EXPERT OPINION

T-cell co-stimulatory blockade in transplantation: two steps forward one step back!

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Introduction: The concern about nephrotoxicity with calcineurin inhibitors led to the search of novel agents for immunosuppression. Based on the requirement of T-cell co-stimulatory signals to fully activated naïve T cells, it became clear that blocking these pathways could be an appealing therapeutic target. However, some unexpected findings were noticed in the recent Clinical Trials of belatacept,not on. Including a higher rate of rejection, which warranted further investigation with some interesting concepts emerging from the bench.
Areas covered: This article aims to review the literature of the B7:CD28 co-stimulatory blockade in transplantation, including the basic immunology behind its development, clinical application and potential limitations.
Expert opinion: Targeting co-stimulatory pathways were found to be much more complex than initially anticipated due to the interplay between not only various co-stimulatory pathways but also various co-inhibitory ones. In addition, co-stimulatory signals have different roles in diverse immune cell types. Therefore, targeting CD28 ligands with cytotoxic T lymphocyte antigen-4 (CTLA4)-Ig may have some deleterious effects, including the inhibition of regulatory T cells, blockade of co-inhibitory signals (CTLA4) and promotion of Th17 cells. Co-stimulatory independence of memory T cells was another unforeseen limitation. Learning how to better integrate co-stimulatory targeting with other immunosuppressive agents will be critical for the improvement of long-term graft survival.

Keywords: co-stimulation, rejection, tolerance, transplantation

Expert Opin. Biol. Ther. [Early Online]

1. Introduction

Transplantation has significantly advanced in the past 50 years due to the emergence of novel immunosuppressive drugs [1]. The discovery and clinical application of calcineurin inhibitors (CNIs) played a major role in improving 1-year graft survivals and in decreasing the rates of acute rejection when combined with antiproliferative agents, steroids and induction therapy [2]. Nonetheless, CNIs were shown to have significant side effects, including vasoconstriction and nephrotoxicity. Indeed, Ojo *et al.* reported that the prevalence of significant renal dysfunction (glomerular filtration rate [GFR] < 30 ml/min) at 5 years after non-renal solid organ transplantation was: 21.3% among intestine recipients, 18.1% among liver recipients, 15.8% among lung recipients, 10.9% among heart recipients and 6.9% among heart-lung recipients [3]. Renal biopsy studies among these recipients have shown that CNI-related injury is a common finding [4,5]. Therefore, the development of CNI-free regimens became an important goal for further improving long-term renal graft outcomes and preventing chronic kidney disease (CKD) in non-renal organ transplant recipients. Mammalian target of rapamycin inhibitors were considered as great potential substitutes, but their use has been limited by their significant side effects [6].

10.1517/14712598.2013.845661 © 2013 Informa UK, Ltd. ISSN 1471-2598, e-ISSN 1744-7682

Article highlights.

- The non-redundancy and compensatory function of different co-stimulatory pathways in T-cell biology poses a challenge in targeting individual components for the induction of transplant tolerance.
- CTLA4-Ig may have deleterious effects in the immune response due to the inhibition of Tregs and the promotion of Th17 cells, though the intensity, timing and context of blockade are important in determining the ultimate outcome.
- Preserving co-inhibitory signals, such as B7:CTLA4, should be attempted in order to promote long-term alloimmune regulation. More selective anti-CD28 antagonists could be promising alternatives.
- Memory T cells are more resistant to co-stimulatory blockade due to a lower threshold for activation and less dependence on co-stimulation; therefore, taming these cells require a complementary immunosuppressive strategy.
- Despite a higher rejection rate in the high-intensity belatacept arm, recipients treated with belatacept had comparable graft survivals to cyclosporine group and better renal function at 5 years after transplant.

This box summarizes key points contained in the article.

Advancement in the understanding of the alloimmune response has generated great excitement on selectively manipulating immune cell co-signaling receptors with monoclonal antibodies and/or fusion proteins.

The rejection of a transplant organ is orchestrated by T cells, which become activated after receiving an antigen-specific signal in combination with additional co-stimulatory signals [7]. The antigen-specific signal is provided by the interaction of major histocompatibility complex (MHC)–peptide complex on antigen-presenting cells (APC) with the T-cell receptor (TCR) on T cells. This primary signal is not sufficient to determine the fate of the T cells. Additional co-stimulatory signals are critical to fully activate a naïve T-cell. This observation was first noted in the 1980s and provided the knowledge essential for the development of potential co-stimulatory targets for immune regulation (**Figure 1**) [1,7].

2. Co-signaling receptors: stimulation versus inhibition

The group of co-stimulatory molecules significantly expanded in the past 30 years and the term became inadequate once co-signaling receptors were found to also inhibit T cells, such as cytotoxic T lymphocyte antigen-4 (CTLA4). Currently, the preferred term is T-cell co-signaling pathways [8]. The best-characterized positive co-signaling pathway is the B7:CD28. In both mice and humans, CD28 is constitutively expressed on all naïve $CD4^+$ and $CD8^+$ T cells [9] and it can interact with two ligands, B7.1 (CD80) and B7.2 (CD86), expressed on APCs. B7:CD28 interaction enhances TCR signaling, leading to full activation and expansion of T cells. Blocking this pathway results in anergy and/or apoptosis of responding T cells [10]. B7:CD28 signal specifically increases the transcription and mRNA stability of interleukin-2 (IL-2) [11], elevates the expression of anti-apoptotic molecules such as Bcl-XL [12] and decreases the threshold of TCR activation [13].

Other positive co-signaling pathways include: ICOS:ICOS-L, CD40:CD40L and OX40:OX40L [7,14-17]. These pathways appear to be non-redundant and are important in different phases of the immune response and may have dominance in certain cell subtypes. Similar picture is also seen in the family of co-inhibitory pathways, which are composed of programmed death ligand (PDL):programmed death-1 (PD1), B7:CTLA4, TIM3:Galectin9, among others [14,18-26]. The complexity of the co-signaling receptors led to difficulties in the selection and development of ideal therapeutic targets. One of the challenging characteristics of co-signaling receptors is their promiscuity, since a single receptor may interact with different counter-receptors. This is exemplified by the possible interaction of B7.1 with CD28, CTLA4 and PDL1, eliciting different outcomes depending on the predominant interaction. Moreover, signaling may occur in both directions such as via B7.1 to APCs and CTLA4 to T cells [27,28]. The final outcome of the APC–T-cell interaction depends on the integration of all these signals. Therefore, the expression of receptors and their affinity to counter-receptors are critical in determining the fate of T cells. Herein, we will discuss the clinical and basic immunological principles of the co-stimulatory pathway B7:CD28, focusing on its current and future potential as therapeutic target for immune modulation as well as its limitations.

3. Therapeutic co-stimulatory targets in transplantation

3.1 CTLA4-Ig (abatacept and belatacept)

Initial animal studies clearly showed that CD28 receptor was the most powerful co-stimulatory signaling receptor, and based on its selective expression on T cells, it became an obvious therapeutic target. However, initial attempts failed to develop an effective CD28 blocking antibody [29], since most of the antibodies targeting CD28 were actually agonists, leading to TCR-independent T-cell activation.

Attention was turned to the CD28 ligands – B7.1 (CD80) and B7.2 (CD86) – expressed on APCs. Blocking monoclonal antibodies against B7.1/2 were capable of delaying renal allograft rejection in nonhuman primates [30,31]. A recombinant fusion protein, CTLA4-Ig (abatacept), was developed by fusing the extracellular domain of human CTLA4 with an immunoglobulin heavy chain tail [32]. 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* [32]. Subsequent testing of CTLA4-Ig revealed its efficacy in protecting the allograft against acute rejection in MHC mismatched murine models of cardiac and islet cell transplantation [33,34], though it lacked the same efficacy in

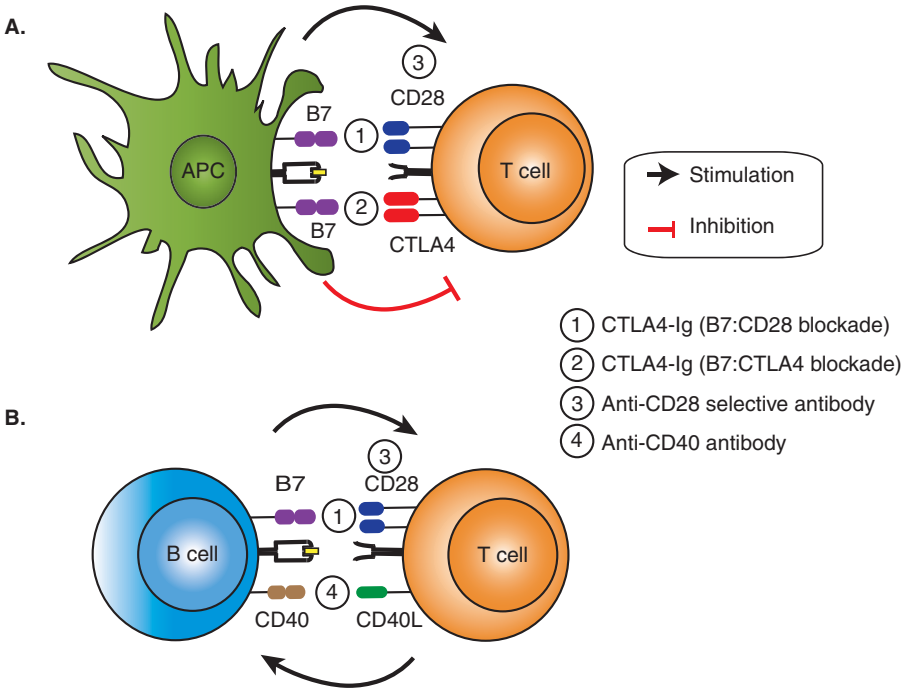

Figure 1. Illustration of co-stimulatory signals and their targets in transplantation. A. On antigen encounter, B7 ligands expressed on APCs interact with CD28 receptors on T cells, leading to full T-cell activation. Blockade of B7 ligands via CTLA4-Ig [1] interrupts this signal and promotes T-cell anergy and/or apoptosis in the context of TCR stimulation. However, CTLA4-Ig may also affect inhibitory signals from B7:CTLA4 pathway [2]. More selective agents targeting CD28 [3] could block the stimulatory signal, while preserving the inhibitory one via CTLA4 [2]. **B.** Co-stimulation is also important for B-cell activation and differentiation. B cells receive stimulatory signals from CD40L:CD40 interaction [4] and also signals to follicular T cells via B7:CD28. Blockade of CD40 inhibits B-cell proliferation, survival, isotype switching and GC generation. For illustrative purpose, a limited number of interactions are shown.

primate transplant models [35,36]. One of the potential reasons for the lack of significant effect was the affinity of the antibody, since CTLA4-Ig was 100-fold less potent in inhibiting B7.2 compared to B7.1 co-stimulation [37]. 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; belatacept). This novel fusion protein had a higher affinity to both B7.1 and B7.2, translating into a 10-fold increase in biological potency [37]. Belatacept (Bristol-Myers Squibb, New York, NY, USA) led to long-term allograft survival in nonhuman primates when used in combination with mycophenolate mofetil (MMF) and steroids [37].

In a Phase II clinical trial, belatacept was compared to cyclosporine in kidney transplant recipients co-treated with basiliximab induction therapy (IL-2 receptor monoclonal antibody) and maintenance immunosuppression with steroids and MMF [38]. Belatacept was non-inferior to cyclosporine in the prevention of acute rejection and it was superior in regard to GFR at 1 year after transplantation, presumably by the absence of CNI-induced vasoconstriction and nephrotoxicity [38]. Subsequently, two pivotal Phase III clinical trials were conducted in kidney recipients of standard criteria deceased and living donors (BENEFIT) as well as in extended-criteria donors (BENEFIT-EXT). Two dose intensities of belatacept were tested in combination with MMF and steroids [39-41]. Despite similar graft survival and superior renal function, the belatacept groups had a significantly higher rate of acute rejection, especially in the intensive arm receiving more frequent doses (22 vs. 7% on cyclosporine arm) [39]. Moreover, these rejections were more severe than the ones with cyclosporine (majority with grades IIA or higher). Based on the 1-year graft outcomes, the less intense belatacept regimen was approved by the FDA. However, the unexpected higher rate of acute cellular rejection, especially in the more intensive regimen, was intriguing and suggested some unexpected consequences of therapeutic B7:CD28 blockade in kidney transplant recipients. Similar findings were also identified in a Phase II trial in liver transplant recipients, in which high rejection rates were identified leading to early termination of this trial [42].

3.2 Potential limitations of CTLA4-Ig

3.2.1 Memory T-cell resistance

Memory T cells develop after exposure to blood transfusions, pregnancies, prior transplantation or infections. The

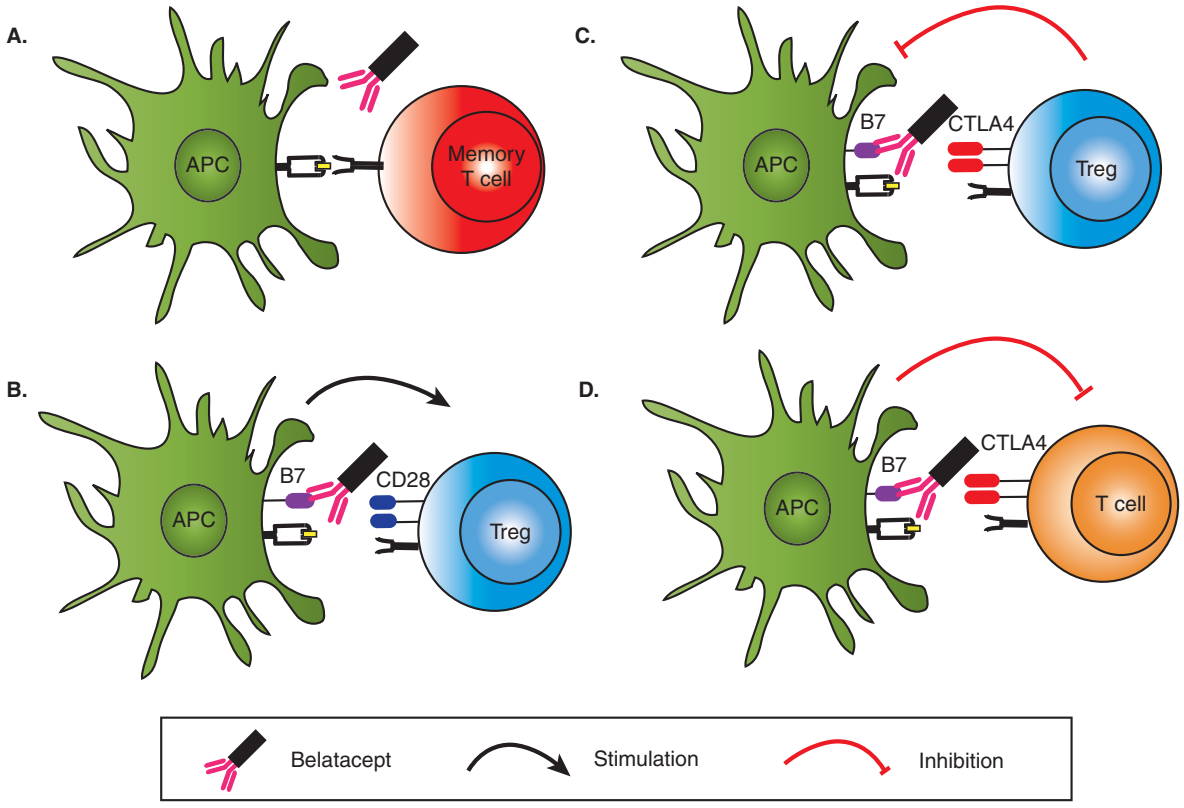

Figure 2. Illustration of potential limitations of CTLA4-Ig as an immunosuppressant. A. B7:CD28 pathway is critical for activation of naïve T cells on antigen presentation. However, memory T cells do not have the same requirement and may be activated by APCs without B7:CD28 signal. This could lead to failure to control the immune response with CTLA4-Ig in patients with memory T cells against the donor. **B.** CD28 signal is essential for the generation and maintenance of Tregs, and blockade of B7:CD28 can affect the number of Tregs. The final outcome of the immune response depends on the balance of effectors T cells and Tregs, raising concern on the long-term effect of B7:CD28 blockade on Tregs in transplantation. The inhibitory pathway B7:CTLA4 can also be affected by CTLA4-Ig and this pathway has been shown to be important for Treg suppressive function (**C**) and for APC-T-cell inhibition (**D**).

latter is believed to arise from cross-reactivity of the antibody to microbial antigen/self MHC complex and allogeneic MHCs [43-45]. Further, 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 [46,47]. Memory T cells have a lower threshold for activation and are less dependent on co-stimulation (including CD28 signal) [48] and therefore are more resistant to co-stimulatory blockade (**Figure 2A**) [49].

Indeed, Kitchens *et al.* showed that memory $CD8^+$ T cells are resistant to co-stimulatory blockade but targeting integrins, such as leukocyte functional antigen-1 and very late antigen-4, may overcome this resistance and inhibit graft rejection in mice [50]. Similarly, the combination of CTLA4-Ig with a selective memory T-cell agent (CD2-specific fusion protein alefacept) was shown to improve allograft survival in nonhuman primates [51], opening potential new venues in targeting memory cells. Nonetheless, targeting these cells carry their own risks since they play a key role in the immunity against infectious diseases.

3.2.2 Deleterious effect on regulatory T cells

Regulatory T cells (Tregs) have emerged as important players in the inhibition of the immune response and for the induction of tolerance [52]. Several groups have published initial exciting results with cell-based therapy with Tregs in tolerance induction [53,54]. Importantly, the development and homeostasis of natural Tregs is directly dependent on B7:CD28 co-stimulation and deficiency on this pathway significantly decreased the amount of Tregs in rodents [55,56]. This is a concern since blocking this pathway could potentially affect Treg generation in addition to effector T cells (**Figure 2B**).

Indeed, administration of CTLA4-Ig significantly decreased the number of Tregs in naïve mice by affecting its homeostatic proliferation [57]. While CTLA4-Ig prevented rejection in a fully allogeneic mismatched model, it accelerated rejection in a MHC class II mismatch model, in which

long-term allograft survival is dependent on Tregs [57]. This accelerated rejection was associated with a marked reduction in thymus-induced Tregs and led to a higher effector:Treg ratio in secondary lymphoid organs and in the allograft. Therefore, B7:CD28 signal is not only important for the activation of pathogenic effector T cells but also important for the generation of Tregs, being the balance of effector T cells and Tregs that ultimately determines the fate of an allograft [58]. Consequently, the outcome of the immune response on CTLA4-Ig administration is dependent on the type of immune response, including the degree of human leukocyte antigen (HLA) mismatch, sensitization and memory response. In addition, the timing and intensity of blockade play a key role. The latter can be illustrated by the observation that a very strong CD28 signaling may actually suppress the induction of Tregs [59]. Therefore, co-stimulatory signals most likely do not function as an *on and off* switch and optimal intensities of co-signals may vary among different cell subsets and is highly context-dependent [8]. The higher rate of rejection in certain patients points to possible underlying factors influencing the response to CTLA4-Ig that must be further explored in order to better use this novel agent.

3.2.3 Non-redundant co-stimulation

Though B7:CD28 seemed to be the dominant co-stimulatory pathway, additional co-stimulation, such as CD40:CD40L and OX40:OX40L, may play a non-redundant role in the alloimmune response. CD40:CD40L co-stimulatory molecules are members of the TNF-TNFR superfamily, and signaling through this pathway is critical for the humoral response [19]. CD40 ligation stimulates B-cell proliferation, survival, isotype switching, formation of the germinal center (GC) and memory B-cell generation. Mice deficient in CD40L or CD40 are unable to generate a primary or a secondary antibody response to a T-cell-dependent antigen and do not form GCs and are deficient in generating antigen-specific memory B cells [60]. Clinical development of a CD40L blocking antibody was halted due to safety concerns after the development of thromboembolic events in both primate studies and Phase I clinical trials [61]. This unexpected complication is likely related to the expression of CD40L on platelets, which promotes platelet aggregation when bound by anti-CD40L mAb [62]. The significant beneficial effect of targeting this pathway on transplant survival prompted the search of alternative options to circumvent problems associated with anti-CD40L, including the development of blocking antibodies against CD40, not expressed on platelets [63-68]. Indeed, the combination of CTLA4-Ig with a CD40 blocking antibody showed promising results in suppressing alloantibody production in a nonhuman primate islet cell transplantation model [69].

OX40:OX40L engagement is another potent co-stimulatory signal that activates T effector cells, supporting their survival, differentiation and transition to memory phenotype [19]. In order to obtain long-term graft survival in a stringent transplant model, Vu *et al.* blocked OX40, CD28 and CD40L co-stimulatory molecules and were able to induce long-term skin graft survival [70]. In particular, memory T cells seem to be sensitive to OX40 blockade. Dissecting the role of different co-stimulatory pathways in different cell subtypes is challenging but will be essential for the optimization of co-stimulatory blockade in transplantation.

3.2.4 Blockade of inhibitory signals

Co-stimulatory molecules were discovered to share counter-receptors with each other and some of these receptors demonstrated capability of inhibiting rather than activating T cells. For example, CTLA4 was found to be structurally related to CD28 and to bind to the same ligands on APCs (B7.1 and B7.2) as CD28, though with greater affinity [32]. However, B7 interaction with CTLA4 leads to inhibition of T-cell activation, through activation of protein phosphatase 2 and blockade of AKT phosphorylation [71]. In addition, CTLA4 receptor can interact with B7.1/B7.2 ligands on APCs and reduce the expression of these ligands by trans-endocytosis of the B7:CTLA4 complex into T cells [28]. Last, CTLA4 signaling can increase the expression of indoleamine 2,3-dioxygenase (IDO) on APCs – a potent inhibitory molecule [72].

CTLA4 plays an essential role in immune homeostasis as evident by the lethality of CTLA4 deficiency in mice due to the development of an aggressive multiorgan autoimmune disease at 3 weeks of age [73]. In addition, CTLA4 expression on Tregs is critical for Treg function, as proved by a conditional knockout approach to CTLA4 on Tregs (**Figure 2C**) [74]. Blockade of CTLA4 with an anti-CTLA4 antibody has been shown to precipitate rejection and prevent induction of allograft tolerance in the transplant setting [15], reinforcing the important role of CTLA4 signaling in inhibiting the alloimmune response. An agonistic agent to CTLA4 could potentially promote tolerance and improve graft survival; however, attempts of developing this agent have been so far unsuccessful. In sum, CTLA4 is an important inhibitory signaling pathway and its blockade by CTLA4-Ig could affect the regulation of the alloimmune response (**Figure 2D**).

PDL1 was also found to bind B7.1 and inhibit the immune response [26,75-77]. Selective blockade of B7.1:PDL1 enhanced chronic injury in a single MHC mismatched murine cardiac transplant model [23]. This was associated with an increase in IFN-γ and IL-6 cytokine production by allo-stimulated splenocytes and a decrease in Tregs [23]. Interestingly, PDL1 expression is found not only on hematopoietic cells but also on non-hematopoietic cells such as the endothelium and its expression is upregulated on transplantation [20]. Therefore, PDL1 may play an important role in inhibiting the immune response locally in the graft. Consequently, preserving B7:CTLA4 and B7.1:PDL1 signal should be important for long-term immune regulation and B7 blockade with CTLA4-Ig may affect potential regulatory pathways in addition to stimulatory ones, raising the question if more selective blockade of CD28 would yield better outcomes in

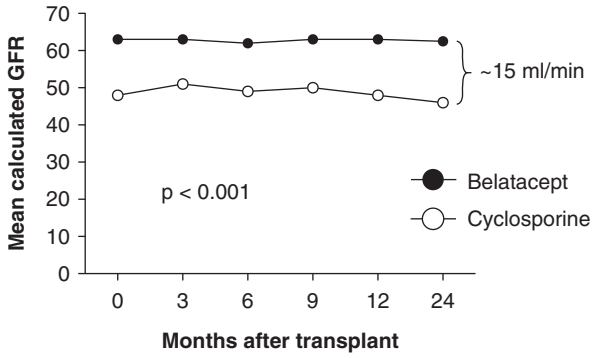

Figure 3. Graphical representation of renal function over-time in belatacept- and cyclosporine-treated groups in the BENEFIT trial. Mean GFR (95% CI) was calculated with the MDRD equation. Since less intensive as against more intensive belatacept groups had similar eGFR through the study, values were combined in one line.
Adapted from [41].

transplantation. Nonetheless, further studies are needed to clarify the relative physiological importance of B7.1: PDL1 in human immune homeostasis compared to other co-inhibitory pathways such as PD1:PDL1.

3.2.5 *Generation of Th17 cells*

Th17 cell is a subtype of $CD4^+$ T cells that produce significant amounts of IL-17 and promotes neutrophil activation and immunity to extracellular pathogens. Th17 cells have also been found to be major players in certain autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis [78,79]. In transplantation, Th17 cells have been associated with allograft rejection [80]. Neutralization of IL-17 via blocking antibodies improved graft survival in a rat cardiac allograft model and inhibited transplant arteriosclerosis in a mouse model of aortic transplantation [81,82]. The primary concern is that Th17 cells are resistant to current available immunosuppression and especially resistant to co-stimulation blockade [83,84]. In fact, CTLA4-Ig facilitated both murine and human Th17 differentiation *in vitro* [85]. In addition, CTLA4:B7 interaction inhibited Th17 cell differentiation and suppressed the development of Th17-mediated autoimmunity [86]. Confirming these findings, our group found that CTLA4-Ig-treated mice had an increase in Th17 cells infiltrating the allograft in a mouse cardiac transplant model when compared to controls [57]. However, neutralization on IL-17 was not able to improve graft survival [57]. Collectively, these findings suggest that B7:CD28 blockade with CTLA4-Ig may favor Th17-cell differentiation, though the exact importance of this T-cell subtype in acute and chronic rejection in humans still remains to be determined.

3.2.6 *Risk of infection and malignancies*

Transplant recipients with Epstein–Barr virus (EBV)-negative status prior to transplantation had a higher risk of developing post-transplant lymphoproliferative disease (PTLD) with belatacept treatment. PTLD presented with a predominant central nervous system involvement and high mortality rate [87]. Viral immunity is dependent on effector CD8 cells and the recent work by Dolfi *et al.* suggests that CD28 co-stimulation is critical to potentiate the CD8 effector antiviral response, preventing apoptosis and maximizing viral clearance [88]. Therefore, blockade of B7:CD28 may negatively affect the effector viral response against EBV infection leading to a chronic infected state that ultimately promotes the development of post-transplant lymphoproliferative disorder. As a consequence of the above clinical observation, EBV-negative status was considered a contraindication for belatacept use and a risk evaluation and mitigation strategy was implemented. Though other general infection rates were similar between groups, tuberculosis was more frequent in belatacept groups, in particular in patients from endemic tuberculosis areas such as Brazil.

3.3 Latest trials with CTLA4-Ig

Three-year follow-up studies of the BENEFIT and BENEFIT-EXT trials confirmed the similar rate of patient and graft survivals and superior renal function in the belatacept groups compared to cyclosporine, despite the significantly higher number of acute rejection episodes in the more intensive belatacept regimen. Belatacept-treated patients maintained a better renal function at 3 years, with a calculated GFR of 21 ml/min and 11 ml/min higher than cyclosporine group in the standard and extended-criteria donor, respectively (**Figure 3**) [40,41]. Preliminary report of the 5-year follow-up study with belatacept reinforces the long-term observations of a better renal function in the belatacept groups (stage 1 and stage 2 CKD on most patients) compared to cyclosporine (predominance of stage 3 CKD) [89]. The potential explanation for the discordance between acute rejection rate and long-term renal function may involve several factors. The first one might be related to the lack of adverse impact from early cellular rejection, despite common presumption. This benign course of cellular rejection is only present in the absence of concomitant donor-specific antibodies (DSA) and/or antibody-mediated rejection (AMR). Second, belatacept may have an inhibitory effect on DSA generation, potentially mediated by its effect on T-follicular helper cells and this may account for the better long-term outcomes. It is clear now that AMR and its chronic presentation with transplant glomerulopathy is the most common etiology of graft failure after the first year of transplantation [90]. Few patients on the belatacept-treated group developed DSA (3%) compared to 8% on cyclosporine (CSA). Based on the low numbers of each group, further studies are needed to confirm those findings. Third, belatacept is not nephrotoxic and lacks the vasoconstrictive effect on the kidneys and therefore may minimize non-immunological graft injury. The vasoconstriction appears to be a critical factor since the difference in estimated glomerular filtration rate (eGFR) is noted from the start of the

transplant (**Figure 3**). Fourth, the compliance achieved by monthly intravenous infusions of belatacept ensures adequate immunosuppression, which is important for the prevention of DSA formation and chronic AMR [91]. Last, the better metabolic profile including blood pressure and lipids control have a beneficial impact on cardiovascular disease and may ultimately preserve graft function.

Another potential approach for belatacept use would be the conversion from CNI to belatacept after 6 – 12 months in patients with CNI intolerance or in patients with low immunological risk. This was tested on an open-label, Phase II trial, in which kidney transplant recipients > 6 months but < 36 months after transplant were randomized to either switch to belatacept or to continue on CNI treatment [92]. About 85 patients were enrolled on each group and renal function was slightly superior on the belatacept group compared to CNI (study not powered to show a statistically significant difference). About 7% of patients in the switch group developed acute rejection, while none developed acute rejection in the CNI group. Graft and patient survival were equivalent. In addition, other combinations including belatacept with sirolimus have been tried with success in nonhuman primates [93] and in humans [94], though larger cohorts are needed to validate those results. In summary, belatacept carries a great potential as a component of immunosuppressive regimen with low toxicity profile and the benefit of adherence, though the use of this agent has been limited by concerns of the high acute rejection rate and the long-term potential significance of these events.

3.4 Selective CD28 blockade

The development of a CD28 blocking antibody has been challenging since depending on the epitope targeted on the CD28 receptor, different effects were seen on T cells including: polyclonal activation of T cells in the absence of TCR signaling (superagonist anti-CD28 antibodies); enhancement of co-stimulation in the setting of TCR signaling (conventional anti-CD28) and true blocking anti-CD28 antibodies (monovalents anti-CD28 antibodies), which lack the crosslinking effect on CD28.

Superagonist anti-CD28 antibodies were found to actually have some protective effect in animal models of solid organ transplantation [95,96], likely through the expansion of Tregs. However, a Phase I clinical trial on humans for the TGN1412 CD28 superagonist antibody induced a massive cytokine storm that led to severe and life-threatening adverse effects [29]. The true antagonist antibody against CD28 was developed from monovalent fragments (Fabs) of a conventional anti-CD28 antibody. These monovalent fragments were capable of inhibiting T-cell activation, proliferation and cytokine production [97]. This selective approach not only blocked CD28 co-stimulation but also spared the B7:CTLA4 co-signal that promotes immune regulation [98] and is essential for Treg function [74]. In a heart transplantation model in mice, monovalent CD28 antagonist delayed acute rejection and inhibited chronic rejection when combined with CNI or anti-CD40L antibody [99]. In nonhuman primate models of heart and kidney transplantation, similar CD28 fragment in combination with CNI prevented acute and chronic allograft rejection [98]. CD28 fragment-treated primates had decreased effector T-cell proliferation and function with a predominant regulatory phenotype as evident by higher IDO expression and intragraft regulatory cells' infiltration [98]. Newer generation of anti-CD28 Fab' antibody fragments are being developed, including a PEGylated form FR104, with improved pharmacokinetics and predominant antagonist effect [100]. FR104 significantly suppressed human T-cell proliferation and cytokine production *in vitro* and had no significant CD28-stimulatory properties *in vitro*, even after crosslinking with secondary antibodies or in the presence of anti-CD3 antibodies [100]. By sparing CTLA4 and PDL1 co-inhibitory signals, FR104 might lead to higher therapeutic responses compared to CTLA4-Ig. However, due to the complexity of the co-stimulatory pathways, only human transplantation trials will be able to confirm or deny this hypothesis.

4. Expert opinion

The development of co-stimulatory blockade therapies is one of the greatest examples of modern translational medicine, in which the initial discovery of the biology of T-activation led to the development of a therapeutic target, initially tested in different animal models up to human trials, with modifications of the molecule along the way. The outcome was the approval of belatacept by the FDA in 2011. However, the complex interplay between different co-stimulatory and co-inhibitory pathways as well as their roles in diverse immune cell types raised a number of challenges. The presence of non-redundant, parallel and compensatory co-stimulatory pathways made it clear that targeting a single pathway will be ineffective for the induction of transplantation tolerance.

Despite the exciting results of improved graft function on the belatacept groups compared to cyclosporine in the 3-year BENEFIT trials, most transplant centers have been skeptical and worried about the high acute cellular rejection rate. Various centers are attempting combination protocols involving the use of low-dose tacrolimus during the first year as a preventive measure, and the Clinical Trials in Organ Transplantation (CTOT)-15 study, under the leadership of Dr Christian Larsen, is actively recruiting patients for a protocol of optimization of belatacept with thymoglobulin, MMF and steroids with minimization of tacrolimus (NCT01790594). Prior CTOT-10 study, which enrolled kidney transplant recipients on a protocol with alemtuzumab induction, MMF and no steroids with or without tacrolimus withdrawal, was prematurely terminated due to poor outcomes (NCT01436305). The success in achieving a lower acute rejection rate will be critical for the acceptance of belatacept in kidney transplant centers. Though we, as transplant physicians, love to discuss how we must improve long-term graft survivals, we are reluctant to accept high cellular

rejection rates in the short term, despite no clear evidence of potential long-term harm.

Further study on the subset of patients treated with belatacept that develop acute rejection will be essential in order to better learn what exactly is precipitating the rejection and how we could better select patients. Is memory response the critical factor? Functional assays, such as ELISPOT with donor stimulators [101], and immune phenotyping of recipients' immune cells prior to transplantation might help in determining if any specific immunological characteristics may account to the differences.

We are victims of our own short-term success in transplantation. Developing better biomarkers of allograft injury and learning to wisely use our available immunosuppression armamentarium will be critical for improving long-term outcomes. Recent findings that urinary CXCL9 protein levels could predict earlier transplant kidney injury may facilitate the monitoring of post-transplant course [102], yielding modifications of the immunosuppressive regimen accordingly. Based on the complexity of the immune system, it is naïve to expect that one biomarker will be enough to predict an outcome on co-stimulatory blockade in transplantation. Building an immunological network composed of molecular and cellular components will be required to better understand the totality of the immune response. System biology is a novel field that uses bioinformatics to generate computer models [103]. To build a biological network, one has to start by inputting profiles of cellular transcripts that occur after manipulation of a particular molecule, such as with CTLA4-Ig [104]. These models may also predict the consequences of multiple drugs' targeting as with immunosuppressive protocols used in our transplant recipients. This approach has yielded successful outcomes in the oncology field [105]. Whether transplantation will have similar success remains to be determined.

Belatacept has many attractive features including the adherence factor, since nonadherence is a major risk for the development of chronic AMR. The favorable metabolic profile is also appealing since almost 50% of kidney grafts are being lost by patients' death due to cardiovascular disease. Improving the selectivity and strength of targeting co-stimulatory blockade antibodies will improve their clinical efficacy in transplantation. Further, the role of B cells in chronic rejection has been increasingly recognized [106], 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. In sum, the future of co-stimulation targeting in kidney transplantation will involve the combination of different agents with the goal to inhibit effector/memory T cells, T-follicular helper cells and antibody production, while promoting regulatory cells and limiting single drug toxicity.

Declaration of interest

Research grant from the American Heart Association was given to LVR, National Institute of Health (NIH) Grant RO1 AI51559 was awarded to MHS, and PO1AI56299 was provided to MH Sayegh and AH Sharpe. The authors of this manuscript have no conflict of interest to disclose.

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