Published in final edited form as:

Am J Transplant. 2012 October ; 12(10): 2588–2600. doi:10.1111/j.1600-6143.2012.04180.x.

THE ROLE OF CO-STIMULATORY MOLECULES IN DIRECTING THE FUNCTIONAL DIFFERENTIATION OF ALLO-REACTIVE T HELPER CELLS

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Abstract

Co-stimulatory molecules are a heterogeneous group of cell surface molecules that act to amplify or counteract the initial activating signals provided to T cells from the T cell receptor (TCR) following its interaction with an antigen/major histocompatibility complex (MHC), thereby influencing T cell differentiation and fate. While co-stimulation was previously thought to be indispensable for T cell activation at all stages of development, it is now known that the requirements for co-stimulation, and co-stimulatory molecules involved, vary according to the stage of T cell differentiation. The ability to influence T cell fate is of paramount interest in the field of transplantation as we seek therapeutic options that inhibit detrimental alloimmune responses whilst simultaneously promoting allograft tolerance. As with many immune mechanisms, there is a degree of functional overlap between certain co-stimulatory molecules, while some have diametrically opposite effects on different T cell subsets despite sharing common ligands. This is a critical point when considering these molecules as therapeutic targets in transplantation, as blockade of a co-stimulatory pathway, while desirable in itself, may prevent the ligation of an essential regulatory co-inhibitory molecule. This review discusses the T helper cell lineages pertinent to transplantation and the co-stimulatory molecules involved in their differentiation.

Keywords

Co-stimulatory Molecules; Alloreactive T cells; T cell development

Introduction

Co-stimulatory molecules belong to three major families, namely the immunoglobulin (Ig) superfamily, the tumor necrosis factor (TNF) – TNF receptor (TNFR) superfamily and the emerging T cell Ig and mucin (TIM) domain family (Table 1); they may additionally be classified according to the nature of the signal they provide. They cannot activate T cells independently, but are critical to the functional naïve T cell response, the nature of which depends upon the outcome of integration of these stimulatory or inhibitory signals(1, 2). Upon activation, the naïve T cell develops into an effector cell, and may subsequently develop into an effector or central memory cell(3); depending upon the nature and duration

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Disclosures:

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

of antigenic stimulus, it may later display markers of exhaustion. Co-stimulation is critical to the naïve T cell response: in the absence of CD28 ligation, TCR stimulation of naïve T cells induces T cell anergy *in vitro*, precluding an effective T cell response and promoting tolerance(4). Knowledge of the different co-stimulatory pathways involved in the various stages of the immune response is crucial: it is now apparent that memory T cells resist many of the therapeutic strategies employed to target naïve alloimmune responses, while there is increasing interest in the feasibility of manipulating signals involved in T cell exhaustion for therapeutic benefit in chronic viral infections and malignancy. The focus of this minireview will be directed to the role of co-stimulation in the functional differentiation of effector T helper cells and the alloimmune response.

Effector T cells

CD4⁺ T cells, or T helper cells, play a central role in the determination of the adaptive immune response in the settings of autoimmunity, allergy, and alloimmunity. Upon interaction with their cognate antigen via the TCR, naïve CD4⁺ T cells can differentiate into various lineages, including the classical effector T helper type 1 (Th1) and type 2 (Th2) subsets, and the more recently identified Th17, T follicular helper (Tfh), Th9 and induced regulatory (iTreg) T cell populations(5–9) (Figure 1). These subsets are functionally distinct and may be identified by the production of characteristic cytokines (Table 2). The differentiation decision is influenced by a variety of factors, including the cytokine milieu in which this interaction occurs, the strength of the TCR signal(10) and affinity of the TCR for the antigen(11), and the nature of the co-stimulatory molecules providing the second signal to the TCR complex(1). CD4⁺ T cell differentiation was, until recently, considered to be a terminal event. However, considerable plasticity is now recognized to exist amongst many T helper subsets(12); indeed, Tregs and Th17 cells have been shown to be capable of reciprocal differentiation(13, 14).

Th1 cells

Th1 cells were identified in the original paradigm of T cell differentiation(15), and despite the subsequent identification of additional T cell subsets, remain as relevant to the immune response as first thought. There exists a large body of data demonstrating that Th1 cells significantly contribute to the alloimmune response and processes of allograft rejection(16, 17).

CD40L (CD154), a member of the TNF – TNFR family, has been widely reported to influence Th1 differentiation(18). CD40L is upregulated upon high antigen dose T cell activation, and following ligation of CD40, promotes the secretion of inflammatory cytokines including TNF and IL-12, and increases the expression of MHC molecules and the costimulatory molecules CD80 and CD86(19). CD40, constitutively expressed on B cells, macrophages and dendritic cells, is itself increased upon activation, promoting CD40/CD154 interactions and intensifying antigen-specific signaling. Interruption of the CD40-CD40L pathway, achieved by use of anti-CD40L monoclonal antibodies or genetic knock-out mice, initially showed great promise in transplantation, significantly enhancing allograft survival and preventing acute rejection in rodents(20, 21) and primates(22, 23), an effect associated with inhibition of Th1 responses and deviation to a Th2 phenotype(24). Furthermore, anti-CD40L mAbs have been shown to act synergistically with other therapies including CTLA4-Ig(20), and, when given in concert with donor specific transfusion, to generate chimerism in the absence of bone marrow conditioning, leading to donor-specific tolerance(25). Interestingly, the combination of anti-CD40L with the immunosuppressive agents cyclosporin A (CsA) and methylprednisolone, but not rapamycin or mycophenolic acid, inhibited anti-CD40L mAb-induced cardiac allograft survival in a murine transplant model(26), thought to be due to marked inhibition of CD40L expression by CsA and

methylprednisolone(26); in another study, use of calcineurin inhibitors or anti-IL-2R were also shown to abrogate anti-CD40L-induced graft survival(27). Rapamycin, however, demonstrated marked synergy with anti-CD40L, achieving indefinite graft survival in all recipients, with significant reduction in the frequency of alloreactive IFN- γ -secreting cells and inhibition of chronic rejection(27); this effect was not diminished by the addition of calcineurin inhibition(27). While these interactions have clear clinical implications, efforts to develop anti-CD40L antibodies for clinical use were unfortunately hindered by the unexpectedly high incidence of thrombotic complications in both non-human primate studies and phase I clinical trials(28), thought to be due to the interruption of CD40L signaling on platelets(29). Efforts have now focused on the development of anti-CD40 monoclonal antibodies; early results in transplantation are promising(30).

Although not found on naïve CD4⁺ T cells, TIM-1 is rapidly expressed upon their activation(31, 32), and is capable of orchestrating the differentiation of several Th lineages depending upon the avidity with which it is engaged(33, 34). Interestingly, following differentiation, TIM-1 expression is maintained by Th2, but not Th1 or Th17 cells(32, 35). In a mouse model of multiple sclerosis, the use of a high-affinity agonistic anti-TIM-1 mAb enhanced both the Th1 and Th17 response(33); conversely, use of an antagonistic anti-TIM-1 antibody in a murine transplant model improved the survival of fully MHC-mismatched cardiac allografts, an effect dependent on the presence of Tregs and associated with inhibition of the alloreactive Th1 response but maintenance of the Th2 response(36). A recent study also demonstrated that use of a recombinant human TIM-1 fusion protein (TIM-1-Fc) inhibited the proliferation of TIM-4⁻ CD4⁺ cells, an effect associated with inhibition of AKT and ERK1/2 phosphorylation(37). Furthermore, TIM-1-Fc inhibited the proliferation of alloreactive CD4⁺ cells and led to significant prolongation of allograft survival in a murine cardiac transplant model, with inhibition of the Th1 response but preservation of both Th2 and nTreg function, effects thought to be achieved by the binding of Tim-1-Fc to a receptor other than TIM-4(37).

CTLA-4 (CD152) is a receptor structurally related to CD28 that also binds to the ligands B7-1 and B7-2, albeit with much greater affinity(4). CTLA-4 is not constitutively expressed on conventional T cells, but is up-regulated upon activation in a CD28-dependent manner(38, 39). CTLA-4 directly antagonizes CD28 signaling by inhibiting Akt(40), providing negative co-stimulatory signals that inhibit T cell proliferation, IL-2 production and cell cycle progression. Indeed, signaling through CTLA-4 inhibits both Th1 and Th2 differentiation, and is also thought to enhance the susceptibility of effector T cells to suppression(41). Further insights into its immuno-regulatory activity were provided by a recent study which demonstrated that CTLA-4 captures its ligands B7-1 and B7-2 from other cells by trans-endocytosis, leading to their degradation inside CTLA-4 expressing cells, and preventing their ligation with CD28(42). CTLA-4-Ig, a fusion protein that contains the extracellular CTLA-4 domain and the Fc portion of IgG1 and potently inhibits CD28 signaling, was shown to be highly effective in prolonging allograft survival in multiple rodent models(43, 44), although combination therapy with donor splenocyte transfusion was required to induce tolerance in most models(45). Investigation of potential interactions with other immunosuppressive agents failed to demonstrate any abrogation of allograft survival following the addition of calcineurin inhibition, methylprednisolone or rapamycin(27). However, disappointing results seen upon translation of therapy into primate models prompted the development of Belatacept, a higher affinity mutant form of CTLA4-Ig, which has now been approved for use in transplantation. Use of Belatacept in human transplant recipients has not been without complication, with higher rates of severe acute rejection reported in the initial trials(46); as this is thought to be primarily related to effects on the Treg population, it will be discussed in more detail later.

TIM-3, originally identified on activated Th1 cells(47), is now known to be expressed by cells of both the innate and adaptive immune systems(35, 48). Galectin-9, the identified ligand of TIM-3(49), is widely expressed in various tissues, but in the non-activated immune system, is predominantly found on CD4⁺25⁺ Tregs and naïve CD4⁺ T cells. TIM-3, in contrast, is not expressed by naïve CD4⁺ T cells, but is expressed upon their activation and differentiation to the Th1 phenotype(47, 50). The TIM-3 – Galectin-9 pathway has been shown to act as a negative T-cell costimulatory pathway: Galectin-9 is upregulated by IFN- γ , and upon interaction with TIM-3, preferentially inhibits Th1 cells by promoting cell death, thought to be due to a combination of apoptosis and necrosis(49). TIM-3 blockade accelerates Th1-mediated autoimmune disease(50); conversely, TIM-3 has been shown to negatively regulate the secretion of Th1 and Th17 cytokines in human studies, but, in contrast to murine TIM-3, does not appear to induce cell death(51). Our group has previously shown that TIM-3 blockade, achieved by use of a blocking antibody RMT3–23, accelerates allograft rejection in murine transplant models in the absence of CD80/CD86 or CD28 signaling(52), an effect both dependent on the presence of CD4⁺ T cells and associated with expansion of the allospecific CD4⁺ T cell population(52). TIM-3 blockade was also associated with enhanced Th1 polarization and increased frequencies of alloreactive IFN- γ -, IL-17-, IL-6- and Granzyme B- producing cells(52).

There is a significant body of data to demonstrate that the PD-1 – PDL-1 pathway is centrally involved in the alloimmune response, and acts to promote engraftment(53–55). PD-1, PDL-1 and PDL-2 are prominently expressed in acutely rejecting cardiac allografts(55), although there is evidence that each plays a distinct role: blockade of either PD-1 or PDL-1 accelerated allograft rejection in CD28^{-/-} mice recipient of a fully MHC-mismatched cardiac allograft(56), but only PDL-1 blockade precipitated rejection in WT mice recipient of a fully MHC-mismatched(56) or single MHC Class II-mismatched(53) cardiac allograft, indicating relative contributions to the alloimmune response(2, 53). Furthermore, donor, but not recipient, expression of PDL-1 was critical to allograft survival and the prevention of vasculopathy in a single MHC-mismatched cardiac transplant model(57), while donor endothelial PDL-1 expression was shown to be essential for the maintenance of allograft tolerance induced by CTLA4-Ig in a fully MHC-mismatched model(54). PDL1-Ig acts synergistically with both CsA and anti-CD154 to prolong graft survival in models of cardiac(55) and islet(58) transplantation, respectively. Conversely, PDL-1 blockade has been shown to enhance alloreactive Th1 differentiation(59), while there is also evidence that PDL-1 functions in a PD-1-independent manner to mediate apoptosis of alloreactive T cells(59). Interestingly, B7-1 has been recently identified as an additional ligand for PDL-1, with evidence that this interaction functions as a bi-directional inhibitory pathway(60). Furthermore, specific blockade of the PDL-1 – B7-1 pathway has been demonstrated to precipitate allograft rejection independent of PD-1 signaling, with enhanced Th1 and Th2 alloreactivity(53).

Although thought to have a central role in Th2 differentiation, CD28, OX40 and TIM-1 instead promote Th1 differentiation upon strong TCR signaling(10), the production of IFN- α or IL-12(61), or high affinity binding(33), respectively. These molecules will be discussed in more detail below.

Th2 cells

The role of Th2 cells in the alloimmune response is unclear: while there is some evidence to suggest that Th2 cells may play a regulatory role(62), there are also data that indicate their involvement in the rejection response(63, 64).

CD28 signaling has been shown to play an important role in the differentiation of naïve T cells to the Th2 lineage(65), a mechanism dependent on the presence of IL-4 and achieved

via the induction of GATA-3(66). It is also dependent on the strength of signaling through the TCR: weak TCR signaling in concert with CD28 indeed promotes IL-4 and, consequently, Th2 cells(10); strong TCR signaling, however, promotes the production of IFN- γ and the differentiation of Th1 cells(10). Much of the data regarding the contribution of CD28 signaling in transplantation is provided by studies investigating the use of CTLA4-Ig, which competes with CD28 for binding to CD80/86. However, there are also data in animal models regarding the use of agonistic anti-CD28 antibodies, which have been shown to prolong allograft survival in rodent models of transplantation(67). Recent progress has been made in the development of a chimeric human/primate mAb which selectively blocks CD28 signaling without agonistic or superagonistic activity, and was shown to inhibit both acute and chronic rejection in non-human primates, with evidence of synergistic activity when combined with CsA(68).

OX40 plays a central role in the differentiation of naïve CD4⁺ T cells to the Th2 lineage, an effect mediated initially by IL-4R-independent induction of nuclear factor of activated T cells c1 (NFATc1), and subsequently by an IL-4R-dependent effect on GATA-3(69). OX40 is known to act on naïve T cells in concert with CD28, and indeed, the initial effects of OX40 on IL-4 transcription are dependent on intact CD28 signaling(69); moreover, previous data have demonstrated that deficiency of CD28 results in impaired Th2 responses(65). However, production of IFN- α or IL-12(61) has been shown to divert Th2 differentiation, instead promoting Th1 responses. Interestingly, while OX40 signaling has also been shown to promote the Th2 differentiation of human naïve CD4⁺ T cells *in vitro* (70), it did not inhibit IL-12-mediated upregulation of IFN- γ production(70). Furthermore, stimulation of OX40 via an agonistic anti-OX40 mAb in a murine cardiac transplant model, wherein the absence of CD40-CD40L signaling had achieved tolerance, precipitated rejection with evidence of both Th1 and Th2 donor-reactive responses, mediated by CD8⁺ and CD4⁺ T cells, respectively(71). Isolated blockade of OX40 signaling in rodent models of transplantation has little effect on allograft survival(72). However, the combination of anti-OX40L mAb with rapamycin resulted in significant enhancement of allograft survival compared to rapamycin alone, although in contrast to hCTLA4-Ig, failed to demonstrate any such enhancement when combined with CsA(72). Furthermore, OX40 signaling has been shown to have a critical role in CD28- and CD40L-independent rejection: use of a blocking anti-OX40L mAb in the absence of CD28/CD40L signaling, achieved by use of double deficient mice or blocking antibodies, leads to significant prolongation of skin graft survival(73), while OX40 blockade was shown to significantly prolong both cardiac and skin graft survival when combined with CD28-B7 blockade, inhibiting both alloreactive IFN- γ production and the generation of activated/effector lymphocytes(72).

ICOS, a member of the Ig superfamily, is inducibly expressed upon cell activation, and has complex links to both CD28 and CTLA-4: ICOS is upregulated upon CD28 co-stimulation, although ligation of ICOS-L leads to down-regulation of CD86 on APCs(74); conversely, CTLA-4 signaling inhibits ICOS expression. Interestingly, despite the role of CD28 co-stimulation in ICOS expression, ICOS co-stimulation is an important mechanism for T cell activation in the absence of CD28 signaling(75). ICOS has been reported to regulate both Th1 and Th2(76, 77), and, more recently, Th17 differentiation(78); it appears to be more crucial for the Th2 lineage, with evidence that it functions via enhancement of IL-4R-mediated signaling(79), although the requirements for ICOS signaling may depend on the experimental model used and the timing of signaling. Indeed, a recent study of ICOS-deficient patients revealed impaired polarization to Th1, Th2 and Th17 subsets, with further deficiencies in CD4⁺ effector and central memory subsets(80). In transplantation, the expression of ICOS has been shown to be markedly up-regulated in allografts undergoing both acute and chronic rejection(76), while ICOS blockade significantly prolonged allograft survival in a fully MHC-mismatched murine model(75, 76). The timing of therapy was

shown to be important, with delayed blockade shown to effect the greatest prolongation of graft survival(75); mice defective in either STAT-4 or STAT-6 signaling (defective Th1 and Th2 responses, respectively) displayed a similar tempo of rejection to their WT counterparts, although only STAT-4^{-/-} mice demonstrated prolonged allograft survival upon ICOS blockade, indicating that this effect is dependent upon an intact STAT-6 pathway, and, by extension, an intact Th2 response(75). ICOS blockade has further been shown to act in concert with anti-CD40L, preventing the development of chronic rejection seen with anti-CD40L therapy in the absence of DST(76), while the combination of ICOS blockade and a short course of CsA effected permanent engraftment of fully mismatched cardiac allografts with normal histology at day 100(76).

In addition to its role in Th1 and Th17 differentiation, the TIM-1 – TIM-4 pathway is also involved in Th2 differentiation. Interestingly, data from autoimmune and atopic models indicate that, in addition to strength of signal provided to TIM-1 (via different affinity antibodies), the differentiation decision appears to be influenced by the specific anti-TIM antibodies used, indicating that distinct TIM-1 epitopes have widely varying effects. While administration of a high affinity agonistic antibody in a model of EAE enhanced Th1 and Th17 responses and aggravated disease severity, use of a lower affinity antibody promoted Th2 responses and inhibited disease development(33). In models of allergy, the upregulation of TIM-4 on intestinal(81) and bone marrow-derived DCs(82) *in vivo* (following exposure to Staphylococcus enterotoxin B or cholera toxin and peanut extract, respectively), has been shown to promote Th2 polarization and intestinal allergy. Little data exists regarding its role in transplantation.

TIM-2, a TIM molecule to date identified only in mice, is primarily expressed by activated CD4⁺ T cells, particularly those of Th2 phenotype. Despite this, however, TIM-2 is believed to be a negative regulator of Th2 cells in models of allergy and autoimmunity(83). There is little data regarding the role of TIM-2 in transplantation.

Th17 cells

In the setting of transplantation, there is increasing evidence that Th17 cells contribute to the rejection response(84, 85), although many studies have focused on the contribution of IL-17, which may be secreted by cell types other than Th17(86). Our group has previously shown Th17 cells to be capable of mediating a severe, accelerated form of vasculopathy in a cardiac transplant model which lacks Th1 cells(84); a recent study further demonstrated Th17 cells to be capable of mediating rejection in the absence of other lymphocyte populations and despite neutralization of IL-17A(85). Overall, the exact role of Th17 cells in normal alloimmune conditions, in which the Th1 response dominates, remains to be determined.

In addition to its role in other T helper subsets, CD28 co-stimulation is reportedly involved in Th17 differentiation, although this may reflect its general role in T helper cell activation(18, 78). More recently, the use of anti-CTLA-4, which potentiates CD28 responses, was shown to promote Th17 differentiation *in vitro* and *in vivo*, while the use of CTLA-4-Ig, and consequent CD28 blockade, inhibited Th17 differentiation(87). However, in another mouse model, CD28 ligation with an anti-CD28 mAb was itself demonstrated to inhibit Th17 differentiation of naïve cells, but appeared to enhance IL-17 production from previously-differentiated Th17 cells(88), while CTLA4-Ig facilitated both murine and human Th17 differentiation(88).

The role of ICOS signaling in Th17 differentiation has yet to be fully determined. Along with CD28, ICOS has been shown to be involved in Th17 cell development both *in vitro* and *in vivo*(78), with ICOS signaling capable of inducing optimal IL-17 secretion from Th17

cells(78). More recent data has demonstrated that ICOS is also important in the maintenance of the Th17 phenotype and expansion of previously differentiated Th17 cells, the mechanism of which involves the induction of c-maf and upregulation of the stimulatory cytokine IL-21(89). Its role in the differentiation decision, however, is controversial. While shown to be crucial for Th17 maintenance, as discussed above, ICOS was not a prerequisite for Th17 differentiation in a murine model(89); a recent study, however, reported ICOS to play a vital role in both the differentiation and expansion of human Th17 cells(90), while, conversely, evaluation of T cell subsets in ICOS-deficient patients revealed impaired polarization to Th1, Th2 and Th17 subsets, with further deficiencies in CD4⁺ effector and central memory subsets(80).

TIM-1 was initially thought to be primarily involved in Th1/Th2 differentiation. However, more recent data has shown that TIM-1 also plays a role in the differentiation of Th17 cells. Use of an agonist anti-TIM-1 mAb in an islet transplantation model prevented the development of tolerance, and *in vitro*, was shown to convert existing Tregs into IL-17-producing cells(34). Furthermore, inhibition of TIM-1 signaling by anti-TIM-1 was shown to overcome the resistance to tolerance mediated by IL-17-producing CD8 T cells in the absence of CD28 and CD40L signaling(91). Interestingly, a recent study has also provided evidence that TIM-4 inhibits both the differentiation of naïve CD4⁺ T cells to the Th17 lineage and the production of IL-17 in Th17-polarized cultures in a TIM-1-independent manner(92).

As discussed earlier, our group has previously shown that TIM-3 blockade, achieved by use of a blocking antibody RMT3–23, accelerates allograft rejection in murine transplant models in the absence of CD80/CD86 or CD28 signaling(52), an effect associated with both enhanced Th1 and Th17 polarization and increased frequencies of alloreactive IFN- γ -, IL-17-, IL-6- and Granzyme B-producing cells(52). Inhibition of TIM-3 *in vitro* also increased production of IL-17 from effector T cells(52). TIM-3 also inhibits the secretion of Th17 cytokines in human studies, but, in contrast to murine TIM-3, does not appear to induce cell death(51).

In addition to its role in Th1 differentiation, recent autoimmune data have demonstrated a role for the CD40L – CD40 pathway in Th17 differentiation(18). In the setting of strong antigenic stimulation, CD40 ligation was shown to promote the secretion of IL-6 by DCs via pattern recognition receptors *in vitro*, which, when combined with TGF β , provided the optimal milieu for Th17 differentiation(93). Furthermore, CD40^{-/-} mice were shown to be incapable of mounting a normal Th17 response despite high antigen stimulation and were completely protected from the development of EAE(93).

The effect of OX40 on Th17 differentiation in transplantation is unclear. There are *in vitro* data indicating that OX40 inhibits Th17 cell induction, an effect mediated by IFN- γ (94); however, this data does not concur with *in vivo* studies demonstrating that OX40 activity is necessary for the Th17-mediated diseases EAE(95) and RA(96).

Tregs

Natural regulatory T cells (nTregs) are formed in the thymus and are characterized by the expression of the transcription factor FoxP3. Adaptive or induced Tregs (iTregs) may also be generated in the periphery from CD4⁺CD25⁻ cells in the presence of TGF β (7). Tregs are critical to the development and maintenance of self-tolerance, and, in transplantation, are indispensable to the induction and maintenance of allograft tolerance.

CD28 is critical to the development of natural Tregs in the thymus(97), and mice deficient in CD28 or either of its ligands have been shown to have significantly reduced numbers of

natural Tregs(97–100); CD28 costimulation is also involved in the generation of Tregs from CD4⁺CD25⁺ T cells through the production of IL-2(101), although this requirement is not absolute(102). However, high levels of CD28 costimulation have been shown to both inhibit the generation of iTregs and promote the development of T effector cells through a mechanism involving Lck signaling, independent of IL-2(103). *In vivo*, mice deficient in CD28 show prolonged acceptance of fully MHC-mismatched cardiac allografts(100, 104), but paradoxical accelerated rejection of single MHC Class II-mismatched cardiac allografts, accompanied by both an increased ratio of T effector cells to Tregs and increased production of Th1 and Th2 cytokines(100). Use of a superagonistic anti-CD28 mAb in rodent models was shown to preferentially activate and expand functional Tregs both *in vitro* and *in vivo*(105, 106); unfortunately, attempts to translate this therapy into clinical use were disastrous: in Phase I clinical trials, six healthy volunteers developed a massive cytokine storm upon administration of a superagonistic anti-CD28 mAb(107).

CTLA-4 (CD152), as discussed above, plays a pivotal role in negative regulation of the CD28 pathway and Th1/Th2 cell differentiation. While inducibly expressed on conventional T cells, it is constitutively expressed by Tregs, and is central to their normal homeostasis and function(4); indeed, mice deficient in CTLA-4 develop catastrophic lymphoproliferation and inflammation due to unchecked CD4⁺ T cell activation by environmental antigens. It remains controversial whether CTLA4 mediates its effects through cell-intrinsic or -extrinsic mechanisms, or both (4); there are data to indicate that it may involve inhibition of the Erk and Akt pathways; additionally, B7 ligation by CTLA-4 induces the enzyme indoleamine 2, 3-dioxygenase (IDO) which inhibits T cell proliferation and has been implicated in the maintenance of tolerance in a variety of settings(108). Furthermore, as previously discussed, a recent study provided new insights into its immuno-regulatory activity by demonstrating that CTLA-4 acts in a cell-extrinsic manner to capture its ligands B7-1 and B7-2 from other cells by trans-endocytosis, leading to their degradation inside CTLA-4 expressing cells, and preventing their ligation with CD28(42); this was shown to be true for both activated effector cells and Tregs(42). The development and approval of belatacept(109), a mutant form of CTLA4-Ig, for clinical use in transplantation renders these mechanisms particularly important: as CTLA-4-Ig functions by competing with CD28 for B7-1/2 binding, it would also block the CTLA-4/B7 interaction, and, consequently, inhibitory cell-intrinsic signaling. The recent identification of B7-1 as an additional ligand for PDL-1 is also of concern, as regulatory signaling mediated by PDL-1 through this pathway(53, 60) would also be blocked by CTLA4-Ig. Indeed, initial results from the Phase III belatacept trials reported an unexpected increase in early acute rejection in the belatacept-treated group(46), causing significant concern. A recent paper from our group demonstrated that use of hCTLA4-Ig significantly inhibited the generation of nTregs in naïve mice(110), and, as a corollary to data discussed above, prevented rejection in a fully-MHC mismatched cardiac transplant model, but accelerated cellular rejection in a MHC-Class II mismatch model, in which long-term graft survival is dependent upon the emergence of Tregs(110). Evaluation of a small cohort of renal transplant patients treated with anti-IL-2R and randomly assigned to belatacept or calcineurin inhibitor maintenance therapy, revealed a significant, albeit transient, reduction in the frequency of circulating CD4⁺CD25⁺FoxP3⁺ Tregs in both groups which was attributed to treatment with anti-IL-2R(111); however, no reduction in Treg frequency or potency was seen in either group long-term(111, 112).

In addition to its inhibitory effects on alloreactive effector cells, the PD-1 – PDL-1 pathway is centrally involved in both the differentiation and maintenance of FoxP3⁺ Tregs, resulting in increased generation of Tregs, sustained FoxP3⁺ expression and enhanced suppressor function, effects achieved by blockade of the Akt/mTor pathway and enhancement of PTEN expression(113). Notably, while CTLA-4 also mediates its inhibitory effects via blockade of Akt signaling, the PD-1 – PDL-1 pathway has been shown to operate independently of

CTLA-4(114). Furthermore, specific blockade of the PDL-1 – B7-1 pathway was also shown to effect a reduction in the Treg population, indicating its involvement in Treg homeostasis(53).

The interaction of TIM-3 with its ligand Galectin-9 is critically involved in Treg function. In a murine cardiac transplant model, TIM-3 was shown to be constitutively expressed on natural Tregs(52) and while Galectin-9 is expressed upon naïve CD4⁺ T cells and CD4⁺CD25⁺ Tregs in the non-activated system, and, indeed, may potentially be near-universally expressed, it is notable that, upon activation, the expression of Galectin-9 on T cells is down-regulated on the effector population but is maintained on Tregs(47, 50). TIM-3 blockade has been shown to both impair the suppressive capacity of nTregs and prevent the generation of allospecific adaptive Tregs(50), culminating in abrogation or prevention of induced peripheral tolerance(47, 50). Conversely, the administration of soluble Galectin-9 has been shown to promote allograft survival in models of skin(115) and cardiac(116) transplantation, an effect accompanied by attenuated secretion of Th1 and Th17 cytokines and promotion of Tregs(116).

GITR is constitutively expressed on Tregs and inducibly expressed by activated T cells, B cells, macrophages and NK cells. GITR is capable of activating signaling pathways that trigger apoptotic cell death, and is therefore considered to be a promising therapeutic target in cancer and autoimmune disease. It is also thought to be involved in the regulation of FoxP3⁺ Tregs(117). Its precise role in transplantation is unknown.

OX40 signaling negatively influences Tregs, and has been shown to inhibit the TGF- β -mediated induction of FoxP3⁺ Tregs from both naïve CD4⁺ T cells(118) and CD4⁺ T effector cells(119). Furthermore, OX40 ligation leads to decreased gene expression of FoxP3, while OX40 stimulation of Tregs abrogates the suppressor functions of natural FoxP3⁺ Tregs(119, 120). Notably, there is no difference in Treg number or suppressive capacity between OX40 KO and WT mice, demonstrating that OX40 is not involved in the homeostatic regulation of Tregs(119).

The use of different agonistic anti-TIM-1 antibodies in murine models of allergy, allo- and auto-immunity has revealed TIM-1 to be variously involved in the differentiation of Th1, Th2 and Th17 cells, while inhibiting both the commitment to and suppressor function of the Treg phenotype(32–34); accordingly, use of an agonist antibody prevented the induction of allograft tolerance in an islet transplantation model(34).

Tfh cells

T follicular helper (Tfh) cells are a subpopulation of Th cells primarily involved in providing help to B cells, regulating the development of effector and memory B cells, thereby controlling antigen-specific B cell immunity(121); their exact role in the alloimmune response has yet to be determined. Tfh cells express the chemokine receptor CXCR5 and the co-stimulatory molecules ICOS, CD40L and PD-1(121, 122); the expression of OX40 has also been identified among some mouse strains(123, 124).

ICOS signaling has been shown to be centrally involved in the induction of Tfh cells via upregulation of the transcriptional repressor Bcl-6(125) and the maintenance of germinal centres(124). ICOS deficiency is associated with decreased Tfh cell development(126) and profound defects in antibody production, isotype switching and antibody production in both murine models and humans(124, 127). Notwithstanding this, the presence of Tfh cells, albeit limited, and Bcl-6 upregulation in ICOS^{-/-} mice suggests that the requirement for ICOS signaling is not absolute, although clearly their function is grossly impaired. Recent data suggests that there is some functional overlap between ICOS and CD28, as ICOS has been

shown to be capable of substituting for CD28, reversing the Tfh and B cell defects seen in CD28-deficient mice(128).

The CD40 – CD40L pathway is known to play a critical part in humoral immunity and in the provision of T cell help to B cells. Interruption of the CD40 – CD40L pathway in mice or humans has been shown to result in impaired humoral immune responses, including loss of germinal center formation, impaired isotype switching and failure of memory B cell responses(121). Specifically, however, deficiency of circulating Tfh cells has been demonstrated in humans with CD40L mutations(126).

The role of OX40 costimulation in the function and maintenance of Tfh cells is controversial. Interruption of the OX40 – OX40L interaction was only demonstrated to negatively impact Tfh development in isolated mouse models(124); however, other studies have shown such an interruption to prevent the accumulation of Tfh cells in the follicular regions of lymph nodes, indicating a role in Tfh migration, while the maintenance of OX40 expression on effector Tfh cells is thought to suggest a role in the determination of cell fate upon effector Tfh-B cell interactions(123, 129).

Tfh cells also express PD-1, which is up-regulated due to Bcl-6 expression(130), although its role in the differentiation or maintenance of the lineage is incompletely understood. The highest levels of PD-1 are found on GC Tfh cells(131), while GC B cells express PDL-1. Interruption of the PD-1 – PDL-1 interaction via PD-1 blockade was shown to enhance Tfh cell number, although with reportedly variable effect on GC formation(132, 133).

Th9 cells

Th9 cells, a recently discovered T helper subset, are characterized by the production of IL-9 and IL-10. Shown to promote tissue inflammation(6, 9), they have been implicated in asthma and autoimmune disease(134). Their role in transplantation is, as yet, unknown. Interestingly, the combination of TGFβ and IL-4 was shown to direct Th9 differentiation(6, 9), although the mechanism by which this is achieved requires further elucidation: one hypothesis suggests that the Th9 phenotype is generated by TGFβ-induced “reprogramming” of Th2-differentiated cells(9), another that IL-4 inhibits the induction of FoxP3 by TGFβ, instead generating Th9 cells(6). The transcription factors IRF-4(134) and STAT-6(6) are required for their differentiation, but it is unclear whether a Th9-specific transcription factor exists, or indeed which co-stimulatory molecules play a role therein. We await the outcome of further investigation into these matters, and indeed their role in transplantation, with interest.

Conclusions

Co-stimulatory molecules continue to hold great promise as therapeutic targets in transplantation. However, a thorough understanding of how these pathways co-operatively function to direct T cell differentiation is critical, as, given their complex relationships and shared ligands, blockade of a co-stimulatory pathway, while desirable in itself, may prevent the ligation of an essential regulatory co-stimulatory molecule.

Acknowledgments

C.N.M. is supported by a Basic Science Fellowship Grant from the American Society of Transplantation, and was the recipient of a Research Bursary from the Irish Nephrology Society. This work was supported by NIH grant 1R56AI089777-01A1.

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Figure 1.

An overview of T helper cell differentiation. The T helper 1 (Th1), Th2, Th17, Th9, Tfh and Treg lineages are shown, along with the transcription factors and cytokines required for their respective differentiation; their subsequent cytokine production is also indicated.

Table 1

Co-stimulatory Molecules: Their Ligands and Expression

Co-stimulatory Molecule	Ligands	Family	Expression
CD28	B7-1 (CD80); also binds CTLA-4(4), PDL-1(60) B7-2 (CD86); also binds CTLA-4(4)	IgG - CD28/B7	Constitutive - all naïve CD4 & CD8 T cell subsets (4)
CTLA-4	B7-1 (CD80)(4) B7-2 (CD86)(4)	IgG - CD28/B7	Constitutive - Tregs(4) Inducible - activated T cells(4)
ICOS	ICOS-L(2)	IgG - CD28/B7	Inducible(135)
PD-1	PDL-1(136); also binds B7-1(60) PDL-2(136)	IgG - CD28/B7	PDL-1: Constitutive & Inducible - APCs, T cells, Tregs, non-hematopoietic cells (4, 136) PD-1: Inducible - activated CD4/CD8, activated B cells, NK cells & macrophages(136), Tfh cells(122)
CD27	CD70(2)	TNFR/TNFR	Constitutive - naïve T, B & NK cells(2) Inducible (CD70) - T & B cells(2)
CD30	CD30L(2)	TNFR/TNFR	Constitutive - Tregs(2) Inducible - activated T effector/memory(2)
CD40L	CD40(2)	TNFR/TNFR	Inducible - activated T cells, NK cells, eosinophils, platelets (2)
OX40	OX40L (61)	TNFR/TNFR	Inducible - activated T cells (61)
GITR	GITRL (2)	TNFR/TNFR	Constitutive - Tregs (2) Inducible - activated T effector cells, B cells, NK & macrophages (2)
TIM-1	TIM-1(48) TIM-4(48)	TIM	Inducible - activated CD4 & CD8 T cells(31, 32)
TIM-2	H-ferritin(48) ?Semaphorin 4A (Sem4A)(48)	TIM	Inducible - activated T cells (Th2), splenic B cells (48)
TIM-3	Galectin-9 (49)	TIM	Constitutive - Tregs (52) Inducible - Innate: (NK cells, DCs & monocytes - humans; mast cells, macrophages & DCs - mice) Adaptive: (subset activated CD4 & CD8 T cells, terminally differentiated Th1 cells, Th17 cells)

Table 2

The functions and differentiation characteristics of T helper cell lineages

Lineage	Physiological Function	Disease State	Co-stimulatory molecules that Promote Lineage Differentiation	Co-stimulatory molecules that Inhibit Lineage Differentiation
Th1	Cellular immunity against intracellular pathogens Viral immunity	Hypersensitivity Autoimmunity	CD40L (18) TIM-1 (33) CD28(10) OX40 (61) ICOS(76, 77, 79) TIM-1(33) TIM-4(81, 82)	TIM-3 (47, 50–52) CTLA-4(41) PDL-1(59)
Th2	Humoral immunity B-cell help - regulate B cell activation and antibody class switching Extracellular parasitic / helminthic infections	Allergy Atopy	CD28(10, 65, 66) OX40(61, 69, 70) ICOS(76, 77, 79) TIM-1(33) TIM-4(81, 82)	CTLA-4(41) PDL-1(53) TIM-2(83)
Th17	Mediate responses to extracellular bacteria and fungi	Autoimmunity	CD28(18, 78, 87) ICOS(78, 89, 90) TIM-1(34) CD40L(18, 93) OX40(95, 96)	TIM-3(51, 52) TIM-4(92) CTLA-4(87)
Th9	Helminthic infections	Atopy Autoimmunity	?	
Tfh	Provide help to B cells Regulate the effector & memory B cell response Role in germinal center formation & switched antigen-specific antibody responses		CD28(128) ICOS (124–126, 128) CD40L(121, 126, 129) OX40(123, 124)	PD-1, PDL-1(130, 132, 133)
Treg	Regulation/suppression of the immune response Critical for maintenance of self- tolerance		CD28 – nTregs (97–100) CTLA-4 (4) PD-1/PDL-1(53, 113) TIM-3(47, 50) GITR(117)	CD28 - iTregs(103) OX40(118–120) TIM-1(32–34)