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T-cell co-stimulatory blockade in kidney transplantation: back to the bench

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It is believed that blocking positive T-cell co-stimulatory pathways should lead to long-term graft acceptance. Despite the exciting initial achievements in experimental animal models, targeting co-stimulatory pathways has shown to be much more complex in the clinic. In addition to multiple binding partners, some co-stimulatory interactions have been found to be inhibitory in nature, whereas others were demonstrated to be important in the development of regulatory T cells. Moreover, memory T cells have been shown to be resistant to co-stimulation blockade. Herein we focus on the B7:CD28 pathway and describe the evolution of targeting this pathway with cytotoxic T-lymphocyte antigen-4-Ig from bench to clinic. We also attempt to address possible causes for the unexpected high rejection rate observed in the phase III clinical trials with belatacept, using experimental data obtained from basic science research.

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T cells have a central role in allograft rejection. The specificity of the alloimmune response is determined by the interaction of the major histocompatibility complex (MHC) molecule on antigen-presenting cells with the T-cell receptor (TCR) on T cells, consisting of 'signal 1'. However, in order to fully activate a naïve T cell, a second antigen-independent co-signal must be delivered by co-stimulatory molecules (signal 2). Therefore, co-stimulation has a key role in determining the outcome of the T-cell encounter with the alloantigen, with important therapeutic applications in transplantation.^{1,2}

The best characterized co-stimulatory pathway is the B7:CD28 pathway. In both, mice and humans, CD28 is constitutively expressed on all naïve CD4⁺ and CD8⁺ T cells,³ and it can interact with two ligands, B7.1 (CD80) and B7.2 (CD86), expressed on antigen-presenting cells (Figure 1a). In the presence of TCR signaling, B7:CD28 interaction leads to full activation and expansion of T cells, whereas blocking this pathway results in anergy and/or apoptosis of responding T cells (Figure 1b).⁴ In the transplant setting, initial studies in experimental animal models with blockade of B7 ligands revealed promising results. However, the continuous expansion of co-stimulation knowledge led to some unexpected findings.⁵ Additional co-stimulatory molecules were discovered that shared ligands with each other, and some of these receptors demonstrated capability of inhibiting rather than activating T cells. For example, cytotoxic T-lymphocyte antigen-4 (CTLA4) was found to be structurally related to CD28 and bind to the same ligands on antigen-presenting cells (B7.1 and B7.2) as CD28; however, its interaction with B7 ligands led to inhibition of T-cell activation (Figure 1c). Moreover, other positive co-stimulatory pathways were discovered with non-redundant and compensatory roles in T-cell activation.⁵ Currently, it is clear that the integration of multiple positive and negative co-stimulatory signals ultimately determines the outcome of the T-cell response.⁵ In this review, we will focus on the B7:CD28 co-stimulatory pathway and discuss the evolution of this therapeutic target from bench to bedside, and then back to the bench, in an attempt to understand and explain recent clinical outcomes in kidney transplantation.

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B7:CD28 BLOCKADE: FROM BENCH TO BEDSIDE

After an alloantigen encounter, B7:CD28 signaling was shown to help in fully activating T cells by increasing transcription

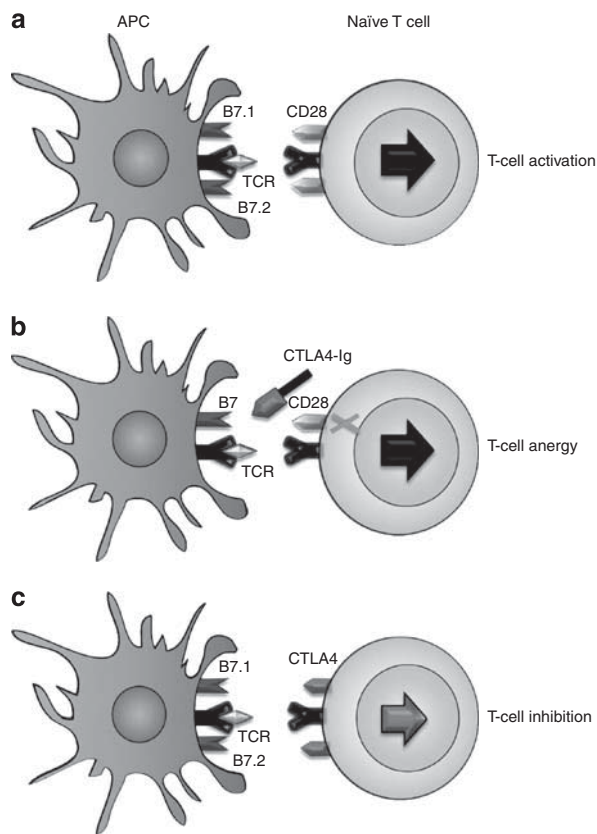


Figure 1 | Role of co-stimulation in T-cell activation. (a) Upon antigen encounter, B7.1/B7.2 ligands expressed on antigen-presenting cells interact with CD28 receptors on T cells, leading to full T-cell activation. (b) In the absence of B7:CD28 interaction, T cells become anergic and/or apoptotic in the context of T-cell receptor (TCR) stimulation. This can be seen, for example, with blockade of the B7 ligands by CTLA4-Ig. (c) Co-stimulatory pathways can also be inhibitory as in the case of B7:CTLA4, which is able to suppress T-cell activation. It is the balance between positive and negative co-stimulatory pathways that will ultimately determine T-cell outcome. APC, antigen-presenting cells; CTLA4, cytotoxic T-lymphocyte antigen-4.

and mRNA stability of interleukin-2 (IL-2),⁶ elevating the expression of antiapoptotic molecules such as Bcl-XL,⁷ and decreasing the threshold of T-cell receptor activation.⁸ After failed attempts to develop an effective CD28 blocking antibody,⁹ researchers were able to successfully target CD28 ligands with antibodies against B7.1 and B7.2.^{10,11} In the early 1990s, a recombinant fusion protein, CTLA4-Ig, was developed by fusing the extracellular domain of human CTLA4 with an Ig heavy chain tail.¹² This antibody had a higher affinity to the B7 ligands than CD28, and was shown to be a powerful inhibitor of T-cell activation *in vitro*.¹² Subsequent testing of CTLA4-Ig revealed its capability of protecting the allograft against acute rejection in MHC-mismatched cardiac transplantation, as well as in islet cell transplantation in murine models.^{13,14} Despite its potent effect, CTLA4-Ig alone was incapable of inducing tolerance in non-human primates,^{15,16} requiring additional immunosuppression to promote graft survival.¹⁷

Further mechanistic studies revealed that CTLA4-Ig was 100-fold less potent in inhibiting B7.2 co-stimulation compared with B7.1 co-stimulation,¹⁸ possibly explaining its lower-than-expected potency *in vivo*. Therefore, a modification of this antibody was undertaken, with substitution of two amino acids within the B7.2 binding domain, creating a second generation of CTLA4-Ig (LEA29Y), which was shown to have higher affinity to both B7.1 and B7.2, translating into a 10-fold increase in biological potency.¹⁸ LEA29Y, later named belatacept (Bristol-Myers Squibb, New York, NY), was tested in non-human primates and showed superior prolongation in renal allograft survival as monotherapy, when compared with the first-generation CTLA4-Ig, and led to a marked improvement in survival when used in combination with other immunosuppressive regimens such as mycophenolate mofetil and steroids, or an anti-IL-2 receptor antibody.¹⁸

On the basis of these encouraging results, belatacept moved to a phase II clinical trial to evaluate the efficacy of this drug in kidney transplant recipients in comparison with cyclosporine (CsA).¹⁹ During this trial, recipients were assigned to receive either an intensive or a less-intensive regimen of belatacept, compared with CsA. Induction therapy consisted of basiliximab (IL-2 receptor monoclonal antibody), and maintenance therapy included steroids and mycophenolate mofetil. The results of this trial suggested that belatacept was non-inferior to CsA and it could potentially have a beneficial effect on glomerular filtration rate at 1 year after transplantation, presumably by the absence of calcineurin inhibitor-induced nephrotoxicity.¹⁹ Subsequently, a phase III clinical trial (BENEFIT) was published, in which an unexpected higher rate of acute rejection was observed in the belatacept group, especially in the intensive arm receiving more frequent doses (22 vs 7% on CsA arm).²⁰ Moreover, these rejections were more severe than the ones with CsA, with most of them with the Banff grades of IIA or higher. Nevertheless, the belatacept groups had a similar graft survival at 1 year and demonstrated superior renal function when compared with CsA.²⁰ Overall, the unexpected higher rate of rejection, especially in the more intensive regimen, was intriguing and suggested some unexpected consequences of the intensive B7:CD28 blockade that required further investigation.

TARGETING B7:CD28 PATHWAY: BACK TO THE BENCH Regulatory T cells

Although targeting B7:CD28 pathway to improve graft survival was becoming a reality, basic knowledge of this pathway and of the alloimmune response kept expanding. The discovery of regulatory T cells as a sub-population of T cells with inhibitory function and capability of controlling the alloimmune response generated great enthusiasm in the transplant community, as inducing this sub-population of T cells could potentially lead to tolerance development.²¹ Indeed, several groups published initial exciting results with cell-based therapy with regulatory T cells (Tregs) in tolerance

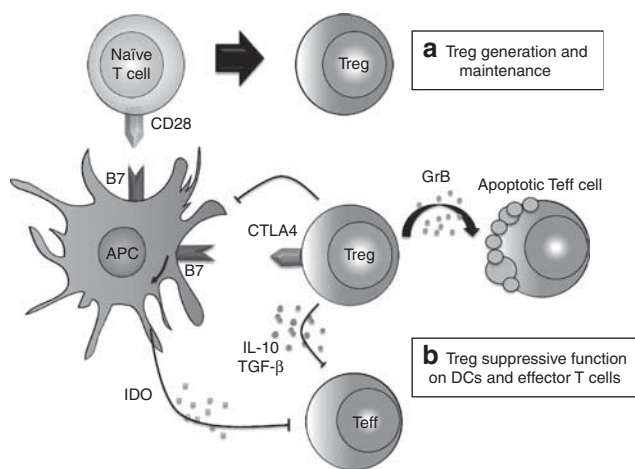


Figure 2 | Regulatory T cells and co-stimulatory pathways.

(a) B7:CD28 pathway was demonstrated to have a major role in the generation and maintenance of regulatory T cells (Tregs), leading to some concerns on the effect of long-term B7:CD28 blockade on Tregs in transplantation. (b) Another concern arose from the discovery that Tregs use the inhibitory pathway B7:CTLA4 to suppress dendritic cell (DC) function via induction of indoleamine 2,3-dioxygenase (IDO) and inhibition of the maturation of DCs (not shown). However, Tregs also exert their suppressive function through other mechanisms, such as secretion of inhibitory cytokines (e.g., interleukin (IL)-10, transforming growth factor (TGF)- β) and granzyme B (GrB), possibly representing parallel pathways of immune regulation. APC, antigen-presenting cells; CTLA4, cytotoxic T-lymphocyte antigen-4; Teff, effector T cell.

induction.^{22,23} Importantly, the development and homeostasis of Tregs was shown to be directly dependent on B7:CD28 co-stimulation, and deficiency on this pathway significantly decreased the amount of regulatory T cells in rodents (Figure 2).²⁴ This was a concern, as blocking this pathway could potentially affect Treg generation. Recently, in a single MHC class II-mismatched model of murine cardiac transplantation, in which allografts survive long term because of the emergence of Tregs that inhibit alloreactive T cells, the deficiency of either B7 or CD28 in recipients paradoxically led to an accelerated rejection.²⁵ This effect was related to the significantly lower number of Tregs in the deficient mice, tipping the balance toward more T-effector/memory cells rather than Tregs.

Therefore, B7:CD28 signal is important not only for the activation of pathogenic effector T cells but also for the generation of regulatory T cells, being the balance of effector T cells and Tregs that ultimately determines the fate of an allograft.²⁶ In an attempt to understand the observations above in rodents, we could hypothesize that in a fully allogeneic mismatched model, the pool of alloreactive T cells is much greater in size than on a single mismatched model; therefore, blockade of B7:CD28 is especially efficient in suppressing the activation and decreasing the alloimmune response in the former (Figure 3a). However, if the pool of alloreactive T cells is smaller, such as in the single mismatch model described above, B7:CD28 blockade could have a more deleterious effect in Tregs than on effector T cells, tipping the

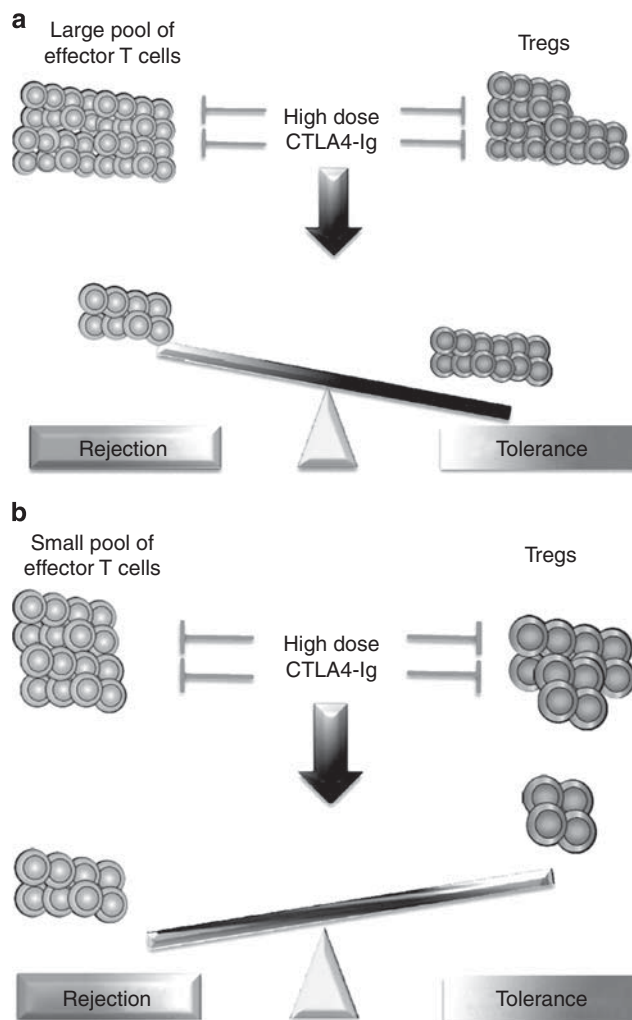


Figure 3 | The pool size of alloreactive T cells and the effect of co-stimulation blockade.

(a) In the setting of a large pool size of alloreactive T cells, blockade of B7:CD28 pathway by CTLA4-Ig leads to a predominant effect on effector T cells, tipping the balance toward regulatory T cells (Tregs)/tolerance. (b) When the number of alloreactive T cells is smaller, blockade of B7:CD28 leads to a dominant inhibitory effect on the generation of regulatory T cell, leading to more effector T cells than Tregs, and precipitating rejection in the transplant setting; CTLA4, cytotoxic T-lymphocyte antigen-4.

balance toward the pathogenic side and precipitating rejection (Figure 3b). The pool size of the alloreactive T cells could potentially correlate in humans with different scenarios, such as in deceased donor recipients and in sensitized patients vs first living-related transplants in a non-sensitized recipient (Figure 3).

Although an initial small study did not reveal a significant decrease in peripheral regulatory T cells of patients treated with belatacept in comparison with CsA,²⁷ this finding requires further evaluation, as the degree of mismatch, the sensitization of the recipient, the dose, and timing of belatacept must be taken into account, given that some recipients with well-matched grafts might suffer from the deleterious effects of B7:CD28 blockade on Treg generation.

B7:CTLA4 pathway

The failure of the CTLA4-Ig to uniformly induce tolerance may also relate, at least in part, to its blocking capacity of B7:CTLA4 interaction, which has been shown to be a critical inhibitory co-stimulatory signal.²⁸ In fact, CTLA4-deficient mice develop a severe systemic autoimmune disorder leading to death at several weeks of age,^{28,29} demonstrating a key role of CTLA4 in maintaining self-tolerance. In addition, blockade of CTLA4 with an anti-CTLA4 antibody has been shown to precipitate rejection and prevent induction of allograft tolerance in the transplant setting,³⁰ reinforcing the important role of CTLA4 signaling in inhibiting the alloimmune response.

CTLA4 is constitutively expressed on Tregs and has a 20-fold higher affinity than CD28 for both B7.1 and B7.2 ligands.³¹ In addition to the direct effect of Tregs on effector T cells by the secretion of inhibitory cytokines (e.g., IL-10) and apoptotic effects through granzyme B, the function of Tregs was also shown to be dependent on CTLA4 expression³² (Figure 2b). CTLA4 on Tregs can interact with B7.1/B7.2 ligands on antigen-presenting cells and downregulate the expression of these ligands while upregulating the expression of indoleamine 2,3-dioxygenase, a potent inhibitory molecule.³³ An agonistic agent to CTLA4 could potentially promote tolerance and improve graft survival; however, attempts of developing this agent have so far been unsuccessful. Overall, CTLA4 is an important inhibitory signaling pathway, and its blockade by CTLA4-Ig could affect the regulation of the alloimmune response.

Th17 cells

After antigen encounter, naïve T helper (Th) cells might differentiate into different subtypes according to signals delivered by antigen-presenting cells and the cytokines present in the microenvironment.³⁴ The subtypes of Th cells are characterized by diverse cytokine productions, with Th1 cells producing predominantly interferon- γ , whereas Th2 cells secrete IL-4. Donor-specific T cell responses after transplantation are typically dominated by interferon- γ -producing T cells (Th1).³⁵ More recently, T cells that produce IL-17 were discovered and showed an association with allograft rejection.³⁶ The concern about Th17 cells is that they have been reported to be resistant to current available immunosuppression, and especially resistant to co-stimulation blockade.^{37,38} In fact, CD28 co-stimulation reduced the frequency of Th17 cells, whereas CTLA4-Ig facilitated both murine and human Th17 differentiation *in vitro*.³⁹ In addition, CTLA4:B7 interaction was also demonstrated to inhibit Th17 cell differentiation and suppress the development of Th17-mediated autoimmunity.⁴⁰ Collectively, these findings suggest that B7:CD28 blockade might favor Th17 cell differentiation with potential concern for allograft outcome; however, the true role of IL-17 in the alloimmune response in humans still needs to be clarified.

Memory T-cell resistance

Memory T cells are lymphocytes that have been previously activated and possess a unique capacity to generate rapid

effector functions upon rechallenge with antigen. This capacity is related to their lower threshold for activation, less dependence on co-stimulation, and enhanced trafficking/adhesion mechanisms, being especially important in the response to infectious organisms.⁴¹ Humans develop alloreactive memory T cells after exposure to blood transfusions, pregnancies, or prior transplantation. More recently, it has been proposed that alloreactive memory T cells can also be generated by exposure to pathogens and environmental antigens, because of the resemblance of allogeneic MHC and microbial Ag/self-MHC complex (cross-reactive response).^{42,43} Finally, T-cell-depleting induction therapies used in transplantation have been shown to promote homeostatic proliferation of non-depleted T cells and these proliferating cells carry a memory phenotype.^{44,45}

The presence of memory T cells has important clinical relevance, as higher frequency of alloreactive T cells before transplantation correlate with an increased risk of rejection.^{46,47} Furthermore, these memory T cells are more resistant to B7:CD28 co-stimulation blockade⁴⁸ and, consequently, targeting solely this pathway might be ineffective in inducing tolerance. Indeed, the previous observations of effective results of CTLA4-Ig in naïve recipients with naïve T-cell repertoire in a laboratory-controlled environment (rodents) and failure of the same agent in promoting tolerance in recipients with memory cells (non-human primates) suggest a key role of these cells in tolerance resistance to co-stimulation blockade. Confirming this, the combination of CTLA4-Ig with a selective memory T cell agent (CD2-specific fusion protein alefacept) was shown to improve allograft survival in non-human primates,⁴⁹ opening potential new avenues in targeting memory cells. Nevertheless, targeting these cells carry its own risks as they have a key role in immunity against infectious diseases.

FUTURE OF TARGETING CO-STIMULATORY PATHWAYS IN THE CLINIC

Co-stimulation has a central role in T-cell activation, and targeting this pathway has become a reality in transplantation, consisting of a true translational research. However, the complex interplay between different co-stimulatory pathways and the function of these pathways in different cell types raised a number of challenges, and it is now clear that targeting a single pathway will likely be ineffective for the induction of transplantation tolerance.

To improve long-term graft survival, avoiding calcineurin inhibitor nephrotoxicity and long-term cardiovascular and metabolic side-effects are important goals. A recent open-label phase II trial has demonstrated that switching from a calcineurin inhibitor-based therapy to a belatacept-based regimen at 6 months after transplant is feasible and well tolerated, demonstrating potential improvements in renal function at 12 months.⁵⁰ This latter switch could be beneficial as it will require a less-intensive dose of belatacept in face of the smaller pool of alloreactive T cells later after transplant, and might be associated with a lower rejection rate.

Another experimental approach with remarkable results is the combination of CTLA4-Ig with a T-cell-depleting agent such as thymoglobulin. In a stringent transplant model in rodents, this combination tipped the balance of Tregs/Teff in favor of Tregs, promoting regulation and favoring graft survival.⁵¹ Moreover, the development of newer synergistic agents targeting negative co-stimulatory pathways such as PD-1:PD-L1 could enhance immune regulation and promote tolerance.⁵² Finally, the role of B cells in chronic rejection has been increasingly recognized,⁵³ and the generation of selective agents that are capable of decreasing alloantibody production and generating regulatory B cells will likely lead to considerable improvements in graft outcomes.

Overall, the future of co-stimulation targeting in kidney transplantation will most likely involve the combination of agents with different mechanisms of action, with the goal of inhibiting pathogenic lymphocytes and promoting regulatory ones, limiting single-drug toxicity, and possibly achieving the Holy Grail of transplant tolerance.

DISCLOSURE

All the authors declared no competing interests.

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