CD28SA expanded Tregs showed enhanced suppressive activity (increased IL-10 production) and migrated to inflamed tissues without causing cytokine release syndrome.[139-142] Treg expansion by CD28SA is dependent on paracrine IL-2 from CD28SA-stimulated Tconv cells.[142] Accordingly, CD28SA expanded Tregs are highly effective in the treatment/prevention of disease in rodent models of autoimmunity, like EAE,[141] experimental autoimmune neuritis[143]

and T1D,[144] as well as inflammation, e.g. arthritis,[145,146] glomerulonephritis[147] and trypanosomiasis-associated inflammation.[148] Furthermore, CD28SA expanded Tregs prolong graft survival in experimental models of solid organ (renal/heart) transplantation.[149,150]

CD28/CD80/86 (B7.1/2) manipulation in pre-clinical allogeneic hematopoietic stem cell transplantation. A single dose of CD28SA (clone JJ316) administration *in vivo* to Lewis rats induced a selective 4-fold expansion of Tregs over Tconv. Adoptive transfer of anti-CD28 mAb treated splenocytes in a rat transplantation model reduced lethality and suppressed GvHD. Importantly, this therapeutic effect was mediated by expanded Tregs detected in high levels after transfer of anti-CD28 mAb-treated lymphocytes.[151] Moreover, this mAb was utilized to treat F1 rat recipients at different time-points pre- or post aHSCT. GvHD mortality was absent or suppressed when recipients were treated with anti-CD28, three days before T-cell transfer or on day 0, respectively. In contrast, no protective effect was observed in recipients treated on day 7 or 10. Importantly, GvHD lethality reduction was mediated by anti-CD28SA selective Treg expansion in an antigen-specific manner.[152] In a murine major-MHC mismatched aHSCT model, adoptive transfer of unfractionated polyclonal activated donor Tregs, with CD28SA (D665) significantly reduced GvHD-associated clinical signs and histopathological changes. Notably, this mAb administered to recipients or *in vitro* pre-stimulated donor T cells conserved a potent anti-lymphoma (GvL) response.[153] The CD28SA *in vitro* approach may have clinical potential for patients because an anti-human CD28 mAb (TGN1412) induced a cytokine storm during the first in human clinical study.[154]

IL-33/ST2

IL-33/ST2 targeting to manipulate Tregs in vivo. IL-33, an IL-1 family member, is constitutively expressed in epithelial and endothelial cells at barrier sites where it functions as an endogenous danger signal in response to tissue damage. IL-33 is also a pleiotropic cytokine found in fibroblastic reticular cells of secondary lymphoid organs. It binds to the ubiquitously expressed IL-1R accessory protein (IL-1RAcP) and the more selectively expressed receptor ST2. The ST2 receptor is constitutively expressed on innate (mast cells, ILC2s, eosinophils, basophils, NK cells) and adaptive immune cells (Tregs, Th2, NKT) and up-regulated upon activation on Th1 and cytotoxic T cells. A soluble form of ST2 can be produced by alternative splicing and serves as a decoy receptor to limit IL-33 signaling.[155,156] Notably, the quantitative differences in ST2 expression among different T-cell subsets potentially could lead to competition for IL-33. Tissue Tregs, which express constitutively high levels of ST2, could therefore sequester IL-33 from inflammatory cells which would give them an advantage over effector T cells in situations of limited IL-33. Soluble ST2 released from intestinal tissue has recently been identified as an important biomarker of GvHD that is highly predictive for early post-HSCT mortality.[157]

Several reports have shown that administration of IL-33 leads to a ST2 dependent expansion of Tregs *in vivo*.[158,159] $ST2^+$ Tregs represent an activated subset of Tregs and are preferentially expressed in non-lymphoid tissues, like lung (20-30% of Treg), gastrointestinal tract (GI) (approx. 20%), and liver (50-60%).[160-162] $ST2^+$ Tregs exhibit superior suppressive function compared to $ST2^-$ Tregs.[161] In a model of allogeneic heart transplantation, IL-33 expanded $ST2^+$ Tregs migrated to the graft and prolonged survival.[163,164] Furthermore, in a model of collagen induced rheumatoid arthritis, IL-33 expanded $ST2^+$ Tregs suppressed clinical and histological signs of arthritis.[165] In addition, IL-33 signaling is apparently crucial for liver Treg expansion, accumulation and suppression of infection after murine CMV infection.[166] A combination of IL-33 together with IL-2 administration has a synergistic effect with regard to Treg expansion.[56] This group also generated a hybrid fusion protein between those 2 cytokines, IL-233, which bears the activities of both. IL-233 treatment significantly increased the number of Tregs in blood, spleen and renal compartments and prevented ischemia reperfusion injury more efficiently than a mixture of IL-2 and IL-33.

IL-33/ST2 manipulation in pre-clinical allogeneic hematopoietic stem cell transplantation. In aHSCT, the IL-33/ST2 axis has recently emerged as a novel therapeutic target for GvHD. In particular, high levels of IL-33 and ST2 were detected in recipients post conditioning, contributing to lethal aGvHD *via* donor TH1 alloimmune responses.[167] Therefore, transplants involving either $IL\text{-}33^{-/-}$ recipients, $ST2^{-/-}$ donor T cells or IL-33 antagonist (ST2-Fc fusion protein) administration resulted in increased recipient survival.[167] Conversely, *in vivo* administration of peri-aHSCT IL-33 (day -10 to day 4) to recipients induced a diminution of clinical GvHD scores and prolonged survival. This protection against GvHD was promoted by IL-33-expanded recipient $ST2^+$ Tregs (persisted after total body irradiation) which controlled M1 macrophage activation and reduced effector T-cell levels. The underlying mechanism of IL-33/ST2-induced Treg proliferation was mediated by activation of p38 MAPK.[168] Subsequent studies found that transfer of $ST2^{-/-}$ donor Tregs affect diminished protection of GvHD after an MHC-mismatched aHSCT.[167] We are unaware of any ongoing clinical trials regulating the IL-33/ST2 pathway *in vivo* to manipulate Tregs.

"Unintentional" effects on the Treg compartment: potential impact in hematopoietic stem cell transplantation

While direct targeting of molecules on the Treg cell surface can induce their expansion, unintentional but clinically useful procedures in HSCT including extracorporeal photopheresis (ECP),[169] donor stem cell mobilization,[170-172] PTCy,[173,174] azacytidine,[175] JAK1/2 inhibitors[176,177] and ROCK1/2 inhibitors[178,179] also affect the Treg compartment although to a lesser extent than intentional interventions. Although Tregs have demonstrated promising results in regulating GvHD, combining their regulatory activity with other strategies being used in the clinic may further improve transplant outcomes for patients. Therefore, if strategies partially diminish or do not interfere with Tregs, augmenting the compartment should be useful. For example, in addition to alloreactive Tconv deletion, Tregs are reportedly needed for optimal PTCy (day 3-4) GvHD prophylaxis.[180,181] Treg expansion subsequent to PTCy may, therefore, augment the effectiveness of this reagent. In contrast, if strategies elevate Tregs this should provide additional benefit and further enable synergistic expansion. For example, azacytidine (AzaC), like PTCy, preferentially inhibits Teff *versus* Treg proliferation, but additionally converts Teffs to Tregs *via* hypomethylation of the Foxp3 promoter.[175] Pre-clinical and clinical studies have

demonstrated that post ECP, Treg levels in mouse and humans are elevated, suggesting that these effects on the Tregs might be a primary mechanism underlying the relative success of this clinical approach.[169] Regarding stem cell mobilization, studies in primates and healthy HSCT donors showed an increase in Treg frequency using plerixafor.[171,172] Development of balanced JAK1/JAK2 targeted inhibitors has led to promising clinical results in aHSCT.[176] Notably, baricitinib is significantly more potent than ruxolitinib in preventing GvHD and demonstrates high Treg expansion by preserving the JAK3-STAT5 IL-2R pathway.[177] Additionally, the Rho kinase inhibitors which down-regulate ROCK1/ROCK2 inhibiting inflammation have also shown promise in suppressing GvHD.[178,179] ROCK2 inhibition leads to pSTAT5 upregulation inducing an increase in Tregs.

Cellular "cross-talk" with Tregs can also result in an expansion of this population. For example, host iNKT cells were shown to induce *in vivo* donor Treg proliferation *via* IL-4 while preventing lethal aGvHD in mice.[182] Subsequently, reports showed that donor iNKT cells diminished aGvHD and cGvHD through the expansion of donor Tregs.[183,184] These approaches are currently being examined in clinical trials of α-galactosylceramide, which has been shown to increase numbers of Tregs in model systems by expanding iNKT cells.[185]

Future perspectives

Approaches manipulating Tregs *in vivo* continue to advance. Systemic administration of IL-2 and other compounds can elevate the peripheral compartment for extended time-periods without apparent alteration of global immune function. Notably, not all reagents have equivalent efficacy in inducing and maintaining Treg expansion, which is a result of the specific receptor targeted and reagent persistence. For example, administration of modified IL-2 compared with free IL-2 results in greater Treg frequency in large measure due to prolonging the half-life of the cytokine.[45,180,186] This has fostered increasing numbers of novel IL-2 constructs including fusokines, muteins and IL-2/receptor fusion proteins.[54,181,187,188] A recent study reported human IL-2 complexed to a human anti-IL2 mAb with *in vivo* enhancing activity.[189] The development of highly CD25 specific signaling reagents with increased persistence suggests *in vivo* delivery to selectively target Tregs *versus* Tconv may lead to more effective therapeutic application during disorders where effector cells are present.

To date, approaches targeting Tregs have focused primarily on systemic (*vs*. local) homeostasis. Local manipulation of Tregs might provide an effective strategy for the treatment of widespread (systemic lupus erythematosus, etc.) as well as regional (e.g. GI diseases, encephalitis) inflammation. Treg expansion in the conjunctiva/ocular adnexa can be induced targeting CD25,[41] or more potently through CD25 and TNFRSF25 (*Copsel et al., unpublished data, 2019*). Such strategies may be useful to treat ocular GvHD and uveitis. In the context of HSCT, conditioning using targeted total lymphoid irradiation promotes selective survival of Tregs locally, for example the GI. Subsequent administration of reagents may therefore "selectively" manipulate local intestinal Tregs which could be particularly effective in inhibiting GvHD.[190,191]

Allogeneic hematopoietic stem cell transplantation is primarily administered to patients with hematologic malignancies. There has been increasing usage of checkpoint inhibitors (CI) including PD-1 for the treatment of these cancers and their effect on Tregs remains controversial. Studies have suggested that the PD-L1/PD-1 pathway drives Treg stability/function and expansion.[6,192,193] while others have reported Treg inhibition.[194,195] Clinical improvement of experimental GvHD was associated with increased PD-1 levels on Tregs consistent with the hypothesis that this pathway promotes Treg-mediated tolerance.[196] Stimulation of PD-1 on Tregs in hematologic tumors have been reported to promote the inhibition of effector T cells, reducing anti-tumor immune responses.[193] Although early reports noted that severe GvHD may result from administering pembrolizumab post HSCT in patients with hematologic malignancies,[197] evaluation using CI after PTCy deletion of alloreactive T cells should be considered. In addition to promoting CD8 anti-tumor specific T cells, the success of this therapeutic approach may also depend on direct effects of PD-1 inhibitors on Tregs, since increasing or decreasing their numbers/function could positively or negatively modulate anti-tumor immunity. Taken together, since no single prophylactic treatment including adoptive Treg transfer is likely to abolish GvHD, we suggest combining *in vivo* Treg expansion strategies with promising reagents being translated to clinical aHSCT is feasible and could provide a significant advance in the field.

Conclusions

The Treg compartment can now be manipulated *in vivo* as a consequence of intentionally targeting identified receptors. Pre-clinical and clinical studies contributed by a large number of laboratories have directly targeted these cells and reported the ability to efficiently augment their numbers and function *in vivo*. While there is an increasing number of molecules which effectively expand Tregs, for the moment IL-2 is the only FDA-approved compound in use in GvHD clinical trials. It is not surprising that efforts to improve *in vivo* IL-2 efficacy through the generation of modified IL-2 molecules are currently underway. Other strategies stimulating different signaling pathways promoting Tregs have shown promise in preclinical models. Targeting TNF receptors for example, TNFRSF25 alone or together with LD IL-2 has demonstrated extremely potent Treg expansion and improved function. Combination approaches should, therefore, be investigated for potential clinical application.

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