Costimulation blockade: towards clinical application

Timothy A. Weaver[1,2], Ali H. Charafeddine[1], Allan D. Kirk[1]

[1]The Transplantation Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892, [2]Howard Hughes Medical Institute – NIH Research Scholars Program, National Institutes of Health, Bethesda, Maryland 20892

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1. ABSTRACT

Organ transplantation is an increasingly successful therapy for many forms of organ failure, but its success depends upon drug therapies to prevent immunologic destruction of the transplanted organ also known as rejection. Most therapies designed to prevent rejection alter the immune system in a rather broad, antigen independent way, and thus alter protective immunity as well as immune responses directed against the transplanted organ. Over the past 3 decades, however, it has been realized that a class of surface molecules known as costimulatory receptors are required to generate a fully productive immune response, and that blockade of these receptors during allo-antigen recognition can be used to influence the immune system's future response to that particular allo-antigen. Costimulation blockade has thus been developed as a specific field of interest towards achieving improved antigen specific control over transplant rejection while minimizing broad attenuation of protective immunity seen with conventional immunosuppressives. This field has grown rapidly in the past decade and is now poised to become a valuable therapeutic option for transplant clinicians. This review will outline the basic premise of costimulation biology, review the seminal experimental basis for its use in preventing organ rejection, and discuss the relevant data derived from its initial use in clinical transplant trials. Specific attention will be focused on two major costimulatory pathways, the CD28/CD80-CD86 and the CD40-CD154 pathways, and the clinically applicable data supporting their validity as therapeutic targets. Newly discovered costimulatory pathways will also be discussed as potential therapeutic targets for future clinical drugs.

2. INTRODUCTION

Organ transplantation today remains the preferred treatment for most causes of end-stage organ failure but remains dependent on the use of immunosuppressive drugs to prevent immune mediated organ injury known generally as rejection. Current immunosuppressive therapies have significantly reduced the incidence of acute graft rejection, however the incidence of chronic immune mediated allograft injury has not been significantly impacted, and consequently, long-term graft survival rates have remained stagnant over the past decade (1). The incomplete efficacy of current immunosuppressive therapies relates to two major factors; 1) many agents non-specifically inhibit T cell activation, clonal expansion, and immune system effector function, leading to immune attenuation extending beyond that required to prevent rejection, thus precipitating infectious and cancerous morbidities; and 2) current drugs require daily administration. This is expensive, requires adherence to daily drug regimens, and the chronic exposure results in drug toxicities exacerbating cardiovascular disease, hypertension, hyperlipidemia, and new onset diabetes.

The chronic drug approach to maintenance immunosuppression has been based on a premise that immune system alloreactivity is a fixed entity, continuously at odds with a transplanted organ. However, significant contributions by Bretcher and Cohn, Lafferty and Cunningham, Schwartz, June, and many others, discussed within this review, have greatly advanced our understanding of allorecognition, pointing out that immune responsiveness in general, and alloreactivity specifically, is plastic. The nature of the initial encounter between an antigen and the immune system can lead to lasting, specific effects facilitating the antigen's elimination, or importantly, its persistence. This realization that specific immune responses can include perpetuated aggression or tolerance has its underpinnings in the biology of costimulation signaling and its role in the development of an immune response. This knowledge, coupled with major technological advancements in protein engineering, has led to the creation of many novel fusion proteins and monoclonal antibodies (mAb) specific for costimulatory receptor/ligand interactions. With the right timing of administration and combination of allo-specific peptide the new biological agents promote a more narrowed approach towards alloimmune response modification (2,3).

The importance of costimulation biology to transplantation is now emerging from a theoretical concern to one of therapeutic practicality. After more than two decades of basic and translational research in animal models, agents specific for costimulation pathways are now making their way to the clinic for testing (4) and providing reasonable evidence that costimulation manipulation is of potential benefit in human transplantation. Thus, the stage is set for the field to enter a new era of enhanced treatment options. As will become evident in the outline below, the proper utilization of costimulation based drugs will require knowledge of a growing repertoire of costimulatory pathways with both immunostimulatory and inhibitory

characteristics, each containing unique temporal, spatial, and functional expression characteristics extending beyond the T cell to interactions involving antigen presenting cells (APCs), B cells, endothelial cells, platelets, and parenchymal cells (see Figure 1). Although current trials are now involved in the translation of single costimulation-specific agents, it is probable that the optimal development of these agents will involve the integration of multiple agents at specific time points, targeting dependent costimulatory signals required in the regulation of alloimmunity to effectively facilitate a lasting tolerant state. In addition, as will be discussed, the recognition that costimulation mediated effects may be subject to change with changing immune perturbations such as infections also highlights the need to develop novel analytical immunologic and pharmacologic assays to monitor and characterize the specifics of an individual's immune homeostasis. Although costimulation blocking therapeutics can be influentially directed towards an allo-antigen by simultaneously inducing initiation of alloimmunity, so can be the same towards a concurrently present pathogenic antigen and thus, risking the neutralization of protective immunity.

This review will provide a historical perspective on costimulation based therapies followed by a review of key studies advancing our understanding of the costimulation pathways which have reached the clinic in some form; the B7/CD28 pathway and the CD40/ CD154 pathway. The translation of these two major costimulation pathways from the bench to the clinic will be discussed emphasizing nonhuman primate and clinical trials. In addition, the many novel costimulation pathways that help color an immune response and have shown themselves to be relevant in transplantation, but are not nearly so developed toward the clinic, will be touched upon briefly throughout this review. Lastly, we will discuss the challenges and obstacles faced in moving current and future co-stimulatory therapies to the clinic.

3. HISTORICAL PERSPECTIVES

3.1. Conceptual Developments

In 1970, Bretscher and Cohn reported that B-cells would die if exposed to antigen in the absence of a secondary cell signal, termed 'help'. With this they established the Associative Recognition Model and introduced a new paradigm of two signal modeling in lymphocyte regulation. This model added the requirement of a contextual signal to a signal of specificity for the generation of an appropriate immune response (5). In 1974, Lafferty and Cunningham, expanded this concept to T cells, postulating that in addition to a T cell receptor - antigen recognition (Signal 1), a second signal (Signal 2) now known as costimulation, was needed for a T cell to become fully activated (6). These signals were proposed to be provided by accessory cells with specialized antigen presenting capacity so as to provide an element of control over immunity. Importantly, they went on to suggest that Signal 1 stimulation alone would induce tolerant T cells (7). While insightful, these ideas remained conceptual for some time. For the next decade an abundance of research
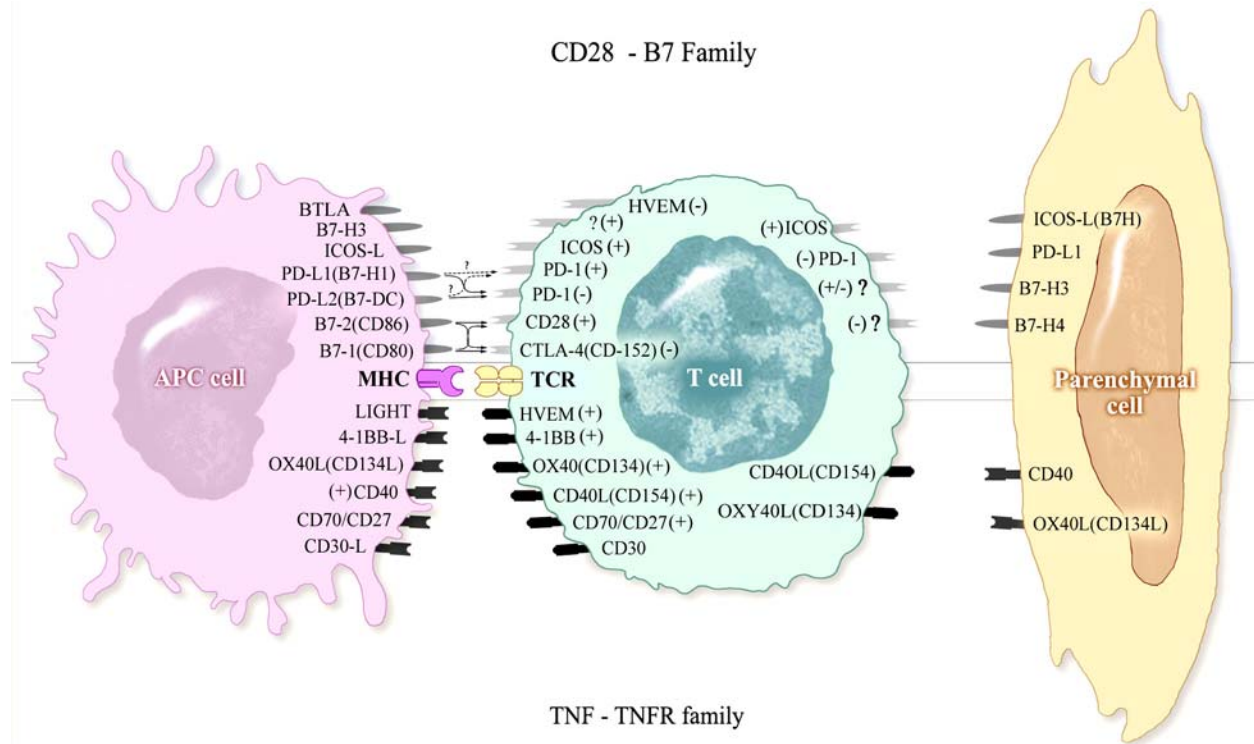
Figure 1. Lymphoid and parenchymal costimulatory molecules and their ligands. Depicted are the major (conventional) and novel costimulatory molecules discussed in this review. These costimulation molecules belong to two main families, the CD28 – B7 Family located within the top half of the figure and the TNF – TNFR Family located in the bottom half of the figure. It is now clear that the repertoire of costimulatory pathways contains both immunostimulatory and inhibitory specific pathways as well as some ambiguous pathways with the possibility of doing both. Each pathway contains unique temporal, spatial, and functional expression characteristics extending beyond the T cell to encompase interactions involving antigen presenting cells (APCs), B cells, endothelial cells, platelets, and parenchymal cells. Note: TCR-MHC complex binding is also found between the T cell and parenchymal cells as well as binding between the CD28 & CTLA-4 / B7.1 / B7.2 pathways, which are not pictured in this figure.

was conducted on Bretscher and Cohn's 'help' signal as a T cell function with less attention focused on a potential costimulation role of APCs. At the time, emphasis focused on the idea of a 'help' signal provided by antigen specific T-helper cells, while the contribution of costimulation provided by APCs lacking antigen specificity was not as appreciated. An immune response initiated by antigen non-specific scavenging APCs, thought to be constitutively active at that time and presenting phagocytized material indiscriminately, did not clearly fit with the immunologic theory of the day which was focused on the recognition and discrimination of self - nonself antigens (8).

Two main developments helped move costimulation from theory to practice and underscored the role of the APC in regulating antigen specific immune activation. First, Schwartz and colleagues in 1987, serendipitously uncovered direct experimental evidence that T cell activation required a second costimulatory signal (Signal 2) (9). Studying the nature of Signal 1, they attempted to stabilize the MHC/peptide complexes by using gluteraldehyde to fix antigen specifically loaded APCs. They found that these fixed APCs, while still containing nominal antigen, no longer functioned to stimulate T cell clones and actually induced an inactive or anergic state in

some cells. It is important to note that the only T cells affected were those engaged in a Signal 1 interaction, convincingly suggesting that this phenomenon was antigen specific and that a Signal 2 was necessary for T cell activation. The second major development focused on the constitutive state, functional nature, and stimulatory requirements of APCs. Work by Janeway *et al.* in 1989, demonstrated that, contrary to the prevailing theories of the time, APCs were constitutively quiescent and become activated through the engagement of pattern recognition receptors that recognize evolutionarily conserved pathogen-associated molecular patterns on bacteria (10). Activated APCs then up-regulated Signal 2 costimulatory molecules, began acquiring and processing pathogenic, infectious, and damaged tissue antigen, finally presenting them to passing T cells (11).

Following these advancements, the immunologic and transplantation communities began collecting vast amounts of experimental data on costimulation molecules and pathways, giving way to the full emergence of a two signal paradigm in T cell activation and the idea of antigen specific immune tolerance induction. Further work by Schwartz and Jenkins, reported strong evidence that TCR engagement in the absence of effective costimulation

resulted in T cell anergy and/or apoptosis, (12,13) thus providing a potentially significant mechanism to target for the induction of transplant tolerance. Anergy, defined as the specific inability of a T cell to respond when re-exposed to the same antigen was developed as a potential means toward promoting allo-antigen anergy and allograft tolerance (14).

The first major pathways to be identified and characterized as costimulatory were the B7/CD28 and CD40/CD154 pathways. More recently, many additional pathways have been discovered with clear homologous associations with know costimulatory molecules, and the concept of costimulation has spawned a complementary concept of co-inhibition. Together, these pathways have been woven into a view of immunity as a balanced process of activation and resolution (15,16). Furthermore, costimulation molecules have been shown to exist not only on lymphoid but also on parenchymal tissues. Thus, costimulation has grown to be seen as a process by which immune responses occur cognizant of the state of the parenchyma in the maintenance of organism homeostasis (see Figure 1). The last decade has seen considerable work in the manipulation of costimulatory molecules to eliminate allograft rejection. There has been success in increasing long-term survival in large animal models, and these pre-clinical results have given rise to early clinical trials.

3.2. The Characterization of Major Costimulatory Pathways

3.2.1 CD28/B7/CD152

With the emergence of the two signal paradigm of T cell activation, increasing numbers of T cell surface molecules were suggested to influence proliferation and differentiation (17,18). CD28 was the first costimulation molecule to be recognized as such. Initially recognized as a 44 kDa homodimeric glycoprotein expressed on 80% of human peripheral blood T cells, (19,20) the antigen was initially termed T44 and was subsequently designated CD28 in 1987. Gmunder and Lesslauer were the first to report that the binding of bivalent anti-CD28 monoclonal antibodies (mAbs) could augment T cell proliferation after suboptimal doses of PMA (21). Subsequent reports showed CD28 stimulation also caused marked augmentation of T cell proliferation with suboptimal stimulation by TCR cross-linkage (22,23,24). June and colleagues showed the addition of CD28 stimulation induced cyclosporine (CsA)-independent T cell proliferation and enhanced interleukin 2 (IL-2) mRNA transcription and protein secretion in polyclonal stimulated cells (25,26). Furthermore, CD28 stimulation in conjunction with TCR stimulation dramatically augmented cytokine production (27). The identification of a ligand for CD28 came in a report that antibodies to B7 could block the adhesion of B cells to T cells transfected with CD28 (28). Subsequently, it was shown that cells transfected with B7 could provide costimulation signals to antigen or mitogen-activated T cells (29). Together these data suggested CD28 was an obligate costimulatory receptor for the activation of resting T cells, and that it was specifically responsive to the B7 molecules CD80 and CD86 (30,31).

These findings provided specific targets for controlling an immune response, and stimulated a surge in the development of blocking agents and knockout mice. This advance led to rapid gains in our understanding of how manipulating the CD28/B7 pathway could promote long-term allograft survival in animal models (32,33). Additional studies helped uncover the biochemical and molecular basis involved in CD28/B7 interactions (34). Interesting double KO transgenic mice for CD80/CD86 are not able to reject cardiac allografts (35), however fully mismatched CD28 KO mice are still able to develop an immune rejection response (36). The basis for this differential, suggested CD28 independent pathways of rejection exist. At the same time alternative costimulation pathways began to emerge within cell-specific populations, suggesting involvement of CD8 T cells and/or NK-mediated effector cells, in the mediation of CD28-independent triggered immune responses (36,37). In addition to the significant investigational efforts and growing understanding of the importance of novel costimulation pathways in allo- and auto- immune responses, the B7/CD28 pathway continued to be heavily researched; it currently remains the most extensively studied costimulation pathway and thus has evolved the furthest as a clinical therapeutic agent.

The concept of co-inhibition as a partner to costimulation was born in the discovery of CTLA-4, a homolog of CD28 that was initially identified through subtractive RNA hybridization screening of mouse CTL clones (38). Like CD28, CTLA-4 binds both CD80 and CD86 (39) but with markedly higher affinity: 10 and 20 fold higher for CD80 and CD86, respectively (40). However, unlike CD28, CTLA-4 expression is absent on resting T cells and is up-regulated following CD28 induced activation. CTLA-4, now known as CD152, inhibits IL-2 and IL-2R expression, arrests T cells in G1 phase, and inhibits naïve and primed CD4+ and CD8+ T cells (41). It also regulates peripheral T cell tolerance, binding to CD80 and CD86, and inducing expression of idoleamine 2,3 dioxygenase (IDO) in DCs (42). IDO stimulates the degradation of tryptophan limiting the proliferation capacity of T cells (43). Thus, the same ligands that initiate an immune response also prevent its unbridled continuation. When there is excess B7, sufficient CD28 signaling can persist, but given the dominant affinity of CD152, when B7 is limiting (as when APC activation wanes with the clearance of an offending pathogen), the inhibitory effects of CD152 dominate and quell T cell responsiveness.

CD28 is constitutively expressed on over 90% of CD4+ T cells and 50% of CD8+ T cells in humans (44). On the other hand, CD80 is typically absent and CD86 is only expressed at low levels on resting APCs, (31) and both undergo significant upregulation following APC activation. While initial studies suggested that the lack of T cell CD28 ligation (Signal 2) during antigen presentation renders those T cells anergic, subsequent work has shown T cell dependency on costimulation is quantitative rather than absolute. For example, strong TCR signals are less CD28-dependent, and once activated, T cells have reduced needs

for CD28 compared to naïve T cells (45,46). CD28 signaling synergizes with Signal 1, lowering the activation threshold and leading to enhanced proliferation, increased survival thru expression of anti-apoptotic genes, increased cytokine production, differentiation into T helper cells, and enhances B-cell antibody production help. CD28+ T cells may also stimulate Dendritic Cells (DCs) thru CD80 and CD86, activating the production of IL-6 and inhibiting the immunosuppressive metabolism of tryptophan catabolism by IDO (47).

3.2.2. CD40/CD154

The CD40/CD154 pathway was the second major costimulation pathway to be discovered (48,49). By 1984 it was evident that a direct contact between B and T cells is critical for the activation of resting B cells (50). This triggered many research centers to isolate the surface structures responsible for this interaction. CD40, now known as a member of the TNF receptor family, was first recognized for its role in B cell activation (51). Then Bancherau in 1989 showed anti-CD40 mAb with IL-4 or anti-IgM induced B-cell proliferation (52). At the same time it was found that a combination of anti-IgM and anti-CD40 would prevent the rapid and selective apoptosis of germinal center B cells (53). Following these studies, the focus of research shifted towards the role of CD40 in T cell dependent signals necessary for the maturation of germinal B cells.

Next, generation of a murine mAb 5c8, which inhibited contact dependent T-helper function of a T cell line (D1.1) (54) led to the identification and immunoprecipitation of a novel 30-kD surface protein (55) named TBAM. This ligand, variably known as gp39, CD40L and eventually CD154, was subsequently determined to be the functional ligand for CD40 expressed on the surface of activated T cells (56) (57) that promoted T-helper dependent B cell activation. This receptor/ligand interaction was also linked to the initiation of thymus-dependent humoral immune responses (56). In 1993, it was discovered that patients suffering from X-linked Hyper-IgM Syndrome were unable to class switch due to defective CD154 on their activated T cells; uncovering the mechanism to this disease that had been unknown for nearly 3 decades (58,59).

Keeping these previous studies in mind, many people, including Noelle, Aruffo, Gray, and others started to appreciate the important role of CD40/CD154 interaction in the regulation of humoral immune response, and immunity (60). Overall, many *in vivo* experiments with anti-CD154 antibodies and genetic studies from of the Hyper IgM patients as well as analysis of CD154-deficient mice have demonstrated the important role of CD40/CD154 in B cell proliferation, Ig production, isotype switching, and memory B cell development (61,48).

It is now recognized that CD40 is constitutively expressed on APCs, such as B cells, macrophages, dendritic cells (DCs), and thymic epithelia and can be induced on endothelial cells and fibroblasts (48). Initial CD40/CD154 interactions trigger costimulation signals in APCs to a greater degree than T cells. Thus, while CD154 was described as a T cell costimulatory molecule, its effects are more appropriately categorized as an APC activating pathway through CD40. CD40 plays a major role in the maturation of DCs, inducing them to become 100-fold more potent initiators of T cell responses (62,63). This increased augmentation in antigen presentation allows CD40 activated APCs the ability to activate CD8 cytotoxic T cells lymphocytes without further CD4-cell help (64,65). Engagement of CD40 leads to upregulated expression of, CD44, CD80, CD86, and ICAM-1, promotes survival through nuclear factor (NF)-kappaB responsive pathways, and enhances proliferation (62). In DCs, CD40 further augments production of IL-12, a potent inducer of Th1 differentiation, and in macrophages, CD40 increases production of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines, such as TNF, IL-8, and TNFalpha (62,66).

CD154 is expressed both as a type II integral membrane protein and a soluble cleaved cytokine. It is expressed following TCR: antigen engagement, and its expression is augmented by CD28 derived signaling (62). CD154 is most abundant on activated CD4+ cells and its expression coincides with their function as helper cells for B cell responses. T cells produce sufficient amounts of IL-2 which in combination with CD154 drives the proliferation of B cells (67). In the absence of CD28, CD3-CD154 activated T-cells, fail to produce IL2 and undergo apoptosis (68). CD154 can also be identified on CD8+ cells, B cells, eosinophils, mast cells, basophils, dendritic cells, epithelial cells, fibroblasts and endothelial cells (69). Lastly, but perhaps of greatest physiological importance, is the presence of CD154 in platelets (70). CD154 is now known to be contained in platelets and released upon platelet activation. This has been shown to be sufficient to induce an alloimmune response in the absence of any other source of CD154 (71), and is now viewed as a means by which injury and its requisite hemostasis, can initiate antigen uptake and an adaptive immune response (72).

4. CLINICAL DEVELOPMENT OF MAJOR COSTIMULATION PATHWAY INHIBITORS

4.1. CD28/B7 Targeted Studies

Initial studies in rodent models demonstrated that long-term survival could be reliably achieved by inhibition of the CD28/ B7 costimulation pathway. Strikingly, in some cases, inhibition of this pathway as a sole maneuver induced donor specific tolerance (73,74,75). Although the more striking beneficial effects have been shown to be model and strain dependent, (76,77,78) and less success has been seen in more stringent rodent and primate allograft models, (79,80)(81,82) the CD28/B7 pathway remains a critical target for induction of antigen specific tolerance. As would be expected from the role of this pathway, the tolerogenic effects seen in animal models can be augmented by the concomitant administration of donor-specific transfusion (DST) or other means of donor antigen augmentation (83). Blockade of the CD28/B7 pathway has been most commonly achieved through the administration of mAbs directed against CD80 and CD86, or through use

of B7 specific recombinant fusion proteins such as CTLA4-Ig.

CTLA4-Ig has been the most widely used and studied agent for blockade of the CD28/B7 pathway. It is a fusion protein consisting of the extracellular binding domain of CD152 (CTLA4) linked to a modified Fc domain of human IgG1. Like CD152, it binds with higher affinity to CD80/CD86 on APCs than does CD28 and thus serves as a competitive inhibitor of the CD28 pathway (84). Murine forms of CTLA4-Ig have experienced great success in rodents, inhibiting T cell-dependent antibody responses, humoral and cellular immunity, development and progression of chronic rejection, and significantly prolonging transplant organ survival (62). CTLA4-Ig is thought to act primarily on naïve T cells given their dependency on CD28 stimulation for activation. Thus, the agent has been seen as useful in preventing de novo responses, with less effect against established memory responses. However, recent evidence has also been reported suggesting CTLA4 can impact CD4+ memory. These results have shown that CTLA4-Ig significantly blocks Ag-driven memory CD4+ T cell proliferation and expansion, without affecting early recall and activation inducing a shift in the phenotype of the responding population from predominantly T – effector memory {T(EM)} cells in control-treated mice to predominantly central memory T cells, suggesting biased effects of CTLA4-Ig on T(EM) responses (85).

The human form of CTLA4-Ig, Abatacept, has been shown to have modest anti-rejection properties in non-human primate models of islet and kidney transplantation (81)(82). Its effect is likely the result of combined CD80 and CD86 blockade as individual blockade of these molecules with CD80 or CD86 specific mAbs is minimally effective, while the combined effect of CD80 and CD86-specific mAbs mimics that of abatacept (86). A Limited Phase I trial in renal transplant recipients provided evidence that B7 specific mAbs were safe when combined with an immunosuppressant maintenance regimen consisting of cyclosporine, mycophenelate mofetil (MMF), and steroids. However development of this dual mAb approach was stopped for financial reasons before efficacy could be measured (87). In contrast, abatacept has moved into the clinic, not as an anti-rejection agent, but as a drug to combat T cell mediated autoimmune diseases such as psoriasis and rheumatoid arthritis (88,89).

As the initial studies with abatacept in primate transplantation did not achieve the anticipated success, development for a more effective means of CD28/B7 inhibition continued (90). Efforts proceeded to enhance abatacept's binding properties through manipulation of CD152/B7 contact residues, particularly with regard to CD86 (91) as CD86 is constitutively expressed. (33,92) CD86 has been suggested to initiate T cell activation while the inducible CD80 functions more in sustaining a response (93). Furthermore, recent studies suggest CD86 binds preferentially to CD28, while CD80 primarily ligates CTLA-4 (94). Considering abatacept showed less than optimal binding to CD86 (95) and the desired effects seen

in allograft models required blockade of both CD80 and CD86, extensive screening of a library of CTLA4-Ig variants was undertaken. As a result a mutant molecule was identified with two amino acid substitutions (L104E and A29Y), named LEA29Y, now named belatacept, which demonstrated a fourfold slower off-rate for CD86 and twofold slower off rate for CD80 compared to abatacept. In addition it was tenfold more potent at inhibiting T cell proliferation in mixed lymphocyte reaction (90). In non-human primate studies, belatacept, demonstrated prevention of acute rejection and prolongation of renal and islet allograft survival as a monotherapy and in combination with a number of typically used drugs in human transplant immunosuppressive regimens – basiliximab, steroids, MMF, and corticosteroids. Belatacept also inhibited anti-donor antibody formation, thought to contribute to the development of chronic rejection and a major barrier to retransplantation (90).

Belatacept's favorable performance in non-human primates has led to its formal clinical development in transplantation. In the most formalized study of any costimulatory blockade agent in transplantation to date, a phase II multi-center clinical study comparing the safety and efficacy of belatacept versus cyclosporine (also referred to as the Calcineurin inhibitors, CNI, group) in approximately 200 human recipients of de novo renal allografts has been completed. All patients received basiliximab induction, MMF, and corticosteroids. Belatacept – based maintenance therapy, administered as a 30 minute IV infusion every 4 or 8 weeks, demonstrated equivalent efficacy in preventing biopsy-proven acute rejection at 6 months versus cyclosporine-based treatment (6-7% for belatacept versus 8% for CNI) (4). Additionally, belatacept-treated patients showed significant improvements in renal function and reductions in chronic allograft nephropathy compared with CNI-treated patients at 1 year, a finding that may be predictive of long-term graft survival. Furthermore, rates of cardiovascular and metabolic co-morbidities, such as hypertension, dyslipidemia, and post-transplant diabetes, were observed to be lower in the belatacept arm compared to the CNI arm however was not significant. Discontinuations and adverse effects, including infections and malignancies, were similar between belatacept and CNI groups, as were rates of graft loss and death (4). Phase III clinical trials of belatacept have been initiated in renal transplantation, both in primary renal allograft recipients and in recipients using organs from extended-criteria donors. Both trials are utilizing several primary endpoints to show non-inferiority to CNI with respect to acute rejection as well as to assess belatacept's effect on preservation of renal function.

Many novel experimental uses of CD28/B7 inhibitors have been studied. It is clear that this pathway has promise beyond its current use as a maintenance immunosuppressant. Bashuda *et al.*, building on the understanding of suppressor activities of anergic T cells, (96,12) have recently induced long-term renal allograft survival in rhesus monkeys using *ex vivo* generated anergic T cells. Splenic CD4+ T cells from recipient monkeys were harvested, co-cultured with irradiated donor splenocytes in

the presence of anti-CD80 and anti-CD86 mAbs, and then they were re-injected into recipient monkeys after 13 days of cyclosporine (CsA) and cyclophosphamide treatment. Three of six monkeys survived indefinitely. (97) Animals on the same regimen, but re-injected with T cells activated by third party rather donor, soon experienced severe acute rejection. The safety of this protocol makes it a plausible regimen to move towards the clinic.

Despite the occurrence of rejection seen in CD28 KO mice suggesting that additional CD28 independent pathways can play a major role in allograft rejection, researchers have continued to target this molecule directly with antibodies. Recently, anti-CD28 antibodies showed promise in rodent models preventing acute and chronic rejection (98). Anti-CD28 also prevented MHC class II alloantibody production and generation of a B7+ non-T regs with tolerance sustained by IDO and inducible nitric oxide production (99). However, the most recent attempt to bring anti-CD28 antibodies to the clinic has met with sobering complications. A trial with the CD28-specific mAb TGN1412 has resulted in unexpectedly severe complications reminiscent of a cytokine storm with shock, ARDS, and the need for intensive care unit admission (100).

Lastly, strategies to try and crosslink CD152 *in vivo* and mimic its coinhibitory effects in mice (using CTLA-4 antibody agonists) have initially demonstrated that selective ligation of CD152 attenuates *in vivo* T cell responses, prevents development of autoimmunity, and represents a novel immunotherapeutic approach for the induction and maintenance of peripheral tolerance (101). In allograft rejection, CD152 signaling serves to limit the number and activity of CD4 and CD8 T cells responding to allogenic challenge (102). Importantly, CD152 signaling is required for the induction of tolerance by several strategies, including DST and mixed chimerism by bone marrow transplantation (BMT) after nonmyeloablative conditioning with anti-CD154 (103). Although CD152 has protolerogenic effects, harnessing its therapeutic potential is limited by the fact that it is not constitutively expressed. As would be expected antagonistic anti-CD152 augments rather than inhibits immune responses. The later is evident in cancer trials showing autoimmunity associated with anti-CTLA-4 antibody treatment and tumor regression (104,105).

4.2. CD40/CD154 Targeted Studies

Therapeutically targeting the CD40/CD154 pathway in hope of inducing antigen specific tolerance has also been an attractive idea in the transplant community for the last decade. Remarkable results in allograft survival and tolerance induction using anti-CD154 have been observed in rodent models (69). In addition, combined treatment with the administration of allogeneic small lymphocytes permitted indefinite pancreatic isl*et al*lograft survival (106). Anti-CD154 also has been shown to dramatically prevent acute rejection and promoted long-term allograft acceptance in non-human primates (81,107,108), although, in all of these models, the effect has not prevented eventual chronic allograft loss. Given the remarkable ability of

CD154-specific mAbs to prevent acute rejection, significant effort has been directed toward the clinical development of anti-CD154 based approaches.

Unfortunately positive results in animal models have not easily translated into clinical benefit. Clinical trials testing anti-CD154 in autoimmune disease and transplantation were terminated due to an unanticipated elevated incidence of thrombo-embolic complications. Subsequent work has shown that platelets are a major source of soluble CD154 (sCD154) in peripheral blood and that this molecule has a role in the control of thrombo-embolic and inflammatory processes seen clinically (109). These results fostered further investigation into the physiologic role and function of CD154, this costimulation pathway, and their implications to immunity and transplantation.

To briefly summarize these investigations, it was found that thrombin-activated platelets rapidly express CD154 that can interact with CD40 on endothelial cells mediating chemotaxis and upregulation of adhesion molecules, including E-selectin, ICAM and VCAM. This interaction has attractive teleological implications in that it pulls together an adaptive response to trauma with an initial immune activation response (72). Others additionally suggested that sCD154 might activate platelets through CD154/CD40 ligation although these effects are weak (110). CD154 also showed evidence that it can stabilize arterial thrombi by an integrin glycoprotein (GP) IIb/IIIa dependent (CD40-independent) mechanism (111). CD154-/- deficient mice have unstable thrombi, whereas this defect is not observed in CD40-/- mice (112).

Confounding these new findings, an additional study in human renal transplantation have further raised concerns about the efficacy of anti-CD154 mAbs as data showed that five of seven patients treated with hu5c8 experienced rejection episodes (113). Subsequent evidence suggested that CD154 is most relevant in de novo responses and has less of a role in recall responses or heterologous memory responses, (114) therefore, as with CD28/B7 blockade, the appropriate use of CD154-based therapies will be dependent on the adjuvant therapies utilized with it.

Given the need for appropriate adjuvant immunosuppression for use with anti-CD154, significant effort was directed toward combination therapies. Administration of CTLA4-Ig with anti-CD154 has been an attractive option. It has been particularly successful in rodents inducing indefinite acceptance of cardiac allografts and prolonged skin graft survival (75). It has been clearly efficacious but less durable in non-human primates (81) (115). Importantly however, the addition of B7 blockade to CD154 based approaches has consistently delayed the development of donor-specific allo-antibodies compared to anti-CD40/CD154 monotherapies (107,116). In addition, anti-CD154 antibody paired with anti-CD25 has been shown to produce markedly prolonged allograft survival, however not significantly better than animals treated with anti-CD154 alone (117).

More recently, CD154 mAb IDEC-131, was used in combination with rapamycin and a short course DST. This combination was notably successful in prolonging skin graft survival and inducing operational tolerance in a primate renal allo-transplant model (118,119). Similarly, recent work by Larsen and colleagues has shown that anti-CD154/anti-CD28 costimulation blockade combined with rapamycin and BMT significantly improved the length of induced chimerism and tolerant survival in a nonhuman primate model (120). Another anti-CD154 antibody, ABI793, also prolonged renal allograft survival in rhesus monkeys, however, thromboembolic complications were again raised and chronic allograft nephropathy developed after stopping the treatment (121). These results further support a crucial role for anti-CD154 in tolerance induction however these severe complication risks have halted its industry development towards clinical application. Perhaps combining anti-CD154 into a multi-drug regimen (within academia) would help limit the required dose level of anti-CD154, decreasing adverse side affects, and help to bring this therapeutic agent back into developmental favor.

Although the results of targeting the CD154/CD40 pathway through CD154 blockade proved ineffective due to resulting severe complications, recognizing that thrombo-embolic side effects occur in a CD40-independent manner as mentioned above, may suggest that blocking CD40 could be an alterative method to safely target this pathway. To this end, several anti-CD40 agents have been developed, one of which has been evaluated and showed promising results in a primate renal allograft model (122,123). Chi220, a chimeric anti-human CD40 mAb, is another that has been evaluated. Alone, it modestly prolonged islet and renal allograft survival in rhesus macaques. However, it was particularly effective when combined with belatacept (124). Chi220 inhibits B-cell proliferation, however its contribution to ligand blockade, partial agonistic properties, and/or FcR-dependent cell depletion immunosuppressive activities are largely unknown. In this regard it is also important to note that altering glycosylation on CD154-specific mAbs markedly changes its efficacy, suggesting that ligand cross linking may be important to finding a resolution to the anti-CD154 mediated complications mentioned above (125).

In summary, although translation of anti-CD154 has not proceeded as anticipated, the result achieved in animal models has raised the bar for other costimulation therapies. It is clear that modulation of the CD40/CD154 pathway is crucial for the induction of transplant tolerance, thus further investigation is needed to resolve the unfortunate events seen in clinical trials. In addition, historically classified as a costimulatory molecule, it may be reasonable to view CD154 as a molecule that defies the stimulatory/inhibitory classification model and is best viewed as an APC regulatory molecule. Also keep in mind its broad distribution, which suggests it plays a much more important role in orchestrating the immunity than we currently understand (69,72). Lastly, further investigation into therapies targeting CD40 are clearly warranted.

5. NOVEL PATHWAYS CURRENTLY IN ANIMAL MODELS

Although it is clear that the CD28 pathway is critical for physiological T cell activation, CD28 (-/-) mutant mice still induce cytotoxic T cells, reject skin grafts, and mediate delayed type hypersensitivity (126)(127)(128). Thus, the CD28/B7 pathway is not an absolute requirement for mounting an immune response against an allograft and other CD28 independent co-stimulatory pathways have been appropriately implicated. There pursuit has led to the discovery of many additional costimulatory pathways each of which has potential for future clinical exploitation (see Figure 1).

5.1. Costimulation Positive Pathways

5.1.1. ICOS/ICOSL (Bh7)

A CD28 homologue, inducible costimulation molecule (ICOS) is induced after TCR engagement on activated T cells and on resting memory T cells (129). CD28 costimulation enhances ICOS upregulation, which is markedly reduced in the absence of B7. This suggests that this pathway may mediate some of the functions of CD28 (130). ICOS Ligand is constitutively expressed on resting APCs and B-cells, and can be induced on fibroblast and endothelial cells (31). In the transplant setting, rejecting cardiac allografts exhibit increased parenchymal expression of ICOS, and blockade of the pathway prevents the rejection of vascularized allografts in some murine models (131). Combined blockade of CD154 and ICOS results in prevention of chronic allograft vasculopathy in mice (132). This combined blockade also leads to the long-term acceptance of fully allogeneic islet cells (133). Anti-ICOS mAbs also synergize with CTLA4-Ig to induce donor specific tolerance under some conditions (134). The timing of ICOS blockade is an important parameter. Blocking this pathway during the effector phase significantly promotes long-term survival when compared to early blockade (135,136). Furthermore, ICOS-B7h blockade synergizes with DST to promote long-term allograft survival in a class II mismatched skin model (137). Lastly, plasmacytoid DCs were shown to prime IL-10 producing T-regs through ICOS-L (138).

5.1.2. CD134 (OX40)/CD134L (OX40L)

CD134 (OX40) was originally identified on activated rat CD4+ T cells. The gene was later cloned in rats, mice, and humans (139)(140)(141). In humans and rodents, OX40's expression is restricted to activated T cells (142). CD134L (OX40L), a member of the TNF superfamily, is a type II trans-membrane protein (143) found on activated murine B cells (144) human dendritic cells (145), human vascular endothelial cells (146), and HTLV-1-transformed T cells (147). Many studies investigated the role of OX40/OX40L in regulating primary T cell responses. Activation of naïve T cells (both *in vitro* and *in vivo*) results in mild expression of CD134, with a peak after 2-3 days, and down regulation by days 4-5 (148). OX40/OX40L not only induces clonal expansion and survival of CD4 cells in the primary response, but also

mediates a potent positive costimulatory signal that increases effector and memory T cell function (149).

Studies with OX40 (-/-) mice have shown OX40 to be required for T cells to sustain proliferation during the latter phases of a primary immune response. These mice also exhibit diminished Th1 and Th2 cytokine responses (150)(151)(152). Agonistic OX40 mAbs have been shown to reverse an established state of tolerance in mice (153). Using a CD28/CD154 double knockout (DKO) mouse model, blockade of the OX40/OX40L pathway resulted in the induction of long-term skin allograft survival (154). In another model, OX40L blockade in combination with B7 blockade has been shown to achieve long term allograft survival in all rat recipients of cardiac and skin allografts, preventing the expansion and persistence of primed effector alloreactive T cells. However, Anti-CD134L mAb alone does not significantly prolong cardiac allograft survival (155). In the absence of CD28 and CD154 signaling, OX40 blockade is able to inhibit rejection in a presensitized mouse model using adoptive transfer of memory T-cells, generated either by homeostatic proliferation or priming with donor antigen, further strengthening the role of this pathway in mediating effector/memory T-cell subsets (156). Lastly, studies are just beginning to suggest a role for the OX40/OX40L pathway in suppressing T-regulatory cell function during and immune response (157,158).

5.1.3. CD137 (4-1BB)/CD137L (4-1BBL)

CD137 (4-1BB) is present on activated T cells and NK cells (159)(160). Its ligand, CD137L is primarily expressed on dendritic cells, activated B cells, and macrophages (161). Signaling via the 4-1BB pathway is more important for CD8 proliferation and survival than CD4 (162,163). The 4-1BB:4-1BBL pathway, is parallel to the OX40/OX40L pathway in exerting its effects late in the primary immune response by sustaining triggered CD4 and CD8 T cell responses as well as increasing cell division and improving the T cell effector function (164). CD137 blockade has been shown to significantly inhibited rejection of intestinal allografts by CD8+ but not CD4+ T cells in mice (165) and may be an attractive adjuvant for blocking CD28 independent alloimmune responses, particularly those driven by CD8+ T cells (166)(167)(168).

5.1.4. CD27:CD70 (CD27L)

CD27 is a member of the TNF-R superfamily and is involved in T cell activation and development of T cell dependent antibody production (169). CD27 is present on NK cells, however unlike OX40 and 4-1BB, it is present on B-cells and constitutively expressed on naive T cells (170). Similar to these pathways, it is up-regulated by Signal 1, although it peaks after several rounds of cell division, before OX40 and 4-1BB (171). Loss of CD27 correlates with high levels of effector function.

CD70 (CD27 ligand) is found on medullary thymic epithelium and is rapidly upregulated on activated T-cells, B-cells, and dendritic cells. In transplantation, CD70 blockade prolonged survival of fully mismatched, wild-type, murine cardiac allografts recipients and induced long-term survival in CD28-deficient mice as well as significantly prevented the development of chronic allograft vasculopathy. CD27/CD70 blockade had little effect on CD4 (+) T cells, but it prevented CTL mediated rejection, inhibited effector CD8 T cell proliferation/activation, and decreased expansion of memory T cells *in vivo* (172). Therefore making this pathway, like the 4-1BB/4-1BBL pathway, a possible target for the modulation of CD8+ tolerant resistant effector T-cells.

5.1.5. LIGHT:HVEM

Recent studies have shown that herpesvirus – entry mediator (HVEM, a costimulatory TNF receptor) engagement with its endogenous ligand, LIGHT, a member of the TNF family, induces a powerful immune response (173,174). Identification of human cDNA-encoding HVEM was initially discovered during a screening that would allow HSV1 entry into Chinese hamster ovary (CHO) cells (175). Precipitation studies using a HVEM-Fc fusion protein then allowed for the identification of the first HVEM ligand, lymphotoxin-alpha (LF-alpha). The second ligand to HVEM was identified, LIGHT (*l*ymphotoxin-like, exhibits *i*nducible expression, and competes with HSV *g*lycoprotein D (gD) for *H*VEM, a receptor expressed by *T*-lymphocytes; TNFSF14), was found to be homologous to LF-alpha (176). LIGHT, which can also bind to lymphotoxin-Beta receptor, has emerged as a potent initiator of T-cell costimulation signals effecting CTL-mediated tumor rejection, allograft rejection, and graft versus host disease. Constitutive expression of LIGHT leads to tissue destruction and autoimmune-like disease syndromes (177). More recently, LIGHT was further shown to be a regulator of allogenic T-cell activation and allograft rejection in a cardiac transplantation murine model. LIGHT deficient recipients showed a modest increase in allograft survival, while the addition of cyclosporine significantly prolonged cardiac allograft survival. In addition, the authors showed that increased expression of LIGHT and HVEM on allograft infiltrating leukocytes with the absence of expression of these costimulatory molecules on cardiac tissue, suggested any effects seen are a result of LIGHT-HVEM signaling between T-cells (178). In another study, blockade of the HVEM-LIGHT pathway with HVEM-Ig attenuated the development of graft arterial disease through the suppression of cytokine expression and SMC proliferation (179). Clearly more studies are needed with HVEM blocking antibodies, fusion proteins, and HVEM-LIGHT pathway disrupted murine models to specifically understand this pathway's immunologic function. Regardless, recent studies have illustrated the potential importance to targeting LIGHT signaling through HVEM in immune responses.

5.2. Co-inhibitory Pathways

5.2.1. PD-1 – PDL1 / PDL2

PD-1 (programmed cell death receptor-1) and its ligands, PDL1 (B7-H1) and PDL2 (B7-DC), are all members of the B7 costimulatory family. PD-1 was identified by subtractive hybridization screening of a T cell hybridoma undergoing cell death (180). PD-L1 is expressed

on T and B cells, APCs, and a broad range of non-lymphoid cells suggesting a role in peripheral tolerance (31). PD-L1 expression on pancreatic islet cells, but not the expression on APCs, delays the development of autoimmune diabetes in a non-obese diabetic (NOD) mice suggesting PD-L1 plays a role in the regulation of T-cell mediated adaptive-immune response development towards auto- reactive and allo-specific tissues, targeted during trauma, disease, and/or transplantation (181). PD-L2 expression, on the other hand, is limited to APCs, such as dendritic cells and macrophages. These studies would suggest that a deficiency in allograft PD-L1 expression could lead to a decreased ability to negatively influence T-cell allo-reactive signaling, subsequently resulting in allograft rejection. Similarly, auto-pathology such as in autoimmune diseases can become persistently auto-reactive to a specifically targeted tissue deficient in PD-L1 expression.

On the other hand, the ability to enhance PD-L1 signaling (T-cell activation inhibition) to modulate alloimmune responses has been made possible through the use of a PD-L1 – Ig fusion. When PD-L1 – Ig is given with a small, sub – therapeutic, dose of immunosuppressives or costimulation blockade there is a delay in the onset of allogeneic graft rejection in murine models (182,183). PD-L1 blockade however, accelerates acute rejection, but only in the absence of CD28 signaling (184). More recently, PD-L1 blockade has shown to inhibit the expansion and induction of CD4+25+Foxp3+ regulatory T-cells by alloantigen presentation of vascular endothelium (185). Evidence thus far has shown there to be a significant role for the PD1 / PD-L1 pathway in terminating an alloimmune response, either through negative T-cell signaling (leading to T-cell anergy or apoptosis) or expansion of T-regulatory cells. Overall, PD1/PD-L1 pathway offers a specific peripheral locale (tissue or allograft parenchyma), which can be specifically targeted for the regulation of immune responses. Assuming proper temporal expression of PD-L1can be accessed, maximum therapeutic effect could be achieved with smaller doses, reducing risks of adverse effects (186).

5.2.2. BTLA – HVEM
B and T lymphocyte attenuator (BTLA) was more recently discovered and identified as a member of the B7 family that is expressed on activated T-cells, B-cells, and DCs (187). BTLA interacts almost exclusively with HVEM, expressed on naïve T and B-cells, leading to inhibitory signaling and negatively influencing allospecific immune responses (188,189). BTLA – KO mice are characterized by enhanced *in vivo* immune responses, further suggesting an inhibitory function of this pathway (190,191,192). At the moment however, little is known about the significance of this pathway in the development and resolution of alloimmunity. In partially MHC-mismatched mice, BTLA blockade lead to accelerated allograft rejection. Conversely, fully MHC-mismatched mice acutely rejected their grafts despite induction of both PD-1 and BTLA. Unexpectedly, targeting BTLA resulted in an up-regulation of PD-1 expression by alloreactive CD4 and CD8 T-cells, and prolonged allograft survival (193).

However, additional work has recently suggested a role for BTLA activity in sustaining CD4+ T-cell survival (194).

5.2.3. B7-H3 / Unknown Ligand
Another newly identified costimulation molecule, B7-H3, a member of the B7 family, is broadly expressed in non-lymphoid tissues and is up-regulated by inflammation mediators in human DCs, monocytes, T and B-cells, and NK cells. In an alloimmune setting, murine B7-H3 KO, cardiac and islet allograft recipients, showed no survival advantage without intervention. However, administration of rapamycin or cyclosporine significantly improved survival in this group compared to WT mice, suggesting a positive role for B7-H3 in T-cell activation (195). Additional, studies cumulatively support an ambiguous role, showing both positive and negative costimulatory contributions to immune response (196-199). Further clouding our current understanding of this pathway, soluble B7-H3 (sB7-H3) binds to an unknown molecule on activated T-cells that is distinct from CD28, CTLA4, inducible ICOS, and/or PD-1 (31). Further studies are required to identify a ligand receptor for B7-H3, to define the full function of this pathway in alloimmune responses, and to determine the hierarchical importance of the B7-H3 pathway in relation to major costimulation pathways.

5.2.4. B7-H4 / Unknown Ligand
B7-H4 (B7x, B7S1) is the newest member of the B7 family, which has showed to inhibit *in vitro* T-cell proliferation, cycle progression, and cytokine production (200-202). Blockade of B7-H4 *in vivo* promotes enhanced T-cell response development, however, administration of B7-H4 – Ig fusion protein results in an antigen specific impaired T-cell response. B7-H4 expression is limited to peripheral tissues and an inducible only pattern on hematopoetic cells, including T- and B-cells, monocytes, and DCs (201). The receptor for B7-H4 has yet to be identified; however, *in vitro* work has suggested that B7-H4 binds to a receptor on activated T-cells, but not naïve T-cells (31).

Recent studies have shown B7-H4 ectopic expression on APCs induces normal macrophages to begin to exhibit T-cells suppressive characteristics (203). It was then shown that T-regulatory CD4+ cells stimulated B7-H4 expression on APCs, enabling APC suppressive activity through B7-H4 signaling. The role of this pathway in alloimmunity remains largely unexplored, however this negative costimulation pathway provides a promising target for limiting T-cell activation (204).

6. OBSTACLES TO TOLERANCE INDUCTION AND RISKS INVOLVED WITH MOVING CO-STIMULATION THERAPIES INTO THE CLINIC

While insights gained into the mechanism of immune function in mice form the foundation for clinical costimulation modulation, the increased complexity of the immune system of critically ill human transplant recipients leads to barriers to tolerance that do not exist in murine models. For this reason NHP models have evolved to play a critical role in the translational application of costimulation

therapies in the clinic. Important attributes of NHP models that allow them to serve as an investigational bridge to patient care include their high degree of DNA and protein homology to the human system, more complex histories of immune exposures than experimental mice, and their relatively small size facilitating experimental drug dosing and captive breeding for use in preclinical studies (205). Nevertheless, these models are expensive and their necessity has made preclinical progress somewhat slow.

Another barrier to costimulation-based therapies is heterologous immunity, (206) the process by which immunological exposures induce T cells specific for cross-reactive alloantigens (207,208). Heterologous immunity can occur by at least two mechanisms, TCR cross-reactivity or non-specific bystander activation (209)(210). Given a precursor frequency of 1-10% (211,212) it is not surprising that many responses to viral antigens induce cross-reactive allospecific T-cells (206). In addition to data in rodent models of heterologous immunity, evidence in humans also exists. Memory cells specific for an EBV virus peptide (FLRGRAYGL) presented in the context of HLA-B8 have been shown to cross-react with three common allogeneic HLA molecules (213). Others have shown that a higher level of environmentally induced anti-donor memory is associated with a higher rejection rate in clinical transplantation (214).

It has also been reported that infection at the time of transplantation can prevent the induction of tolerance (215,216). Furthermore, tolerance induction through costimulation involves the risk of also inducing tolerance to a concurrent smoldering, persistent, or active pathogenic response. The harboring of a quiescent latent pathogen at the time of tolerance induction, present in a majority of human transplant recipients, adds similar risk of simultaneously losing immunity to the pathogen. This area has remained relatively unexplored experimentally and will be important to address moving forward.

Murine models have clearly shown the importance of complexity of a recipient's immune system in determining the ease or difficulty of inducing tolerance (217). However, parallel studies have yet to be conducted in NHP models (218). While successful control of naïve T-cell response can be achieved through costimulation blockade, inducing tolerance in memory T-cells has proven much more difficult. Recent studies show NK cells, CD8 T cells, and memory-effector responses appear to be less dependent on CD28 and/or CD154 costimulation, and utilize novel co-stimulatory pathways for activation. Furthermore, these novel signals differ in their ability to enhance or inhibit T cell activation, in their temporal and spatial expression patterns, and in their relative importance within the hierarchy of costimulatory signals. Emerging data suggest that costimulatory molecules are also expressed on endothelial cells and parenchymal cells. It has been clearly shown that memory T-cells posses a lower threshold for activation and may not be susceptible to current costimulation modulating therapies.

While current tolerance induction strategies focus on the adaptive immune response there is a growing appreciation from murine studies of the impact of the innate system on transplant rejection (219-222). Toll-like receptors (TLRs) and natural killer (NK) cells have featured predominantly in these analyses, there are currently no humanized reagents designed to directly target TLR or NK-mediated graft rejection in NHP or in patient populations. Controlling innate immunity during transplantation and developing the tools to rigorously examine its impact on NHP transplantation represents a critical unmet need in the field (205).

Further importance should be considered when contemplating the design of a clinical trial, whether integration of current immunosuppressive therapies is applicable. Early fears that conventional drugs may impair tolerance induction, has begun to be borne out, however studies have shown varied results while the optimal treatment regimen remains far from clear (223). Most current studies focus on uni- or bi-dimensional approaches to tolerance induction through costimulation blockade, however the growing body of evidence indicating the complexity of out bred, pathogen-exposed immune systems of primates and humans now demand that we broaden our targeted approaches to include multiple strategies simultaneously. When a multi-drug strategy is used the question of which drug(s) and when to taper them becomes much more complex as well. An additional major barrier to such a multimodal approach to tolerance induction is the fact that many of the reagents are not commercially available. Thus, most experiments most be performed under proprietary restrictions that can make combination therapy difficult (205).

7. CONCLUSION AND PERSPECTIVE

Many experimental studies have revealed an important role for costimulatory blockade/modulation in organ transplantation. Through their extensive investigation, the use of these agents are becoming increasingly well defined. However, as we learn more we also discover that these pathways are much more complex than originally envisioned by a simple two signal model (224). Nevertheless, progress is being made, particularly with CD28 dependent pathway inhibition. Ultimately, the optimal use of costimulatory blockade will require better monitoring tools adapted to these costimulation blocking agents and their effects. Also, it will be important to define the proper combinations of pathways to influence, as single pathway interruption is unlikely to be sufficiently efficacious. While, costimulation blockade already appears to be a powerful treatment strategy, it s crucial that we remain cognizance that there is no costimulation pathway solely alloimmune specific. Thus the attempt to induce allo-antigen specific tolerance carries with it equally compelling risk of inducing tolerance to pathogenic antigens, resulting in severe infectious and/or tumorogenic complications. However, with the proper combination of costimulation agents, dose levels, temporal administration, and congruent administration of allo-antigen; these regimens have clearly shown potential for promoting the

induction of transplantation allograft specific tolerance while minimizing the attenuation of protective immunity and need for chronic immunosuppression.

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Abbreviations: APC: Antigen Presenting Cell, ARDS: Acute Respiratory Distress Syndrome, BMT: Bone Marrow Transplant, BTLA: B and T lymphocyte attenuator, CHO: Chinese hamster ovary, CNI: Calcineurin inhibitors, CsA: Cyclosporine, CTL: Cytotoxic T lymphocyte, CTLA-4: Cytotoxic T lymphocyte antigen – 4, DC: Dendritic Cells, DST: Donor Specific Transfusion, DKO: double knockout, GP: Glycoprotein, HSV1: Herpes Simplex Virus -1, HVEM: herpesvirus – entry mediator, ICAM: Intracellular adhesion molecule, ICOS: inducible costimulation molecule, IDO: Idoleamine 2,3 Dioxygenase, iNOS:

inducible nitric oxide synthase, kDA: kilo Dalton, KO: knock out, LF-alpha: lymphotoxin-alpha, LIGHT: *l*ymphotoxin-like, exhibits *i*nducible expression, and competes with HSV *g*lycoprotein D (gD) for *H*VEM, a receptor expressed by *T*-lymphocytes; TNFSF14, mAb: monoclonal Antibody, MHC: Major Histocompatability Complex, MMF: mycophenelate Mofetil, NK: Natural Killer, PD-1: Programmed cell death receptor-1, PMA: phorbol myristate acetate, SMC: Smooth Muscle Cell, TCR: T cell receptor, T(EM): T-effector memory, TNF: Tumor Necrosis Factor, VCAM-1: Vascular Cell Adhesion Molecule-1 WT: wild-type,

Send Correspondence to: Allan Kirk, M.D., Ph.D., F.A.C.S., Department of Surgery and Emory Transplant Center, Emory University, 101 Woodruff Circle, Suite 5105 WMB, Atlanta, GA 30322, Tel: 404-727-8465, Fax: 404-727-3660, E-mail: allan.kirk@emoryhealthcare.org.