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Advances in Targeting Co-inhibitory and Co-stimulatory Pathways in Transplantation Settings: The Yin to the Yang of Cancer Immunotherapy

Leslie S. Kean¹, Laurence A. Turka^{2,*}, and Bruce R. Blazar^{3,*}

¹Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, The Fred Hutchinson Cancer Research Center and the Department of Pediatrics, University of Washington, Seattle Washington

²Center for Transplantation Sciences, Department of Surgery, and Immune Tolerance Network, Massachusetts General Hospital and Harvard Medical School, Boston, MA

³Division of Blood and Marrow Transplantation, Department of Pediatrics and the Masonic Cancer Center, University of Minnesota, Minneapolis, MN

Summary

In the past decade, the power of harnessing T cell co-signaling pathways has become increasingly understood to have significant clinical importance. In cancer immunotherapy, the field has concentrated on two related modalities: First, targeting cancer antigens through highly activated chimeric antigen T cells (CAR-Ts) and second, re-animating endogenous quiescent T cells through checkpoint blockade. In each of these strategies, the therapeutic goal is to re-ignite T cell immunity, in order to eradicate tumors. In transplantation, there is also great interest in targeting T cell co-signaling, but with the opposite goal: in this field, we seek the Yin to cancer immunotherapy's Yang, and focus on manipulating T cell co-signaling to induce tolerance rather than activation. In this review, we discuss the major T cell signaling pathways that are being investigated for tolerance-induction, detailing pre-clinical studies and the path to the clinic for many of these molecules. These include blockade of co-stimulation pathways and agonism of coinhibitory pathways, in order to achieve the delicate state of balance that is transplant tolerance: a state which guarantees lifelong transplant acceptance without ongoing immunosuppression, and with preservation of protective immune responses. In the context of the clinical translation of immune tolerance strategies, we discuss the significant challenge that is embodied by the fact that targeted pathway modulators may have opposing effects on tolerance based on their impact on effector versus regulatory T cell biology. Achieving this delicate balance holds the key to the

*Correspondence to: Laurence A. Turka, Center for Transplantation Sciences, 149 13th Street, Room 5101, Boston, MA 02129 lturka@partners.org Phone: 617 724-7740. Bruce R. Blazar, University of Minnesota, MMC 366, 420 SE Delaware St, Minneapolis, MN 55455; blaza001@umn.edu Phone 612 626-1926.

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major challenge of transplantation: lifelong control of alloreactivity while maintaining an otherwise intact immune system.

Keywords

Transplantation; Co-stimulation Blockade; Co-inhibitory Blockade

Introduction

The last five years have witnessed tremendous progress in cancer immunotherapy. This has included advances in both antibody- and cell-based therapies, with the success of each of these modalities significantly reliant on their ability to regulate co-stimulatory and co-inhibitory signaling to optimize T cell anti-tumor responses. The prevailing strategies for controlling and enhancing T cell signaling for cancer eradication have focused on modulating both sides of the signaling equation, to enhance T cell activation. Thus, they have harnessed co-stimulatory signaling to enable antigen-driven activation of chimeric antigen receptor (CAR)-T cells, and blocked coinhibitory signaling to re-engage hypofunctional T cells in the tumor microenvironment. In stark contrast to the typically suppressive tumor microenvironment, transplanted allogeneic or xenogeneic tissues or cells can create a pro-inflammatory environment resulting in graft injury or loss for tissue transplants, and graft-versus-host disease (GVHD) or donor cell rejection for donor hematopoietic lineage cell infusions. If the strategy surrounding cancer immunotherapy is focused on harnessing the Yang—the optimized activation and ‘heat’ in T cells, then in transplantation, we seek the ‘cooling’ Yin, in focusing on controlling these same co-stimulatory and co-inhibitory pathways, towards the goal of inducing antigen-specific tolerance in T cells rather than T cell assault.

In what follows we will review the work in the field of transplant tolerance, highlighting the many successes that have been achieved by targeting T cell costimulatory and co-inhibitory pathways, and discuss the challenges that we still face on the road to the ultimate goal of tolerance-on-demand for all transplant patients. In discussing successes and challenges, we will examine the complexity of manipulating T cell co-signaling, given that both conventional, effector T cells (Teffs) are targeted by these approaches, along with regulatory T cell (Treg) populations, for which these manipulations may have opposing biologic effects and, hence, transplantation tolerance outcomes. The search for the ideal pathway, which, when manipulated, could simultaneously tilt the balance towards tolerance in both Teffs and Tregs, remains one of the holy grails of our field of inquiry.

Transplantation tolerance is defined as the lifelong acceptance of transplanted organs or cells, in the absence of any immunosuppressive medications, and with preservation of protective immunity. Immune tolerance thus has three major components, all which must co-exist in transplant patients. Each is critical, and all are inter-related, thus raising the bar needed for achieving transplantation tolerance, especially in large animal models and patients. Efforts to blunt co-stimulation pathways in order to accomplish this goal began in earnest over two decades ago, when CTLA4Ig was first synthesized for the purpose of

precluding a productive immune response in antigen-activated T cells. (1–4) These seminal studies demonstrated that this new way of interfering with T cell co-signaling could potentially down-regulate T cell and antibody responses to xenogeneic (xeno) and allogeneic (allo) tissues and were heralded in the lay press as having reliably achieved the holy grail of tissue transplantation tolerance. Individual costimulatory pathway blockade reports were closely followed by a series of important publications with combination therapy. These included a 1996 study by Dr.

Christopher Larsen and colleagues that demonstrated that the coordinated blockade of B7 signaling (through CTLA4Ig) and CD40/CD154 signaling (through an anti-CD154 monoclonal antibody) could produce long-term tolerance to concurrently transplanted skin and cardiac allografts, while preserving the recipient's ability to reject non-donor allografts transplanted after co-stimulatory pathway blockade had been discontinued.(5) Such reports resulted in the birth of combinatorial co-stimulation blockade for generating antigen-specific graft tolerance, a paradigm that continues to be vigorously pursued in the present day. In what follows we provide an overview of this field, as it relates to immune tolerance induction, and discuss the relevant pathways, cells and clinical applications, highlighting where we've been, and where we need to go to make immune tolerance a reality for the patients we treat.

Simultaneous blockade of B7/CD28 and B7/CTLA-4: Confronting the double edge sword of co-stimulation and co-inhibition

The field of co-stimulation blockade began with the search for molecules that paired with antigen-specific TCR stimulation to optimize T cell activation after cognate antigen recognition. To accomplish this, researchers used agonistic monoclonal antibodies directed at newly identified T cell surface molecules, and identified the immunoglobulin (Ig)-superfamily member CD28 as a critical T cell co-signaling molecule which was required to optimally initiate, amplify and sustain T cell responses.(6) Subsequent studies demonstrated that there were two distinct CD28 ligands, CD80 (B7-1) and CD86 (B7-2) that were expressed as dual coreceptors on antigen presenting cells (APC).(7–11) While this was a central discovery, the complexities of signaling through this pathway and its surrogates is still incompletely understood, yet key to ongoing efforts to target the CD28 pathway to control T cell activation.

The first reagent used to inhibit CD28 signaling was developed by Linsley and colleagues, who developed a soluble CD28Ig, reasoning that it could block CD28 signaling by providing an alternative binding site for CD28 ligands.(12) While the data supported this molecule as the first 'costimulatory blockade' reagent, its affinity for the ligand(s) of CD28 was relatively low such that *in vivo* experiments (and clinical applications) with this reagent were not sufficiently encouraging for further testing. At this time, a molecule with molecular similarity to CD28, named cytotoxic T lymphocyte antigen-4 (CTLA-4), now also known as CD152, was discovered by Dr. Pierre Goldstein at the Pasteur Institute.(13, 14) CTLA-4 was first presumed to act as stimulator of T cell activation. However, further studies by several laboratories subsequently showed that, while CTLA-4 was upregulated during T cell

activation, the signal delivered by CTLA-4 engagement functioned as a negative, rather than positive regulator of T cell function.(15, 16)

Given the shared structure between CTLA-4 and CD28 as well as B7 ligand binding (and prior to its unequivocal identification as a negative regulator of T cell function),(14) a CTLA4Ig fusion protein, consisting of the extracellular domain of CTLA-4 fused to an IgG tail (to prolong its half-life), was developed, with the hopes that CTLA4Ig mediated blockade of positive T cell co-signaling would dominate over blocking the CTLA-4 inhibitory pathway. Dr. Peter Linsley and colleagues indeed showed that CTLA4-Ig was capable of binding B7 at clinically-relevant concentrations and in so doing, inhibited T cell allo-proliferation and T-dependent B cell antibody production.(4) This breakthrough discovery ushered in the era of T cell modulation for clinical control of undesired, aggressive donor and host T- and B- cell immune responses that precluded transplantation tolerance. As briefly mentioned above, in vitro characterization studies with CTLA4Ig were quickly followed with demonstrations of the ability of CTLA4Ig to modulate allo- and xeno-immunity in vivo, including producing striking prolongation of xeno-islet graft survival in mice.(2, 3, 17) While further studies in mice, non-human primate (NHP) and patients have indicated that this agent is not capable of producing tolerance, these first *in vivo* studies were striking in their demonstration of the impact of this first targeted co-stimulation blockade approach in small animal model systems.

Given the ability of CTLA4Ig to impact both T and B cell function, it was seen as a potentially important new therapeutic for auto- as well as allo- immune indications. Murine studies demonstrated striking activity in models of lupus-like disease(18) and collagen-induced arthritis,(19) and more variable results against murine experimental allergic encephalitis (EAE), a preclinical model of a multiple sclerosis (MS)-like disease.(20) The result in EAE is noteworthy in that it foreshadowed future clinical observations with CTLA4Ig in renal transplant (and may be related to the impact that this molecule has on Tregs, discussed in detail below) in that it found that higher doses of CTLA4Ig worsened, rather than improved results with this agent against EAE.(20) The results in murine models of lupus, and especially arthritis, spurred the initial clinical trials of CTLA4Ig, which focused on patients with psoriasis(21) and rheumatoid arthritis (RA). In a series of Phase II and Phase III trials and now with over a decade of follow-up, CTLA4Ig (known as abatacept and marketed as Orencia™) has shown significant clinical activity for patients with RA,(22–26) and was the first FDA-approved co-stimulation blockade agent, approved for use in RA in 2005. Given its significant activity in RA, it is somewhat surprising that CTLA4Ig has not developed a larger sphere of clinical indications, with trials in MS, asthma, Type I diabetes, ulcerative colitis, and lupus not yielding significant enough clinical improvement to lead to efforts to obtain FDA approval for those diseases(27–31) especially in the context of other therapeutic options available for treatment of these diseases. Whether the suboptimal clinical results are due to an unwanted impact of abatacept on CTLA4-mediated co-inhibitory signaling, untoward effects on Tregs in these diseases, or other causes has not been determined. Despite the lack of widespread clinical applications in autoimmunity, the success of abatacept in RA patients supported continuing clinical investigation of B7 blockade with CTLA4Ig for other indications, especially solid organ and hematopoietic stem cell transplantation (HCT). In HCT, our work and that of others in

murine and NHP models suggested that abatacept has biologic activity in preventing GVHD. (32–37) This led to a promising first-in-disease feasibility trial (38) which demonstrated the pharmacodynamic impact of abatacept in controlling T cell proliferation early post-transplant, providing the foundational data for our ongoing Phase II study designed to evaluate the efficacy of abatacept in preventing severe GVHD after either mismatched or matched unrelated donor HCT (Clinicaltrials.gov # NCT01743131).

In solid organ transplant, although the abatacept formulation of CTLA4Ig showed significant promise in murine models, (39–51) including evidence for a critical role in immune tolerance when combined with CD154-based co-stimulation blockade (discussed in detail below), initial studies in NHP renal transplant models were underwhelming. (52, 53) These results may have been mechanistically linked to the fact that CD28 and CTLA-4 compete for B7 ligands (CD80 and CD86) that are required for CD28 activation, and that the interaction of CD80 with both CD28 and CTLA-4 appears substantially better than that of CD86. Because abatacept has decreased affinity for CD86 compared to CD80, CD80/CD86 interactions with CD28 and CTLA-4 may be incompletely blocked with the abatacept formulation of CTLA4Ig. (12) Therefore, the group at Bristol-Myers Squibb, in collaboration with Larsen's group at the Emory Transplant Center decided to create directed mutations in the CTLA4Ig molecule designed to increase its affinity for CD86. (52) The resulting formulation, differing in 2 amino acids from abatacept (known first as LEA29Y and subsequently as belatacept, and now marketed as Nulojix™), increased both affinity and avidity of CTLA4Ig towards both B7 molecules. In so doing, belatacept resulted in increased blockade of both CD28- and CTLA-4 mediated T cell activation *in vitro* and prolonged allograft survival in NHP models when compared to abatacept. (48, 52) A subsequent series of Phase II and Phase III trials in renal transplant patients demonstrated that belatacept led to improved long-term renal functional outcomes after transplant and fewer off-target toxicities. (54) These results have been verified in long-term follow-up studies. (55–57)

Two important observations from the belatacept trials point to important biological complexities of targeting T cell co-stimulation using CTLA4Ig: First was the observation that patients treated with a lower-intensity regimen of belatacept actually had superior graft outcomes than those treated with a higher-intensity regimen. (54) Second was the observation that while overall results were improved with belatacept, patients treated with this drug experienced higher rates of early (reversible) rejection events. (54–57) There are two potential explanations for these observations: First, is the fact that belatacept, like abatacept, not only targets CD28 positive co-signaling but also targets CTLA-4-based co-inhibition. CD28 is a highly expressed but low-affinity receptor, in contrast to CTLA-4, which is in low abundance but is a higher-affinity receptor. At high *in vivo* concentrations, both CD28 and CTLA-4 would be blocked, whereas at lower *in vivo* concentrations, CTLA4Ig would preferentially block the high abundant CD28 receptor and restricting available ligand for the low abundance CTLA-4 receptor. CTLA-4 has a clear regulatory function as evidenced by CTLA-4 knockout mice. (58) These mice develop fatal lymphoproliferative disease at 3–4 weeks of age, suggesting a key role for CTLA-4 in maintaining self-tolerance. By restricting available ligand for the low abundance CTLA-4 receptor at low CTLA4Ig concentrations, tolerance induction induced by CTLA-4 signalling remains more intact than the co-stimulatory CD28 pathway. (59) This points to the knives-

edge that many of these studies may be on, in terms of the balance of CTLA4Ig being a co-stimulation *blocker* or *enhancer*, dependent upon the relative effects on co-stimulatory CD28 and co-inhibitory CTLA-4 pathways, which could lead to disease-specific impacts on efficacy given differential reliance on CD28 versus CTLA-4 signaling in different auto- and allo-immune entities.

A second important consideration also exists, as mentioned at the beginning of this review: this is the impact of CTLA4Ig on CD4⁺CD25⁺FOXP3⁺ Tregs. As one of the key regulatory mechanisms to combat Teffs, Tregs represent a major force for tolerance induction in normal immune homeostasis. These cells have been well documented to express both CD28 and CTLA-4. Their activation augments Treg CTLA-4 expression,(60, 61) critical for the ability of Tregs to prevent autoimmunity, in part by competing for B7 ligands that would otherwise be engaged by CD28 on conventional T cells (Tconv).(58, 62) In addition to the role that Treg-expression of CTLA-4 may have on restraining immune activation, Tregs also rely on CD28 expression in order to maintain their peripheral homeostasis.(63, 64) Thus, an agent like belatacept might well produce an unexpected negative impact on tolerance induction through the impact of CTLA4Ig on CD28 signaling in Tregs. In fact, these data are consistent with results from our group and others that show that CTLA4Ig can lead to decreased levels of Tregs after both solid organ(65, 66) and HCT,(67) which raise the possibility (although this hasn't been directly proven yet) that one of the potential relative contraindications of the use CTLA4Ig may be an undesired negative effect on Treg homeostasis and function.

Direct targeting of CD28: Superior to CTLA4-Ig?

Given the fact that CTLA4Ig can target both co-stimulation through CD28 and co-inhibition through CTLA-4, there has been deep interest for many years in directly targeting CD28 alone rather than targeting B7 ligands (that would affect CD28 along with CTLA-4). However, progress in this field was negatively affected by the tragic Phase 1 trial of TGN1412 in healthy controls, in which super-agonist activation by the cross-linked anti-CD28-specific intact mAb produced life-threatening cytokine storm in all 6 participants.(68, 69) Recently, at least two independent efforts have led to second-generation CD28-targeting molecules that are incapable of cross-linking and have demonstrated broad safety in a number of preclinical settings and in clinical trials. Bristol-Myers Squibb is developing anti-CD28 'domain-specific' antibodies that are pegylated to prolong their half-life.(70) Preclinical work has demonstrated significant in vitro and in vivo activity of these molecules.(71, 72) The clinical formulation, lilizumab, has now completed Phase 1 studies, (73, 74) and is being studied in a Phase 2 trial in lupus as well as in a proof-of-concept study in Sjögren's syndrome.

Led by Dr. Bernard Vanhove, Effimune independently developed a pegylated anti-CD28 Fab', 'FR104', which has now been licensed to Janssen Biotech. FR104 has been shown to have biologic activity in a number of auto-immune and allo-immune settings, including in prolonging renal allograft survival in NHP and in preventing xenoGVHD in a NHP→→mouse model recently developed by our group.(75–81) Importantly, FR104 performed superiorly to belatacept in NHP models of renal transplant(80) and human xeno-GVHD.(76)

The mechanisms that were critical for this improved activity have not been determined, although experiments in both systems suggest that both CTLA-4- and Treg-mediated mechanisms may be operative.(76, 77, 80) FR104 is also now in clinical trials, being studied in patients with RA.

CD40:CD154-directed costimulation blockade: The story of on-target potency being sidelined by an unexpected toxicity

In preclinical studies, there has been no class of molecules that has seen more success in transplant tolerance-induction than those that target CD40: CD154 signaling. CD40 (a member of the tumor necrosis factor receptor (TNFR) superfamily, expressed on most APCs), and its ligand, CD154 (expressed primarily on activated T cells, but also found on other hematopoietic cells, discussed below) are critical for T cell function, as well as for the B and T cell interactions that result in the generation of high affinity antibodies that can be pathological in solid organ transplantation and HCT.(82) Consistent with its TNFR structure, CD40 signaling depends on its interactions with TNFR-associated factors (TRAFs),(83) which when engaged, lead to T cell activation through a variety of secondary signals, including NF- κ B, Jnk and AKT pathways.(82)

The importance of CD40:CD154 engagement likely is due to its critical impact both on T cell activation and on the initiation and perpetuation of the germinal center response in lymph nodes, thus being a critical hub for both T and B cell immunity.(84, 85) The potential translational importance of targeting this pathway has been demonstrated numerous times, in murine, canine and NHP models. The early studies concentrated on blocking CD154, and led to the striking result that a single dose of the MR1 anti-CD154 mAb permitted long-term cardiac transplant acceptance when combined with donor splenocyte infusion, and that anti-CD154 mAb infusion led to skin acceptance in mice.(86, 87) In addition, striking synergy was observed when blockade of CD154 was combined with B7 blockade (using CTLA4Ig), which was demonstrated in numerous publications, including the seminal observation of its ability to lead to tolerance to both heart and skin allografts in the allogeneic Balb/c $\rightarrow$ C3H model.(86) Additionally, a series of studies by the Sykes, Larsen and Blazar laboratories demonstrated the potent effects of anti-CD154 mAb on facilitating donor chimerism in sub-optimally conditioned recipients, attributed to either peripheral host anti-donor Teff deletion followed by central tolerance or alternatively, by peripheral immune regulatory mechanisms. (42, 44, 49, 87–94) Moreover, when anti-CD154 mAb was combined with donor bone marrow, immune tolerance to skin allografts was achieved even in the stringent B6 $\rightarrow$ Balb/c model system.(46, 49, 50)

Several mechanistic studies have provided important insights into the mechanisms of CD154 blockade and tolerance induction. For example, anti-CD154 mAb added to an *in vitro* mixed lymphocyte reaction induced antigen-specific tolerance, measured both *in vitro* and *in vivo* in a GVHD reaction, with evidence that this effect was dependent upon Tregs. (46, 88, 95–98) Notably, activated Tregs express high levels of CD154, although a direct signaling mechanism for CD154 in T cells has not been described. In other studies, investigators demonstrated that anti-CD154-induced tolerance resulted in the abortive expansion of the

alloreactive, T_{eff} pool with a loss of cytokine production, activation marker expression, and absence of memory T cell markers. Additionally, the tolerant alloreactive T cell phenotype induced by CD154 was long-lived but could be reversed by in vivo Treg depletion. (46, 88, 95–98) Dr. Mandy Ford and colleagues further showed that blockade of this pathway not only controlled donor-reactive CD8⁺ T cell responses, but also was associated with the expansion of peripherally-generated antigen-specific CD4⁺FoxP3⁺ Tregs. (99–101) These observations suggest that blockade of CD154 may create the balance that was missing with CTLA4Ig, with pro-tolerogenic effects on both T_{conv} and Treg populations.

The impressive results in mice led to a large number of studies in NHP. In those studies, anti-CD154 antibodies were a critical component for the prolonged acceptance of both bone marrow and renal allografts. (67, 102–105) While the induction of true immune tolerance in mice was not able to be directly extrapolated to NHP, CD154 blockade results were sufficiently impressive that they led to widespread interest in the clinical translation of CD154-directed therapies, as potential adjuncts to both conventional immunosuppression and CTLA4Ig-based therapeutics.

Unfortunately, the clinical translation of the first anti-CD154 antibodies was halted quickly, when thromboembolic complications were noted. This was found to be due to the expression of CD154 on activated platelets, leading to anti-CD154-mediated platelet activation and clotting in treated patients. (106–109) This unfortunate turn of events led the field to explore CD40 blockade as a potential alternative to CD154. While numerous studies have shown that CD40 blockade can also serve to prolong allograft survival in both mouse models and NHP, (110–120) the combined impact on both T_{conv} and Tregs noted for anti-CD154 antibodies has not yet been demonstrated for those blocking CD40.

Nonetheless, the large body of preclinical evidence supporting a central role of CD40:CD154 pathway blockade in prolonging allograft survival has sustained significant interest in CD40 for clinical translation, with a Phase 2 trial of the anti-CD40 antibody ASKP1240 currently underway in renal transplantation (Clinicaltrials.gov # NCT01780844). As with CD28, the advent of single-chain domain-specific antibodies has also presented an important solution to the clinical toxicity observed with anti-CD154 antibodies that were dependent on cross-linking. A domain-specific antibody for CD154 has now been developed, and has shown significant activity in murine models of alloimmunity. (100, 121) These reagents are now being tested in NHP models of solid organ transplant and early-phase clinical trials are being planned.

ICOS/L, a supporting player in germinal center targeting and co-stimulation blockade

Structurally similar to CD28, Inducible T-cell Costimulator (ICOS, also known as CD278) is also an Ig superfamily member, expressed as a homodimer in activated (but not resting) T cells, as well as NK and NKT cells. (122–124) As with CD28, binding of ICOS to its ligand, CD275, results in T cell activation, including expanded T cell proliferation, survival, cytokine production and effector differentiation. (125–127) Consistent with these properties, ICOS blockade inhibited murine acute GVHD induced by CD4⁺ and/or CD8⁺ T cells and

also was shown to promote alloengraftment of donor bone marrow in suboptimally conditioned recipients. Murine experiments also have documented that ICOS is required for T-mediated B cell function, including a critical role in T-follicular helper (Tfh) generation and CD40-mediated Ig isotype switching.(127–129) While the impact of ICOS signaling on T cell function is striking(130) and positive effects have been seen in acute GVHD models, monotherapy mAb-mediated ICOS blockade in a solid organ cardiac allograft model only demonstrated a modest impact, although synergistic activity was seen when ICOS blockade was combined with cyclosporine to produce >100 day survival in a cardiac transplant model. (131) ICOS/L blockade has also been shown to effectively partner with anti-CD40L in prolonging islet allograft survival(132, 133) and blockade of ICOS;ICOSL reduced alloreactivity in an acute GVHD.(134) Moreover, ICOS has been demonstrated to be upregulated on activated T cells in a canine model of chronic GVHD,(135) and in a murine model of chronic GVHD model induced by injecting parental splenocytes into unirradiated F1 recipients, antiICOS mAb inhibited B cell activation, autoantibody production and glomerulonephritis, the primary manifestation of chronic GVHD in that model system.(136) In addition, using a murine multi-organ system model of chronic GVHD with a hallmark of lung and bronchiolitis obliterans that is due to antibody production and deposition, blockade of ICOS in mice with active chronic GVHD restored pulmonary function to the level seen in no-GVHD controls.(137) In that setting, ICOS/L blockade resulted in a significant reduction in germinal center formation, essential for chronic GVHD generation in that model system.

Whether these encouraging results in mice will be translatable to the clinic is unclear. Indeed, a recent study showed that in a NHP renal transplant model, ICOS blockade did not prolong allograft survival, either as a monotherapy or when combined with belatacept.(138) However, combination blockade of this pathway with other potential partners was not tested in this study, nor was a comprehensive examination of its impact on diseases that are more reliant on GC formation. The mixed results notwithstanding, an anti-ICOSL antibody (AMG-557) is currently in clinical trials for lupus (Clinicaltrials.gov # NCT01683695 and NCT00774943), and as such, a direct test of the impact of ICOS pathway blockade in patients is being undertaken.

Targeting OX40:OX40L: An additional avenue for the ‘right’ targeting of Tconv and Tregs?

Given the complexities of the impact that co-signaling pathway blockade makes on both conventional and regulatory T cell populations, the search for the ideal therapeutic continues in the fields of both autoimmunity and transplantation. New studies are identifying a role for OX40 (CD134):OX40L (CD252) blockade in transplant immunomodulation, given that, like CD134 pathway blockade, it may be able to simultaneously control Tconv effector function while potentially permitting Treg homeostasis and function. It has been well-documented that OX40 is upregulated on CD4⁺CD8⁺ cells during T cell activation.(139–142) In several murine acute GVHD models, OX40:OX40L blockade augmented survival associated with anti-host hyporesponsiveness with the major effects being seen on CD4⁺ T cell mediated acute GVHD.(143–147) OX40 is also upregulated on human alloreactive T cells, and depletion of CD134⁺ T cells during an allogeneic mixed lymphocyte culture reaction with

HLA mismatched stimulator cells dramatically reduced anti-host but not anti-third party alloresponses.(148)

In the context of solid organ transplantation, blockade of OX40:OX40L signals was shown to prevent allograft rejection in murine models, in a manner that is nonredundant with blockade of CD28 or CD154.(149) Of note, several of the murine solid organ allograft studies showed that OX40:OX40L blockade did not impact T cell proliferation, but rather, inhibited T cell survival, and thus resulted in fewer cells available to infiltrate and reject allografts.(141, 142) Of significant interest is the fact that in contrast to the positive impact that OX40 ligation makes on Tconv, Vu et al have shown that its ligation on Tregs results in the opposite effect, inhibiting Treg survival(142) and resulting in inhibition of their ability to suppress Tconv activation.(150) These results have led to significant interest in the field, and experiments in NHP investigating the impact of OX40:OX40L are underway in models of both solid organ and HCT, which are documenting that targeting this pathway may be an important avenue for tolerance induction (Kean et al., unpublished results). If these effects in NHP are robust, OX40:OX40L blockade agents are poised to be one of the next agents in the clinical pipeline for controlling auto- and allo-immune activation.

4-1BB/4-1BBL blockade: More central to tumor immunology than transplant immunology?

4-1BB has emerged as a major component of CAR-T cell engineering, (151, 152) as a key intracellular signaling domain that can lead to long-term T cell persistence in vivo after CAR T cell adoptive transfer. However, the impact of blockade of this pathway on solid organ transplant outcomes are, in general, less striking. A member of the TNF family of receptors, 4-1BB (also known as CD137 and tnfrsf9) is expressed on activated Tconv (but not their naïve counterparts), Tregs,(153) as well as being expressed on B cells and dendritic cells. (154–157) An agonistic anti-4-1BB mAb amplified donor anti-host alloreactive T cell responses, accelerating GVHD as well as cardiac and skin allograft rejection, associated with heightened CD8+ T cell proliferation and IFN γ response.(158) In other studies, both CD4+ and CD8+ T cell-mediated alloresponses were regulated by 4-1BB:4-1BBL interactions to approximately the same extent as assessed in acute GVHD and host bone marrow allograft rejection models, (159) and 4-1BB receptor-facilitated CD4+ T cell-mediated alloresponses were partly CD28 independent. Additionally, agonistic 4-1BB antibodies increased the graft-vsleukemia effect of a suboptimal number of donor splenocytes given in a delayed fashion after bone marrow transplantation by bolstering allogeneic responses resulting in leukemia elimination. In other studies, host 4-1BBL deficiency reduced the capacity of the host to resist the GVHD lethality effect of delayed donor splenocyte infusion, which was used to simulate the clinical settings in which donor lymphocytes are given later post-transplant to treat or prevent leukemia relapse.(160) Consistent with these murine data, selective depletion of human alloreactive T cells after a mixed lymphocyte reaction was highly effective in reducing alloresponses. (161) In parent-into-F1 unirradiated GVHD models, blocking anti-CD137 mAb ameliorated acute GVHD, while unfortunately enhancing chronic GVHD associated with reduced CD8+ T cells expansion and IFN γ expression along with enhanced antibody production.(162)

In solid organ allograft models, using 4-1BBL deficient recipients, skin allograft survival was not prolonged, and the combination of 4-1BB and CD28 deficiency only slightly delayed rejection kinetics.(163) These data suggest that 4-1BB was not the main pathway of escape in the absence of CD28 signaling. Despite the lack of striking effect of the genetic absence of 4-1BBL, blockade of the pathway has had positive impact on allograft survival in mice, prolonging corneal, intestinal and cardiac allo-transplants.(164-167) Signaling through 4-1BB and its ligand therefore clearly play a role in alloimmunity; however, its supporting role in this process has thus far hindered its pathway towards the clinic for control of T cell activation, quite distinct from the central role it played in CAR-mediated T cell activation and subsequent tumor clearance.

Blockade of T cell trafficking to secondary lymph nodes and target organs and tissues: impact on transplant outcomes and interactions with co-signaling blockade

One of the most critical steps in both organ transplant rejection and GVHD pathogenesis is the recruitment of activated T cells to target organs. As such, there is high interest in the impact that adhesion and chemokine blockade may have on both processes. Much of the work on adhesion blockade has been pioneered in autoimmune disease, where several targeted therapies have been investigated.(168–173) These include blockade of the lymphocyte function antigen-1 (LFA-1; CD11a) pathway, the alpha4 (CD49d), beta 1 (CD29) integrin very late antigen-4 (VLA-4) pathway, and the sphingosine-1-phosphate receptor (S1P1R) pathway, that controls lymphocyte egress from lymph nodes and can be blocked by the S1P1R antagonist, FTY720.(174–196) While the predominance of the published data suggest important roles for both LFA-1 and VLA-4 pathway blockade in improving GVHD and graft acceptance (176, 177, 197-199) the latter analyzed in murine, NHP and patient trials, the significant risk of infectious disease with both these agents has limited their use in transplant settings.(184) Thus, while LFA-1 blockade has shown some promise in both murine and some, but not all NHP studies, and was also included in several islet transplant clinical protocols, the identification of the risk of progressive multi-focal leukoencephalopathy (PML) in patients treated with the anti-LFA-1 antibody efalizumab for autoimmune disease prompted it to be withdrawn from the market. Similarly, while pre-clinical efficacy was observed with the anti-VLA-4 antibody natalizumab in both autoimmune and transplant settings, it has also been associated with a significant risk of PML. Patient advocacy has retained its approval for MS, but, given this risk, there has been limited adoption for other indications, especially in settings where there are viable alternatives.

As with anti-LFA-1 and anti-VLA4, there is also evidence to suggest that FTY720 can prevent acute GVHD, and at least temporarily prevent rejection as assessed in preclinical murine models of transplantation.(200–214) In murine acute GVHD systems, FTY720 has been shown to prevent GVHD lethality without impairing graft-versuslymphoma or -leukemia effects. Mechanisms in these studies have been attributed to impairing lymphocyte migration, inducing donor anti-host alloreactive T cell apoptosis, and reducing splenic CD11c+ cells along with T cell responder frequencies.(205, 208, 212, 214, 215) In a chronic

GVHD scleroderma model, FTY720 suppressed the immune response by promoting the expansion of Tregs and reducing vascular damage and infiltration of immune cells into the skin. In contrast to these positive results in GVHD in mice, in a large animal DLA non-identical acute GVHD model, FTY720 was ineffective in reducing acute GVHD, though the number of recipients studied was somewhat limited.(213) In the context of non-myeloablative transplants, FTY720 treatment in mice markedly but only transiently inhibited graft rejection due to the emigration of mature graft rejecting T cells from the host thymus upon drug withdrawal. However, when combined with tolerogenic anti-CD154 mAb, graft rejection did not occur upon drug withdrawal.(205) In the canine model, graft rejection rates did not differ from non-treated historical controls.(216) Although clinical studies with FTY720 have been completed and have resulted in drug approval for MS, and there was initially significant enthusiasm for it as a potential novel therapy to prevent GVHD and organ graft rejection, trials in renal transplant patients failed to show an advantage of this drug compared to standard therapy, despite encouraging early results. Thus, FTY720 is no longer being evaluated in this setting.

It should be noted that each of these three modifiers of T cell trafficking have demonstrated more efficacy in autoimmune disorders than in transplant settings. It is still not clear what the mechanistic underpinnings of this observation are; however, one of the important observations in large animal models, similar to the murine model of suboptimal conditioning and bone marrow graft rejection discussed above, has been the rebound effect that occurs when drug was withdrawn. Thus, in both NHP renal(185, 190) and bone marrow transplant (LSK unpublished), rejection occurred rapidly after withdrawal of anti-LFA1, ostensibly from alloreactive cells that were restrained from reaching their targets but not deleted or tolerized by the anti-integrin treatment. While patients treated long-term with these agents might benefit from the chronic inhibition of T cell trafficking, this strategy would be expected to increase the cumulative risk of infectious toxicities.

As with the adhesion and egress inhibitors, there has been significant interest in blocking chemokine–chemokine receptor interactions to improve transplant outcomes. Of the many chemokines that could be targeted, CCR5 has been investigated most thoroughly, after having been shown to be a central molecular mediator of T cell trafficking to GVHD target organs, including the GI tract.(217, 218) In preclinical studies, CCR5 blockade has shown protection against GVHD in sublethally irradiated recipients, associated with reduced homing to the intestinal Peyer’s patches in some models using knockout donor T cells(217, 219), and, somewhat paradoxically, acceleration of GVHD in heavily but not lightly irradiated recipients.(219, 220) Based upon the positive data in murine non-myeloablated recipients, the CCR5 inhibitor maraviroc (NCT00948753), an FDA approved drug designed to limit CCR5-mediated HIV entry into CD4 T cells, was given for 1 month post-transplant, along with standard of care pharmacological agents, to high-risk patients receiving matched unrelated or 1 antigen mismatched unrelated donor grafts. Although acute GVHD of the skin was not eliminated, patients given maraviroc had a low incidence of GI and liver GVHD which constituted one of the first demonstrations of a target-organ-specific effect of a biologic anti-GVHD prophylaxis regimen.(221) The durability of the impact of maraviroc on GVHD control is still undetermined, however, and there may be conditioning-regimen-specific effects in patients as seen in rodent models.(219) This agent is now being

investigated as part of a large multicenter study sponsored by the BMT-CTN, (Clinicaltrials.gov # NCT02208037) where issues of conditioning and durability of the GVHD protective effect will be investigated more thoroughly.

LFA-3 (CD58): CD2 Blockade: A missed opportunity?

In the last several years, there have been multiple demonstrations of the potential importance of targeting LFA3:CD2 with an LFA-3/Fc fusion protein to control T cell (and NK cell) activation and effector function, with several important lines of evidence suggesting that it might preferentially target memory rather than naïve T cells. These have included *in vivo* studies of antiCD2 mAb in rodent xenogenic islet and allogeneic cardiac transplant models, (222) as well as *in vitro* studies of the human allo-response(223), and translational studies in NHP demonstrating a role for CD2 blockade in renal transplant.(224) The clinical benefits of anti-CD2 mAb could be seen in studies in both bone marrow transplant(225) and Type I diabetes(226, 227) indicating the potential importance of this pathway in controlling T cell mediated disease. However, although alefacept is currently FDA approved for the treatment of psoriasis, the parent company for the marketed version of the drug decided to discontinue marketing this drug in 2011 for financial reasons, making future clinical applications uncertain. Given the wide array of indications in which this agent may have significant activity, and its potentially unique pre-disposition for memory, rather than naïve T cells, this likely represents a missed opportunity, and one that the transplant and autoimmune communities would welcome revisiting.

The Central Role of Tregs in Transplant Tolerance

In solid organ transplant models, it is clear that achieving long-term allograft survival and/or tolerance through the manipulation of co-signaling pathways is associated with an expansion of Tregs.(228). What is less clear, however, is whether this is a specific result of blocking costimulatory pathways *per se*, or rather, a secondary effect of antigen presentation in an environment where inflammation has been deliberately suppressed, promoting the expansion of preexisting Tregs (tTregs) and the induction of *de novo* induced pTregs. Obviously, these are not mutually exclusive hypotheses; however there is some evidence in favor of the latter. For example, recent studies indicate that CD28 is an important homeostatic survival signal for Tregs(229) and thus CTLA4Ig would interfere with this survival signal. We suggest that promotion of Tregs in the setting of costimulatory blockade likely is an indirect effect of inhibition of inflammation, which promotes TGF- β . In addition, inflammatory cytokines are able to activate the PI-3 kinase pathway in T cells, which, when unopposed, inhibits Treg differentiation and promotes destabilization of preexisting Tregs.(230) Thus dampening inflammation may promote Tregs via multiple mechanisms. In BMT, it is increasingly clear that Tregs can play a major role in controlling unwanted allo-reactivity, and can function both to prevent acute GVHD and treat chronic GVHD. Thus, our group and others have shown that Treg adoptive transfer early after transplant is safe, feasible and has efficacy in reducing acute GVHD, both in murine models(231–233) and in patient trials.(234–243) Important studies have also shown that adoptive transfer of these cells can also function to promote engraftment(244) and do not impair GVL.(245–248) These data underscore the central role that Tregs play in both establishing and maintaining successful engraftment, and

in restraining alloreactivity. Thus, in an era of manipulation of T cell co-signaling with targeted therapies, choosing those that ‘walk the fine line’ of restraining effector T cell activation while supporting Treg reconstitution and function will likely be necessary in order to achieve immune tolerance after both solid organ and HCT.

The key role of memory T cells in escaping co-stimulatory pathway blockade and approaches to prevent Tmemory cell escape

As noted above, CD28 is required for optimal activation, function and survival of naïve T cells, with a particularly important role in CD4 T cell function. However in a number of studies (albeit not all), memory T cells have been shown to be relatively independent of CD28 costimulation.(249) Indeed, mouse models of solid or tissue transplantation have clearly demonstrated that costimulatory blockade is relatively ineffective in animals harboring pre-existing anti-donor specific T cells.(250) This presents a particularly problematic clinical situation since it can be anticipated that a large number of patients, even without evidence of prior antigenic exposure or preexisting anti-donor humoral immunity, will have memory T cells capable of recognizing donor antigen. This is likely due to a variety of factors, most prominently heterologous immunity(251, 252) and the frequent expression of more than one TCRab heterodimer on human T cells(253) (the latter implying that a T cell with anti-donor alloantigen specificity based on one TCR might have memory phenotype/function acquired due to prior exposure to the antigen recognized by its second TCR).

Studies to overcome pre-existing memory have focused on non-CD28 mediated pathways. Pre-clinical studies in mice suggest that OX-40 may be a useful target.(254), and data in non-human primates points to CD2 as a potential useful target for memory T cells.(224) As noted above, this has been supported by an early phase clinical study in type I diabetes suggesting that using LFA3Ig (a soluble CD2-ligand) may deplete Teffector/memory cells and/or promote T cell exhaustion.(226)

The Co-Inhibitory Pathways: Turn them off to fight cancer, turn them on to fight rejection and GVHD

The significant success in re-awakening anti-tumor T cell responses through checkpoint blockade has opened up new treatment possibilities for patients for whom conventional chemotherapeutic approaches are inadequate. In the clinic, these approaches have focused on blockade of two prominent inhibitory pathways: CTLA-4 and PD-1 (programmed death-1). Given the success of blockade of both of these pathways in activating T cell immunity, there has been much interest in activating these pathways for tolerance induction. We’ve discussed the CTLA4 pathway at length above, which plays a central role in the function of both Teffs and Tregs. Unfortunately, an agonistic reagent that specifically activates CTLA-4 is not available in the clinic, therefore, agonism of this pathway for immune tolerance-induction is not currently a viable clinical goal.

The other behemoth of the checkpoint blockade pathways for cancer immunotherapy is the PD-1 pathway. PD-1 was described as a novel Ig supergene family member by Professor Tatsuku Honjo in 1992.(255) PD-1 is expressed on dendritic cells, NK, NKT cells and on activated T cells. In BALB/c mice, PD-1 deficiency can result in autoimmune cardiomyopathy, consistent with its negative regulatory function.(256) The first identification of the critical importance of this pathway in antigen-responsive T cells *in vivo* was made by Dr. Rafi Ahmed's group, which showed that PD-1 is upregulated on exhausted T cells that accumulate during chronic infection.(257) Further studies showed that working through both immunoreceptor tyrosine-based inhibitory and switch motifs, PD-1 recruits SHP phosphatases leading to the inactivation of multiple T cell signaling pathways.(258)

Experiments with knockout mice for PD-1 and one of its ligands, PD-L1, as well as with PD-1 and PD-L1 blocking antibodies, demonstrated the necessity of this pathway for costimulation-blockade-mediated allograft acceptance.(259–261) Additionally, acute GVHD studies indicate a major role for PD-1 in down-regulating lethality, as shown with PD-1 KO donor T cells and anti-PD-1 blocking mAbs, which were demonstrated to function via an IFN γ -dependent mechanism.(262–264) In addition, in a sublethally irradiated minor antigen mismatched model of sclerodermatous chronic GVHD, blockade of the PD-1 pathway worsened disease.(265) These data reinforce the central role that PD-1 pathways play in T cell activation. While this has yielded several important new drugs to augment T cell responses by blocking this pathway, unfortunately, no agonists of PD-1 have yet been developed to inhibit T cell auto- or allo-activation, and thus it is not possible to manipulate this pathway clinically to improve solid organ or bone marrow transplant outcomes..

PD-L1 vs PD-L2: Predominance of PD-L1 due to more promiscuous tissue expression?

In its negative-regulatory functionality, PD-1 is known to interact with both PD-L1 and PD-L2, and the relative importance of these ligands has been a matter of significant investigation. PD-L1 (B7-H1, CD274) is expressed constitutively on hematopoietic cells and nonhematopoietic cells and upregulates after activation. PD-L2 (B7-DC, CD273) expression is restricted primarily to DCs, macrophages, and cultured bone marrow -derived mast cells. The broad PD-L1 expression suggests an important role in inhibiting immune responses in lymphoid and nonlymphoid organs.

Using a TCR transgenic system, we demonstrated that only blockade of PD-L1, and neither PD-1 nor PD-L2 abrogated skin graft tolerance in a transplant model with MHC Class II disparity.(266) In the same study, we found that in an adoptive transfer model, both PD-1 and PD-L1 were critical early after transfer, with PD-L1 becoming the predominant inhibitor of T cell allo-proliferation as time progressed. This effect was dependent on the presence of CD4 $^{+}$ Tregs, suggesting that a multicellular mechanism of transplant acceptance may be in play, with both Tregs and Tregs necessary for the ultimate control of alloreactivity. The prominence of PD-L1 signaling in a model of Class II-mediated alloreactivity was also demonstrated by Yang et al., who found that in ClassII-disparate cardiac allograft model, signaling through PD-L1 was critical for allograft acceptance, but blockade of neither PD-1

nor PD-L2 had a negative impact on engraftment.(267, 268) Moreover, PD-L1 was found to be critical for the induction of Tregs.(269) In acute GVHD studies, PD-L1 has been shown to be the dominant host PD-L1 ligand that controls apoptosis of donor T cells, Teff expansion, function, and metabolism.(270) This is also true when delayed lymphocyte infusions are given. The expression on non-hematopoietic cells likely substantially contributes to this dominance, resulting in a markedly diminished GVL effect after delayed lymphocyte infusion.(264, 271, 272) Recent studies also indicate that PD-L1 expression on donor T cells regulates acute GVHD lethality, further complicating interpretation of the use of anti-PD-L1 mAbs.(273) Indeed, in an autoimmune-like minor histocompatibility antigen mismatched GVHD model, host APC PD-L1 reacting with CD80, was shown to augment donor Treg survival and expansion, suppressing GVHD.(274, 275)

Another potential explanation for the predominance of PD-L1 signaling in controlling allograft acceptance came from the discovery that PDL1 (and not PD-L2) can interact with CD80 in addition to PD1.(276) In acute GVHD, PD-L1/CD80 interactions induce apoptosis of donor T cells in a PD-1 dependent manner, resulting in amelioration of GVHD. Similarly, PD-L1/CD80 interactions can control diabetogenic Teffs. Thus PDL1 may provide a parallel track for the control of alloreactivity that renders it as a central player in allograft survival. Evidence for this role was further strengthened by the identification of a monoclonal antibody that specifically targets the PDL1:CD80 binding motif, providing a structural basis for the previously identified biologic activity. (268)

The downside of blocking co-inhibitory receptors: unwanted T cell activation, GVHD, autoimmunity

While this review is focused on manipulation of co-stimulatory and co-inhibitory molecules to control rather than stimulate T cell function, it is important to consider the impact of these agents on both auto-and allo-immunity, since patients at risk for both these processes are now being treated with checkpoint blockade agents. There have now been several reports of immune activation syndrome in patients treated with both anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab). These include evidence for pulmonary, skin, cardiac, hepatic, GI and bone marrow disease.(277–282) The range of clinical severity of these diseases is large, and unfortunately includes some patients who have required extensive hospitalization and treatment with broad-acting immunosuppressive drugs, with patient death occurring in some instances. However, there are also numerous reports of patients with de novo inflammation or flare of known autoimmune disease being successfully treated with conventional immunosuppression while continuing to receive check-point blockade. Indeed, early-phase data showed that administration of ipilimumab was feasible in patients with recurrent hematologic cancers after allogeneic HCT, and resulted in durable responses, especially of extramedullary acute myeloid leukemia, although immune-mediated toxic effects and GVHD occurred.(283) In other studies, PD-1 pathway blocking reagents have been used to treat relapse post-allo-HCT, although early data does point to higher acute GVHD incidence and severity in treated patients.(284) It is still not clear what the impact of combining these two opposing therapies will be on tumor biology, as a large enough study has not yet been

undertaken, but, at least in melanoma and small cell lung cancer, pre-existing autoimmunity is not considered an absolute contraindication for treatment with checkpoint blockade.

The issue of intercurrent allo-activation during checkpoint blockade or CAR-T therapy is also critical to the design of relapse-prevention strategies for patients that have undergone allogeneic BMT. In these patients, there is concern about the risk of induction or exacerbation of GVHD with checkpoint blockade, and it is not currently offered clinically. Although murine studies have suggested that GVHD can occur with allogeneic CAR therapy in the setting of transplant,(285) this has not been a major problem in patients, and many post-allo-BMT patients are treated with CAR-T cells.(286) However, there is concern with giving CAR-T cells in post-transplant patients who are being treated for GVHD, and this is not currently standard practice, given the concern of exacerbating GVHD with these therapies. There is also a concern for negatively impacting the efficacy of CAR-T cells when patients are receiving treatment with steroids and other agents to control GVHD. Engineering CAR-T cells to be resistant to these agents may increase the application of this therapy post-transplant. However, given the potential hazard of having a T cell population that cannot be controlled by conventional immunosuppression(287), having a suicide trigger engineered into these T cells is considered a necessary safety criteria.(288, 289)

Co-inhibitory ligands other than CTLA-4 and PD-1

In addition to CTLA-4 and PD1-, another of the major co-inhibitory molecules, LAG-3, has also become a major target for checkpoint blockade, with trials of anti-LAG-3 antibodies in glioblastoma, other solid tumors as well as hematologic malignancies.(290–292) In immune tolerance studies, its expression on induced Tregs is upregulated by exposure to IL-27 and is critical for the optimal function of these cells in controlling autoimmune diseases.(292, 293) In acute GVHD, studies have shown that LAG-3 KO Tconv induce significantly more severe GVHD than wild-type Tconv and that the absence of LAG-3 on CD4 but not CD8 T cells is responsible for worsening GVHD.(294) Notably, Tregs from LAG-3 KO mice were functionally intact. While there is significant interest in modulating LAG-3 to enhance control of both auto-and allo-immunity, no targeted agents to upregulate this molecule yet exist.

More limited data exist for B7-H3 (CD276), a B7 family member overexpressed in a variety of solid tumors, which can be associated with poor prognosis. In allograft rejection, different studies have demonstrated either the promotion of rejection or prolongation of allograft survival by B7-H3 suppression of Th1 responses.(295, 296) In acute GVHD, B7-H3 expression by donor and host cells negatively regulates GVHD lethality.(297) Similar to LAG3-3, clinical agonistic reagents are not available. Notably, both resting and activated murine Tregs were found to lack B7-H3 messenger RNA.(297, 298)

Similar to B7-H3, B7-H4 is another B7 family member that is a negative regulator of T cell responses. This molecule is expressed on tumors and can be associated with a poor prognosis. Although GVHD studies have not been reported to date, B7-H4 expression has been shown in multiple studies to prolong islet allografts and in CD28deficient recipients, B7-H4 blockade resulted in accelerated cardiac allograft rejection associated with increased

frequencies of IFN γ , IL4 and granzyme B producing splenocytes.(299) Moreover, the efficacy of CTLA4Ig in promoting allograft survival was diminished in mice that did not have an intact B7-H4 pathway. B7-H4 agonistic reagents are not clinically available at this time.

2B4 (CD244, SLAMf4) is also a central co-inhibitory molecule. Although it was originally identified on NK cells, it is clear that 2B4 is upregulated on activated T cells.(300–303) While delineation of the major function of 2B4 has proven complex, (and has included its associated with both T cell activation and inhibition), studies from Dr. Arlene Sharpe's laboratory,(304) and from Dr. Mandy Ford and others provide strong evidence for its role as a co-inhibitor of CD8+ T cell alloresponses in vivo.(72, 305) Although the 2B4 pathway has not yet been harnessed for the promotion of tumor immunity, these studies suggest that it may be a viable candidate, especially in combination with either PD-1 or CTLA-4 blockade.

T cell immunoglobulin mucin-3 (TIM-3) was also found to be a critical player in transplantation tolerance when the Kuchroo laboratory demonstrated that anti-TIM3 blocking antibodies prevented transplant tolerance by interfering with Treg function.(306, 307) TIM-3 is expressed on pathogenic T cells in acute GVHD and its ligand, galectin-9 is up-regulated in inflamed tissues.(308) Tim-3/galectin-9 blockade accelerates acute GVHD, while conversely, galectin-9 transgenic recipients have demonstrated reduced acute GVHD. Paradoxically, TIM-3/galectin-9 blockade in recipients of Treg-depleted grafts reduced GVHD due to activation-induced T cell death as a result of increased IFN γ production. In skin allograft recipients, activation of the TIM-3/galectin-9 pathway negatively regulates CD8+ alloreactive T cells and prolongs allograft survival,(309, 310) a finding also observed with cardiac allografts.(311, 312) TIM3+ Tregs, which are frequently PD-1+, were found to be a short-lived Treg subset that can restrain skin allograft rejection responses.(313) Unlike CTLA-4 and PD-1, an agonistic reagent does exist for TIM3, with its ligand, Galectin-9 recently being shown to promote acceptance of solid organ allografts.(314–316) Thus, TIM3 represents a molecule of considerable interest for both the cancer immunotherapy and transplant tolerance fields, with promise as both immune modulator and immune stimulator.

The special role of large animal models in understanding co-signaling in transplantation

Most of the pathways and molecules involved in T cell co-signaling have been first identified in murine models, using both genetic knock-out technologies and antibody- and small-molecule based blockade. While these murine studies have been absolutely critical to the progress in the field, canine models(135, 213, 317–322), and NHP models(323) have been central to the translation of co-stimulation blockade to the clinic, in both solid organ and bone marrow transplant. The best example of this is with the FDA approval of belatacept for clinical use in renal transplantation. In the path to the clinic, Bristol-Myers Squibb closely collaborated with academic investigators to first design the higher avidity version of CTLA4Ig that belatacept represents(52), and then performed the key IND-enabling studies in NHP prior to launching pivotal clinical trials. These NHP studies closely predicted both the success of belatacept in being a true calcineurin-sparing agent (with all of

the attendant advantages in terms of renal and cardiovascular function that accompanies a calcineurin-free regimen) and also predicted the belatacept-resistant rejection that has been observed clinically. For belatacept, the cycle of murine→→NHP→→patient and back to murine and NHP studies has been realized, with the detailed investigation of belatacept-resistant rejection being vigorously pursued in both murine and NHP models. While a clear solution to this issue has not yet been identified, ongoing studies in mice and NHP focused on identifying the causative mechanisms, and using a range of agents designed to overcome these early rejection responses is underway.

The utility of NHP models in moving co-stimulation blockade to the clinic has also been demonstrated in the field of BMT. While the success of both abatacept in RA and belatacept in renal transplant should have predicted the utility of these approaches in bone marrow transplant (along with the murine data that supported a role for this pathway in the pathogenesis of acute GVHD), (32–37) until our demonstration of the activity of CTLA4-Ig in preventing GVHD in an NHP model,(33) there was hesitation in the field to use *in vivo* co-stimulation blockade as a novel approach for GVHD prevention. (There had been one trial of ex-vivo exposure of bone marrow products to either CTLA4Ig or anti-B7 antibodies, (324) but this was not followed with significant adoption by the BMT field). The demonstration in NHP that combination co-stimulation blockade could significantly protect recipients from GVHD provided the necessary rationale for clinical translation of this strategy to patients.(38) We are now completing a large Phase II study (Clinicaltrials.gov# NCT01743131) designed to determine both the benefits and risks of the addition of abatacept to standard-of-care GVHD prophylaxis in both adult and pediatric transplant patients.

Importantly, while NHP studies proved critical in identifying both the efficacy and the breakthrough rejection of belatacept in renal transplantation, not all clinical toxicities have been correctly identified in NHP studies. A major example of this is the fact that studies in cynomolgus macaques, one of the most prevalent of the NHP models used, did not identify the risk of uncontrolled T cell activation and cytokine storm that accompanied clinical use of the cross-linking anti-CD28 agonist targeting the C'D loop of CD28, TGN1412(68), likely due to the lack of expression of CD28 on CD4+ effector cells in macaques, and its increased expression on the human counterpart.(325–327) Vanhove and colleagues have subsequently shown that baboons represent a much more predictive NHP model for testing the toxicity of reagents targeting CD28, (328) and used this model to provide compelling data for the safety of their single-chain anti-CD28 reagent, FR104. They have gone on to demonstrate the potential efficacy of this therapeutic (originally developed by Effimune, now licensed to Janssen) in a number of NHP models of transplantation(76–81, 328), and it has now completed Phase 1 trials in RA (Clinicaltrials.gov # NCT02800811). BMS has also developed a similar single-chain anti-CD28 antibody, 'Lulizumab', which has also been moved into early phase clinical trials for Sjogrens syndrome and lupus (Clinicaltrials.gov # NCT02843659 and NCT02265744).

The importance (and also the limitations of NHP models) in facilitating the translation of new therapeutics to the clinic have also been demonstrated with antiCD154 and anti-CD40 antibodies. In murine tolerance models, strategies targeting CD154 have long been

demonstrated to be amongst the most potently tolerogenic, for both solid organ and BMT applications. These striking results in mice were recapitulated in NHP models,(104, 329–331) where anti-CD154 antibodies were found to be critical components of calcineurin-sparing regimens for many solid organ transplant models as well as for bone marrow chimerism-induction. Given the many positive attributes of targeting this pathway, both in terms of its impact on effector cells as well as on Tregs, there was much enthusiasm for translation to the clinic, and two trials were opened, one in renal transplant (Clinicaltrials.gov # NCT00001857) and one for patients with lupus nephritis.(332) However, both clinical trials were halted shortly after their initiation, given an unusually high rate of thromboembolic complications in treated patients. Subsequently, Cosmi and colleagues reported a new regimen that incorporated antiCD154 antibodies in NHP that also demonstrated the thromboembolic events;(109) however, many previous studies had failed to identify this complication. The combined lessons with anti-CD28 and anti-CD154 have pushed the field of NHP transplantation research to evolve more stringent practices for evaluating the potential toxicity and efficacy of therapeutics prior to clinical translation, and the road to the clinic through primate investigation has continued to be robust.

Following the identification of the thrombosis risk with anti-CD154, there was a significant push to develop and evaluate anti-CD40 reagents in NHP for use in transplantation, and these studies suggested that targeting CD40 had significant efficacy, on par with what was observed for CD154-directed therapies. These NHP results are now being translated to the clinic, with the first anti-CD40 antibodies being tested for psoriasis and renal transplantation (clinicaltrials.gov # NCT01585233, NCT01780844 and NCT02217410). In addition, the thromboembolic complications with the conventional anti-CD154 antibodies have now been overcome, with domain-specific, single-chain antibodies, (rigorously determined to not induce thrombosis), having been developed. These are being re-introduced into the fields of both autoimmunity and transplantation with high hopes for significant efficacy without toxicities.

The experience with both anti-CD28 and anti-CD154 antibodies have reinforced the contention that no animal model is a perfect surrogate for carefully conducted clinical trials. However, the strengths of NHP models have been critical for the streamlining of translation of co-stimulation blockade to the clinic for autoimmunity and transplantation. These studies permit combination therapies that are more difficult to interrogate in murine models, given significant differences in pharmacokinetics and in receptor:ligand expression patterns in mice compared to humans. They also permit the direct evaluation of reagents that are developed for human use, which usually cross-react with NHP targets but rarely do so with their murine counterparts. Finally they permit intensive monitoring of both the blood and transplant target organs (eg kidney, heart, skin, GI tract) such that both the peripheral and tissue-specific immunologic consequences of transplant and novel therapeutic strategies can be fully assessed. This is essential for both the evaluation of safety and efficacy, with the proviso that careful attention be paid to any species-specific heterogeneity of target expression, as occurred with TGN1412.

In order to maximize their utility for clinical transplantation, the field of NHP experimentation has greatly benefited from recent advances that help assure that the immune

milieu of these transplants is understood in the greatest detail possible. One significant example is the establishment of a new 'gold standard' in transplant studies, where the degree of MHC disparity between donors and recipients is now expected to be defined, and equivalent amongst different treatments in any transplant series.(102, 333) Until 2010, this degree of information about the MHC was not included in transplant planning, which opened up these studies to the potential confounder of serendipitously close MHC matching leading to transplant outcomes that were more successful than they otherwise would have been (or vice versa with highly disparate transplant pairs). Our group and others created a new paradigm in NHP transplant planning by applying first microsatellite-(102, 333) and now MHC-allele-level typing to multiple NHP colonies(334), with the NIAID providing critical support for this effort, by funding the detailed MHC typing of their sponsored macaque colony. In addition to the rigorous MHC matching that is now possible in NHP transplant experiments, the detailed annotation of the rhesus transcriptome(335, 336) has allowed a systems approach to understanding the impact that new therapies make on the T cell transcriptional networks controlling transplant outcomes. This has led to new insights about the breakthrough alloreactivity (336) that occurs with both standard and CTLA4Ig-based immunomodulation for GVHD prevention, as well as the identification of new potential targeted therapies to control the T cell activation that leads to GVHD.(337) These advances have helped solidify the depth of analysis that is possible with NHP models of T cell co-signaling, thereby helping to optimize the ability of these models to most efficiently advance novel targeted therapeutics to the clinic.

Conclusions and Perspectives

In this review, we have discussed the co-signaling pathways that lead to T cell activation. However, our focus has not been on controlling these pathways in order to activate T cells, or awaken them from senescence (what one might call harnessing the 'Yang' in T cells) but rather, we have concentrated on the field of immune tolerance, a field that is focused on the Yin, or the specific 'cooling' of T cells, to control T cell activation and to lead to lifelong acceptance of allografts, without the need for ongoing immunosuppression. This work has its philosophical underpinnings in the landmark work of Billingham, Brent and Medawar, over 60 years ago, which introduced the concept that under some conditions, life-long acceptance of foreign cells and tissues could occur.(338) As we have learned more about the complex molecular networks that control T cell activation, and T cell tolerance, two things have happened:

First, we are more and more able to manipulate T cell programming to achieve our goals (whether they be T cell activation or T cell tolerance) and second, we are increasingly aware of the extremely delicate balance that exists, that makes these manipulations extremely difficult to fully control. This has been shown in both the fields of cancer immunotherapy and in immune tolerance work. In cancer immunotherapy, we have seen toxicities directly attributable to our unleashing of T cell activation in a manner that has many fewer brakes than exist in the natural setting. In immune tolerance, we have seen the consequences of 'over-cooling' of T cell immunity, which can lead to defects in protective immunity against both infectious pathogens and tumor cells. Moreover, our increasing awareness of the impact of manipulating T cell co-signaling pharmacologically, affecting both effector and regulatory

T cells, has led to an understanding that we must induce immune tolerance on a ‘knives edge’: We must seek to understand in exquisite detail the complex systems that control T cell programming such that we can simultaneously induce tolerance in all T cell subpopulations, both effector and regulatory. The ongoing work in murine NHP and patient trials are geared toward just this goal: understanding the programming that naturally induces tolerance, and recapitulating this with targeted therapies such that we induce just the right amount of Yin for our patients undergoing transplantation.

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