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Mechanisms of tolerance induction: blockade of co-stimulation

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Induction of tolerance to transplantation antigens is believed to be a promising way to achieve long-term allograft survival without a deleterious immunosuppressive regimen. T-cell activation, which is an essential feature of graft rejection, requires a first signal provided by T-cell receptor (TCR) ligation and a second signal provided by engagement of co-stimulatory molecules with their respective ligands on antigen-presenting cells. The coordinated triggering of these two independent signalling systems ensures the full T-cell activation, including proliferation and acquisition of effector function. TCR occupancy in the absence of co-stimulatory signals leads to a sustained loss of antigen responsiveness called clonal anergy, which could be of major importance in transplantation. *In vivo*, co-stimulation blockade was indeed shown to allow for long-term allograft survival in several transplantation models. However, the current continuous identification of new co-stimulatory molecules suggests that a functional redundancy of the system exists and that tolerance to transplantation antigens might be achieved more easily through the combined blockade of two or several co-stimulatory signals.

In this review, we analyse the biological effects of the disruption of some co-stimulation pathways *in vitro* and *in vivo* and discuss their potential interest for tolerance induction.

Keywords: T cells; antigen-presenting cells; tolerance; co-stimulation; transplantation

1. INTRODUCTION

Since the seminal experiments of Medawar, Billingham and Brent 50 years ago, tolerance induction to complex and composite 'antigens', such as allografts, has been a major goal in immunobiology and transplantation science. Ironically, at the beginning of a new century, it appears more and more obvious that conventional immunosuppression, despite having allowed organ transplantation to be performed as a routine procedure, has had an impact only on the early phase of the recipient anti-graft immune response without modifying significantly the rate of graft losses after one year. Interestingly, only tolerance induction seems to result in a state of graft acceptance with minimal histological symptoms of chronic rejection, a characteristic that has been proposed as a modern definition of the tolerant state.

Despite important advances in the protocols inducing both central and peripheral tolerance and a better understanding of the mechanisms operating during induction and maintenance of the phenomenon, our capacity to induce tolerance has been restricted for decades to the permissive rodent models, except for deletional 'central' tolerance following induction of macrochimerism in partially bone-marrow-ablated hosts, a manoeuvre which is still considered to carry an unacceptable risk for most human recipients, despite promising developments in experimental models.

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It is only recently, following the molecular identification of co-stimulatory molecules and the development of weakly immunogenic new bioreagents, that the concept of tolerance induction has actually become a preclinical reality. Indeed, the characterization of new T-cell coactivation markers has progressively extended the field of tolerance-induction models in rodents, and the blockade of some co-signals has, for the first time, provided evidence that a stage of operational tolerance, close to what is observed in rodents, can be achieved in the primate (Kirk *et al.* 1999) and therefore possibly in man. Furthermore, these important results obtained with anti-CD40 ligand (CD40L) antibodies *in vivo* (Larsen *et al.*, this issue) are likely to be synergistic with other agents or with other more complex methods (such as donor cell infusion, as shown in rodents).

In this short review, we analyse the effects of the blockade of some co-stimulation molecules *in vitro* and *in vivo* and discuss their potential interest for tolerance induction.

2. TARGETING THE B7–CD28 CO-STIMULATION SYSTEM

The co-stimulatory pathway that includes B7, CD28 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) plays a key role in regulating T-cell activation. The B7–CD28–CTLA-4 pathway consists of two B7 ligands, B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells (APCs), and their two receptors, CD28 and CTLA-4 on

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T cells. CD28 is expressed constitutively on T cells and promotes cell division and interleukin-2 (IL-2) production. CTLA-4 is upregulated after T-cell activation. CTLA-4 is able to transmit an intracellular signal that reduces activation through the T-cell receptor (TCR), and has a 20-fold higher binding affinity for B7 molecules than does CD28. Therefore CTLA-4 competes with CD28 for ligand binding and its upregulation on activated T cells is thought to stop the T-cell response. Receptors of the B7–CD28–CTLA-4 pathway are thus promising therapeutic targets but can also deliver opposite signals. Preventing their interactions clearly reduces T-cell responses but whether this system can be manipulated to induce tolerance in man is not yet known.

(a) *Inhibition of B7 versus CD28*

Blocking B7 molecules has been achieved with CTLA-4 immunoglobulin (CTLA-4-Ig) and with anti-B7 antibodies. CTLA-4-Ig is a soluble recombinant protein consisting, in the extracellular domain, of CTLA-4 fused with CH2 and CH3 domains of the Fc domain of IgG. Blocking B7 molecules prevents B7 interactions with both CD28 and CTLA-4, which may lead to opposite effects. This manoeuvre, which respects the interaction between the major histocompatibility complex (MHC) and the TCR in the absence of a co-signal, has been shown to induce non-responsiveness of T cells, *in vitro*. Since CTLA-4 transmits inhibitory signals, it has been suggested that allowing CTLA-4–B7 interactions could bring an advantage towards inhibition as compared with a B7 inhibition, which would also prevent CTLA-4 being engaged. Inhibiting solely signals transmitted by CD28 could therefore be a solution for inducing tolerance. In agreement with this concept, Perrin *et al.* (1999) showed that monovalent Fab fragments of anti-CD28 antibody blocked the clinical symptoms of experimental autoimmune encephalomyelitis in mice in a model where the disease is induced by myelin administration, or by transfer of T cells from a diseased animal.

In a heart transplantation model in the rat, however, Dengler *et al.* (1999) showed that administration for several days of a modulating anti-CD28 antibody did not prevent but only delayed allograft rejection, suggesting that exposing CD28-negative alloreactive T cells to their antigens does not result in the generation of regulatory cells that in turn depress alloreactive clones. The protocol followed in this specific experiment does not exclude the possibility, however, that T cells primed in the absence of CD28 signalling die or stay non-responsive, and that newly generated T cells from the thymus that arise at a time when CD28 inhibition is no longer present, restore the immune response. In addition, and in apparent contradiction to the postulate that blocking CD28 alone would inhibit T-cell activation, are the data obtained with CD28 knockout (KO) mice, which reject skin allografts promptly, and despite presenting a reduced immune response, it is sufficient to reject cardiac allografts (Kawai *et al.* 1996). Immunology of transplantation has provided us with many examples of the redundancy of T-cell activation mechanisms and this observation argues for the existence of alternative T-cell activation pathways. Newly described co-stimulatory pathways such as B7 related protein inductible T-cell co-stimulator (B7RP–ICOS) may compensate for CD28 deficiency, as well as factors such as IL-6 and tumour necrosis factor-α (TNF-α) that are able to promote $CD8^+$ T-cell growth in the presence of a vigorous antigenic stimulation (Sepulveda *et al.* 1999). In addition, T-cell mitogens whose receptors share the common cytokine receptor gamma chain such as IL-7, IL-9 and IL-15 can mediate T-cell proliferation in the absence of either IL-2 or IL-4 (Li *et al.* 1999).

In vitro, Van Gool *et al.* (1999) have demonstrated that anti-B7 antibodies in a primary mixed lymphocyte reaction (MLR) inhibit proliferation and cytokine secretion in an antigen-specific manner in secondary stimulation. This suggests that T-cell clones that reacted in the absence of a B7-mediated stimulation become unresponsive. The question of whether T-cell signalling in the absence of B7 resulted in T-cell anergy or apoptosis has been approached in different ways. Scheipers & Reiser (1998) have shown that cross-linking CTLA-4 on resting $CD4^+$ T cells with agonistic antibodies blocks the cell cycle without inducing apoptosis. Actually, cross-linking CTLA-4 on resting $CD4^+$ T cells blocks transition from G_0 to G1 and induces the anti-apoptotic factor Bcl-xL (Blair *et al.* 1998). In contrast, cross-linking CTLA-4 on activated $CD4^+$ T cells induces a Fas-independent cell death (Scheipers & Reiser 1998). *In vivo*, alloreactive T cells injected into irradiated murine recipients actively multiply and become increasingly susceptible to apoptosis with divisions (Li *et al.* 1999). CTLA-4-Ig administration reduces proliferation and enhances the susceptibility to apoptosis of dividing cells. This could be one explanation of how CTLA-4-Ig allows for allograft survival in several transplantation models, without clear evidence for induction of tolerance through the generation of immunoregulatory T cells.

(b) *Evidence for delivery of a negative signal through CTLA-4*

That signals mediated through CTLA-4 negatively regulate T-cell activation has been now molecularly and functionally demonstrated. In addition to its competition with CD28 for binding to B7, CTLA-4 inhibits TCR signalling by binding to TCR-ζ and inhibiting tyrosine phosphorylation after T-cell activation (Lee *et al.* 1998). It acts through the recruitment of phosphatases that dephosphorylate molecules in the CD3 complex (Bachmann *et al.* 1999*b*). Accordingly, CTLA-4 engagement leads to a decreased accumulation of nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1) in the nucleus (Fraser *et al.* 1999), which results in an inhibition of CD3–CD28-mediated IL-2 accumulation. Moreover, CTLA-4 stimulates an increase of IκB-α (inhibitor of nuclear factor κB) and a decrease of nuclear RelA (unidentified product of the RelA gene), which is normally translocated into the nucleus after stimulation through CD3 and CD28 (Pioli *et al.* 1999). This leads to a suppression of the production of multiple cytokines produced by both Th1 and Th2 cells, including IL-2, IL-3, IL-4, IL-10 and interferon-γ (IFN-γ) (Alegre *et al.* 1998). On the other hand, CTLA-4 inhibits the production of cyclins (D3, cdk3, cdk6) (Brunner *et al.* 1999). Thus CTLA-4 ligation inhibits early events in T-cell activation, and acts both on IL-2 production, and directly on proliferation. As such, it antagonizes the actions of CD28 on

the TCR signal and on cell division. Indeed, CD28 ligation amplifies TCR-induced ZAP-70 (zeta associated protein 70 kDa) activity and association of Vav with ZAP-70. This regulates the Rac-1-associated GTP–GDP exchange activity of Vav and downstream pathways leading to PAK-1 (P21 activated kinase 1) and p38 mitogen-activated protein kinase (MAPK) activation (Salojin *et al.* 1999). That p38 MAPK inhibitors block IL-2, IL-4 and IFN-γ induced by CD3–CD28 engagement confirms the involvement of this molecule. In addition, CD28 regulates T-cell cycle entry and progression through the G1 phase in an IL-2-independent manner, resulting in activation of cyclin D2-associated cdk4–cdk6 and cyclin E-associated cdk2. Mechanistically, it has been demonstrated that inhibition of T-cell responses trough CTLA-4 cross-linking occurs only when co-engaged with the TCR during T cell–APC interaction (Griffin *et al.* 2000).

(c) *Activity of CTLA-4 in naive and primed T cells*

Metz *et al.* (1998) have shown that naive CD4^{+} T cells do not contain much CTLA-4. They suggest that CTLA-4 prevents memory T-cell activation by regulating the low-intensity TCR engagement that is thought to be involved in maintenance of memory cells. Naive T cells with blocked CTLA-4, using anti-CTLA-4 antibodies or T cells from CTLA-4$^{-/-}$ mice, proliferate normally upon stimulation, but more vigorously than in wild-type T cells in a secondary stimulation (Chambers *et al.* 1998). This enhanced proliferation could be due to reduced cell death, increased expression of survival factors or to altered cell-cycle regulation. Data showing that cross-linking of CTLA-4 with agonistic antibodies on resting human T cells upregulates the anti-apoptotic gene *Bcl-xL*, also indicate that rescue from apoptosis may be a mechanism of the enhanced proliferation observed in secondary stimulation when CTLA-4 is engaged (Blair *et al.* 1998).

(d) *Modification of Th1–Th2 balance*

The result of CTLA-4 stimulation is not limited to a reduction of IL-2 production and proliferation. CTLA-4 may actually reduce TCR signalling to the extent that cytokine production is shifted and not inhibited. CTLA-4 engagement leads T cells to produce transforming growth factor-β (TGF-β) (Chen *et al.* 1998). Blocking CTLA-4 *in vitro* in a mixed lymphocyte reaction (MLR) with Fab fragments from anti-CTLA-4 antibodies, while promoting IL-2 and IFN-γ production in priming, polarizes responding cells to a Th2 phenotype in a secondary stimulation (Kato & Nariuchi 2000). In contrast, in the absence of Fab of anti-CTLA-4 in the primary MLR, T cells synthesize high amounts of IL-2 and IFN-γ and little IL-4 and IL-5, in the secondary response. This shows that the co-stimulation through CTLA-4 influences polarization of naive CD4^{+} T cells towards the Th1 subset and inhibits Th2 cell differentiation (Oosterwegel *et al.* 1999). This effect can, in part, be attributed to the enhancement of TGF-β production. Arguing against this concept, however, are the experiments of Alegre *et al.* (1998) showing that CTLA-4 stimulation suppresses both Th1 and Th2 cytokine production. It may be that stimulation of CTLA-4 indeed transmits a negative signal irrespective of the Th1–Th2 status of the cells, leading to a suppression of transcriptional activity and to a reduced cytokine production, but that simultaneous stimulation through CD28 counterbalances this inhibition to a level where a shift towards production of Th1 but not Th2 cytokines is produced. If simultaneous stimulation through CD28 is too weak and CTLA-4 dominates, the shift towards the synthesis of Th2 cytokines occurs.

In an intestinal allograft rejection model, blockade of the CD28–B7 pathway with CTLA-4-Ig, which preferentially blocks interaction of CD28 with B7 and therefore favours the remaining interaction of B7 with CTLA-4, inhibits rejection in wild-type and CD8 KO mice with reduced levels of IFN-γ and TNF-α, and increased levels of IL-4 and IL-5. Guillot *et al.* (2000) showed that adenovirus-mediated CTLA-4-Ig expression leads to the permanent acceptance of heart allografts, with increased levels of IL-13 and a decrease in IFN-γ expression, which illustrates a switch of the response to a Th2 phenotype. In CD4 KO mice, CD8^{+} cells reject allografts and are resistant to inhibition through CTLA-4-Ig. In that case the shift to Th2 differentiation is not observed. CD4^{+} and CD8^{+} cells thus present different co-stimulatory requirements that may be related to the upregulation of alternate co-stimulatory molecules, including for example B7RP–ICOS, expressed on certain lymphocytes such as activated and memory cells.

3. TARGETING THE CD40–CD40L PATHWAY

Initially identified as critical in the T-dependent humoral response, CD40–CD40L interactions are now thought to have a more general implication in immune regulation as demonstrated by the impairment of the antigen-specific priming in CD40L-deficient mice (Campbell *et al.* 1996; Kamanaka *et al.* 1996; Soong *et al.* 1996). CD40 is a type I transmembrane protein and its extracellular domain is highly homologous to the TNF receptor (TNFR) family. This molecule is expressed on APCs such as dendritic and B cells and on many other cell types (monocytes, endothelial and epithelial cells) involved in immune as well as in inflammatory responses (for a review see Van Kooten & Banchereau 2000). Its ligand, CD40L, a member of the TNF family, is expressed on the surface of activated T cells (CD4^{+} and some CD8^{+}), mastocytes, eosinophils and platelets.

(a) *Functional consequences of CD40–CD40L interaction*

As demonstrated in CD40L- and CD40-deficient mice, this co-stimulation pathway has important effects on both humoral and cellular responses. CD40 ligation is considered to be essential to potentiate presentation capacities of APCs (for a review see Mackey *et al.* 1998) and to drive them to a full maturation state (Banchereau & Steinman 1998). Signal transduction via CD40 ligation has been mostly studied in B cells. The intracytoplasmic domain of the CD40 molecule contains two binding sites for the TNFR-associated factors (TRAFs) (Pullen *et al.* 1998), the association with TRAF-2 being particularly important, since it triggers the CD40-induced nuclear factor κB translocation required for CD80 and intercellular adhesion

molecule 1 (ICAM-1) expression (Hsing & Bishop 1999). CD40 cross-linking also activates the Janus activation kinase-signal transducers and activators of transcription (STAT) pathway and other transcription factors such as AP-1 or NFAT (Francis *et al.* 1995) controlling important gene regulations. APC activation through CD40 triggers the upregulation of MHC class II and of several co-stimulatory molecules (CD80, CD86, lymphocyte function-associated molecule 3 (LFA-3), and CD134). Moreover, IL-12 production by dendritic cells and some B-cell subsets (Schultze *et al.* 1999) is very sensitive to CD40 ligation and results in a Th1 skewing of T cells.

The CD40 pathway also plays a pivotal role in CTL priming by helper T cells. Indeed, CD40 ligation on professional APCs allows for a subsequent CTL priming, since an activating anti-CD40 antibody can substitute for $CD4^+$ T-cell help in the generation of CTLs *in vitro* (Bennett *et al.* 1998; Ridge *et al.* 1998; Schoenberger *et al.* 1998). So, despite the fact that some stimuli, such as TNF-α or lipopolysaccharide, may contribute to generate CD4-independent CTL priming, dendritic cells relieve the signals from helper T cells to $CD8^+$ T lymphocytes through CD40L–CD40 interactions. This argues in favour of a model of sequential interactions between $CD4^+$ T cells, APCs and $CD8^+$ T cells.

The CD40–C40L system also regulates several B-cell functions, according to their maturation state. CD40 activation enhances B-cell proliferation, induces telomerase activity in memory cells (Hu *et al.* 1997) and controls isotype switching in concert with the cytokine environment. Genetic alterations of the CD40L gene (Notarangelo & Peitsch 1996) are associated with the hyper-IgM syndrome, characterized by the absence of the other Ig isotypes. Finally, CD40 activation on endothelial or epithelial cells induces the production of chemokines and cytokines in response to activation by IL-1 or IFN-γ *in vitro*, and therefore is likely to be involved in some inflammatory processes and in graft rejection.

Whether and how CD40L cross-linking affects T-cell functions is less well documented, but it has been demonstrated to contribute to the development of helper function (Van Essen *et al.* 1995), to enhance cytokine production and to give rise to terminal T-cell differentiation *in vitro* (Poudrier *et al.* 1998).

(b) *Agonistic CD40 ligation can boost immune responses*

In some situations, such as anti-tumour immunity, viral infections or immunodeficiency syndromes, one would need to boost the immune response. The use of agonistic monoclonal antibodies (mAbs) has provided some substantial information about the regulations involved in the establishment of a tolerant state, and about the role of the CD40–CD40L pathway in these mechanisms.

In a murine model of *Leishmania major* infection, Ferlin *et al.* (1998) demonstrated a protective effect associated with the induction of Th1 cytokines. The treatment, combining an anti-CD40 and a vaccinating peptide, restores antigen-specific responses, spares the expending capacity of clonotypic $CD8^+$ cells and reduces tumour development and metastasis (Diehl *et al.* 1999; Sotomayor *et al.* 1999). The antibody used as an adjuvant of the vaccination illustrates the importance of the CD40–CD40L pathway in CTL priming. Furthermore, co-injection of a DNA coding for a trimeric form of CD40L and the targeted antigen has been shown to enhance a specific protective response (Gurunathan *et al.* 1998). Thus, unwanted tolerance states can be broken through the provision of an activating signal to $CD40^+$ cells, which in turn, promote a specific response able to trigger the regression of established tumours.

(c) *Tolerance induction by disrupting the CD40–CD40L pathway*

The possibility that co-stimulation blockade may induce antigen-specific tolerance is currently being extensively explored, especially in the field of transplantation. *In vitro*, CD40–CD40L blockade does not inhibit a primary MLR response. MLRs using stimulator cells from CD40-deficient mice are unaffected, except when B cells are used as APCs (Ozaki *et al.* 1999). Similarly, a blocking anti-CD40L antibody only slightly reduced proliferation and cytotoxicity in the human MLR. In this model, additional blockade of the B7–CD28 pathway through anti-B7 antibodies was required to achieve anergy of the responder population (Van Gool *et al.* 1999).

Recent investigations have dealt with the respective role of B7–CD28 and CD40–CD40L pathways in T-cell responses, *in vivo*, and suggest that the latter could be more involved in the amplification than the initiation of the immune response (Howland *et al.* 2000). The difficulty in affecting T-cell responses only by disrupting CD40–CD40L interactions was confirmed by several *in vivo* studies. In mice, donor-specific tolerance to skin allografts can be achieved only when donor-specific transfusion or depleting anti-CD8 mAb is added to the anti-CD40L antibody treatment (Durham *et al.* 2000; Honey *et al.* 1999). In these models, anti-CD40L antibodies alone are likely to be sufficient to tolerize $CD4^+$ but not $CD8^+$ lymphocytes. This is confirmed by the study of Iwakoshi *at al.* (2000) describing the peripheral deletion of allospecific $CD8^+$ T cells by a treatment combining donor-specific blood transfusion (DST) and blocking anti-CD40L, whereas neither DST nor anti-CD40L alone did so. Similarly, anti-CD40L antibodies induce tolerance to fully mismatched cardiac allografts in STAT-$4^{-/-}$ mice, unable to mount Th1 responses (Kishimoto *et al.* 2000).

In addition, if one considers the absence of chronic rejection as the ultimate definition of a tolerant state, the combination DST–anti-CD40L has been demonstrated to prevent arteriosclerosis development in a mouse cardiac allograft model. In this model, in contrast to anti-CD4 mAbs, anti-CD40L induced a Th2 skewing that correlated with the expression of anti-apoptotic genes (*HO-1*, *Bcl-xL* and *A20*) within the graft (Hancock *et al.* 1998). However, although a humanized anti-CD40L monotherapy may not induce tolerance to donor antigens, in the strict sense, such a treatment significantly increased allograft survival in non-human primates. In rhesus monkeys, prolonged treatment with a humanized anti-CD40L allowed islet and kidney allografts to survive long-term, and was associated with donor-specific hyporesponsiveness (Kenyon *et al.* 1999; Kirk *et al.* 1999). These models are described in more detail in Kirk *et al.* (this issue).

4. TARGETING LFA-1 AND CD2 MOLECULES

(a) *Inhibition of LFA-1–ICAM-1 interaction*

Some molecules with co-stimulatory effects were first identified as belonging to the adhesion molecule family (here also called adhesion receptor family (for a review see Frenette & Wagner 1996)). Integrins are heterodimeric membrane glycoproteins that exist in inactive interaction states (Lollo *et al.* 1993). Activation of T cells by various stimuli results in transformation of inactive molecules (i.e. no binding to counter-receptors) to molecules with high affinity for their ligand. Several stimuli activate LFA-1 conformation including CD2, CD3 and CD28 (Mobley *et al.* 1994; Van Kooyk *et al.* 1989) or chemokine (Detmers *et al.* 1990). LFA-1 reacts with domain 3 of ICAM-1 (Diamond *et al.* 1991). However, when ICAM-1 is inserted into a lipid monolayer under solution flow, neither resting nor activated leucocytes attach (selectins are responsible for this attachment) (Williams 1991). Then, an 'outside in' co-signalling occurs that can affect T cells (activation, proliferation or apoptosis (Matsumoto *et al.* 1994)).

Leucocytes from mice lacking CD11a (a ligand for LFA-1) display a defect in homotypic aggregation and proliferation following allo- or mitogen stimulation (Schmits *et al.* 1996). Although these animals can produce functional CTLs, they do not reject immunogenic tumours. Disruption of this gene affects also natural killer (NK) cell function. In addition, as has been noted for sometime (Chan *et al.* 1998), various T- and B-cell functions, such as mitogen- or antigen- (including allogeneic cells) induced proliferation, T-cell-mediated cytolysis, B-cell aggregation and cytokine production are affected to various extents by anti-LFA-1. According to the epitope targeted by the anti-LFA-1 used, *in vitro* results are different. For instance, only anti-CD18 blocks the proliferation of anti-influenza T-cell clones, whereas anti-CD11a does not. The effect of these antibodies can also vary according to the type of APC used (Schmits *et al.* 1996).

Blockade of LFA-1 could influence differentially Th1 and Th2 cytokines, with a 'paradoxical' increase in IL-4 and IL-15 blocked by anti-CD28 in the same experiment (Salomon & Bluestone 1998).

Studies with LFA-1-deficient monocytes indicate that LFA-1 also acts in concert with complement receptor 3 to mediate trans-endothelial migration of these cells (Andrew *et al.* 1998). Both ICAM-1 and -2 are involved in this migration (Shang & Issekutz 1998). Migrations through epithelia are also involved.

Wulfing & Davis (1998) have demonstrated a novel mechanism of co-stimulation involving both CD28 and LFA-1 but also CD2 (Shaw & Dustin 1997), in the formation of an immune 'synapse' (Dustin & Shaw 1999), which might be specially relevant for molecules that function as both adhesion and co-signal molecules on T cells. These 'synapses' bring closely together receptors and ligands and generate an 'occlusive barrier' that excludes large molecules from the contact area, allowing low-affinity TCRs to interact better with the MHC–peptide complex. This new paradigm for immunological co-stimulation, which has been constructed around the central role of membrane contact formation in T-cell activation, exemplifies the intricate relationship between adhesion and co-stimulation.

Anti-LFA-1 has been shown to interfere with the rejection process. In mice, in a protocol of treatment of ongoing heart rejection (C57 BL/6 to BALB/c), anti-LFA-1 prolonged survival from 8 days to 17 days (Chan *et al.* 1998), and combination with an anti-α4 integrin further prolonged graft function to 28 days with a reduction of cellular infiltrate. Although a state of operational tolerance has been obtained using a combination of anti-LFA-1 and anti-ICAM-1 in the mouse (Isobe *et al.* 1992), this has not been reproduced in other species (Brandt *et al.* 1997). Tolerance to protein has been reported in mice (Benjamin *et al.* 1988). Antibodies to the LFA-1 epitope specific for the binding of ICAM-1 prolonged (without inducing tolerance) survival of xenotransplanted islets in B6 diabetic mice with a strongly decreased infiltrate (Zeng *et al.* 1994). A perioperative short course of anti-LFA-1 (but not of anti-CD2) induced unresponsiveness (but not tolerance) against allogeneic islets in mice (Gotoh *et al.* 1994).

In large animals, anti-LFA-1 can only moderately prolong allograft survival time. Anti-LFA-1, anti-vascular cell adhesion molecule-1 or a combination of them did not prolong kidney graft survival in an ovine model (Grooby *et al.* 1998). In a rabbit model, injection of anti-CD18 significantly reduced infiltration by polymorphonuclear (PMN) cells and T lymphocytes but not by macrophages (Keizer *et al.* 1985). In a primate model, an anti-ICAM-1 was shown to significantly delay kidney allograft rejection and reverse ongoing rejection (Cosimi *et al.* 1990). In man, an anti-CD11a (25.3) has been shown to reduce the overall grading of steroid-resistant grade III–IV acute graft-versus-host diseases with a partial response in 80% of patients (Stoppa *et al.* 1991), and to prevent rejection of a mismatched bone marrow graft in patients with immune deficiency (Fischer *et al.* 1986, 1991). In addition, it decreased the incidence of early acute rejection occurring in patients with delayed graft function in a randomized study in kidney recipients (Hourmant *et al.* 1996; Le Mauff *et al.* 1996). However, no definitive conclusion could be reached owing to the small cohort size and the study design. The same anti-LFA-1 was not efficient in reversing an ongoing acute kidney rejection in man.

(b) *Targeting CD2*

CD2 was one of the very first identified co-signal molecules able to enhance the TCR recognition signal upon binding to its LFA-3 (CD58) ligands (Springer *et al.* 1987), whereas antibodies specific for CD2 inhibit the T-cell response to antigen. CD2 and its ligands (CD58–CD48 and possibly CD49) belong to the CD2 family of glycoproteins, including also SLAM (signalling lymphocytic activation molecule), CD84 and lymphocyte antigen 9 (LY9 2B4) 2B4 can be suppressed; all molecules with homotypic adhesion properties (Van der Merwe 1999).

Comparative studies suggested that human T cells might be much more dependent upon CD2 activation pathways than murine ones. Recent solution of the crystal structure of the CD2–CD58 complex has led to the hypothesis that CD2 might position at an optimal distance the APC and T-cell membrane to allow full

TCR–MHC interaction (close contact zone), contributing to the 'immune synapses' (see § 4(b)). In this model, the CD2–ligand interaction occurs later than the LFA-1–ICAM one, the complex of which spans a much longer distance in the membrane than CD2–CD58 or TCR–MHC. The position of CD2–CD59 in the synapse suggests that it may act as a barrier for interfering moieties through cytoskeleton interaction.

However, CD2 also displays part of it effect through specific signals conveyed by its cytoplamic tail, which can also be mobilized by specific combination of mAb (Hunig *et al.* 1987). CD2 signalling acts through regulation of different tyrosine kinases in the TCR–CD3 complex or independently of them.

CD2 KO mice have been found to have an almost normal immune response. However, more refined studies have shown lower responses against lymphocytic chorio meningitis virus (LCMV)-derived peptide (Bachmann *et al.* 1999*a*). As for mice lacking ICAM-1, the effect is more pronounced when low-affinity binding peptides are used. The polyclonal T-cell hyporesponsiveness that persists long after membrane CD2 recovery follows incubation with antibodies suggests that some anti-CD2 mAbs also deliver an inhibitory signal (Guckel *et al.* 1991). It is interesting to note that the blockade of CD2 and LFA-1 co-stimulation pathways could result in a T-cell anergy state mimicking that described following blockade of CD28 or B7 activation (Bell & Imboden 1995). Furthermore, LFA-3-Ig fusion molecules also inhibit TCR activation in conditions where the activation is not CD2 dependent in terms of a co-signal. Using human leucocyte antigen DR7 (anti-DR7) CTLs anergized by inhibition of the B7 co-signal, Boussiotis *et al.* (1994) showed that an anti-CD58 can reverse the anergy, suggesting that CD2 was also involved in this process. The cross-talk between different co-signal molecules is further complicated by the fact that transcription of a subtle CD2 epitope (CDR2) is controlled by IL-2. Therefore, recognition of antigen by TCR and CD2 ligation can combine to deliver a state of activation or inhibition.

Anti-CD2 antibodies with the property of inhibition *in vivo* (modulating or not, depleting or not) delay allograft rejection to various extents in rodents and induce a tolerant state when combined with a blockade of signal I (anti-CD3) (Chavin *et al.* 1993) or of a second anti-co-signal (CTLA-4-Ig). Combinations of an anti-CD2 and CD48 can also result in indefinite graft survival (Qin *et al.* 1994). Interestingly, anti-CD2 synergized with immunosuppressive molecule (Tacrolimus®) (FK506) but not with rapamycin to induce tolerance in a heart allograft model in mice (Chavin *et al.* 1994).

Most of our knowledge on anti-CD2 effects in man is derived from the use of LO-CD2a, a mAb strongly inhibiting MLR at low concentrations (less than $20\,\mathrm{ng\,ml^{-1}}$) without an agonist effect on cytokine production. Furthermore, T cells incubated *in vitro* in the presence of LO-CD2a are refractory to a second stimulation (Latinne *et al.* 1996). *In vivo*, in a rejection prophylaxis protocol (Nizet *et al.* 2000), administration of this antibody with a triple regimen (steroid, azathioprine and cyclosporin A) resulted in a 25% acute rejection incidence at two years, versus 60% in controls. Its administration was associated with a strong decrease in peripheral T-cell count and low circulating NK cells. Multifactorial events including apoptosis, surface modulation, and antibody-dependent cellular cytotoxicity are likely to contribute to this effect (Cosimi *et al.* 1981). Interestingly, blocking anti-CD2 antibody, which does induce lymphopenia *in vivo*, has not been shown to be efficient in the primate (Kaplon *et al.* 1996), whereas LFA-3-Ig fusion protein displayed significant immunosuppressive action. A humanized LO-CD2 mAb has been obtained but no clinical data have been produced yet.

5. CONCLUSION

Our review has not considered all the numerous molecules that are described as having some co-stimulation effects on T cells or APCs. We have tried to deal with some recent progress in the field of tolerance induction through co-stimulation blockade alone or in combination with other perioperative treatment.

Recent evidence suggests that the anti-CD40L co-stimulation pathway may be a preferential target *in vivo*. Indeed, some preliminary evidence has shown continuing kidney allograft survival several months after anti-CD40L treatment withdrawal in the primate (see Kirk, this issue). Interestingly, the best results were obtained without a pharmaceutical agent blocking calcineurin (and possibly antagonizing apoptosis of activated T cells). However, two decades of active research in the field of tolerance induction using bioreagents targeting co-stimulatory molecules illustrate how flexible the immune response is, with many redundant pathways. This suggests that more effort is necessary, especially by combining targets. Recent progress in this direction, such as by association of B7 pathway blockade, substitution of calcineurin inhibitors by rapamycin, or by adding donor cell infusion to anti-CD40L treatment, is encouraging. As for modern immunosuppression, which now allows more than 90% of allografts to survive for more than one year, only a combination of treatments with synergistic effects may allow tolerance induction to be achieved. Finally, owing to the success of current immunosuppressive strategies in preventing acute rejection, the prevention of chronic rejection will be the most important target in the field of tolerance induction.

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