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DOI:
10.1097/TP.0b013e31826d4672

Document Version
Peer reviewed version

Citation for published version (Harvard):
Kinnear, G, Jones, ND & Wood, KJ 2013, 'Costimulation blockade: current perspectives and implications for therapy', *Transplantation*, vol. 95, no. 4, pp. 527-35. https://doi.org/10.1097/TP.0b013e31826d4672

Costimulation blockade: Current perspectives and implications for therapy

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Word Count: Abstract = 162; text 3322

Key words transplantation; biological therapeutics; immunosuppression; T cell activation; rejection; immunoregulation; CTLA-4

3 Figures + 1 Table

Abstract

T cells must be activated before they can elicit damage to allografts, through interaction of their T cell receptor (TCR) with peptide-MHC complex, and through accessory molecules. Signalling through accessory molecules or costimulatory molecules is a critical way for the immune system to fine tune T cell activation. An emerging therapeutic strategy is to target selective molecules involved in the process of T cell activation using biological agents, which do not impact TCR signalling, thus only manipulating the T cells which recognise alloantigen. Costimulatory receptors and their ligands are attractive targets for this strategy and could be used both to prevent acute graft rejection as well as for maintenance immunosuppression. Therapeutic agents targeting costimulatory molecules, notably belatacept, have made the progression from the bench, through non-human primate studies and into the clinic. This Overview describes some of the most common costimulatory molecules, their role in T cell activation, and the development of reagents which target these pathways and their efficacy in transplantation.

T cell activation: a potential target for therapeutic blockade

T cells play a central role in the immune response towards an allograft (1) therefore interfering with the process of T cell activation has the potential of prolonging allograft survival through modulation of the alloresponse. Naïve $CD4^+$ and $CD8^+$ T cells must become activated in order to acquire effector functions which can result in graft damage (2). Activation of a naïve T cell is a tightly regulated process which requires three distinct signals. Signal 1 determines the specificity of the immune response and involves the interaction between a given T cell receptor (TCR) on a T cell and a MHC-peptide complex on antigen presenting cells (APC) which generates a signal that is transmitted through the adjacent CD3 complex (3, 4). Additional, so-called second signals are generated through other cell surface molecular interactions, known as costimulation (5). The third signal is delivered from an APC to the T cell by means of cytokines. A number of cytokines have been implicated as providing signal 3, including IL-12 as it is able to promote Th1 differentiation (6).

The accumulation and integration of intracellular signalling molecules from these 3 pathways triggers T cell gene transcription including IL-2, anti-apoptotic molecules, such as Bcl-2, molecules. Together such signals lead to clonal expansion and survival of antigen-specific T cells and influence the effector functions acquired by T cells as they differentiate, such as granzyme B a molecule that plays a role in the function of cytotoxic T cells (CTL or Tc). Without costimulatory signals during the activation process, T cells become anergic and refractory to further stimulation by antigen (7). Therefore targeting costimulatory pathways in the setting of transplantation has the potential to alter the evolution of an immune response to an allograft and prevent rejection.

Costimulation

Figure 1 shows examples of costimulation pathways involved in the T cell response. Although different costimulatory molecules can have over-lapping functions, which molecules act to co-stimulate T cell responses appears to depend on the stage of differentiation of the responding T cell as well as the availability of corresponding ligands (Table 1) (8). This feature highlights the high degree of redundancy which has evolved to enable effective regulation of an immune response. Given the significant role played by costimulation in T cell activation, costimulation blockade provides a promising adjunctive or alternative therapy to the currently licenced immunosuppressive drugs used to prevent graft rejection.

Diversity of costimulatory molecules

Costimulatory molecules can be categorized by both their functional properties and structure. Based on functionality, costimulatory molecules can be divided into those participating in positive costimulatory pathways promoting T cell activation, survival and/or differentiation, or negative costimulatory pathways which antagonise signals from the TCR resulting in suppression of T cell activation and termination of the immune response. Classification by structure divides costimulatory molecules into four distinct groups: (1) immunoglobulin (Ig) superfamily members (e.g. CD28); (2) tumour necrosis factor receptor (TNFR) family members (e.g. CD40); (3) cell adhesion molecules or integrins (e.g. LFA-1); T cell Ig domain and mucin domain (TIM) molecules. Figure 2 and Table 1 shows a number of the best characterized costimulatory molecules in the Ig and TNFR superfamilies. These two families

will be the focus of this Overview, but it is important to note that this is not a definitive list of receptor-ligand pairs able to provide costimulation.

Therapeutic strategies in experimental models of transplantation

Novel immunosuppressive drugs and strategies are required for the prevention of allograft rejection to reduce the side effects associated with current immunosuppressive drug therapeutic regimens and to provide better control of the low grade immune response that leads to late allograft loss. A number of new agents have been developed which target immunological pathways in rejection for example; 1) modulation of cell surface molecules such as costimulatory molecules 2) inhibition of signalling cascades 3) inhibition of T cell proliferation and 4) modulation of cell trafficking. There are many different reagents being developed to target these pathways (9), and this Overview will focus on the development of agents which impact the costimulatory pathways described above.

CD28/CTLA4:CD80/CD86 pathway

The CD28-CD80/CD86 axis was the first costimulatory pathway to be defined and is therefore the best characterised to date (10-12) (Table 1; (13, 14)). After T cell activation, another receptor for CD80/CD86 is upregulated; CTLA4 (CD152) which although structurally homologous to CD28, has a ~20 fold higher affinity for CD80/CD86 and thus can out-compete CD28 for CD80/86 binding (Table 1; (15)). The CD28/CTLA4;CD80/CD86 axis has a dual function in the T cell response (Figure 3). In contrast, CTLA4 inhibits the T cell response by limiting CD28-CD80/CD86 interactions, decreasing IL-2 secretion and promoting indole-amine 2,3-dioxygenase (IDO) expression by APC upon ligation of CD80/86 that in turn promotes the expansion of regulatory T cells by modulating tryptophan

catabolism (16-18). Based upon these properties, a receptor Fc fusion protein, CTLA4-Ig, has been developed to block CD80/86 and thereby inhibit T cell costimulation (15, 19).

The importance of the CD28 costimulatory pathway in allogeneic responses was first demonstrated *in vitro* by using an anti-CD28 monoclonal antibody (19, 20) or CTLA4-Ig fusion protein (21, 22). However, using $CD28^{-/-}$ mice, Kawai *et al* demonstrated that the signals generated through CD28 were critical for the proliferation of alloreactive T cells *in vitro,* but that *in vivo* skin allograft rejection could occur in the absence of CD28 (23). In rodents, blockade of the CD28:CD80/CD86 pathway by CTLA-4-Ig, was shown to prevent acute allograft rejection, but this finding was found to be model and strain dependent (22, 24-26) due to the redundancy in the immune response. CTLA4-Ig also prevented the development of anti-donor antibody responses and resulted in long-term survival of islet, cardiac and renal transplants in rodent models (21, 27-29) (Figure 3). These data provide a rationale for combination therapies within the clinical setting.

CD40:CD154 pathway

The role of the CD40:CD154 pathway in immunity became clear when hyper-IgM syndrome was found to be a direct result of a mutation in the gene encoding CD154 (30). The effects of CD40 on the immune response are mediated by a signalling cascade which is initiated when it binds its ligand CD154 (CD40L) (Table 1; (31, 32)); a CD28 independent event (33). Initial efforts were aimed at blocking the CD40:CD154 interaction by use of monoclonal antibodies specific for CD154; an approach that showed promise in transplantation models in rodents (34-36) and in non-human primates (NHP) (37-39). Anti-CD154 has a preferential impact on effector T cells by inhibiting their activation and therefore proliferation, while also enriching the Treg population (40).

In preclincal studies it was found that rhesus monkeys given anti-CD154 mAb for 5 months as part of an induction therapy followed by 5 further monthly doses accepted kidney allografts for over a year after treatment was discontinued. However, the allografts were eventually rejected suggesting that tolerance was not achieved (38, 39). In addition, anti-CD154 (IDEC-131) alone significantly prolonged cardiac allograft survival in cynomolgus monkeys, while graft survival was further extended with the introduction of anti-thymocyte globulin in addition to anti-CD154 but as in previous studies did not induce tolerance (41).

More recently, reagents which target CD40 rather than CD154 have been developed. Anti-CD40 was found to synergise with CTLA-4-Ig to promote long term allograft survival in mouse models of skin and bone marrow transplantation (42). Anti-CD40 (4D11) showed significant prolongation of renal allograft survival in NHPs and prevented the development of alloantibodies (43) suggesting that blockade of the CD40:CD154 pathway still may contain promise in humans (44).

ICOS:ICOSL pathway

Another member of Ig superfamily is inducible costimulator (ICOS; CD278) (Table 1; (45-47)). In a full-MHC mismatch mouse cardiac allograft model Ozkaynak *et al* showed that blockade of ICOS in combination with either cyclosporine or anti-CD154 prevented chronic rejection (48). However, if donors and recipients were mismatched for minor histocompatibility antigens only, blockade of ICOS during the T cell priming phase accelerated rejection, while blockade during the effector phase of the alloimmune response prolonged graft survival (49). This may be explained by ICOS being critical for the function of effector/memory T cells as well as regulatory T cells (50). Co-blockade of ICOS:ICOSL

and CD40:CD154 (see above) results in indefinite cardiac allograft survival with a significant reduction in chronic allograft vasculopathy and therefore chronic rejection (51). These data suggest that preventing ICOS signals alone will be insufficient to induce long term allograft survival and tolerance, therefore combining interruption of ICOS-ICOSL interactions with blockade of other costimulatory pathways may be an important step forward if ICOS blockade is going to reach its full therapeutic potential.

PD-1:PD-L1/L2 pathway

Like CTLA-4, PD-1 (CD279) is also a member of the Ig superfamily that has co-inhibitory activity (Table 1; (52)), and is important in suppressing T cell activation and preventing autoimmunity. PD-$1^{-/-}$ mice develop strain specific autoimmunity, demonstrating a role for PD-1 in negatively regulating the immune response (53, 54) and in maintaining peripheral tolerance to self-antigens.

Administration of blocking monoclonal antibodies against PDL1, but not PD-1 or PDL2, in a MHC Class II mismatched skin graft model, resulted in accelerated rejection due to selective prevention of T cell apoptosis, increased alloantigen driven T cell expansion and promotion of Th1 differentiation (55). Gao *et al* used a PDL1-Ig fusion protein and found that it prevented allograft rejection and allowed the induction of tolerance when combined with anti-CD154 or sub-therapeutic doses of rapamycin (56). These data suggest that the PD-1 ligands may mediate opposing effects as a result of their differential tissue distribution.

OX40:OX40L pathway

The TNFR superfamily member OX40 (CD134) is transiently induced on both $CD4^+$ (57) and $CD8^+$ (58) T cells after activation (Table 1) which is involved in the late expansion phase

of effector T cells, as well as promoting memory T cell generation (59, 60). Blocking the OX40-OX40L pathway (using an OX40-Ig fusion protein) in a mouse model of cardiac transplantation was found to result in prolonged cardiac allograft survival when donor and recipient were mismatched at a minor histocompatibility antigen loci but not across a full MHC mismatch (61). In contrast, in a full-MHC mismatch model where TCR transgenic $CD8^+$ T cells were adoptively transferred into syngeneic T cell deficient recipients, anti-OX40 was able to significantly prolong skin graft survival, although tolerance was not achieved (62). OX40:OX40L has been suggested to be utilised differentially by effector and regulatory T cells (Treg). For example, blockade of OX40:OX40L inhibits the generation of an optimal effector T cell pool by promoting activation induced cell death (59, 62), whilst concomitantly aiding the induction of Treg (63, 64). These data provide a clear precedent for the utilisation of OX40-OX40L blockade in transplantation however this appears to be contingent on suboptimal or low frequency T cell responses.

41BB:41BBL pathway

Another inducible costimulatory molecule in the TNFR superfamily is 4-1BB (CD137), and ligation of 4-1BB to its ligand (41BBL) (Table 1; (65, 66)) preferentially promotes $CD8^+$ T cell proliferation, and survival compared to $CD4^+$ T cells (67). In a transplantation setting, agonistic 4-1BB monoclonal antibodies have been shown to accelerate cardiac and skin rejection (67). Furthermore, blockade of 4-1BB prolonged intestinal allograft survival where rejection was mediated by $CD8^+$ T cells but not where rejection was caused by $CD4^+$ T cells (68). Cho *et al* showed that 4-$1BB^{-/-}$ recipients had only a minor impairment in their ability to acutely reject fully-MHC mismatched cardiac allografts (69). These data suggest the 4-1BB-4-1BBL pathway could be targeted where $CD8^+$ T cells exercise CD28/CD154 independent rejection.

CD27:CD70 pathway

The final TNFR family member to be discussed in this review is CD27 and its interaction with ligand CD70 (Table 1; (70). CD70 expression is dependent on TCR stimulation and TLR stimulation (71). The CD27-CD70 interaction has been implicated in T cell development, T cell activation and T cell dependent antibody production by B cells (72). Blockade of CD27-CD70 pathway prolongs allograft survival in fully MHC mismatched cardiac allografts in wild type recipients (73). But in $CD28^{-/-}$ recipients CD70 blockade induced long term survival and prevented the development of chronic allograft vasculopathy (74). This synergy was mediated by the effector/memory alloreactive $CD8^{+}$ T cells, while little effect was seen on the $CD4^{+}$ T cell function.

LFA-1:ICAM and VLA-4:VCAM pathway

Integrins have a number of roles in the immune response; T cell recirculation, migration into inflammatory sites, stabilisation of T cell-APC interactions and providing costimulatory signals. Experimental transplantation models have shown that blockade of the LFA-1-ICAM interactions with anti-LFA-1 monoclonal antibodies can result in prolonged survival of islet (75) and cardiac (76) allografts. In a murine cardiac allograft model, anti-VLA4 reduced the incidence and severity of arterial intimal thickening which is closely associated with chronic rejection (77). Anti-VLA4 and anti-LFA-1 administered together displayed potent synergy in a murine islet model, which resulted in significant graft prolongation compared with either of the monotherapies (78). Kitchens *et al* have shown anti-VLA4 or anti-LFA-1 can abrogate the resistance of memory T cells to costimulation blockade (79). Anti-VLA4 acts by blocking T cell trafficking to the graft, while anti-LFA-1 was also able to block T cell trafficking but also could impact memory T cell recall function (79).

TIM family

TIM molecules are an emerging family of cell surface type 1 transmembrane glycoproteins which have recently been shown to have important immunological functions as costimulatory molecules (80). The TIM family regulate a wide variety of immune responses and can provide positive signals to T cells which can enhance T cell activation (81), proliferation (82) and cytokine production (81). In particular, costimulation via TIM-1 abolishes the generation and suppressor function of Treg by reducing FoxP3 expression. Agonistic TIM-1 mAb enhances Th17 differentiation, therefore suggesting TIM-1 plays a critical role in the delicate balance between Treg and Th17 (83) or autoimmunity and tolerance. Blockade of TIM family proteins may provide a viable strategy for the amelioration of autoimmune and inflammatory diseases. For example, anti-TIM-1 mAb and rapamycin synergise to prolong cardiac allograft survival by inhibiting Th1 responses (84). This strategy may also benefit from combination therapy with other costimulation blockade.

Obstacles to tolerance induction and clinical translation

There is a growing body of evidence in rodent models to suggest that blocking costimulation can lead to tolerance induction (35, 85), however, data from NHP studies is more complex, and suggests that tolerance induction is more difficult to achieve in higher animals including humans (37-39, 86). These differences can be explained in part by the increased complexity of the higher vertebrate immune system and the increased diversity of pre-existing immunity due to exposure to environmental antigens (87). Although outbred laboratory animals have a wider diversity of exposure to such antigens, they are still less immunologically educated compared to humans. Differences can also result due to basic observations such as size;

vastly different number of total cells able to respond as well as differences in drug absorption and clearance. Despite these differences, large animal models remain the best way to gain knowledge before initiating a safe and ethically robust clinical trial.

The leap from bench-side to bedside

Targeting costimulatory molecules has great potential in transplantation for preventing rejection as the therapy will only impact T cells undergoing activation. As a result the specificity of immunosuppression achieved using this therapeutic approach has the potential to be increased compared to current small molecule immunosuppressive drugs.

Belatacept (human CTLA4-Ig fusion protein; Nujolix®) has been approved for clinical use for the indication of prophylaxis of organ rejection in adult kidney transplant recipients (FDA in June 2011; EMA in April 2011). Belatacept was developed as a second generation CTLA4-Ig after another version of the fusion protein (abatacept) proved sub-optimal in NHP transplant models (88). Structurally belatacept and abatacept differ only in two amino acids, which allows for more potent binding to its ligands by belatacept resulting in more efficacious inhibition of T cell activation. The FDA has approved belatacept to be used in conjunction with basiliximab induction, mycophenolate mofetil (MMF) and corticosteroids as maintenance immunosuppression (89). The efficacy of belatacept in *de novo* kidney transplantation was assessed in two open label, randomised, multicentre phase II/III clinical trials, named BENEFIT (Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial). At 1 year, the incidence of acute rejection was higher in those patients treated with belatacept (90). The rates of infection and malignancy were comparable between both arms, while the metabolic and cardiovascular risk profiles were

better in the belatacept treated patients (91). Rejection episodes were associated with a failure of belatacept to suppress a subset of alloreactive T cells, i.e. memory T cells (92). Memory T cells have a reduced reliance on costimulation and therefore are more resistant to costimulation blockade (93-96). Development of reagents to target memory T cells (such as integrin antagonists (79)) in addition to costimulation blockade may provide a useful tool to overcome the increased incidence of rejection observed with belatacept. Memory T cells therefore pose a potential barrier to regimens such as costimulation blockade which hope to lead to tolerance induction and graft acceptance within the clinic.

In addition some patients who were treated with high dose belatacept also developed post-transplantation lymphoproliferative disorder (PTLD) (90, 97-99). The FDA approval of belatacept is therefore limited for use only in Epstein-Barrvirus (EBV) seropositive patients due to increased risk of PTLD in EBV-seronegative patients. The PTLD observed was mainly of the central nervous system (89). Belatacept has only been approved for use in adult kidney transplantation, as there was increased graft loss and mortality in liver transplant recipients (100).

Other reagents which target CD28 have also been developed (101, 102). A CD28-superagonist, TGN1412, showed enhanced expansion of regulatory T cells in preclinical studies and was taken into a Phase I clinical trial. However, 6 healthy volunteers treated with TGN1412 experienced a massive expansion of inflammatory T cells that resulted in a cytokine storm. The trial was terminated as all volunteers experienced multi-organ failure (103). Despite this, the development of antibody mediated selective costimulatory blockade remains a highly attractive therapeutic strategy as specifically blocking CD28 for example,

would still allow CTLA-4 signalling potentially enhancing T cell suppression. Monovalent Fab fragments (Fv) which selectively block CD28 have been developed to prevent signals through CD28 whilst allowing negative signals via CTLA-4 to remain intact. In a full MHC mismatch cardiac transplant mouse model, CD28 single chain Fv (scFv) fragments have been shown to significantly prolong allograft survival (104) and Poirier *et al* demonstrated that blocking CD28 in NHPs increased the number of Treg in the periphery as well as in the graft, which resulted in the prevention of renal allograft rejection (105).

The interruption of the CD40-CD154 interaction also has the potential to prevent rejection and promote long-term graft acceptance, as demonstrated by various rodent (32, 34, 36) and NHP studies (37, 38). Phase I-II trial evaluating anti-CD154 for the prevention of renal transplant rejection had to be terminated prematurely, due to thromboembolic complications as a result of activated platelets expressing CD154 (106). Therefore future plans for the development of anti-CD154 remain unclear. Antibodies targeting CD40 are being developed as they have been shown to have a similar efficacy in NHP kidney models without the thromboembolic side-effects seen with anti-CD40L mAbs (43, 107).

The immunomodulatory properties of integrins have encouraged the development of clinical antagonists to treat autoimmunity. In a phase I/II open label multi-centre trial, the efficacy and safety of efalizumab (anti LFA-1; humanised IgG1) was tested in renal transplant recipients that also received cyclosporine/rapamycin/steroids or cyclosporine/MMF/steroids. The patient and graft survival was comparable between the treatment arms at 6 months (108), but a significant subset of patients (30%) developed post-transplant lymphoproliferative disease (PTLD) (109), while chronic use resulted in progressive multifocal

leukoencephalopathy (PML) in some patients (110). This raises concerns over the safety and future uses of eflalizumab, particularly in the treatment of conditions such as psoriasis, although its short term use may be more acceptable in the field of transplantation. Indeed in a pilot trial in renal transplantation patients treated with efalizumab had a low incidence of rejection (108). Also many of the current immunosuppressive agents used in transplant recipients carry similar risks for PML (110, 111).

Future opportunities

The use of costimulation blockade in clinical transplantation has been accelerated by the clinical trial data for Belatacept, as well as its recent approval for renal transplantation. One of the important unmet goals for therapeutic strategies in transplantation is to reduce the toxic side effects of calcineurin inhibitors and other immunosuppressive drugs, while also lowering the risk of acute and delayed rejection. A number of new pathways and molecules have been investigated for this purpose including costimulation.

Blockade of costimulatory interactions have been found to modulate immune responses following T cell activation. However, specific pathways may or may not be utilised by particular subsets of T cells or are important only at specific stages in an immune response. Clearly this presents challenges to the ‘one size fits all’ concept for immunosuppression as it suggests that it may be necessary to use different combinations of therapeutic agents tailored to the immunological challenges faced by individual donor recipient pairs and that this may need to be modified as the immune response progresses. Therefore a combination of costimulation blockade may provide a therapeutic advantage over individual reagents. For

example anti-CD154, CTLA-4 Ig and an integrin antagonist (e.g. anti VLA-4 or anti-LFA-1) prolongs allograft survival in mice (79). Despite the success of costimulatory molecule blockade in rodent models, the key to success in the clinic will be to define the expression patterns of individual costimulatory molecules and their ligands within the allograft as well as in the secondary lymphoid tissue in order to understand the implications of targeting costimulatory molecules more precisely.

Acknowledgments

The authors would like to thank all the people involved in the field of costimulation, for moving our understanding and immunologic knowledge forward. Our own work has been supported by The Wellcome Trust, Medical Research Council, BBSRC, British Heart Foundation, Kidney Research UK and the European Union RISET and TRIAD projects. GK has been supported by an MRC-CASE studentship.

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FIGURE LEGENDS:

Figure 1: Diversity of costimulatory molecules in the different stages on the immune response.

Figure 2: A simplified diagram of the costimulatory molecules which are important in providing signal 2 for T cell activation. TNFR and Ig superfamily co-stimulatory signals appear to overlap in the activation of MAP kinase cascades, PKB, and activation of the transcription factor NF-κB. Concomitant activation of the transcription factors NF-κB, AP-1

and NFAT are critical for the transcription of genes that promote T cell activation such as IL-2.

Figure 3: Proposed model of the mechanism action of CTLA-4-Ig.

Table 1: Expression and function of costimulatory pairs in the immune response to an allograft