

The role of CD154 in organ transplant rejection and acceptance

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CDl54 plays a critical role in determining the outcome of a transplanted organ. This simple statement is amply supported by experimental evidence demonstrating that anti-CDl54 antibodies are potent inhibitors of allograft rejection in many rigorous transplant models. Unfortunately, despite intensive investigation over the past ten years, the precise mechanisms by which antibodies against CDl54 exert their anti-rejection effects have remained less obvious. Though originally classified with reference to B-cell function, CDl54–CD40 interactions have also been shown to be important in T cell–antigen-presenting cell interactions. Accordingly, CDl54 has been classified as a T-cell co-stimulatory molecule. However, mounting data suggest that treatment with anti-CDl54 antibodies does not simply block co-stimulatory signals, but rather that the antibodies appear to induce signalling in receptor-bearing T cells. Other data suggest that anti-CDl54 effects may be mediated by endothelial cells and possibly even platelets. In fact, the current literature suggests that CDl54 can either stimulate or attenuate an immune response, depending upon the model system under study. CDl54 has secured a fundamental place in transplant biology and general immunology that will no doubt be the source of considerable investigation and therapeutic manipulation in the coming decade.

Keywords: CDl54; Tcell; B cell; immunosuppression; tolerance

1. INTRODUCTION

Antibodies directed against CDI54 have been shown to prevent acute allograft rejection in many rodent and primate models. The apparently generalizable antirejection effect of anti-CD154, covering primarily and secondarily vascularized organs as well as cellular grafts, combined with its minimal toxicity in preclinical trials, has stimulated considerable interest in this molecule and the biology of its associated pathways. Since its discovery, CD154 (originally described as TBAM, gp39 and subsequently CD40 ligand) has been shown to have an impact on a variety of immune effector functions. Importantly, it has defied classification as an exclusively stimulatory or inhibitory molecule, but rather would appear to be best viewed as a regulatory element. This paper will outline the physiological biology of CDI54 as well as the results of preclinical trials aimed at manipulating CDl54 to prevent allograft and xenograft rejection.

2. THE PHYSIOLOGICAL FUNCTION OF CD154

(a) Physiological CD154 expression

CDl54 is a 32–39 kDa cell surface glycoprotein that is most abundant on activated CD4⁺ T cells. It has also been identified on CD8⁺ T cells, B cells, eosinophils, mast cells, basophils and dendritic cells (Lauzon *et al.* 1988; Armitage *et al.* 1992; Gauchat *et al.* 1993*a,b*, 1995). More

recently, CD154 has been identified on other cell types, including epithelial cells, fibroblasts and endothelial cells (Mach et al. 1997; Hollenbaugh et al. 1995). Additionally, pre-formed CD154 has been demonstrated to be present within the intracellular stores of platelets and to be expressed on the platelet surface upon activation (Henn et al. 1998). The broad distribution of CD154 suggests that it plays an important role in the coordination of immune events. Aside from CD154's well-studied effects on T-cell function, however, the functional role the receptor plays on these other cell types is neither well studied nor understood.

CDl54 expression is tightly regulated on T cells via controlled production and degradation (receptor-mediated endocytosis, proteolytic cleavage and lysosomal degradation) (Roy et al. 1993; Castle et al. 1993; Lederman et al. 1992; Yellin et al. 1994). Exposure of a T cell to its cognate antigen alone can increase the T cell's CDl54 expression, but the concomitant effects of CD28 signalling significantly enhance CD154 expression (Johnson-Leger et al. 1998). Upon ligation, CDl54 is internalized by receptormediated endocytosis thus serving to control its expression. Although CDI54 is normally expressed on the cell surface as heteromultimeric complex (usually as a trimer, Hsu et al. 1997), shorter soluble forms of CD154 are released. Furthermore, the soluble CD154 forms are biologically active and can bind CD40, suggesting that CD154 can also act as a bona fide cytokine (Graf et al. 1995). Depending upon the local conditions, this soluble CD154 could serve to amplify or abrogate specific immune

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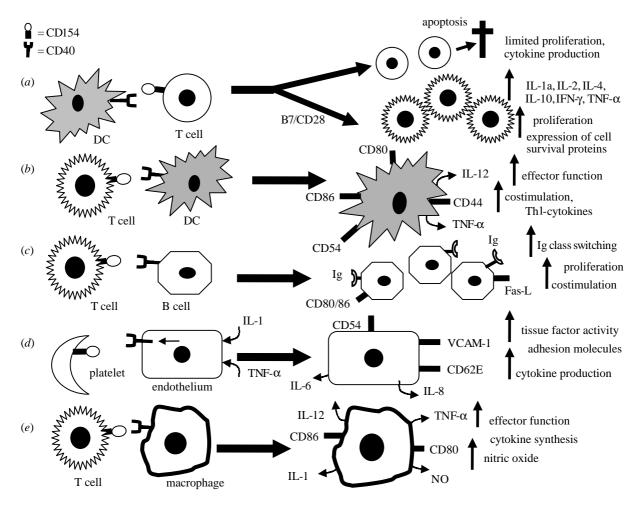


Figure 1. Biological effects of CD40–CD154 interaction. (a) Co-ligation of the TCR and CD154 on naive T cells, in the absence of significant CD28–B7 interactions, results in modest proliferation and cytokine production but ultimately apoptosis in some T-cell clones. Co-stimulation through both the CD28–B7 and CD40–CD154 pathways leads to optimal proliferation, expression of cell activation molecules, cell survival genes and cytokine expression. (b) Stimulation through CD40 in dendritic cells results in an enhancement of their effector functions characterized by upregulation of the co-stimulatory receptors CD80 and CD86 in addition to cell adhesion molecules and Th1-type cytokines. (e) Similarly, interactions between CD154 and CD40 on activated B cells leads to clonal expansion and Ig-class switching and the increased expression of CD80, CD86 and Fas-L. (d) IL-1 and TNF-α drive CD40 expression in endothelial cells. Interaction of CD154, possibly on platelets, and CD40 expressed on endothelial cells mediates the upregulation of adhesion molecules IL-6 and IL-8. (e) CD154 on activated T cells augments effector functions in CD40-expressing macrophages, resulting in release of proinflammatory cytokines and nitric oxide and the induction of the co-stimulatory receptors CD80 and CD86.

responses beyond areas where surface expression is detected.

CD154's physiological ligand is CD40, a 50 kDa homotrimeric complex of type II integral membrane glycoproteins expressed mainly on professional antigenpresenting cells (APCs) (e.g. dendritic cells, macrophages) and endothelial cells (reviewed in Van Kooten & Banchereau 1997, 2000). CD40 has also been demonstrated on B cells. Both receptors have the general motif of the tumour necrosis factor- α (TNF- α) and TNF receptor family. The general distribution of CD154 and CD40 suggests that the role of this receptor–counter receptor pair is to coordinate immune activation in response to antigen exposure.

(b) CD154 signal transduction

When CD154 binds its natural ligand, a cytosolic signal is generated (Van Kooten & Banchereau 1997; Brenner et al. 1997; Koppenhoefer et al. 1997; Blair et al.

2000). The precise mechanism by which the signal is generated is unclear. As the intracytoplasmic portion of CD154 is relatively small (22 amino acids), an association with a currently undefined signal transduction complex is likely. Though small, CD154's cytoplasmic domain is highly conserved (82% identical in human and mouse) (Farrah & Smith 1992), further suggesting functional relevance. Ligation of CDI54 on Jurkat T cells triggers activation of the JNK-p38 kinase pathways, an action that appears to be mediated by the src-like tyrosine kinases $p56^{lck}$ and Rac-1. CDl54 activation has also been shown to result in neutral sphingomyelinase induction (Brenner et al. 1997; Koppenhoefer et al. 1997). Additionally, CD154 signalling has been associated with a phosphatidylinositol second messenger system and with activation of both serine-threonine protein kinases and mitogen-activated protein kinases. Transcription factors such as nuclear factor-κB (NF-κB) and c-Jun are induced following ligation of CD154 (Van Kooten & Banchereau 1997). CD154-mediated signalling also appears to be calcium dependent and sensitive to perturbations in calcineurin-dependent signal transduction pathways (Blair et al. 2000; Klaus et al. 1994; Fuleihan et al. 1994). Consequently, immunosuppressive drugs such as cyclosporine and tacrolimus abrogate many CD154 effects and may also limit its surface expression. These calcineurin phosphatase inhibitors probably inhibit transcription of the CDl54 gene in a manner similar to that demonstrated for interleukin (IL) 2 (Jain et al. 1995). As will be discussed below, this feature has significant implications for the therapeutic application of anti-CD154 in transplantation.

(c) CD154 and B-cell function

CD154 was originally described as an important mediator of humoral immune functions, in particular, antibody class switching (Aversa et al. 1994). Mutations in the CD154 gene have been found to result in the X-linked hyper-IgM syndrome, a malady characterized by an inability to class switch antibody isotypes (Korthauer et al. 1993). Afflicted individuals present with normal to high levels of IgM and an absence of IgG, IgA, IgE or memory B cells. As a consequence, these individuals are immunocompromised and have an increased susceptibility to opportunistic infections with encapsulated bacteria, as well as intracellular pathogens such as Pneumocystis carinii and Cryptosporidium (Levy et al. 1997). The defect is predominantly one of T-cell help, as B cells from these patients can function normally when cocultured with wild-type T cells expressing CDl54 (Van Kooten & Banchereau 1997).

There is a clear physiological role for CDI54 in mediating the differentiation and apoptosis of B cells, as well as in facilitating T-dependent B-cell help (figure 1) (Grammer et al. 1995, 1999). CDI54+ T cells interact with B-cell CD40 and induce B-cell proliferation and plasma cell differentiation, particularly in the lymph node germinal centres. These events are associated with the production of IL-12 (Cella et al. 1996) and T helper (Th) 2 cytokines such as IL-4, IL-10 and IL-13. Following this activation and differentiation, B cells produce IgM antibody and subsequently undergo immunoglobulin class switching and affinity maturation (Aversa et al. 1994; Van Kooten & Banchereau 1997). Interaction of the T cell's CD154 with the B cell's CD40 has also been shown to upregulate Fas expression on the B cell (Van Kooten & Banchereau 1997). This provides a potential mechanism for response termination through Fas-mediated apoptosis. In contrast, others have found that signals mediated by CD40 act to prevent apoptosis in WEHI-B cells, presumably by IL-13-mediated upregulation of the cell survival protein Bcl-xL (Lomo et al. 1997; Zhang et al. 1996). CD40-CD154 interactions may therefore potentiate APC survival during the generation of an immune response, while limiting the survival of effector B cells. The precise events governing whether a response is augmented, sustained through a memory response or terminated remain unclear, though interactions within the germinal centre with activated dendritic cells probably substantially influence the ultimate outcome. The involvement of CDl54 in both immune response augmentation and termination is also apparent in T-cell functions as detailed below. This presents an attractive physiological theme,

namely, that immune responsiveness mediated through CDl54 is transient and effectors activated through this pathway activate with the intention of dying. Such a hypothesis fits well within the physiological role of immune function.

(d) CD154 and T-cell function

Initiation of an immune response in antigeninexperienced T cells requires direct physical contact with professional APCs. The two-signal model of T-cell activation first proposed by Bretscher & Cohn (1970) and later modified by Lafferty (Lafferty et al. 1978) states that in addition to antigen recognition, T cells require costimulatory signals. The co-stimulatory signal(s) are provided by interactions between co-receptors on the effector cells and their ligands (often counter-receptors) expressed by APCs, as well as by soluble factors such as cytokines. Co-receptors increase the avidity of APCs and T-cell binding to enhance signals generated through the T-cell antigen receptor (TCR). They also provide synergistic signals that modulate T-cell expansion and effector function. Often, the functional outcome depends on a balance between opposing effects, as is the case with events mediated through the CD28 and CTLA-4 (CD152) co-receptors. That is, both CD28 and CD152 bind to the same B7 family of ligands (CD80 and CD86) and yet B7-CD28 interactions activate T cells, and B7-CD152 interactions downregulate immune responses. CDI54 seems to be clearly associated with the enhanced expression of many co-stimulatory events.

The role of CD154 in T-cell responses was first documented in CD154-deficient mice. These reports demonstrated that antigen-specific T-cell responses were impaired and that CD154 expression on T cells was required for the in vivo priming of CD4+ cells (Lederman et al. 1992; Grewal et al. 1995). It was subsequently shown that CD40 ligation on APCs greatly augments their ability to interact with effector and regulator T cells. CD40 ligation upregulates major histocompatibility complex (MHC) class II and thus facilitates antigen exposure to the TCR (figure 1). APC to T-cell adhesion is further facilitated by the increased expression of CD54. Furthermore, the T-cell co-stimulatory molecules CD80 and CD86 are induced (Caux et al. 1994a,b; Kennedy et al. 1994). Additional studies have demonstrated that an increase in IL-12 production, in concert with upregulated APC CD80-86 expression, is critical in facilitating cell-mediated immunity by enabling the APC to drive CD8⁺ T-cell differentiation into cytotoxic T lymphocytes (CTLs) (Schoenberger et al. 1998; Ridge et al. 1998; Bennett et al. 1998). We and others have demonstrated that CDI54 can also assert profound effects upon the T cell itself, functioning as a direct co-stimulatory molecule (Blair et al. 2000; Peng et al. 1996; Cayabyab et al. 1994).

Our in vitro work demonstrates that co-ligation of the TCR and CD154 leads to T-cell proliferation that is cyclosporine sensitive and CD28 independent (Blair et al. 2000). Purified CD4⁺ T cells stimulated with solid phase anti-CD3-CD154 resulted in enhanced production of immunomodulatory cytokines such as TNF- α and IL-10. In this respect, CD3-CD154-stimulated T cells behaved like CD3-CD28-stimulated T cells. Strikingly, however,

and unlike CD28 mediated co-stimulated T cells, CD3–CD154 mediated T-cell activation did not induce IL-2 production or secretion. Furthermore, after several days, anti-CD3–CD154-mediated activation was followed by apoptosis in a significant population of cells, possibly due to the absence of IL-2 induction. We also observed, however, that anti-CD3–CD154 co-ligation did not induce anti-apoptotic Bcl-2 and Bcl-xL expression, and thus may favour activation-induced cell death through apoptosis. Together, these results suggest that CD154 may impart early and direct effects on CD4+ T cells driving short-term T-cell effector responses that do not independently lead to the development of immunological memory.

As a whole, these studies provide evidence that CD40-CD154 interactions influence the amplification of an immune response through the stepwise upregulation of cytokines and both adhesion and co-stimulatory molecules. CDl54's ability to regulate a response may depend on the availability of additional co-receptors and/or their cognate ligands, as well as the activation status of the T cell. The polarization of these receptors and their cognate ligands within an 'activation cap' could provide the means by which CDI54 is associated with downstream signal transduction pathways (Janes et al. 2000). These signals would therefore mediate cellular decisions to differentiate into effector or memory cells. Teleologically, this makes sense: in the absence of additional co-stimulation, regulation of short-term responses through CD154 ligation could control damage from a prolonged immune response.

(e) CD154 in APC activation and mobilization

Activation of peripherally based APCs results in their migration into secondary lymphoid organs. These cells are potent immunostimulatory cells that carry relevant antigens from the periphery to the draining lymph nodes where their interaction with T cells is facilitated. T cells are primed in the T-cell zone of secondary lymphoid organs, following contact of the antigen-loaded MHC molecules on the APC with the TCR-CD3 complex on T cells (Guery et al. 1996). Thus, the development of an immune response to antigen is facilitated first by the homing of antigen-expressing cells to a specialized site of antigen recognition, and second by the subsequent presentation of this antigen to T cells leading to their activation. Strategies that impair either of these events could serve to block many immune events, including allograft rejection.

To date, there has been no direct evidence that CD40 signals induce APC migration into the secondary lymphoid tissue, though it is likely that a link exists. However, several studies have shown that CD154 is a potent activator of APCs and that it imparts critical signals necessary for the development of their effector and regulatory functions (Schoenberger et al. 1998; Ridge et al. 1998; Grammer et al. 1995, 1999; Van Essen et al. 1995) (figure 1). Ligation of CD40 is a critical step in the maturation of fully competent APCs. Following CD40 ligation facilitated by interaction with an activated CD4⁺ T cell's CD154, APCs adopt a phenotype in which they are particularly effective at developing a naive CD8⁺ T cell into an effector CTL (Schoenberger et al. 1998; Ridge et al. 1998). This allows for CD4+ T cells to interact with and activate an APC without the requirement that

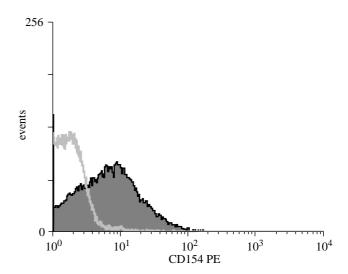


Figure 2. Thrombin activated platelets express CD154. Flow cytometry data using FITC-labelled anti-CD154 are shown for resting human platelets (non-shaded histogram) and platelets incubated with thrombin (solid histogram).

antigen-specific CD8+ T cells be immediately present. As such, an activated APC can await the arrival of antigenspecific CD8⁺ T cells in the secondary lymphoid tissues. Signals through CD40 stimulate monocytes to secrete various cytokines, including TNF-α, IL-1, IL-6, IL-8, IL-12 and IL-15, as well as chemokines, matrix metalloproteinase and nitric oxide that together work to foster T-cell activation and maturation (Mackey et al. 1998). These signals act bidirectionally on both the T cells and the APCs to orchestrate responses to antigen. Furthermore, they determine whether a response will progress towards immunity or towards tolerance. Nonprofessional APCs can also be stimulated to become efficient presenters of activating signals and antigen. Small resting B cells are dependent on CD154-expressing Tcells in order to progress through the cell cycle, upregulate cytokine and co-stimulatory receptors and differentiate into competent APCs (Buhlmann et al. 1995; Evans et al. 2000).

(f) CD154 as a potential bridge between vascular injury and immune activation

One of the most intriguing potential physiological roles for CDl54 has been suggested by the striking upregulation of the molecule on activated platelets (Henn et al. 1998; Kirk 1999a). Thrombin-activated platelets rapidly express CDl54 that can interact with CD40 on endothelial cells mediating chemotaxis and upregulation of adhesion molecules, including E-selectin, ICAM and VCAM (figure 2). This interaction has attractive teleological implications in that it pulls together an adaptive response to trauma with an initial immune activation response. In doing so, events likely to be associated with antigen exposure (trauma, vascular disruption) induce both haemostatic pathways and immune activation pathways involved in chemotaxis and antigen uptake with a single blood-borne element.

The platelet is uniquely suited to play a critical role in both haemostasis and immune activation in that its activation is tightly controlled and limited to areas of endothelial damage. Upon activation, platelets express integrin receptors and become adherent to subendothelial proteins such as fibronectin, collagen and thrombin (Barnes et al. 1998; Gawaz et al. 1998; Massberg et al. 1998; Gear et al. 1997; Bombeli et al. 1998). Activated platelets degranulate locally, inducing formation of a haemostatic plug and stimulating leucocyte chemotaxis. The chemotactic effects have, in the past, been attributed to vasoactive amines, but now appear to be the result, at least in part, of CD154-mediated binding to CD40-expressing endothelial cells in that anti-CD154-stimulated endothelial cells express both adhesion molecules and chemotactic signals like IL-8.

Just as thrombosis must be tightly regulated to avoid systemic coagulation, immune activation must also be controlled so as to avoid a systemic septic syndrome and/or autoimmunity. It is tempting to speculate that, following vascular trauma, CD154 expressing platelets not only interact with endothelial cells, but also induce tissuebased APCs to engulf antigen and migrate to regional lymphoid tissue, thereby activating an adaptive immune response. Evolutionarily, tissue that is traumatized is more likely to have been infiltrated with offending antigens and therefore is fertile ground for antigen uptake, maximizing the likelihood that foreign antigen as opposed to autoantigen will be presented.

3. CD154 IN THE CONTEXT OF TRANSPLANTATION

(a) Data demonstrating the anti-rejection effects of CD154 manipulation

Anti-CD154 antibodies have been shown to prevent allograft rejection in many diverse transplant models. Parker and colleagues first demonstrated in 1995 that anti-CD154 could prevent allograft rejection (Parker et al. 1995). Using a streptozotocin model for experimentally induced diabetes, this group found that co-administration of inactivated donor lymphocytes (chosen as a rich source of donor MHC antigen with low co-stimulatory potential) plus anti-CD154 beginning one week before and for seven weeks following an allogeneic islet transplant permanently prevented graft rejection. They proposed that the graft-sparing mechanism was similar to that observed when the B7-specific fusion protein CTLA-4-Ig was used in a similar model (Rossini et al. 1996). That is to say, they proposed that the anti-CD154 was a co-stimulation blocking strategy in that the anti-CD154 prevented B7 upregulation on the transfused donor lymphocytes, thereby preventing CD28-mediated co-stimulation. This allowed the MHC molecules present on the donor lymphocytes to interact with the host's donor-specific T cells without receiving the required B7-CD28 costimulatory signal (Lafferty et al. 1978).

Several subsequent reports confirmed the efficacy of anti-CD154 therapy in prolonging rodent skin, cardiac, islet and marrow allografts (Rossini et al. 1996; Blazer et al. 1997; Hancock et al. 1996; Larsen et al. 1996a,b; Lu et al. 1997). All showed that a brief course of therapy at the time of transplantation led to 'indefinite' (usually defined as more than 100 days) allograft survival. This was a clear departure from studies using conventional immunosuppressive agents and energized the field of allograft tolerance considerably.

Unfortunately, some strains of rodents, for example C57Bl/6 mice, have been shown to be relatively resistant to the potent effect of anti-CDl54-based therapies (Larsen et al. 1996a,b; Trambley et al. 1999, 2000). In response to this, Larsen and colleagues demonstrated that treatment with CTLA-4-Ig was synergistic with anti-CDl54 therapy (Larsen et al. 1996a,b). Indefinite engraftment of heterotopic cardiac allografts and greatly prolonged survival of skin allografts was achieved using only perioperative anti-CDl54 plus CTLA-4-Ig. Neither agent alone was effective in the stain combination evaluated in this study. This study was also important because it demonstrated that the graft sparing effect of anti-CDI54 plus CTLA-4-Ig was inhibited by cyclosporine. This suggested that the co-stimulatory pathway modifying reagents were not simply preventing T-cell activation (as does cyclosporine) and that the co-stimulatory effect might in fact be an active one. More recently, anti-CD154 has been paired with mixed-chimerism approaches in rodent allotransplant models with considerable success (Durham et al. 2000; Wekerle et al. 2000). Anti-CD154 has been shown to facilitate haemopoietic stem cell engraftment without the need for ionizing irradiation or toxic conditioning. Studies directed by Sykes (Wekerle et al. 2000) and by Larsen (Durham et al. 2000) have shown that co-administration of high dose donor marrow and anti-CD154 leads to multilineage mixed chimerism without the need for additional 'space-making' therapies. Organs transplanted at the time of marrow infusion indefinitely engraft. Furthermore, these animals accept subsequently placed donor-specific grafts while rejecting thirdparty grafts. Indefinite graft survival has been achieved with therapy initiated at the time of transplantation, and thus the approach could be applicable to most forms of clinical transplantation. Furthermore, animals have withstood challenge with second donor specific allografts. This has been the most successful rodent approach to date in terms of durability, and promises to be an important addition not only to solid organ transplantation but also to bone marrow transplantation.

The strain sensitivity of murine transplant studies represents a significant problem in interpreting the generalizability of rodent protocols (Trambley et al. 2000). Moreover, the literature is replete with immunological therapies successful in the rodent but not in higher species. For this reason, studies in large animals have been critical in preparing transplant therapies for the clinic. For biologicals (e.g. antibodies, receptors and other molecules with strong species specific specificity), the testing ground of choice has been the non-human primate (Kirk 1999b). Several studies by our group and others, for example, have evaluated CDI54-based therapies in nonhuman primates. In general, co-stimulatory strategies using CTLA-4-Ig that have been extraordinarily effective in rodents have been ineffective in rhesus and cynomolgus models of renal and islet transplantation (Kirk et al. 1997; Levisetti et al. 1997). Similarly, monoclonal antibodies directed specifically at the B7 molecules CD80 and/or CD86 have been shown to prolong graft survival modestly without inducing tolerance to vascularized organs (Ossevoort et al. 1999a,b; Kirk et al. 2001a). Thus, the primate model has been important in tempering enthusiasm for many therapies.

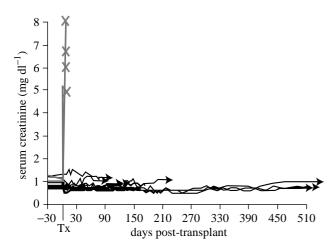


Figure 3. Anti-CD154 monoclonal antibody (hu5C8) therapy prevents acute rejection in non-human primates. Survival in days post-transplant and renal function as determined by serum creatinine (mg dl⁻¹) following unmodified allogeneic renal transplantation (group c, grey lines terminating in X), or following transplantation with anti-CD154 treatment (group i, black lines) is shown. Solid arrows indicate animals that remain alive and well. Tx, time of renal transplantation and native nephrectomy.

We have tested a humanized monoclonal antibody directed against CDl54 (hu5C8) in a juvenile rhesus monkey model of renal allotransplantation (Kirk et al. 1997, 1999). This is a rigorous model system in that animals are specifically mismatched at class I and class II and then segregated to insure that donor and recipient pairs are high responders in mixed lymphocyte reaction (MLR). Furthermore, the animals are bilaterally nephrectomized so as to be completely dependent on the allograft for survival. Untreated animals in this model uniformly terminally reject in five to eight days. Using this model, treatment with CTLA-4-Ig for two weeks prolonged graft survival to 20-30 days post-transplant. Similar results are achieved by using anti-CD80 or anti-CD86, while a combination of these agents leads to a mean survival of ca. 100 days (Kirk et al. 2001a). In contrast to the tepid results from B7 blocking experiments, treatment with anti-CD154 has been remarkably successful in nonhuman primates.

In our experience, treatment with hu5C8 at a dose of 20 mg kg⁻¹ given on days 0, 3, 10, 18 and 28 uniformly prevents acute renal allograft rejection in rhesus monkeys (Kirk et al. 1999) (figure 3). More remarkably, allograft survival can be extended dramatically by continuing to re-dose animals with $20 \,\mathrm{mg}\,\mathrm{kg}^{-1}$ monthly. Many animals maintain excellent allograft function for years after the withdrawal of hu5C8, and the animals lose MLR reactivity to their donor but not to third-party animals. Thus, in rhesus monkeys, hu5C8 appears capable of both preventing allograft rejection and establishing a long lasting state of donor-specific hyporesponsiveness that is not dependent on continuous immunosuppressive medication.

Animals treated with hu5C8 cannot be said to be truly tolerant. All animals develop a donor-specific IgG response early after transplantation (Kirk et al. 1999). Furthermore, while a large percentage of animals enjoy long lasting graft survival after the cessation of drug therapy, some do acutely reject coincident with the clearance of hu5C8 from the circulation (Kirk et al. 2001b). Most animals that do not undergo acute rejection have begun to show histological evidence of chronic allograft nephropathy, possibly as a result of an ongoing alloantibody response.

Despite the late allograft failures, the durability of anti-CD154 therapy has been impressive when compared with conventional immunosuppression (Kirk 1999a,b). Its effectiveness has been best in the renal model and more tenuous in other organ systems. Kenyon and colleagues have shown that monotherapy with hu5C8 dosed as described above can prevent islet cell rejection in baboons (Kenyon et al. 1999a) and in rhesus monkeys (Kenyon et al. 1999b). The baboon data are unique in that they were associated with significant CD4+-cell depletion, an effect not seen in other species. Graft survival has continued in animals as long as antibody is present in the circulation. Thereafter, most animals have lost their grafts despite evidence for donor-specific MLR hyporesponsiveness. These graft losses have not been clearly attributable to alloantibody responses. Though not tolerant, it is fair to say that these results in islet transplantation provided a substantial boost to the field and rekindled enthusiasm for this approach to type I diabetes.

Pierson and colleagues have used hu5C8 in a small series of cynomolgus monkeys receiving heterotopic cardiac allografts (Pierson et al. 1999). This study used a dosing regimen that was significantly less intense than that used in our studies. Even with the lower doses, animals had prolonged survival. The trend was that larger doses of antibody led to improving survival. Longterm survival was not achieved in any animal.

We have investigated the contribution of dose and duration of therapy to hu5C8 efficacy (Kirk et al. 2001b), and have shown that graft survival is clearly dose dependent. Despite uniform success using induction dosing at 20 mg kg⁻¹, achieving trough serum levels well in excess of 300 µg ml⁻¹, at 10 mg kg⁻¹ we have seen rejection during therapy. At 5 mg kg⁻¹ animals experience rejection with a time-course (but not intensity) that is similar to untreated animals. This requirement for high levels suggests that hu5C8 is functioning as a blocking agent and that either the affinity of the antibody is significantly less than that of the natural CD154 ligand (i.e. CD40), that there is a large amount of CD154 to be blocked, or that high circulating antibody levels are required to reach a local compartment where the anti-CDl54 effect needs to be present.

Interestingly, the duration of therapy does not necessarily correlate with the outcome following drug withdrawal (Kirk et al. 2001b). We have tested the $20 \,\mathrm{mg\,kg^{-1}}$ induction regimen combined with monthly maintenance using treatments regimens lasting 2 weeks, 1, 3, 6 and 12 months. Long-term rejection-free survival has been realized in animals receiving courses of therapy as short as one month, while rejection after the cessation of therapy has been seen even in animals treated for as long as one year. This is consistent with an effect that is mediated early after transplantation, and our bias is to believe that the important regulatory events that lead to prolonged graft survival occur at the time of initial

antigen exposure. Though rejection can occur at any time after transplantation, tolerance in all likelihood is the result of events taking place during the first antigen encounter.

(b) Data addressing the mechanism of CD154-based therapy

As discussed above, CDl54 is involved in several aspects of immune regulation. *In vitro* studies have offered many tantalizing possibilities to explain the anti-rejection effects of anti-CDl54. The role of CDl54 in APC activation clearly may be important in initiating antigen presentation to T cells. Similarly, its presence on platelets and the known ability of platelet-bound CDl54 to activate endothelial cells suggests that a critical role may be to initiate chemotactic and adhesion signals at the site of vascular trauma. Additionally, data concerning the role of CDl54 on activated T cells suggests that CDl54 may participate both in T-cell activation and activation-dependent apoptosis. There are *in vivo* data to support each of these possibilities, and it is likely that all contribute to the dramatic effects seen in allotransplant models.

One dominant theme that has emerged in recent years is that the pro-tolerant regulatory events evoked by treatment with anti-CDl54 are active. In our experience, anti-CD154's effect on graft survival is hindered by the concomitant use of steroids or tacrolimus (figure 4) (Kirk et al. 1999). This is in keeping with murine data from Larsen et al. (1996a,b), data from Wells et al. (1999), from Li et al. (1999), and from Hancock and colleagues (Smiley et al. 2000) showing that cyclosporine and/or glucocorticoids inhibits the efficacy of anti-CD154 therapy. Mechanistically, these data strongly suggest that CDl54's effect requires a calcineurin dependent pathway. A most logical explanation is that some TCR signal is required to mediate this effect, and it has long been our assertion that any antigen-specific event must involve an antigen receptor.

The finding that cross-linked CDI54 induces a transient co-stimulatory signal that is calcineurin sensitive and leads to effector cell apoptosis highlights another potential mechanism of action (Blair et al. 2000). That is, CD154 cross-linking at the time of antigen exposure may induce an antigen-specific cell death. This would be consistent with several observations. CD154-deficient animals undergo rejection and are resistant to treatment with anti-CDl54 (D. Dean, personal communication). The expression of CDl54 on T cells is prevented by treatment with cyclosporine (Fuleihan et al. 1994; Smiley et al. 2000), and anti-CD154 is ineffective at preventing rejection in the absence of T-cell CDl54 expression. Furthermore, crosslinking mechanisms typically require supersaturating concentrations of antibody, and high doses of antibody have been required in all models tested to date. Recent data from Larsen and colleagues have shown that anti-CD40 is not as effective as anti-CD154 in preventing allograft rejection in primates (Larsen et al. 2000). This, too, is consistent with a signalling as opposed to a blocking mechanism. Using a chimeric anti-CD40 antibody, C220, graft survival was modestly prolonged in a manner that, unlike anti-CDl54, was not synergistic with CTLA-4-Ig. B-cell depletion was a unique feature of this therapy. It is thus likely that preventing CD40-CD154

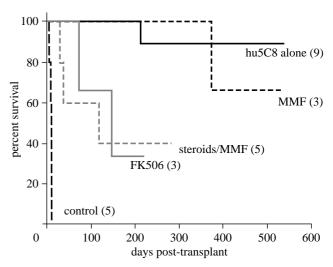


Figure 4. Conventional immunosuppressants worsen the long-term outcome following transplantation with hu5C8. Rejection-free survival following renal allotransplantation is shown for animals without therapy (control, n=5, dark grey longer-dashed line), or using six months of anti-CD154 alone (n=9, solid black line), anti-CD154 plus chronic mycophenolate mofetil and chronic steroids (steroids/MMF, n=5, grey shorter-dashed line), anti-CD154 plus chronic mycophenolate mofetil and a five day course of steroids (MMF, n=3, black shorter-dashed line), or anti-CD154 plus tacrolimus (FK-506, n=3, solid grey line).

interactions may contribute to the overall anti-CDI54 effect, but the durability of the graft sparing effect induced by anti-CDI54 is enhanced by CDI54-mediated activation dependent apoptosis.

Anti-CDl54 does not appear to induce global T- or B-cell depletion. Flow cytometry data have failed to show any reduction of CD3-, CD4-, CD8- or CD20-expressing cells in the rhesus model (Kirk et al. 1999). These findings strengthen the evidence suggesting that T-cell function is involved in the anti-CDl54's graft-sparing mechanism. Markees and colleagues have presented tantalizing murine model data to this end in that CD4-cell depletion actually breaks long-established tolerance induced by anti-CDl54 therapy (Markees et al. 1998). This does not negate the possibility that depletion is occurring at an oligoclonal level, but rather implies that any depletion that occurs is regulated and specific.

Following early experiments, investigators speculated that inhibition of B7 upregulation was critical to the anti-CDl54 effect. While we feel that this may play some role in the effect, it is not a dominant one. We have recently shown that very effective blockade of B7 molecules fails to promote graft survival in a way that approaches anti-CDl54 (Kirk et al. 2001a). Similarly, CTLA-4-Ig treatment alone does not produce an effect as consistent as anti-CD154 (Kirk et al. 1997; Pearson et al. 2001). The addition of B7 specific agents has been synergistic in most models, suggesting that there may be a role for B7 blockade. Since B7 blockade does not inhibit the effect of anti-CD154, it is unlikely that the major mechanism of action is dependent on B7-CTLA-4 engagement (Iwakohi et al. 2000). Also, Larsen and colleagues demonstrated that B7 is upregulated in grafts from anti-CD154 treated animals (Larsen et al. 1996a,b).

Data from several laboratories have suggested that intragraft lymphoid function may contribute to the maintenance of tolerance (Hamano et al. 1996; Burlingham et al. 2000). Histological data from anti-CDl54 treated animals with long-surviving renal allografts support this. T cells infiltrate non-rejecting allografts early in the posttransplant period without engaging in destructive behaviour (Kirk et al. 1999). Whether these cells are detrimental effectors that have been arrested prior to final maturation, or are regulatory cells that are actively limiting the cytotoxic effects of other cells, remains unclear. Interestingly, these cells are arranged in a pseudo-follicular pattern that typically organizes around a CD83⁺ dendritic cell (Kirk et al. 1999). Furthermore, apoptosis can be detected in these follicles suggesting that cells are dying (A. D. Kirk, unpublished data). Thus, CD154 is not dependent on preventing T-cell access to the allograft, and some access may in fact be salutary.

The literature has recently refocused on the mechanism of rejection when CDI54-based therapies fail. Again, a common theme has emerged. Most allografts fail as a result of persistent alloreactive CD8⁺ cells (Trambley et al. 1999; Iwakohi et al. 2000; Jones et al. 2000). Similarly, reduction of CD8⁺ cells generally improves the outcome of anti-CDl54 therapy while CD4+-cell depletion inhibits the effect (Markees et al. 1998; Iwakohi & Mordes 2000). A corollary to this observation is that as immune memory is expanded with increasing environmental exposure, resistance to anti-CD154 may increase. This is due to a heightened ability to mobilize CD8+ CTL clones on secondary exposure to antigen through pathways that are probably not CD154 dependent. For instance, older primates treated with anti-CDl54 appear to be less likely to have prolonged allograft survival as compared with juvenile animals, again highlighting the possible role of immune memory in the success or failure of anti-CD154based therapy (A. Kirk and C. Larsen, unpublished data). Furthermore, CD154 expression is more prevalent on CD4⁺ T cells. As such, the pro-apoptotic effects of anti-CD154 may not eliminate CD8⁺ cells as efficiently. Thus, it may be that for the success of anti-CD154 to be generalizable, some depletion of existing alloreactive CD8⁺ cells will be required.

(c) In vivo consequences of interrupting the CD154 pathway

The striking appeal of anti-CDl54-based therapy has been its apparent innocuousness when used to prevent allograft rejection in non-human primates. Studies in models usually sensitive to immunosuppressive complications have been notably free from problems. For instance, we observed no delayed wound healing, infection or malignancy in our primate studies. In fact, no model has demonstrated any oncogenic effect of anti-CDl54, nor has any role in wound healing been reported. However, no agent with such potent *in vivo* effects can be without its side-effects, and it is clear that CDl54 is important in host immunity (Grewal *et al.* 1997).

Interestingly, though data have shown that antiviral immunity is impaired by treatment with anti-CDI54, a sufficient antiviral immune response appears to occur in some situations despite the complete absence of CD40 or CDI54 (Szomolanyi-Tsuda *et al.* 2000). Of particular

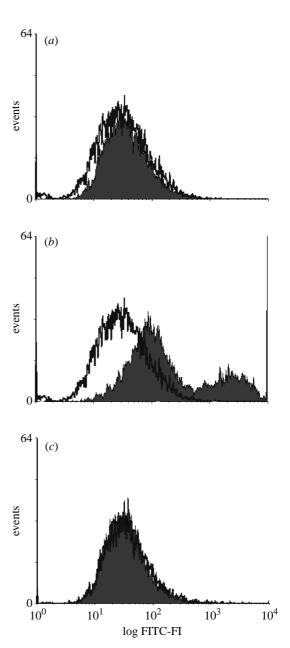


Figure 5. Anti-CD154 prevents human platelet mediated porcine endothelial activation. Shown are porcine endothelial cell flow cytometry studies using FITC-labelled isotype control antibody staining (non-shaded histogram), and FITC-labelled E-selectin antibody (solid histogram) under three conditions: (a) resting endothelial cells; (b) endothelial cells activated by CD154 expressing activated human platelets; and (c) endothelial cells treated with anti-CD154 prior to exposure to CD154 expressing activated human platelets.

interest are studies by Welsh and colleagues, showing that the presence of a pathogenic virus at the time of transplantation can, in many cases, induce anti-CDl54-resistant rejection rather than put the recipient at risk for infection (Welsh *et al.* 2000). This is consistent with the premise that the immune system is considerably more redundant in its defence against environmental pathogens than in its disdain for a transplanted organ.

Recently, concern has been raised regarding the thrombogenic potential of anti-CDl54-based therapies (Kawai *et al.* 2000; Kirk & Harlan 2000). This concern

has been heightened by the occurrence of several thromboembolic events in early clinical trials using one form of anti-CDl54. Indeed, the presence of CDl54 on platelets and atherosclerotic endothelial cells provides a potential mechanism for pro-thrombotic events. However, to date, no in vitro data have been forthcoming (despite intense research in the area) demonstrating a pro-coagulant effect of anti-CDl54 antibody. There is no evidence that anti-CD154 induces platelet aggregation or activation (H. Xu, unpublished data). This, combined with a relative paucity of adverse events in clinical trials using a different source of anti-CD154, suggests that the observed effects may be based on the antibody preparation rather than the specificity. Clearly, the potential for thrombotic events must be defined before broad clinical application of anti-CDl54-based therapies is possible.

(d) CD154 in xenotransplantation

Cellular interactions between human T cells and porcine endothelial or lymphoid cells are similar to their interactions with allogeneic cells (Kirk et al. 1993). Consequently, anti-CD154 functions with efficacy similar to that seen in allospecific mixed lymphocyte culture and may be effective in limiting xenospecific T-cell mediated rejection (Tadaki et al. 2001). However, unlike allografts, discordant xenografts are subjected to delayed xenograft rejection (after first overcoming complement and natural antibody-mediated hyperacute rejection). This process must be overcome prior to the consideration of T-cell mediated rejection.

Delayed xenograft rejection is characterized by the activation of xenograft endothelium, fibrin deposition, microvascular thrombosis and monocyte-macrophage infiltration (Platt et al. 1998). Thus, it has been hypothesized that xenograft endothelial cell activation plays a critical role in xenograft rejection. In vivo studies have demonstrated CD62P expression on xenograft endothelium in concert with infiltrating platelets, suggesting that an interaction between platelets and xenograft endothelial cells may play an important role in endothelial cell activation. We have specifically focused on xenogeneic CD40-CD154 interactions in vitro (Xu et al. 2000). Cultured porcine aortic endothelial cells demonstrate upregulation of CD62E following co-incubation with human Dl.1 T cells expressing CDl54. Blocking with anti-CD154 (but not anti-CD4, CD8 or CD80) prevents subsequent porcine endothelial cell activation.

We have therefore proposed that platelet activation can be induced by vascular trauma during a surgical procedure or by the binding of natural antibody and complement on xenograft endothelium. As a result, activated platelets expressing CDl54 may interact with xenograft endothelial cell and induce endothelial cell activation. To that end, we have demonstrated that activated human platelets induce porcine endothelial cell activation (as measured by E-selectin upregulation), and that anti-CD154 monoclonal antibody treatment can prevent this platelet-mediated endothelial cell activation (figure 5). These data suggest that a critical component governing the interaction between porcine endothelial cells and activated human platelets is CD154. This also strengthens the rationale for the use of CD154-directed therapy in discordant xenografts.

4. FUTURE INVESTIGATION

Many aspects of CDl54 biology deserve heightened in vitro scrutiny. Certainly, the role of this pathway on APC mobilization remains under-investigated. This is particularly true in light of recent data further establishing that the migration of an APC to regional secondary lymphoid tissue is necessary for allosensitization (Larsen et al. 1990a,b,c; Lakkis et al. 2000). Similarly, the importance of CDl54 in CTL maturation and general T-cell regulation deserves further consideration, particularly as it relates to response termination. Finally, the role of CD154 in thrombosis and platelet function will undoubtedly require additional study.

Many in vivo studies will be critical in establishing a clinically applicable strategy for the use of anti-CDI54 in transplantation. Particularly appealing protocols include the use of rapamycin in combination with anti-CD154 so as to establish a conventional regimen that will complement rather than inhibit the anti-rejection effect. In addition, there is a critical need to expand the promising rodent studies, combining bone marrow or other donor antigen infusions with anti-CDl54, into nonhuman primates.

The enthusiasm surrounding the use of CDI54-directed therapies is supported by the exciting animal data generated to date. Perhaps more than any other single agent, anti-CD154 antibodies offer the potential for long-term rejection-free allograft survival without the requirement for continuous immunosuppressive therapy. The transplant community stands willing and able to move this approach forward but is now impeded by a limited availability of antibody for preclinical study. Citing corporate liability and a limited ability to profit from tolerancebased transplant studies, companies which have developed clinical grade CD154-specific antibodies have been reluctant to proceed with aggressive investigation in this field. Though independent groups have created anti-CD154 antibodies, they have been tethered by the complexities of manufacturing the large doses of antibody required for large animal study. It is probable that the current impasse will be resolved, as the nuances of this approach become increasingly understood, and that manipulation of the CD154 pathway will play a role in the next clinical evolution of transplantation.

This paper is US Government work in the public domain in the United States

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