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William H. Kitchens Jr. Date

Costimulatory Blockade-Resistant Transplant Rejection: Mechanistic Characterization and Evaluation of Novel Immunomodulatory Regimens

By

William Henry Kitchens Jr.

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Immunology and Molecular Pathogenesis

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An abstract of
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Abstract

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By William H. Kitchens Jr.

The success of belatacept in late-stage clinical trials inaugurates the arrival of a new class of immunosuppressants based on costimulatory blockade (CoB), an immunomodulatory strategy that disrupts signals required for alloreactive T cell activation. Despite having improved renal function, kidney transplant recipients treated with belatacept experienced increased rates of acute rejection. This finding has renewed focus on costimulatory blockade-resistant rejection, a process likely mediated by subsets of T cells with diminished requirements for costimulation, such as memory and Th17 T cells. To study the contribution of memory T cells to CoB-resistant rejection, we developed a murine transplant system that models a donor-specific memory $CD8^+$ T cell response. After confirming that alloreactive memory T cells can mediate CoB-resistant rejection, we then demonstrated that these donor-specific memory T cells require intact VLA-4 and LFA-1 integrin pathways to mediate rejection. Indeed, the resistance of memory T cells to CoB was abrogated when costimulatory blockade was coupled with either anti-VLA-4 or anti-LFA-1. Mechanistic studies revealed that in the presence of CoB, anti-VLA-4 impaired T cell trafficking to the graft, whereas anti-LFA-1 predominantly attenuated memory T cell effector responses. We extended our findings to both a murine transplant model of polyclonal heterologous memory alloresponses, as well as to a non-human primate kidney transplant system. Next, we evaluated whether Th17 T cells participate in CoB-resistant rejection, exploiting the requirement of IL-23 for Th17 T cell proliferation. We showed that combined CoB and anti-IL-12/23 can significantly prolong survival of murine skin and vascularized cardiac allografts. Further work with selective IL-12 and IL-23 blockade indicated that IL-23 blockade was responsible for the enhanced efficacy of CoB. Combined costimulatory and IL-12/23 blockade inhibited alloreactive T cell proliferation and promoted immunodeviation away from Th1 and Th17 alloresponses. Finally, we extended our findings in a non-human primate kidney transplant system using rhesus macaques treated with belatacept and ustekinumab (humanized anti-IL-12/23), demonstrating prolonged graft survival compared to recipients treated with belatacept monotherapy. Given that integrin antagonists and ustekinumab are in clinical use, these findings have significant translational potential for future clinical transplant trials with belatacept.

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Chapter 1: **Introduction**

Clinical and scientific advances in organ transplantation have revolutionized the therapy for end-stage organ disease over the last fifty years. However, the modern immunosuppression regimens that have underpinned this progress carry a steep cost. Specifically, the calcineurin inhibitors (such as cyclosporine and tacrolimus) which comprise the backbone of modern transplant immunosuppression regimens are associated with an array of deleterious side effects such as hypertension, hypercholesterolemia, hyperkalemia and nephrotoxicity (1). Current immunosuppression regimens are also non-targeted, leaving transplant patients vulnerable to severe infectious complications and at elevated risk for post-transplant lymphoproliferative disease and other malignancies (2). Perhaps most significantly, the dramatic improvement in short-term transplant outcomes over the past 25 years has not precipitated a similar advance in long-term allograft function. Whereas the incidence of acute rejection within the first year after kidney transplantation is now <15%, the half-life of kidney allografts has stagnated for the past two decades (3). Indeed, over 50% of kidney allografts obtained from deceased donors will no longer function 9 years after transplantation, and even living-donor kidneys have a half-life of less than 12 years (3). Given the nephrotoxicity of calcineurin inhibitors, improving these long-term transplant outcomes may necessitate the development of radically new immunosuppression regimens.

Costimulatory blockade: a novel immunosuppression strategy with clinical potential

Costimulatory blockade has emerged as one of the most promising novel immunosuppression strategies designed to avoid these detrimental effects of traditional anti-rejection drugs (4-6). It involves the disruption of critical costimulatory receptor interactions

such as CD40/CD154 and CD28/B7, which are necessary to fully activate alloreactive T cells (7, 8). These costimulatory receptors play an integral role in the traditional "three signal" model of T cell activation. In this model, "Signal 1" is conferred by engagement of the T cell receptor (TCR) and either the CD4 or CD8 coreceptors, "Signal 2" is provided by signaling through the aforementioned costimulatory receptors, and "Signal 3" is delivered by various cytokine signaling networks. Experimental evidence has long demonstrated that if T cells receive Signal 1 in the absence of Signal 2, these T cells will become anergic rather than activated (7). Thus, in the presence of costimulatory blockade, alloreactive T cells (which receive Signal 1 by interaction with the graft itself or by graft antigens presented by recipient antigen-presenting cells) should be anergized. Consistent with this hypothesis, costimulatory blockade significantly prolongs transplant survival in many different experimental transplant systems employing immunologically naïve recipients (9, 10).

Many of these early transplant experiments with costimulatory blockade utilized the CD28 antagonist CTLA-4Ig, a soluble fusion protein consisting of the extracellular domains of the inhibitory receptor CTLA-4 fused with an immunoglobulin tail (9, 11). CTLA-4Ig has higher affinity for CD80 and CD86 (the ligands for CD28) than CD28 itself, allowing it to serve as a competitive inhibitor of CD28. Success in these preclinical models ultimately spurred the development of belatacept, a second-generation CD28 antagonist for use in transplant immunosuppression (12, 13). Belatacept differs from CTLA-4Ig fusion protein in two amino acids, resulting in a fusion protein with between 3 and 10 times the affinity for CD80 and CD86 compared to CTLA-4Ig (12). In June 2011, belatacept became the first entirely new transplant immunosuppressant to receive clinical approval in almost twenty years (13).

The significant clinical potential of costimulatory blockade (CoB) was evident in the recently published BENEFIT study, a phase III clinical trial which demonstrated significantly improved renal function in kidney transplant recipients treated with belatacept compared to patients treated with conventional cyclosporine-based immunosuppression regimens (14, 15). Paradoxically, however, treatment with belatacept was associated with a higher incidence and severity of acute rejection in these patients. Thus, costimulatory blockade must fail to tolerize a subset of alloreactive T cells, enabling them to mediate acute rejection in some patients. **While the mechanisms responsible for this failure of costimulatory blockade to prevent acute rejection are not fully elucidated, we hypothesized that it might be mediated by subsets of alloreactive T cells with diminished requirements for costimulation.** To characterize the mechanisms of costimulatory blockade-resistant rejection, we therefore focused on two subsets of T cells known to possess diminished requirements for costimulation: memory T cells and Th17 T cells.

Memory T cells as a barrier to costimulatory blockade

Accumulating evidence suggests that alloreactive memory T cells may play a critical role in mediating this CoB-resistant transplant rejection (16, 17). Compared to naïve T cells, memory T cells have a lower threshold for TCR activation, respond to lower concentrations of antigen, and circulate freely throughout the periphery, where they may encounter the allograft. Most importantly, memory T cells possess a lower costimulatory threshold than naïve T cells, and in experimental transplant systems, alloreactive memory T cells have proven resistant to costimulatory blockade (18-22). Besides their contribution to CoB-resistant rejection, these donor-specific memory T cells are of broader interest to the

transplant community, as pre-transplant levels of donor-reactive memory T cells are associated with acute rejection and worsened long-term graft function, even in patients treated with calcineurin inhibitors (18, 23-25). Thus, understanding the origins of alloreactive memory T cells and the mechanisms by which they contribute to transplant rejection is essential for improving the clinical outcomes of organ transplants, especially considering the increasing prominence of CoB as an immunosuppression strategy.

Alloreactive memory T cells can arise from prior exposure to donor MHC, whether through a failed prior transplant, blood transfusion or pregnancy. More recently, several groups have described how alloreactive memory T cells can arise in transplant recipients without prior exposure to donor MHC through the process of heterologous immunity. Heterologous immunity is a by-product of infection, whereby a subset of pathogen-specific memory T cells can cross-react with donor antigens, enabling their recruitment into a rejection response (26). Recently published findings have highlighted the significant contribution of heterologous immunity to alloreactive memory responses in humans, finding that >40% of T cells raised against common viruses possess alloreactive potential (27).

Integrin antagonists as a means to target alloreactive memory T cells

In order to dissect the immunologic pathways employed by these memory T cells during costimulation-independent activation, we sought to identify critical molecules utilized by memory T cells to mediate transplant rejection in the presence of costimulatory blockade. We initially chose to target integrins, the heterodimeric cell surface adhesion molecules found on a variety of immune cells, including T cells, B cells, macrophages and neutrophils (28-30). Integrins mediate adhesion between these immune cells and other cells in their environment, playing vital roles in both leukocyte activation and trafficking to sites of

inflammation. Two prototypic integrins are LFA-1 (leukocyte function-associated antigen-1, an $\alpha_1\beta_2$ integrin) and VLA-4 (very late antigen-4, an $\alpha_4\beta_1$ integrin). LFA-1 in particular has been shown to play a vital role in the formation of an immunological synapse between T cells and antigen presenting cells (APCs) (31). Both LFA-1 and VLA-4 have also been implicated in the "arrest" of rolling lymphocytes at sites of inflammation and the subsequent transendothelial migration of T cells into this inflamed tissue (29, 32, 33). These immunomodulatory properties of integrins spurred the clinical development of integrin antagonists against both LFA-1 (efalizumab) and VLA-4 (natalizumab) to treat various autoimmune diseases (34, 35). Specifically, efalizumab was approved by the FDA for the treatment of psoriasis and natalizumab has found use in both multiple sclerosis and Crohn's disease patients.

Integrin antagonists in transplantation

While the initial clinical applications of integrin blockade were focused on autoimmunity, multiple experimental and even clinical trials have emerged over the last decade supporting the use of these therapies in the clinical realm of transplantation (36). Early *in vitro* experiments demonstrated that LFA-1 blockade specifically blocked alloreactive human T cell proliferation in a mixed lymphocyte reaction (37). Furthermore, monotherapy with either LFA-1 or VLA-4 antagonists proved efficacious in prolonging graft survival in a variety of murine transplant systems, including skin (38), cardiac (39-42) and islet (41, 43-48) allograft models. In addition to suppressing acute rejection, integrin blockade was also found to diminish chronic rejection in a murine model of cardiac allograft vasculopathy (49-53). Combined integrin blockade with both anti-VLA-4 and anti-LFA-1 demonstrated potent synergy in both a murine corneal transplant model (54) and a murine islet transplant

system, with islet grafts lasting >60 days compared to 7-9 days with integrin antagonist monotherapy (55). To further augment the efficacy of integrin blockade, several investigators coupled it with standard costimulatory blockade drugs such as anti-CD154 or CTLA-4 Ig, achieving prolonged graft survival in a variety of murine transplant systems (56-62). Dual integrin/costimulatory blockade was even shown to prolong survival of xenografts in murine recipients, including porcine islets (63-66) and porcine dopaminergic neurons (67). This regimen of dual costimulatory and integrin blockade was also recently utilized successfully in a primate islet transplant system (using belatacept and efalizumab), demonstrating a substantial prolongation in islet graft survival (68). All of these encouraging preclinical studies established the critical groundwork that informed later human clinical trials with these integrin antagonists for transplantation.

The initial clinical trials of LFA-1 antagonists in transplantation utilized a mouse anti-human CD11a monoclonal antibody (odulimomab); small pilot studies with this monoclonal were mixed (69-71), but at least one study demonstrated that induction therapy with odulimomab was as effective as rabbit anti-thymocyte globulin in preventing acute rejection (72). Subsequent multicenter trials utilized efalizumab, a fully humanized IgG1 anti-LFA-1 monoclonal antibody. In one early multicenter trial, patients were randomized to either high (2 mg/kg) or low (0.5 mg/kg) dose efalizumab in new renal transplant recipients who were treated with either half-dose cyclosporine/sirolimus/prednisone or routine cyclosporine/MMF/prednisone immunosuppression regimens (73). Despite using half-dose cyclosporine and sirolimus, the cumulative rejection rates with these efalizumab-based regimens (10.4%) were comparable to historic controls with full-dose calcineurin inhibitor-based regimens. However, in the subset of patients receiving the high dose of efalizumab coupled with conventional full-dose cyclosporine/MMF/prednisone, almost

30% of patients developed post-transplant lymphoproliferative disease, a concerning development (of note, none of the patients treated with either low-dose efalizumab regimens or high-dose efalizumab with half-dose conventional agents developed PTLD in this study). More recently, efalizumab was used successfully by two different groups to promote engraftment and insulin-independence in recipients of islet transplants from single donors (74, 75).

In this current series of experiments, we demonstrated that combined costimulatory and integrin blockade (with either anti-VLA-4 or anti-LFA-1) can prolong transplant survival in an experimental transplant system that recapitulates allograft rejection by donor-specific memory T cells. Through mechanistic studies, we showed that anti-LFA-1 predominantly suppresses memory T cell effector responses, whereas anti-VLA-4 impedes trafficking of alloreactive memory T cells to the grafts. We also validated the efficacy of combined costimulatory and LFA-1 blockade in a fully allogeneic transplant system that models allograft rejection by a heterologous memory immune response. Finally, we extended our findings by proving that natalizumab (humanized anti-VLA-4) synergizes with belatacept to prolong kidney transplant survival in a non-human primate transplant system.

Th17 T cells: a newly defined T cell subset

While memory T cells are certainly likely to participate in costimulatory blockade-resistant transplant rejection, they are not the only subset of T cells known to possess diminished requirements for costimulation. Indeed, recent research findings have elevated another potential candidate that may play a major role in the resistance to costimulatory blockade: Th17 T cells.

It has been appreciated for over 20 years that $CD4^+$ helper T cells are not homogeneous, but instead may be stratified into distinct functional subsets based on the cytokines they produce. The "classical" $CD4^+$ T cell subsets include Th1 and Th2 T cells (76). Th1 cells differentiate in response to IL-12 from NK cells and dendritic cells, produce copious amounts of IFN-γ and are essential for mounting immune responses against a variety of intracellular bacteria and viruses. Th2 cells, in contrast, produce IL-4, IL-5, IL-9 and IL-13 and are thought to predominantly contribute to the evolution of humoral immune responses and anti-parasite immunity. Over the past decade, a variety of other $CD4^+$ T cell subsets have also been discovered, including the $FoxP3^+$ regulatory T cells (77), IL-10-producing regulatory Tr1 cells (78), follicular helper T cells (79), IL-9-producing Th9 cells (80, 81), and IL-22-producing Th22 cells (82). Finally, a novel subset of $CD4^+$ T cells that produce IL-17A has been described in both mice and humans, and these have been designated Th17 cells (83-92).

The surface phenotype, differentiation requirements and effector molecules possessed by these Th17 cells truly distinguish them from more conventional helper T cell subsets such as Th1 and Th2 cells. Unlike T cells from other subsets, these Th17 T cells express the CD161 surface marker and a unique pattern of chemokine receptors ($CCR6^+CCR4^+$, with variable CXCR3 expression) (92, 93). In terms of their effector mechanisms, these Th17 cells produce a variety of pro-inflammatory cytokines including IL-17F, IL-21, IL-22, IL-23 and GM-CSF (94, 95). However, despite this variety of secreted effector molecules, the prototypic cytokine produced by Th17 T cells is definitely IL-17A. IL-17A was first cloned in 1993 (96, 97), and it is the founding member of the IL-17 cytokine family, which also includes IL-17B, IL-17C, IL-17D and IL-17F (98). Only IL-17A and IL-17F are expressed by T cells, and both serve as potent pro-inflammatory effector

molecules with neutrophil chemoattractant properties (99-102). In addition to the $CD4^+$ Th17 cells, IL-17A is also produced by subsets of $CD8^+$ T cells (so-called Tc17 cells), γδ T cells, NK cells and macrophages (95, 103).

Over the past five years, much has been learned about the development and differentiation of this novel T cell subset. Th17 differentiation is dependent on the expression of specific transcription factors, RORγt (104, 105) and RORα (106). Additionally, expression of the STAT3 transcription factor by innate immune cells was found to be vital for the production of cytokines necessary for Th17 differentiation (107).

The cytokine signals responsible for polarizing activated T cells towards a Th17 differentiation pathway remain controversial, with potential differences existing in the polarization requirements of murine versus human Th17 cells, as well as possible differences in the *in vitro* versus *in vivo* differentiation requirements of these cells (108). Based on studies of murine Th17 T cells, it was initially determined that TGF-β and IL-6 are critical factors for the differentiation of Th17 cells (85, 86, 109). Specifically, IL-6 was thought to inhibit the expression of FoxP3 (the transcription factor critical for Treg development) and instead induce the expression of RORγt and RORα (86, 104, 106). TGF-β was theorized to promote Th17 polarization indirectly through its inhibition of Th1 and Th2 differentiation (110, 111). However, initial studies with naïve human Th17 cells discounted a role for TGF-β, finding instead that IL-1β and either IL-6 or IL-23 are the essential differentiation factors (112-115). Deepening the controversy, some later studies postulated that TGF-β arising from platelet contamination could have confounded these early findings regarding human Th17 cells; these newer papers determined that human Th17 differentiation does require TGF-β (116-119). Several recent studies, however, have validated the earlier findings that

human Th17 differentiation is TGF-β-independent, and indeed determined that even *murine* Th17 differentiation requires only IL-1β and IL-6 (or IL-23) (110, 120, 121). At least one report, however, found that autocrine TGF-β may play a role in stabilizing the commitment to the Th17 phenotype after initial differentiation (122), perhaps explaining some of the earlier results. To further obscure the differentiation requirements of Th17 cells, one recent study determined that the cytokines required to polarize Th17 cells are tissue specific, with IL-6 being essential for Th17 priming in the skin and mucosal surfaces, but not in the spleen (123). Considering the obvious conflicts between studies, further research will be essential to resolve these ambiguities regarding the *in vivo* differentiation requirements of Th17 cells in mice and humans.

While the role of TGF-β in Th17 polarization is controversial, abundant evidence now supports the participation of IL-23 in Th17 immune responses. Early studies discounted a role for IL-23 in the initial differentiation of Th17 T cells, as naïve T cells do not express the IL-23 receptor (107). However, in activated T cells, IL-23 can serve as a strong polarization factor towards the Th17 lineage (124). IL-23 was also implicated in the proliferation of established Th17 cells. Experiments using either IL-23 neutralizing antibodies or IL-23 receptor-deficient T cells demonstrated that in the absence of IL-23 signaling, Th17 cells failed to maintain IL-17 expression and had reduced proliferation (125). IL-23 is vital for the production of pro-inflammatory IL-22 by Th17 T cells (126). Additionally, IL-23 is now thought to be critical for the development of pro-inflammatory "pathogenic" Th17 T cells, whereas *in vitro* culture of Th17 T cells with TGF-β elicits the development of non-pathogenic IL-10-producing Th17 T cells (121, 126).

Despite the somewhat opaque nature of their origins, Th17 T cells make many important contributions to host defense, especially to mucosal immunity (127, 128). Mice deficient in the IL-17 receptor show enhanced susceptibility to *Klebsiella pneumonia* pulmonary infection (129), *Toxoplasmosis gondii* (130), and *Candida albicans* (131). Mice that are double-deficient for IL-17A and IL-17F are additionally susceptible to systemic *Staphylococcus aureus* infection (132). However, IL-17A was not critical for host defense against intracellular pathogens such as *Mycobacterium tuberculosis* or *Listeria monocytogenes* (133), which may depend more on Th1 immunity. However, IL-17 may indirectly participate in the immune responses against some intracellular pathogens (such as *Francisella tularensis* and *Chlamydia muridarum*) by inducing dendritic cells to produce IL-12 and thus promoting Th1 responses against these pathogens (134, 135). In addition to their role in promoting neutrophil infiltration of infected tissues, Th17 T cells possess several other effector mechanisms that facilitate host defense. IL-17 induces several antimicrobial genes (such as β-defensins) in infected epithelium (136). IL-17 also participates in antimicrobial humoral immunity be directly promoting B cell isotype switching and germinal center formation (137-140).

Although Th17 cells certainly play a major role in protective immunity, given the pro-inflammatory properties of IL-17, it is perhaps of little surprise that Th17 T cells are central players in a variety of autoimmune diseases. Most autoimmune diseases were initially thought to be mediated by Th1 responses. Perhaps the best early evidence implicating Th1 T cells in autoimmunity came from *in vivo* studies of experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis. It was demonstrated that mice deficient in the IL-12 p40 subunit were resistant to EAE induction; given that IL-12 is essential for Th1 induction, this was interpreted as providing a link between autoimmunity and Th1 T cells (141). However, this link was challenged by the finding that mice deficient in IFN-γ (the

primary Th1 cytokine) actually developed *more* severe autoimmune disease in a variety of experimental models such as experimental autoimmune encephalitis (EAE) (142), experimental autoimmune uveitis (143) and collagen-induced arthritis (144). The earlier results utilizing IL-12p40$^{-/-}$ mice were radically re-interpreted with the discovery of IL-23, a cytokine comprised of a novel p19 subunit coupled with the IL-12p40 subunit shared with IL-12 (145). It was subsequently determined that IL-23 (a cytokine vital for Th17 immune responses) rather than IL-12 mediates the onset of EAE (87). Subsequently, Th17 cells have been linked to numerous human autoimmune diseases including rheumatoid arthritis (88, 146-148), psoriasis (149-151), Crohn's disease (152-155) and multiple sclerosis (156). Targeting Th17 responses with ustekinumab, a humanized anti-IL-12p40 monoclonal antibody that also blocks the IL-23 signals vital for full Th17 responses, has proven clinically effective for the treatment of plaque psoriasis (157-159), psoriatic arthritis (160) and Crohn's disease (161).

Th17 cells and transplantation

Given their potent pro-inflammatory effector functions and their link to autoimmunity, many groups have theorized that Th17 T cells may participate in transplant rejection. Although a definitive role for Th17 cells remains unproven, accumulating evidence suggests that Th17 immune responses may indeed contribute to allograft rejection. Some of the earliest evidence came from close examination of clinical specimens from transplant patients. As early as 1997, some groups reported that IL-17 mRNA transcripts could be identified in allograft biopsies from renal allografts undergoing acute rejection (162). Immunofluorescent staining of rejecting renal allograft biopsies also identified the presence of IL-17, which was absent in both pre-transplant biopsies and biopsies of normal

kidney (163). The urinary sediment of renal transplant patients with biopsy-proven early acute rejection also often contains mononuclear cells with IL-17 transcripts (164). In addition to its potential role in renal transplant rejection, IL-17 has been associated with clinical rejection of liver (165) and lung (166) transplants. Importantly, many of these early clinical studies only identified an association between IL-17 and transplant rejection; as several different cell types can produce IL-17, a definitive link between Th17 cells and allograft rejection could not be established. Indeed, one recent study involving renal transplants undergoing acute antibody-mediated rejection demonstrated that renal tubule epithelial cells can themselves produce IL-17, independent of any T cells (167). Another immunohistochemistry study of kidney biopsies from rejecting allografts found that in double-stained slides, almost all the IL-17^{+} cells were mast cells and neutrophils rather than T cells (168). Thus, the precise contribution of Th17 T cells to clinical transplant rejection responses remains incompletely elucidated.

Despite the somewhat ambiguous human clinical data, experimental studies with animal models of transplantation have yielded further clues that Th17 cells may participate in transplant rejection responses. For example, blockade of IL-17 with soluble IL-17 receptor:Fc fusion protein prolonged graft survival in murine heterotopic cardiac allografts (169, 170). Using a similar murine cardiac transplant model, Min *et al.* found that graft-infiltrating neutrophils, Th17 and Tc17 cells all produced IL-17; compared to syngeneic grafts, rejecting grafts also had a skewed Th17 to Treg ratio (171). Other groups utilizing the murine heterotopic cardiac transplant model also confirmed the induction of intra-graft IL-17 early post-transplant, and they found that transplants into IL17A$^{-/-}$ recipients demonstrated reduced early cellular infiltration of the graft, suggesting that IL-17A may play a key role in the early recruitment of neutrophils and T cells into a rejecting graft (103, 172).

Transplant of wild-type grafts into IL-17A-deficient recipients results in markedly less intragraft IL-17 production compared to transplant of the same grafts into wild-type recipients, suggesting that most IL-17 production is mediated by graft-infiltrating recipient cells rather than the parenchymal cells of the graft itself (172). Possible links between Th17 cells and *chronic* allograft rejection have also been demonstrated in renal (168, 173), lung (174-176) and cardiac (177-180) transplants. Perhaps the most convincing data has emerged from heterotopic cardiac transplant experiments utilizing T-BET$^{-/-}$ recipients, which therefore cannot mount Th1 alloresponses (177, 181). Allograft rejection in these T-BET$^{-/-}$ recipients is markedly different than rejection in a wild-type recipient, characterized predominantly by a neutrophilic rather than lymphocytic graft infiltrate. Two different groups found that IL-17-producing lymphocytes are the key mediators of graft rejection in these T-BET$^{-/-}$ recipients, although one group determined that the IL-17 involved in these alloresponses is predominantly from CD4^{+} Th17 cells (177), whereas the other group favored CD8^{+} Tc17 cells (181).

Th17 T cells and costimulatory blockade

Although experimental transplant models suggest that Th17 T cells may contribute to alloresponses, proving a definitive role for Th17 cells in human transplant patients has been challenging, as previously discussed. Part of the ambiguity regarding the role of Th17 cells in human transplantation may arise from the fact that conventional immunosuppressants employed in human patients are highly effective at suppressing Th17 immune responses. For example, corticosteroids potently attenuate many cytokine responses including IL-17 production (182), and glucocorticoids specifically have been shown to suppress Th17 responses in patients with giant cell arteritis (183). Anti-

metabolites such as mycophenolate mofetil also effectively suppress IL-17 production and the polarization of $CD4^+$ T cells into Th17 cells (184, 185). Many reports on calcineurin inhibitors (such as cyclosporine and tacrolimus) have documented inhibition of Th17 immune responses (185-187), although other studies have failed to identify any IL-17 suppression (182). Finally, sirolimus can also block IL-17 production and Th17 differentiation (188).

While these conventional agents can effectively block Th17 differentiation and effector functions, it remains unclear whether newer regimens such as costimulatory blockade can similarly impede the emergence of Th17 alloresponses. Of concern, Th17 T cells are less dependent on conventional costimulatory signals for their activation (181, 189), although secondary costimulatory receptors such as ICOS (190) and OX40 (191-193) may still participate in the differentiation and effector functions of Th17 cells. Because they have diminished requirements for traditional costimulatory signals, Th17 T cells may be uniquely resistant to the suppressive effects of costimulatory blockade with agents such as CTLA-4Ig or belatacept. Indeed, CD28 blockade with CTLA4-Ig actually facilitates the differentiation of murine and human Th17 T cells during *in vitro* culture experiments (194).

Given that Th17 T cells may participate in costimulatory blockade-resistant transplant rejection, we sought to evaluate whether adjuvant immunosuppressants that specifically targeted Th17 T cells could enhance the clinical efficacy of costimulatory blockade. To target these Th17 alloresponses, we exploited the vital role played by IL-23 in the proliferation and effector functions of Th17 T cells. We utilized an anti-IL-12p40 monoclonal antibody that targets the shared subunit of IL-12 (a cytokine vital for Th1 immunoresponses) and IL-23. Combined costimulatory and IL-12/23 blockade significantly prolonged skin and heterotopic cardiac allograft survival in murine transplantation models.

Further experiments revealed that IL-12/23 works predominantly through its effects on IL-23 to prolong graft survival in conjunction with costimulatory blockade. We performed additional mechanistic studies to evaluate the causes of prolonged graft survival with this combined blockade. Finally, we translated our findings to a pre-clinical non-human primate kidney transplant model, showing that combined belatacept and ustekinumab (a humanized anti-IL-12/23 antibody that is clinically-approved to treat psoriasis) yielded significantly longer kidney allograft survival in rhesus macaques compared to either ustekinumab or belatacept monotherapy.

Chapter 2: Methods and Materials

Mice

Adult male 6–8-week-old C57BL/6 mice (NCI-Frederick), BALB/c mice (NCI-Frederick), TCR transgenic OT-I mice (Taconic Farms), μMT mice (Jackson Laboratories), IL-12Rβ2$^{-/-}$ (Jackson Laboratories), T-BET$^{-/-}$ (Jackson Laboratories) and Act-mOVA mice (gifted by Dr. Marc Jenkins, University of Minnesota, Minneapolis, MN) (195) were obtained. All of these knockout and transgenic mice were on a C57BL/6 background. Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

B6.OT-I^{Memory} mouse generation

After quantification of OT-I cells from whole blood of OT-I mice by TruCount bead analysis (BD Pharmingen, San Diego, CA), 10^4 OT-I cells (along with syngeneic carrier splenocytes) were adoptively transferred into each naïve C57BL/6 mouse. Two days later, the mice were infected with 10^4 CFU of LM-OVA (196) by i.p. injection.

Skin grafting

Full thickness tail skin grafts (~1cm^2) were transplanted onto the dorsal thorax of recipient mice. Where indicated, recipients of skin grafts received treatment with costimulatory blockade [500 μg each of hamster anti-mouse-CD154 mAb (MR-1, BioXcell, West Lebanon,

NH) and human CTLA-4 Ig (Bristol-Meyers Squibb, New York, NY)], 250 μg of rat anti-mouse-VLA-4 mAb (PS/2, BioXcell), 250 μg of rat anti-mouse-LFA-1 mAb (M17/4, BioXcell), 250 μg of rat anti-mouse-IL-12p40 mAb (C17.8, BioXcell), 25 μg of goat polyclonal anti-mouse IL-23p19 IgG antibody (G-20, Santa cruz biotechnology), or 100 μg of rat anti-mouse-IL-17A (clone 50104, R&D systems). All monoclonal antibodies were administered i.p. on post-transplant day 0, 2, 4 and 6. Anti-IL-12p40 antibody and anti-IL-17A treatment was continued weekly (without further costimulatory blockade) as maintenance therapy. For the heterologous immunity experiments, integrin antagonists were continued once weekly for the duration of transplant survival.

Flow cytometric analyses for frequency and absolute number

Splenocytes, blood, and/or cells obtained from axillary draining lymph nodes (dLNs) were stained with Thy1.1-PerCP, CD8a-APC, CD11a-FITC and/or CD49d-PE (Pharmingen) for analysis on a BD LSRII flow cytometer (BD Biosciences, San Jose, CA). Absolute numbers of OT-I T cells were determined by TruCount Bead analysis according to manufacturer's instructions. For the heterologous immunity experiments, splenocytes, blood, and/or cells obtained from axillary dLNs were stained with H-2K^{d}-FITC, CD8a-APC and CD4-V500 (Pharmingen). Data were analyzed using FlowJo Software (Tree Star, San Carlos, CA).

Intracellular cytokine staining

For mOVA transplant system experiments, splenocyte suspensions were incubated with 10 nM $OVA_{257-264}$ (SIINFEKL) (Emory University Core Facility) and 10 μg/ml Brefeldin A (Pharmingen). Replicates without peptide were also performed. After 5 hr in culture, cells were processed using an intracellular staining kit (Pharmingen) according to manufacturer's instructions and stained with anti-TNF-PE and anti-IFN-γ-FITC (Pharmingen). The adjusted % dual-producers of TNF and IFN-γ for each sample was calculated by subtracting the % dual-producers from the non-stimulated samples from the matched SIINFEKL-stimulated sample. Outliers (values greater than or less than median $\pm$ 3*SEM) for each group were excluded. For the heterologous immunity ICCS experiments, splenocytes were harvested from the POD#60 BALB/c skin graft recipients, and stimulation was performed with a 2:1 ratio of fresh BALB/c splenocyte stimulators for 5 hours in the presence of brefeldin A, after which intracellular cytokine staining was performed as before. For FoxP3 staining in the heterologous immunity and anti-IL-12/23 experiments, FoxP3-AlexaFluor700 (eBioscience) was used per manufacturer protocol.

CD107a/b degranulation assay

As previously described (197), splenocyte suspensions were incubated in R10 media at 37°C in a 96-well plate (4×10^6 cells/well) for five hours with monensin and anti-CD107a/b-FITC in the presence or absence of 10 nM $OVA_{257-264}$ peptide. After incubation, surface staining with anti-Thy1.1-PerCP and CD8a-Pacific Blue was performed. Degranulation was measured as the adjusted MFI of CD107a/b (peptide-stimulated – unstimulated).

In vivo CTL assay

As previously published (198), CD45.1-congenic splenocyte target cells were labeled with high (1 μM) or low (100 nM) concentrations of CFSE. The $CFSE^{Lo}$ target cells were pulsed at 10 nM $OVA_{257-264}$ peptide; $CFSE^{Hi}$ target cells were incubated without peptide. 10^6 target cells in a 50:50 mixture of unloaded and peptide-loaded target cells were adoptively transferred *i.v.* into each mOVA skin graft recipient. Twelve hours after adoptive transfer, splenocytes were harvested and assessed for CD45.1 expression and CFSE labeling. Outliers (%specific lysis greater than or less than median $\pm$ 2*SEM) were excluded.

Immunohistochemistry

Explanted skin grafts were fixed in OTC and frozen. For experiments utilizing the mOVA transgenic transplant system, sections were stained with anti-Thy1.1 mAb and developed with horseradish peroxidase to visualize infiltrating OT-I cells. Representative images of explanted mOVA skin grafts are shown magnified 40x. For the heterologous immunity experiments and anti-IL-12/23 experiments, explanted skin grafts were fixed in OTC and frozen. Hematoxylin and eosin staining was employed to visualize rejection. Sections were stained with anti-CD3e mAb and developed with horseradish peroxidase. Representative images (of at least 4 transplants per group) are magnified 20X.

Quantitative real-time PCR for OT-I TCR

Explanted skin grafts were homogenized on a TissueLyser II bead mill (Qiagen). mRNA was extracted using a RNeasy Fibrous Tissue kit (Qiagen), and cDNA was generated using a TaqMan RNA-to-Ct 2-step kit (Applied Biosystems). Quantitative real-time PCR for OT-I TCR expression was performed in triplicate using TaqMan Gene Expression Master Mix (Applied Biosystems) and custom primer/probe pairs (Applied Biosystems) specific for the CDR3 region of the OT-I TCR, as previously published (199). Relative quantification was employed using the $2^{-\Delta\Delta Ct}$ method, normalizing OT-I TCR expression against beta-actin expression and comparing to the average normalized OT-I TCR expression in untreated mOVA skin graft recipients (which is set at a normalized value of 1.0).

T cell trafficking experiment

Naïve C57BL/6 recipients were transplanted with mOVA skin grafts. Other C57BL/6 mice previously transferred with OT-I cells were infected with LM-OVA, and after harvesting splenocytes eight days later, effector OT-I cells were counted using TruCount tubes. The equivalent of 10^6 activated effector OT-I T cells were adoptively transferred into each mOVA skin graft recipient following *in vitro* treatment for 30 minutes with an immunosuppressive regimen (100μg/ml for each agent) matching the regimen used to treat the transplant recipient. Twenty-four hours after transfer, skin grafts were explanted and OT-I trafficking was assessed by quantitative rt-PCR for OT-I TCR expression.

Viral infections

Viral infections were conducted by intraperitoneal injection of $2x10^5$ pfu LCMV Armstrong (gifted by R. Ahmed) and 10^6 pfu Vaccinia virus (gifted by J.R. Bennick).

Bone marrow transplantation

Bone marrow recipients were pre-treated with 600 μg of busulfan (GlaxoSmithKline) i.p. The following day, $2x10^7$ BALB/C bone marrow cells (harvested by femur flushing) were adoptively transferred via tail vein injection into the recipients.

In vivo mixed lymphocyte reaction

For the heterologous immunity experiments, splenocytes were harvested on POD#60 from previously sequentially-infected graft recipients treated with different immunosuppression regimens. These splenocytes were labeled for 5 minutes with 10 μM CFSE, and $2\text{-}3x10^7$ of these labeled responders were adoptively transferred *i.v.* into irradiated BALB/c mice (700 rads). Splenocytes were harvested after 72 hours and analyzed by flow cytometry to assess the CFSE dilution and thus proliferation of H-$2K^d$-negative (responder) T cells. For the anti-IL-12/23 experiments, naïve B6.CD45.1 congenic splenocytes were labeled with CFSE, and $2\text{-}3x10^7$ labeled responders were adoptively transferred as before into an irradiated BALB/c mouse, which was then treated on day 0 and +2 after adoptive transfer with either no treatment, anti-IL-12/23 alone, CoB alone or CoB + anti-IL-12/23. Splenocytes were harvested after 72 hours and analyzed by flow cytometry to assess the CFSE dilution and thus proliferation of $CD45.1^+$ (responder) T cells.

Human allostimulation assay

After receiving informed consent, peripheral blood mononuclear cells (PBMCs) were obtained from six human donors to form three responder—stimulator pairings. A 1:1 mixture of responders and irradiated stimulators (3500 cGy) was prepared in triplicate (~10^6 total cells/well). Cells were either left untreated or were treated with belatacept (100 μg/ml, provided by Bristol-Myers Squibb) and/or anti-human-LFA-1 (250 μg/ml, clone TS-1 [BioXcell]). After 6 hours, intracellular cytokine staining was performed as described.

Cytometric bead array determination of cytokines

At the specified timepoint post-transplant, transplant recipients were sacrified and their axillary draining lymph nodes were harvested. If specified, these cells were subjected to magnetic-assisted cell sorting (Miltenyi Biotec) to isolate the $CD4^+$, $CD8^+$, Pan T cells and γδ T cells residing in the draining lymph nodes. The cells were then cultured at a 2:1 stimulator vs. effector ratio with fresh unirradiated BALB/c splenocytes for 48-60 hours at 37°C. After incubation, the supernatants were harvested and either frozen or immediately subjected to a CBA analysis using the mouse Th1/Th2/Th3 cytokine CBA kit, as well as the mouse GM-CSF flexset bead kit (BD Biosciences) per standard manufacturer's protocol.

Non-human primate kidney transplant system in rhesus macaques

All rhesus macaques underwent MHC class I typing by 454 sequencing, and pairs were constructed with maximal antigenic disparity. Whenever possible, transplants were performed in a domino fashion (*i.e.* when the kidney donor of one transplant will become a transplant recipient at a later time-point) in order to minimize the number of required monkeys for transplantation. At the time of transplantation, general endotracheal anesthesia was induced and the transplant was performed with standard techniques (using anastomosis to the abdominal aorta and inferior vena cava). The ureteral anastomosis was fashioned by making a generous anterior cystotomy of the bladder and then performing a small posterior cystotomy through the opened bladder, allowing the ureter to be tunneled in a retroperitoneal position to the posterior wall of the bladder, where it was then spatulated and anastomosed using interrupted 7-0 PDS sutures. All monkeys received belatacept (20 mg/kg *i.v.*) on post-transplant day 0, 3, 7, 14, 28, 35, 42, 56, 70 and 84; monkeys receiving belatacept + ustekinumab also received an additional dose of belatacept on pre-transplant day #7 (in order to minimize the risk that rhesus anti-human antibodies against ustekinumab would develop pre-transplant). Monkeys receiving doses of natalizumab (10 mg/kg *i.v.* per dose) or ustekinumab (7.5 mg/kg *s.c.* per dose) were treated on post-transplant day 0, 3, 7, 14, 21, 28, 35 and 42. Those receiving ustekinumab also received a dose on pre-transplant day #7.

Statistical analyses

Skin graft experiments are presented on Kaplan-Meier survival curves and were compared with log-rank test. All other assays were compared with the Mann-Whitney nonparametric test. Statistical analyses were conducted using GraphPad Prism (La Jolla, CA).

Chapter 3: Integrin Antagonists prevent costimulatory blockade-resistant transplant rejection by $CD8^+$ memory T cells

A novel murine transplant system models rejection by donor-specific memory T cells

To study CoB-resistant rejection, we developed a novel transplant system that models a donor-specific memory T cell response (Figure 1). We adoptively transferred OT-I $CD8^+$ TCR transgenic T cells (200) specific for an ovalbumin (OVA) peptide into naïve C57BL/6 mice. These transferred cells expressed the Thy1.1 marker, enabling us to specifically track them. Mice were subsequently infected with genetically-modified *Listeria monocytogenes* expressing the $OVA_{257-264}$ epitope (LM-OVA) (196), generating recipients containing memory OT-I cells (B6.OT-I^{Memory} mice). Following LM-OVA infection, naïve OT-I T cells rapidly proliferated until post-infection day 8-9, after which they contracted into a stable memory T cell population constituting 0.5-4% of all $CD8^+$ T cells in the blood by post-infection day 28 (Figures 2A-C). After this development of memory OT-I cells, these B6.OT-I^{Memory} mice were re-challenged with skin grafts from mOVA transgenic mice that ubiquitously express OVA in their tissues, thus precipitating rejection by memory OT-I cells (195). We demonstrated that B6.OT-I^{Memory} recipients rejected mOVA skin grafts with second-set kinetics despite costimulatory blockade with CTLA-4Ig and anti-CD154 (Figure 3), thereby recapitulating costimulatory blockade-resistant rejection mediated by donor-reactive memory T cells. Adoptive transfer of MACS-sorted memory OT-I cells was sufficient to mediate this CoB-resistant rejection (Figure 4A), and transplants in B cell-deficient recipients were also promptly rejected, further demonstrating that B cells and/or antibodies are not required for CoB-resistant rejection in this system (Figure 4B). The

inability of LM-OVA infection to induce an anti-OVA antibody response was also confirmed via ELISA assay (Figure 4C).

Combined integrin and costimulatory blockade prolongs graft survival against a donor-specific memory response

Surface expression of both LFA-1 and VLA-4 was upregulated in memory compared to naïve OT-I cells, suggesting that integrin antagonists might target donor-specific memory T cells (Figure 5). To evaluate whether combined integrin/costimulatory blockade could prolong graft survival, we transplanted mOVA skin grafts onto B6.OT-I^{Memory} mice. These graft recipients were treated with costimulatory blockade (CTLA-4Ig + anti-CD154) alone, integrin antagonist alone, or a dual blockade regimen (*i.e.* anti-LFA-1 + CoB or anti-VLA-4 + CoB). Whereas recipients treated with CoB or integrin antagonists alone all displayed accelerated graft rejection similar to untreated controls, the recipients treated with dual blockade regimens displayed significantly prolonged graft survival, with a median graft survival time >100 days (Figures 6A and B, and Table 1). Thus, combined costimulatory and integrin blockade prolongs graft survival, even against a donor-specific memory T cell response. However, donor-specific memory precursor frequencies may impact susceptibility to these combined blockade regimens, as mOVA graft survival was not prolonged when performed on recipients whose frequency of memory OT-I cells was increased dramatically (to ~15% of total CD8$^+$ T cells) by repetitive LM-OVA infection prior to grafting (Figures 7A and B).

Combined integrin and costimulatory blockade does not curtail recall accumulation of donor-specific memory T cells

By what mechanisms do integrin antagonists prolong graft survival in recipients treated with CoB? We first assessed whether integrin antagonists function by curtailing the accumulation of OT-I cells during the memory recall response. We transplanted mOVA skin grafts onto B6.OT-I^{Memory} recipients that were treated with an immunosuppression regimen prior to sacrifice on POD#7. Absolute quantification of OT-I T cells from the memory recall response in the blood, draining lymph nodes and spleen was performed using flow cytometry (201). Despite a decrease in the absolute number of OT-I T cells in the blood in treated versus untreated recipients, there were no significant differences between the absolute number of OT-I T cells in the dual costimulatory/integrin blockade recipient groups compared to treatment with CoB alone (Figure 8A). Similar results were noted in the spleens and draining lymph nodes of mOVA skin graft recipients (Data not shown). Comparing recipients treated with CoB alone and those treated with one of the dual costimulatory/integrin blockade regimens, there was no difference in the frequency of OT-I cells within the total population of $CD8^+$ T cells (Figure 8B). However, an enrichment of antigen-specific OT-I cells was evident only in the draining lymph nodes of recipients re-challenged with an mOVA skin graft, consistent with some of our previous observations.

LFA-1 blockade attenuates donor-specific memory T cell effector responses

As the dual blockade regimens did not impact recall accumulation, we next evaluated whether integrin antagonists might inhibit T cell effector functions during the memory recall response. We again transplanted mOVA skin grafts onto B6.OT-I^{Memory} recipients, which were treated with an immunosuppressive regimen prior to sacrifice on POD#7. Intracellular cytokine staining revealed that immunosuppressive regimens containing anti-LFA-1, unlike those containing anti-VLA-4, substantially decreased the ability of donor-reactive T cells to

become highly active dual-producers of TNF and IFN-γ following stimulation with the $OVA_{257-264}$ epitope (Figures 9A and B). Similarly, a flow cytometric degranulation assay based on surface localization of CD107a/b (197) revealed that LFA-1 blockade (but not regimens including anti-VLA-4) potently hindered cytotoxic granule release by OT-I cells during memory recall (Figures 10A and B). Finally, an *in vivo* CTL assay (198) validated these *ex vivo* findings, demonstrating that anti-LFA-1 significantly suppressed the generation of graft-specific cytotoxicity, whereas regimens containing anti-VLA-4 had only a minimal impact on OT-I effector functions during a memory recall response (Figures 11A and B).

Both VLA-4 and LFA-1 blockade inhibit donor-specific memory T cell trafficking to graft

These results did not explain why combined costimulatory and VLA-4 blockade prolonged graft survival, so we next determined whether combined blockade regimens impact trafficking of T cells from the memory recall response to the graft. We explanted mOVA skin grafts on POD#7 after transplant onto B6.OT-I^{Memory} recipients that were treated with different immunosuppression regimens, and performed immunohistochemistry staining for Thy1.1 to assess the relative infiltration of grafts by memory/effector OT-I cells. Whereas grafts from recipients treated with CoB alone had an abundant OT-I T cell infiltrate, those treated with CoB + anti-VLA-4 had almost no infiltration (Figure 12). To better quantify the degree of OT-I T cell infiltrate in these explanted mOVA grafts, we performed quantitative real-time PCR on graft tissue explanted on POD#7 utilizing a primer-probe pair specific for the OT-I T cell receptor (199). Using this more sensitive assay, a small decrease in OT-I cell infiltration was detected in recipients treated with

regimens containing anti-LFA-1. However, there was a significant decline in memory/effector OT-I infiltration in recipients treated with anti-VLA-4+CoB (Figure 13).

To specifically assess how integrin blockade impacts primed graft-specific effector T cell trafficking, effector OT-I T cells were harvested from LM-OVA-infected mice, blocked *in vitro* with an integrin antagonist, and then adoptively transferred into a naïve C57BL/6 mouse that had received an mOVA skin graft seven days previously (*i.e.* cells were transferred after graft neovascularization). The grafts were explanted 24 hours after adoptive transfer, homogenized and then subjected to rt-PCR analysis to detect OT-I TCR (Figure 14A). Both VLA-4 and LFA-1 blockade were associated with a dramatic decrease in T cell trafficking to the graft (Figure 14B). Interestingly, treatment with anti-VLA-4 in the absence of CoB dramatically impaired trafficking of recently primed effectors (Figure 14B), but not the trafficking of memory T cells and secondary effectors during the recall response (Figures 12 and 13), perhaps reflecting greater dependence of primary effectors on VLA-4 for trafficking into the graft.

Combined LFA-1 and costimulatory blockade prolongs skin graft survival against a heterologous immune alloresponse

To study the impact of combined LFA-1 and costimulatory blockade on transplant rejection mediated by an alloreactive memory response, we utilized a well-defined experimental model of heterologous immunity (26). In this system, naïve C57BL/6 mice are infected with lymphocytic choriomeningitic virus (LCMV), followed by an infection with vaccinia virus six weeks later. These sequential infections generate pathogen-specific memory T cells that are cross-reactive with BALB/c alloantigens (~10^4 allo-crossreactive memory $CD4^+$ and $CD8^+$ T cells per 10^8 splenocytes) (26). Six weeks after the final

infection, the mice receive a simultaneous skin graft and bone marrow transplant from a fully allogeneic BALB/c donor (Figure 15A). While uninfected transplant recipients treated with CoB alone demonstrated indefinite graft survival, sequentially-infected recipients treated with CoB alone promptly rejected their skin grafts with the same kinetics as untreated controls (Figure 15B). Treatment with anti-LFA-1 alone also led to prompt rejection, but treatment with a combined regimen of CoB and anti-LFA-1 enabled prolonged skin graft survival, with a median survival time >100 days (Figure 15B). A donor bone marrow transplant was important for prolonged graft survival in this stringent transplant system, as even uninfected recipients achieved only a 22 day median skin graft survival time when treated with CoB alone in the absence of donor bone marrow (Figure 15B). Similarly, maintenance anti-LFA-1 was required for the duration of transplant, as administration of anti-LFA-1 only during the first 6 days after transplant failed to prolong graft survival (Figure 16).

Whereas grafts explanted from untreated recipients showed a prominent cellular infiltrate, explanted grafts taken either early (day 11) or late (>100 days) post-transplant from recipients treated with combined costimulatory/LFA-1 blockade had no infiltration, closely resembling isografts or grafts explanted from uninfected recipients treated with CoB alone (Figures 17A-D). Further immunohistochemistry with anti-CD3 revealed a lack of T cells in the grafts treated with the combined immunosuppression regimen (Figures. 17E-H)

Combined blockade surmounts barrier posed by heterologous immunity to allogeneic bone marrow engraftment

We also examined BALB/c bone marrow engraftment eight weeks following transplant by assessing for hematopoietic chimerism in the peripheral blood of graft

recipients. Using flow cytometry to determine the expression of donor MHC (H-2K^{d}), we found that sequentially-infected recipients treated with either anti-LFA-1 or CoB alone failed to develop either lymphoid (CD3^{+}) or myeloid (CD11b^{+}) chimerism (Figures 18A and B). In contrast, recipients treated with combined costimulatory and LFA-1 blockade demonstrated durable low-level (1-6%) lymphoid and myeloid chimerism.

Intriguingly, while our earlier published work found coupling CoB to either anti-LFA-1 or anti-VLA-4 could markedly prolong graft survival, treatment with CoB + anti-VLA-4 was ineffective in this current model of heterologous immunity (Figure 19A). Treatment with CoB and anti-VLA-4 also failed to permit bone marrow engraftment and chimerism (Figure 19B), consistent with previous evidence that VLA-4 is critical for homing of lymphocytes to the bone marrow (202-204).

Combined blockade inhibits alloreactive T cell proliferation and effector responses

Further *ex vivo* studies were performed to assess the mechanism by which combined costimulatory and LFA-1 blockade prolongs graft survival. First, we utilized an *in vivo* mixed lymphocyte reaction (MLR) to assess the ability of these different regimens to suppress the proliferation of alloreactive T cells after induction of heterologous immunity. Anti-LFA-1 alone failed to suppress the proliferation of alloreactive splenocytes, while CoB alone had a modest effect (Figures 20A and B). In contrast, combined CoB and anti-LFA-1 demonstrated the most pronounced inhibition of alloreactive recall proliferation, demonstrating the synergy of these regimens (Figures 20A and B). Next, we evaluated how these different regimens impacted alloreactive T cell effector mechanisms. Consistent with our previously published work (205), intracellular cytokine staining for IFN-γ and TNF revealed that following induction of heterologous immunity, transplant recipients treated

with either CoB or anti-LFA-1 alone had a significant reduction in the percentage of splenocytes that were highly-activated double-producers of IFN-γ and TNF compared to untreated recipients (Figures 21A and B). Combined CoB and anti-LFA-1 demonstrated an even more prominent inhibitory effect (Figures 21A and B).

Combined blockade promotes retention of Tregs in draining LNs

Finally, we evaluated whether dominant tolerance mechanisms involving $FoxP3^+$ Tregs could potentially contribute to the observed prolongation in graft survival in the combined integrin and costimulatory blockade recipients. Examining the draining lymph nodes of BALB/c graft recipients in which heterologous immunity had been induced, we found that the percentage of $CD4^+FoxP3^+$ Tregs was significantly higher in recipients treated with combined CoB and anti-LFA-1 at both early and late time-points compared to untreated recipients or recipients treated with CoB alone (Figures 22A and B). Importantly, while this accumulation of Tregs in the draining lymph nodes may contribute to graft survival, it is not sufficient by itself, as a similar accumulation was observed in the recipients treated with anti-LFA-1 alone, despite their early graft rejection (Figures 22A and B).

Combined blockade suppresses cytokine responses of human memory $CD8^+$ T cells

We next extended our findings to human alloreactive memory T cells. Peripheral blood mononuclear cells (PBMCs) were obtained from human responder-stimulator pairs, none of which had prior history of transfusion, pregnancy or solid organ transplant. These responder and stimulator PBMCs were co-cultured along with different immunosuppressant reagents, after which IFN-γ and TNF cytokine production by $CD8^+CD45RA^-$ memory T cells was determined through intracellular cytokine staining. Given the wide variation in

alloreactive T cell precursor frequency between different responder-stimulator pairings (206), the data was normalized against the peak cytokine response obtained with no treatment. Whereas treatment with belatacept alone failed to attenuate the percentage of cytokine producers amongst the alloreactive memory T cell population compared to untreated controls, combined therapy with belatacept and anti-LFA-1 mAb led to a statistically-significant reduction in IFN-γ production by the alloreactive memory T cells, as well as a trend towards lower TNF production (Figures 23A and B). Thus, combined integrin and costimulatory blockade also appears to have efficacy against human heterologous alloreactive memory T cell effector responses *in vitro*.

Integrin and costimulatory blockade prolongs kidney allograft survival in non-human primates

Having validated the efficacy of combined costimulatory and integrin blockade in murine transplant systems (as well as demonstrating the efficacy of this regimen against human memory T cell alloresponses *in vitro*), we attempted to translate the regimen to a preclinical kidney transplant model using rhesus macaques (Figure 24). Because we wished to evaluate a regimen with maximal translational potential, and because efalizumab (humanized anti-LFA-1) is currently not clinically available, we instead utilized natalizumab (humanized anti-VLA-4). Transplant recipients treated with the combined regimen were administered natalizumab (10mg/kg IV) on post-transplant days 0, 4, 7, 14, 21, 28, 35 and 42, as well as belatacept (20mg/kg IV) on post-transplant days 0, 4, 7, 14, 28, 42, 56, 70 and 84. Kidney allograft recipients treated with belatacept monotherapy rejected their allografts with a median survival time of 10 days (rejected on day 8 and 12). This observed rate of rejection is more rapid compared to historical controls with belatacept monotherapy

(previously, MST= 45 days), likely reflecting the more stringent MHC mismatching in our transplant pairs compared to earlier experiments, which did not utilize 454 deep sequencing of the donor/recipient MHC haplotypes (12). In contrast, in limited pilot studies of the combined natalizumab and belatacept regimen, transplant recipients experienced significantly prolonged allograft survival (rejection at day 147, >66 and >60 days). Treatment with combined natalizumab and belatacept is associated with a marked lymphocytosis that resolved upon discontinuation of the natalizumab (data not shown). Encouragingly, treatment with the combined regimen was not associated with cytomegalovirus viremia, providing evidence that this regimen does not cause a devastating suppression of protective immunity (data not shown).

Chapter 4: Th17 cells as a barrier to costimulatory blockade

Anti-IL-12/23 synergizes with costimulatory blockade to prolong murine skin graft and heterotopic cardiac allograft survival

As previously discussed, Th17 T cells possess pro-inflammatory effector functions and are not dependent on traditional costimulatory signals for full activation, rendering them ideal candidates for mediating costimulatory blockade-resistant transplant rejection. To evaluate whether Th17 cells could contribute to costimulatory blockade-resistant transplant rejection, we performed fully allogeneic BALB/c => C57BL/6 skin grafts and treated graft recipients with CoB alone (including anti-CD154 and CTLA-4Ig), anti-IL-12/23 alone, or combined CoB + anti-IL-12/23 (Figure 25A). Because Th17 immune responses are dependent on IL-23, anti-IL-12/23 would be expected to arrest the development of a Th17 alloresponse (in addition to abrogating a Th1 alloresponse, as Th1 cell differentiation requires IL-12). Whereas recipients treated with anti-IL-12/23 alone rejected their grafts with the same kinetics as untreated controls, those treated with the combined regimen of CoB + anti-IL-12/23 showed a significant prolongation of graft survival (Median survival time = 67 days vs. 20 days for CoB alone, $p < 0.0001$).

The efficacy of combined costimulatory and IL-12/23 blockade was next confirmed in a fully vascularized heterotopic cardiac allograft system. We performed BALB/c => C57BL/6 cardiac transplants and treated with CTLA-4Ig alone or combined CTLA-4Ig + anti-IL-12/23 (Figure 25B). It is well-established that full costimulatory blockade with both CTLA-4Ig and anti-CD154 allows almost indefinite cardiac allograft survival, so we elected to use only CTLA-4Ig for costimulatory blockade in this transplant model. Again, we demonstrated that the combined regimen (CTLA-4Ig + anti-IL-12/23) substantially

prolonged cardiac allograft survival (MST= 141 days) compared to recipients treated with CTLA-4Ig alone (MST= 63 days, p= 0.01).

Prolongation of transplant survival with adjunct anti-IL-12/23 is mediated predominantly by IL-23 blockade

Although use of adjunct anti-IL-12/23 enhanced the efficacy of costimulatory blockade in murine transplant models, it remained unclear whether the prolongation of transplant survival is mediated predominantly by IL-12 or IL-23 blockade. We therefore repeated the skin graft experiments using selective IL-12 or IL-23 blockade. For selective IL-12 blockade, we transplanted BALB/c skin grafts into B6.IL-12Rβ2$^{-/-}$ recipients, and then treated graft recipients with costimulatory blockade. For selective IL-23 blockade, we performed BALB/c => C57BL/6 skin grafts and treated the recipients with CoB and a polyclonal anti-IL-23 (raised against the IL-23p19 component, possessing no cross-neutralization with IL-12). Although the B6.IL-12Rβ2$^{-/-}$ recipients demonstrated no prolonged graft survival, those C57BL/6 recipients treated with anti-IL-23 and CoB experienced almost equivalent graft survival as those treated with CoB and anti-IL-12/23 (Figure 26). Thus, the efficacy of anti-IL-12/23 seems to be mediated predominantly by its blockade of IL-23, suggesting a potential role for Th17 cells in mediating CoB-resistant transplant rejection. However, this Th17-mediated rejection may be IL-17A-independent, as the combination of CoB and anti-IL-17A failed to prolong graft survival (Figure 27).

Combined costimulatory and IL-12/23 blockade inhibits alloreactive T cell proliferation

To assess the mechanisms by which combined costimulatory and IL-12/23 blockade can prolong transplant survival, we performed a variety of mechanistic assays. We first assessed the impact of the combined blockade regimen on alloreactive T cell proliferation by performing an *in vivo* mixed lymphocyte reaction. CFSE-labeled C57BL/6$^{CD45.1\ congenic}$ splenocytes were adoptively transferred into sublethally irradiated BALB/c mice, establishing a model of graft-versus-host disease in which the CFSE-labeled responders proliferated against the stimulus of the BALB/c host. After treatment for 72 hours with no therapy, anti-IL-12/23 alone, CoB alone, or combined CoB + anti-IL-12/23, the mice were sacrificed and proliferation of the CFSE-labeled responders was assessed by flow cytometric analysis. As expected, the untreated BALB/c hosts showed significant proliferation of the C57BL/6 responders (Figure 28). Treatment with anti-IL-12/23 alone was associated with almost equivalent allo-proliferation, whereas those treated with CoB alone had significantly reduced responder proliferation in both $CD4^+$ and $CD8^+$ T cells. Treatment with CoB + anti-IL-12/23 demonstrated even further suppression of responder proliferation compared to CoB alone.

Treatment with combined costimulatory and IL-12/23 blockade causes no additional expansion of alloreactive Tregs compared to costimulatory blockade alone

We initially hypothesized that anti-IL-12/23 may help enhance the expansion or accumulation of $FoxP3^+$ regulatory T cells (Tregs) in transplant recipients. IL-23 is required for transdifferentiation of Tregs into Th17 cells, and thus blockade of IL-23 may prevent this transdifferentiation-induced loss of Tregs. Furthermore, as Th17 and Tregs may emerge from a common progenitor, blockade of Th17 proliferation through anti-IL-23 may serve to shunt these progenitors towards a regulatory T cell developmental pathway. Although

treatment with any regimen containing costimulatory blockade caused a loss of *total* FoxP3$^+$ Tregs due to the dependence of Treg development on CD28 signaling (data not shown), we employed the *in vivo* MLR system to specifically assess the impact of these regimens on *allospecific* Treg accumulation. We performed the *in vivo* MLR as previously described, and at the time of sacrifice (72 hours after adoptive transfer of CFSE-labeled responders), we performed intracellular FoxP3 staining to evaluate the percentage of alloreactive T cells (*i.e.* those displaying proliferation by CFSE dilution) that were FoxP3$^+$. In contrast to the effect of costimulatory blockade on *total* FoxP3$^+$ Tregs, we observed that regimens containing costimulatory blockade induced an *increased* accumulation of *allospecific* FoxP3$^+$ T cells (Figures 29A and B). However, contradicting our original hypothesis, we saw no additive benefit of anti-IL-12/23 in promoting the accumulation of allospecific Tregs. However, this result does not fully exclude the possibility that the prolonged graft survival observed with combined costimulatory and IL-12/23 blockade could be partially attributed to dominant immunoregulatory mechanisms mediated by an accumulation of FoxP3$^+$ Tregs.

Combined costimulatory and IL-12/23 blockade induces immunodeviation away from Th1 and Th17 alloresponses

We next hypothesized that IL-12/23 blockade may synergize with costimulatory blockade to induce immunodeviation away from pathologic Th1 and Th17 alloresponses. We again performed BALB/c => C57BL/6 skin grafts, and the recipients were either untreated or administered an immunosuppression regimen (*i.e.* CoB alone, anti-IL-12/23 alone, or combined blockade). We sacrificed the graft recipients on post-transplant day 24, a time point chosen because the majority of recipients treated with CoB alone will have rejected their grafts after 24 days, whereas those treated with combined CoB + anti-IL-

12/23 should have intact grafts. We harvested axillary draining lymph node cells from these recipients, and after a 48 hour co-culture with fresh BALB/c splenocyte stimulators, we performed cytometric bead array analysis on the supernatants for IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A and IL-10. Compared to the recipients treated with CoB alone, those treated with CoB + anti-IL-12/23 had lower levels of Th1 cytokines (*e.g.* IFN-γ) as well as Th17 cytokines (*e.g.* IL-17A) (Figures 30A and B). Interestingly, although recipients treated with CoB alone had less Th1 cytokines in their dLN compared to untreated recipients (Figure 30A), they also displayed markedly *higher* levels of IL-17A (Figure 30B). Thus, it appears that treatment with CoB alone actually *augmented* the production of IL-17A in the draining lymph node. We also validated these conclusions on the mRNA transcript level by performing quantitative real-time PCR on dLN cells harvested from POD#24 BALB/c => C57BL/6 skin graft recipients. Again, we showed that treatment with a combined regimen of CoB + anti-IL-12/23 caused markedly less transcription of IL-17A compared to treatment with CoB monotherapy (Figures 31A and B). As some recent evidence suggests that GM-CSF may be the chief pro-inflammatory mediator released by Th17 T cells, we also examined the production of GM-CSF as a result of allostimulation. We performed skin grafts using BALB/c donors and C57BL/6 recipients, treated with one of our immunosuppression regimens and then co-cultured dLN cells with fresh BALB/c stimulators for 48 hours, after which supernatants were collected for cytometric bead array analysis. In comparison to naïve BALB/c cells, allostimulation resulted in abundant production of GM-CSF (Figure 32). Consistent with a suppression of alloreactive Th17 responses, graft recipients treated with anti-IL-12/23 demonstrated reduced levels of GM-CSF.

Cellular sources of alloreactive IL-17A production

Many different cellular sources of IL-17A have been described, including $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells. To examine the cellular source of IL-17A induced by allostimulation, we performed skin grafts in C57BL/6 recipients, using grafts from BALB/c donors. Skin graft recipients were treated with CoB alone. After sacrifice on post-transplant day 15 (*i.e.* near peak of rejection response), axillary draining lymph nodes were harvested and subjected to magnetic-assisted cell sorting to purify total T cells, $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells. Flow cytometry confirmed >97% purity of the sorted populations (data not shown). Sorted cell populations were co-cultured with fresh BALB/c splenocytes (2:1 stimulator-to-responder ratio), and supernatants were harvested after 48 hours for cytometric bead analysis of IL-17 production. While both $CD4^+$ and $CD8^+$ T cells produced IL-17A in response to allostimulation, $CD4^+$ T cells (*i.e.* Th17 cells) were the dominant source (Figure 33). Very few γδ T cells were obtained from the dLN, and although IL-17 production by these cells was evident by RT-PCR (data not shown), none was detected at a protein level.

Characterization of belatacept-resistant kidney allograft rejection in non-human primates

After demonstrating that Th17 cells may help mediate costimulatory blockade-resistant transplant rejection in murine transplant systems, we next attempted to translate these observations to a preclinical transplant system utilizing kidney transplants in rhesus macaques. We first characterized the mechanisms of kidney allograft rejection in recipients treated with belatacept alone. We performed real-time PCR on kidney biopsies obtained at the time of rejection, and found that (in comparison to naïve kidney specimens) IFN-γ and

IL-17A transcripts were >100-fold enriched in the rejecting kidneys (Figure 34). We also extracted graft-infiltrating lymphocytes from the rejected kidney allografts of belatacept-treated recipients. Characterization of the chemokine receptors expressed by these graft-infiltrating cells showed that many of these cells are $CCR6^+$, consistent with Th17 cells (Figure 35). In contrast, peripheral blood from these same recipients showed almost no $CCR6^+$ lymphocytes. Together, these findings are consistent with the presence of Th17 cells in rejected kidney allografts of recipients treated with belatacept alone.

Belatacept synergizes with ustekinumab to prolong kidney allograft survival in non-human primates

Given the evidence that Th17 cells may play a role in belatacept-resistant transplant rejection, we next assessed whether use of ustekinumab (a humanized anti-IL-12/23 antibody) could augment the efficacy of costimulatory blockade with belatacept in a rhesus macaque kidney transplant model (Figures 36A and B). Kidney allograft recipients treated with ustekinumab monotherapy promptly rejected their kidneys with kinetics similar to historic untreated controls (MST= 7 days, n=2) (Figure 36A). Recipients treated with belatacept monotherapy also demonstrated early allograft loss (rejection at 8 and 12 days). In contrast, recipients treated with combined belatacept and ustekinumab demonstrated significantly prolonged allograft survival (rejection at 158, 149, >105, 51 and 51 days, MST= 149 days) (Figure 36B). Immunohistochemistry analysis of recipients treated with belatacept or ustekinumab monotherapy demonstrated an abundant lymphocytic infiltrate of the kidney allograft at the time of rejection (Figure 37A). Consistent with the markedly prolonged graft survival observed with combined therapy, those recipients treated with combined belatacept and ustekinumab showed significantly reduced tubulitis and arteritis, even 100 days post-

transplant (Figure 37B). The regimen of combined belatacept and ustekinumab was well-tolerated in allograft recipients, with no observed adverse side effects. Weekly monitoring of $CD4^+$ T cells, $CD8^+$ T cells and B cells demonstrated that this regimen caused no lymphocyte depletion (Figures 38A-C). Although there was an early increase in the percentage of effector memory T cells, this quickly plateaued and was not associated with transplant rejection (Figure 38D). Protective immunity also appears to be somewhat preserved by this combined regimen, as no cytomegalovirus reactivation was observed in any of the recipients treated with this combined regimen (Figure 39).

Chapter 5: **Discussion**

Costimulatory blockade may ultimately prove to be a revolutionary new strategy for transplant immunosuppression, offering superior long-term graft outcomes without the nephrotoxicity that encumbers conventional immunosuppressants such as cyclosporine and tacrolimus. However, the immense promise of costimulatory blockade is challenged by the phenomenon of blockade-resistant transplant rejection and the resulting high rates of acute rejection experienced by belatacept-treated patients in the BENEFIT trial (14). In this dissertation, we have explored the mechanisms that underpin CoB-resistant transplant rejection, which we believe is primarily mediated by T cell subsets that have reduced requirements for costimulation. In particular, we have focused on memory T cells and Th17 T cells, as both of these subsets are known to possess diminished costimulatory thresholds. Utilizing a variety of both vascularized and non-vascularized murine transplant systems, we demonstrated that both of these T cell subsets participate in costimulatory blockade-resistant transplant rejection. Furthermore, we coupled traditional costimulatory blockade with adjuvant immunosuppressants that specifically target these subsets, using integrin antagonists (anti-LFA-1 and anti-VLA-4) to suppress alloreactive memory T cells and employing anti-IL-12/23 to attenuate Th17 alloresponses. Finally, we translated these findings to an advanced pre-clinical model, demonstrating that kidney allograft survival is significantly prolonged in rhesus macaques when belatacept is coupled with either natalizumab (humanized anti-VLA-4) or ustekinumab (humanized anti-IL-12/23).

Our initial line of experimentation examined the role played by donor-specific memory T cells in CoB-resistant transplant rejection. We developed a unique transplant system that specifically models CoB-resistant rejection by a donor-specific $CD8^+$ memory T cell recall response. In this transgenic system, $CD8^+$ memory T cells specific for an epitope

of chicken ovalbumin (OVA) are generated through the adoptive transfer of naïve T cells bearing a transgenic TCR specific for OVA, followed by a subsequent infection with OVA-expressing *Listeria*. After the induction of memory, these OVA-specific $CD8^+$ memory T cells are re-challenged with a skin graft from a transgenic mouse that ubiquitiously expresses membrane-bound OVA in its tissues. Using this experimental system, we identified the integrins LFA-1 and VLA-4 as playing important roles in the costimulation-independent donor-specific memory T cell recall response. Antagonists against these integrins synergized with CoB to significantly prolong skin graft survival in this model. These findings are of obvious translational significance, as humanized monoclonal antibodies against both LFA-1 (efalizumab) and VLA-4 (natalizumab) have been developed and are FDA-approved for clinical use against autoimmune diseases. In contrast, several groups have explored other regimens to improve the efficacy of CoB against memory alloresponses, but these have employed reagents that are not clinically relevant (58, 59, 207).

We defined several mechanisms of action for anti-LFA-1 and anti-VLA-4 in prolonging graft survival against a CoB-resistant memory T cell response. As demonstrated by T cell functional assays, VLA-4 antagonism in the setting of concurrent costimulatory blockade dramatically suppresses trafficking of alloreactive memory/effector T cells to the graft, whereas LFA-1 blockade both impairs T cell trafficking to the graft and additionally attenuates T cell effector functions during the memory recall response such as cytotoxicity and cytokine production. These functional assays therefore highlight the distinct immunological pathways employed by integrins during the costimulation-independent activation and function of memory T cells. One recent report also evaluated LFA-1 blockade on early $CD8^+$ memory T cell infiltration of allografts and found that it reduced trafficking to allografts (208). Notably, we also demonstrate that anti-LFA-1 blockade

diminishes trafficking to the graft (Figure 14B), and differences in the degree of impaired trafficking noted in these two studies may potentially be attributed to the different time-points and different target organs (heart vs. skin) evaluated.

Interestingly, although prolonged graft survival was only observed in recipients treated with dual costimulatory/integrin blockade regimens, treatment with anti-LFA-1 alone appeared to have similar effects on memory T cell effector functions, at least as measured in our *in vitro* studies. Importantly, all of these functional assays only evaluated the effect of immunosuppression regimens on the Thy1.1$^+$ graft-specific memory T cells, leaving open the possibility that the addition of CoB enhances graft survival through mechanisms independent of graft-specific T cells. For example, several investigators have found that costimulatory blockade can induce an accumulation of CD4$^+$FoxP3$^+$ Tregs in grafts (209, 210). An alternative hypothesis is that costimulatory blockade may prolong graft survival through independent tolerance-promoting effects on innate immune cells such as dendritic cells, which were not assessed in this report (211, 212). Finally, the synergistic survival benefit conferred by CoB and anti-LFA-1 may be due to augmented inhibition of memory/effector T cell trafficking to the graft, as less donor-specific T cell infiltration is observed in the graft with either combined integrin/costimulatory blockade regimen compared to either integrin antagonist alone (Figure 13).

How does costimulatory blockade synergize with anti-LFA-1 and anti-VLA-4 to inhibit the trafficking of memory/effector T cells to the graft? Several potential mechanisms can be invoked. First, graft infiltration by T cells in CoB-treated recipients is dramatically reduced when neutrophil infiltration of the graft is impaired (213). Given that both LFA-1 and VLA-4 are involved in the transmigration of neutrophils into tissues (214), the synergistic reduction in graft infiltration observed with combined blockade regimens

compared to CoB treatment alone may be due to an effect on these innate immune cells. An alternative explanation is based on the recent finding that although memory T cell effector responses are costimulation-independent, the ability of memory T cells to activate innate immune cells and thereby enhance inflammation and tissue injury remains critically dependent on CD154 signaling (215). As local tissue inflammation augments the expression of integrin ligands such as ICAM-1 and to a greater extent VCAM-1 (29), CoB may synergize with integrin antagonists by inhibiting graft-expression of integrin ligands, further inhibiting integrin-mediated transmigration of memory/effector T cells into the graft. This synergy between costimulatory and integrin blockade may be particularly critical for memory and secondary effector T cell trafficking (Figures 12 and 13), as integrin antagonists alone were sufficient to prevent trafficking of primary effectors (Figure 14).

The mOVA transgenic transplant system utilized in these experiments possesses several notable advantages over older models of memory T cell-mediated rejection, most of which relied upon anti-donor memory induced by the rejection of a primary graft in an untreated transplant recipient. Because the OT-I cells employed in the mOVA transplant system express the Thy1.1 marker, our system enables the graft-specific memory T cells to be specifically identified and tracked, permitting mechanistic studies previously limited with other transplant systems. Additionally, as LM-OVA infection does not induce an appreciable anti-OVA antibody response, our system allowed us to specifically focus on rejection mediated by a memory T cell recall response. In other transplant systems used to study memory alloresponses, the contribution of memory T cells to graft rejection is often confounded by the contribution of anti-donor antibody. Finally, the initial priming stimulus in this system is of limited duration, as LM-OVA infection is completely cleared after 6 days (216). Thus, this system ensures that donor-specific T cells at the time of transplant are true

memory T cells, as they persist for over 30 days by the time of re-challenge despite an absence of antigen.

Despite its advantages, our experimental approach also bore some limitations. First, given that our *in vitro* assays are measured 7 days after re-challenge with an mOVA graft, it is difficult to assess whether the activity of the combined blockade regimen is impacting memory $CD8^+$ T cells themselves or $CD8^+$ effector cells that have differentiated during the memory recall response. Second, in the mOVA transplant system we employ a non-vascularized skin graft model, which may differ from the memory recall responses encountered with vascularized clinical transplants. Next, this system utilized a fully MHC-matched transplant pairing, with rejection targeted against only a nominal antigen (ovalbumin); this may not be relevant to fully allogeneic memory recall responses. Finally, this transgenic transplant system fails to accurately model cross-reactive heterologous immunity, as the epitope used to prime the memory T cells was *identical* to the antigen recognized on the donor graft. In true heterologous immunity, pathogen-specific memory T cells likely recognize a cross-reactive, non-identical donor antigen, for which the T cells may possess altered affinity.

To address some of the limitations of this mOVA transplant system, we next extended our findings to a fully allogeneic transplant system that more closely recapitulates an allo-crossreactive heterologous memory immune response. We utilized an established model of heterologous immunity in which alloresponses are generated against BALB/c donor antigens via sequential infection with LCMV and Vaccinia virus (26). Consistent with our previous results, we found that while CoB alone could not prolong graft survival against a heterologous immunity memory response, an immunosuppression regimen using combined costimulatory and LFA-1 blockade did enable durable graft survival.

In contrast to our earlier results in the mOVA transplant system, however, the regimen of CoB + anti-VLA-4 failed to prolong graft survival in our model of a heterologous memory alloresponse. This difference may be explained by the impact of VLA-4 blockade on bone marrow engraftment. Several groups have demonstrated that VLA-4 is required for T cell and hematopoietic stem cell homing to the bone marrow (202-204, 217), and unlike recipients treated with CoB + anti-LFA-1, those treated with CoB + anti-VLA-4 failed to demonstrate successful engraftment of the BALB/c bone marrow transplant. Establishment of durable mixed chimerism may be required for long-term allogeneic skin graft survival in this very stringent transplant system, explaining why CoB + anti-VLA-4 failed to prolong graft survival. Alternatively, this difference may reflect different integrin utilization between low-affinity memory T cells (*e.g.* cross-reactive T cells generated by heterologous immunity) and high-affinity memory T cells (*e.g.* ovalbumin-specific transgenic T cells utilized in our previous transplant system) (218).

The clinical potential of immunosuppression based on combined costimulatory and integrin blockade is perhaps best reflected both in our non-human primate kidney transplant system and in our *in vitro* human allostimulation experiments. In comparison to belatacept alone, the combination of belatacept and natalizumab significantly prolonged kidney allograft survival in non-human primates. The efficacy of natalizumab in this kidney transplant system stands in contrast to its lack of effect in the murine heterologous immunity transplant model. This discrepancy highlights the differential transplant barrier posed by kidney transplant versus skin grafting (which is a much more stringent transplant system).

Although our study reveals the potential of integrin antagonists as adjunct immunosuppressive agents, enthusiasm for these drugs must be tempered by important safety concerns raised by the early clinical experience with efalizumab and natalizumab, as

several patients receiving chronic therapy with these integrin antagonists developed progressive multifocal leukoencephalopathy (PML) (219-222). PML is an opportunistic viral infection resulting from reactivation of latent JC virus (a human polyomavirus) in the brain of immunosuppressed patients (223, 224). PML is highly lethal, with most patients succumbing to disease after it has developed. The risk of these biologics was first revealed in patients chronically treated with natalizumab, a very small handful of whom began to manifest clinical signs of PML. Importantly, the risk of PML is almost solely confined to those patients receiving long-term integrin blockade, with patients receiving at least 24 monthly infusions of natalizumab estimated to have a 1:1000 risk of developing PML (220). The PML risk signature of natalizumab initially led to its voluntary withdrawal from the market, but vocal support from multiple sclerosis patients led to the reintroduction of natalizumab under tight controls in June 2006. As of July 2010, a total of 61 patients on natalizumab have developed PML (222).

Similar to natalizumab, efalizumab also is associated with an elevated risk of PML and JC virus reactivation. Of the estimated 46,000 patients who had received treatment with efalizumab worldwide since its FDA approval in 2003, a total of four PML cases (three definite and one probable) were reported by 2009, yielding a total PML incidence of 1 in 10,000 (219). However, of those patients treated for $\geq$3 years with efalizumab (which included every patient who developed PML), the estimated incidence rate was 1 in 400 patients (219). On April 8, 2009, Genentech announced that they would voluntarily withdraw efalizumab from the market due to this PML risk, and efalizumab was phased out completely by June 2009.

While a remote risk of developing a fatal neurodegenerative condition may be utterly unacceptable for patients with a relatively benign condition such as psoriasis, it may certainly

be tolerable in the transplant arena, especially if the novel integrin blockade therapy prolongs allograft function compared to conventional immunosuppression. The argument for exploring transplant indications for integrin antagonists is further bolstered by the fact that many drugs currently used to treat transplant patients (such as mycophenolate mofetil and rituximab) actually carry similar risks for PML compared to either natalizumab or efalizumab (219, 225, 226). Furthermore, the likely duration of therapy for integrin antagonists used in a transplant setting would likely prove protective against PML development. The epidemiology of PML in patients with autoimmune diseases treated with integrin antagonists confirms that the risk of PML increases substantially with duration of therapy: the incidence of PML is 0.01 per 1000 patients for those who received 1 to 12 infusions of natalizumab, 1.27/1,000 for those who received $\geq$12 infusions and 1.71/1,000 in those who received $\geq$24 infusions (222). Integrin antagonists would likely be employed as short-term perioperative induction immunosuppression therapy in transplant patients, which should substantially mitigate the risks posed by integrin blockade.

Thus, while the risks of integrin blockade must not be neglected, they certainly do not exclude integrins as a viable target for transplant immunosuppression. The experimentation found in this dissertation offers the potential that combined integrin and costimulatory blockade might be an effective immunosuppression regimen, especially for patients with high frequencies of donor-specific memory T cells who would otherwise be vulnerable to transplant rejection if treated with costimulatory blockade alone. Further primate and clinical trials with combined integrin and costimulatory blockade are certainly merited in the future to evaluate the efficacy and risks of this novel immunosuppression strategy.

An alternative strategy to address the clinical barrier posed by costimulatory blockade-resistant transplant rejection is offered by our more recent line of experimentation. We found that Th17 cells may play an important role in this blockade-resistant rejection, demonstrating that the draining lymph nodes of murine skin graft recipients treated with costimulatory blockade produced copious quantities of IL-17A in response to allostimulation. Interestingly, treatment of skin graft recipients with costimulatory blockade elicted more robust IL-17A production in the draining lymph nodes (dLNs) than no treatment at all. Based on our findings, we hypothesize that traditional costimulatory blockade is highly effective at suppressing the early post-transplant production of Th1 cytokines such as IFN-γ, which are known to potently antagonize the polarization of Th17 T cells (89, 227-229). Thus, by suppressing Th17-antagonistic cytokines, costimulatory blockade unveils a latent Th17 alloresponse that is not evident in traditional acute allograft rejection (Figure 40).

To target these Th17 alloresponses, we coupled costimulatory blockade with anti-IL-12/23 antibody that recognizes the common IL-12p40 subunit. This adjunct immunosuppressant should suppress both Th1 rejection responses (through blockade of IL-12), as well as Th17 alloresponses (through blockade of IL-23). Although a humanized monoclonal antibody targeting IL-12/23 is in current clinical use to treat autoimmunity (230), very few reports of the use of this reagent in transplantation exist in the literature. Cao *et al.* found that anti-IL-23 prevents chronic rejection in a rat orthotopic tracheal transplant system (231). Another group utilized murine anti-IL-12/23p40 antibody as monotherapy and demonstrated a slight prolongation of murine cardiac allograft survival (232). To our knowledge, we are the first group to couple anti-IL-12/23 with costimulatory blockade in a transplant setting.

We validated the efficacy of this combined blockade regimen in both non-vascularized murine skin grafts and vascularized heterotopic cardiac transplants. We demonstrated that this combined regimen potently suppresses allospecific T cell proliferation and induces immunodeviation away from Th1 and Th17 cytokine responses in the dLNs. Furthermore, we examined which pathway (Th1 versus Th17) was predominantly responsible for the synergy with CoB, finding that Th17-selective blockade (with a polyclonal anti-IL-23p19 antibody) secured almost the same prolongation of graft survival as treatment with the full anti-IL-12/23 monoclonal. Importantly, this last finding does not exclude a role for the suppression of Th1 alloresponses in prolonging graft survival.

We unfortunately were unable to optimize intracellular cytokine staining for IL-17A in allostimulated T cells. This failing compels us to be careful of our interpretation of the cytometric bead array cytokine measurements. First, we cannot definitively state that all of the IL-17A we measure is produced by "classical" Th17 T cells. Indeed, our magnetic-assisted cell sorting experiments indicated that although $CD4^+$ T cells are the primary producers of IL-17A after allostimulation, $CD8^+$ Tc17 cells also contribute to the production of this cytokine in the dLN. Even if most of the IL-17A is produced by $CD4^+$ helper T cells, it need not stem from classical Th17 cells, as recent studies have revealed a surprising degree of plasticity in many of the helper T cell subsets, especially Th17 cells. Several studies have shown that in the presence of a conducive inflammatory milieu, Tregs can convert to pro-inflammatory IL-17A-producing Th17 cells (233-235). Additionally, under certain polarizing conditions (such as exposure to IFN-γ or IL-12), Th17 cells will upregulate the T-bet transcription factor and begin producing IFN-γ (236-238). Adoptive transfer of Th17 T cells into lymphopenic hosts will also promote their production of IFN-γ (239, 240). Fate mapping experiments have revealed that IL-17^+ IFN-γ^+ T cells all originally derive from

Th17 cells (241). These dual-producers of IFN-γ and IL-17A may be the chief pathologic mediators of autoimmunity (and potentially of allograft rejection), as they are often found to infiltrate target organs in autoimmune disease models, including the central nervous system in EAE (242) and the colon during experimental colitis models (153, 243). Consistent with this hypothesis that dual-producers of IFN-γ and IL-17A may be particularly harmful to allografts, we found that the dLNs of rejecting skin grafts all produced large quantities of IFN-γ, even despite treatment of recipients with costimulatory blockade.

Importantly, while Th17 alloresponses do seem to contribute to CoB-resistant transplant rejection in our murine transplant systems, our work does not suggest that IL-17A itself is necessarily the chief cytokine mediator of this rejection. Indeed, in a limited pilot study, we failed to prolong graft survival when treating murine skin graft recipients with combined costimulatory and IL-17A blockade. This failure may potentially be attributed to insufficient dosing of the anti-IL-17A monoclonal, or perhaps to redundant signaling through Th17-produced IL-17F. However, an alternative explanation is that some other cytokine effector molecule produced by Th17 cells may be responsible for the transplant rejection. What may this pathogenic cytokine mediator be? Recent studies from several groups have highlighted GM-CSF as being a likely candidate.

It has long been appreciated that Th17 cells produce large quantities of GM-CSF, and that this cytokine helps regulate IL-6 and IL-23 *in vivo* to promote the generation and maintenance of Th17 cells (244, 245). More recently, it was determined that GM-CSF may be the chief pathogenic cytokine produced by Th17 cells in experimental models of autoimmunity (246-248). We found that allostimulated dLN cells from CoB-treated skin graft recipients produced very large quantities of GM-CSF. This abundant GM-CSF production was at least partially suppressed in the presence of anti-IL-12/23, consistent with

the known dependence of Th17 cells on IL-23 for GM-CSF production (247). In future experiments, we intend to use neutralizing anti-GM-CSF monoclonal antibody in conjunction with CoB to see if this regimen will prolong allograft survival as effectively as combined costimulatory and IL-12/23 blockade.

Having successfully demonstrated the efficacy of targeting Th17 T cells to prevent CoB-resistant transplant rejection in mice, we next extended our findings to the pre-clinical rhesus macaque kidney transplant system. In stark contrast to monotherapy with either belatacept or ustekinumab (humanized anti-IL-12/23), combined treatment with both these clinically-available agents demonstrated a significant prolongation of kidney allograft survival in 5/5 treated recipients. This regimen was well-tolerated, as no reactivation of clinical CMV viremia was observed and no overt toxicities were noted.

In conclusion, our experiments provide compelling evidence that memory T cells and Th17 T cells are likely mediators of costimulatory blockade-resistant transplant rejection. Through a careful mechanistic characterization of this phenomenon, we identified clinically-relevant targets to suppress these T cell subsets, utilizing integrin antagonists (anti-LFA-1 and anti-VLA-4) to attenuate memory alloresponses and using anti-IL-12/23 to target Th17 cells. We demonstrated the efficacy of these combined regimens not only in murine transplant systems, but also in an advanced pre-clinical non-human primate kidney transplant model. Given the clinical availability of natalizumab and ustekinumab, our results in the primate transplant system provide compelling justification for future clinical trials of these adjuvant therapies in transplant patients receiving belatacept. It is our hope that these novel combined immunosuppression regimens may permit the long-term benefits of costimulatory blockade to be accrued without the detrimental short-term risks of increased

acute rejection, thus finally realizing the full clinical potential of costimulatory blockade as an immunosuppression strategy.

Figures and Tables

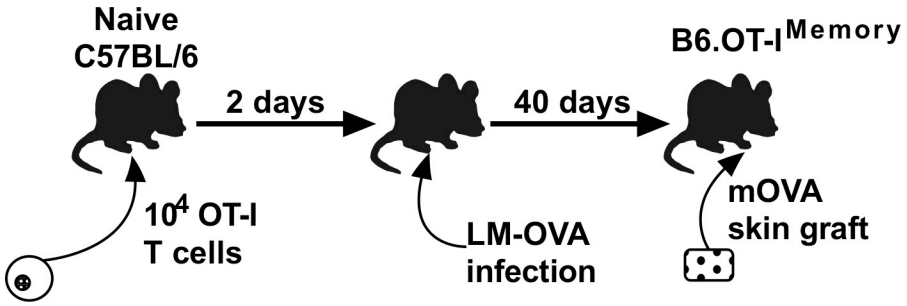

Figure 1: **Schematic of mOVA transplant system to model graft-specific memory responses.** 10^4 $CD8^+$ TCR transgenic T cells specific for ovalbumin (OVA) were adoptively transferred into naïve C57BL/6 mice that were then infected with genetically-modified *Listeria* that expresses the $OVA_{257-264}$ epitope (LM-OVA). Forty days after infection, the recipients were re-challenged with a skin graft from a mOVA transgenic mouse that ubiquitously expresses OVA in all tissues.

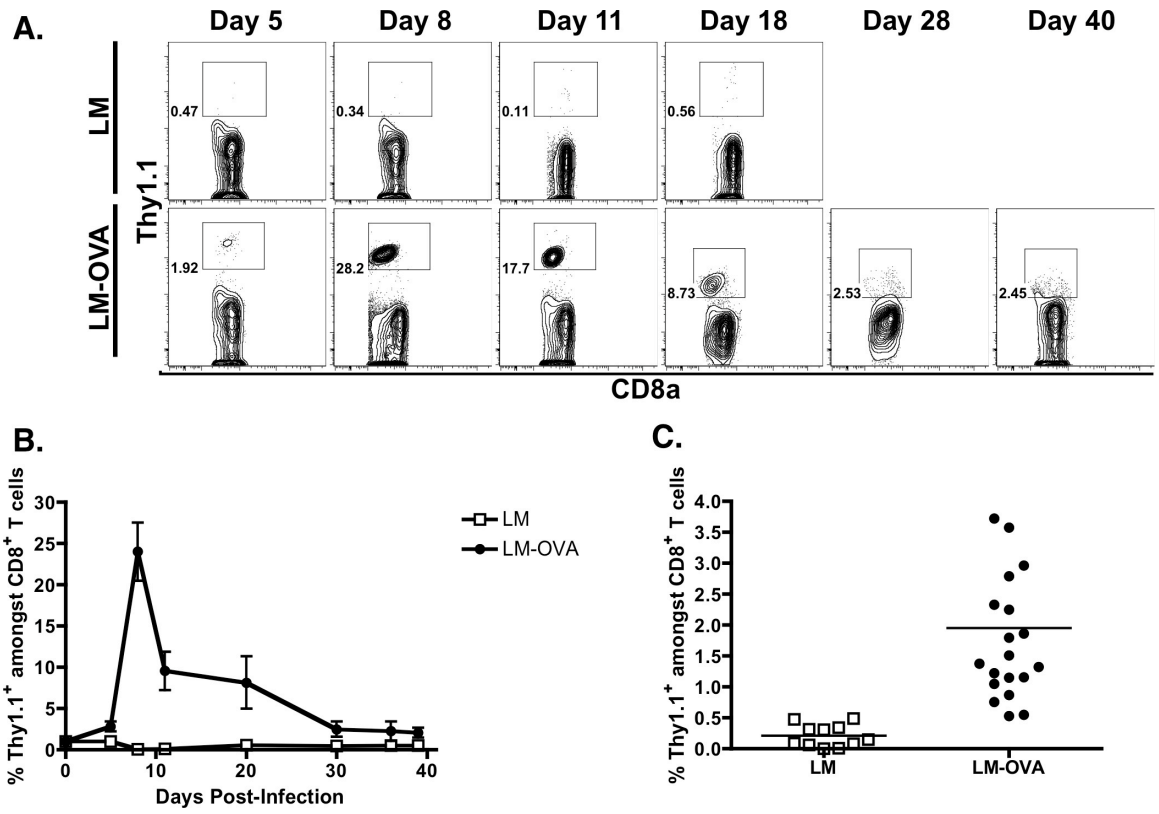

Figure 2: **Kinetics of memory OT-I cell generation after LM-OVA infection**. (A) Representative flow cytometry demonstrating time course of OT-I $CD8^+$ T cell expansion as measured by frequency of $CD8^+$ $Thy1.1^+$ T cells (in box) after wild-type *Listeria* infection (n=2) or LM-OVA infection (n=5). (B) Summary of kinetics of OT-I $CD8^+$ T cell expansion after wild-type *Listeria* (n=2) or LM-OVA infection (n=5). (C) OT-I frequency thirty days after infection with wild-type *Listeria* (n=11) or LM-OVA (n=20). Combined results from four independent experiments for each group are shown. All error bars represent the mean $\pm$ SEM.

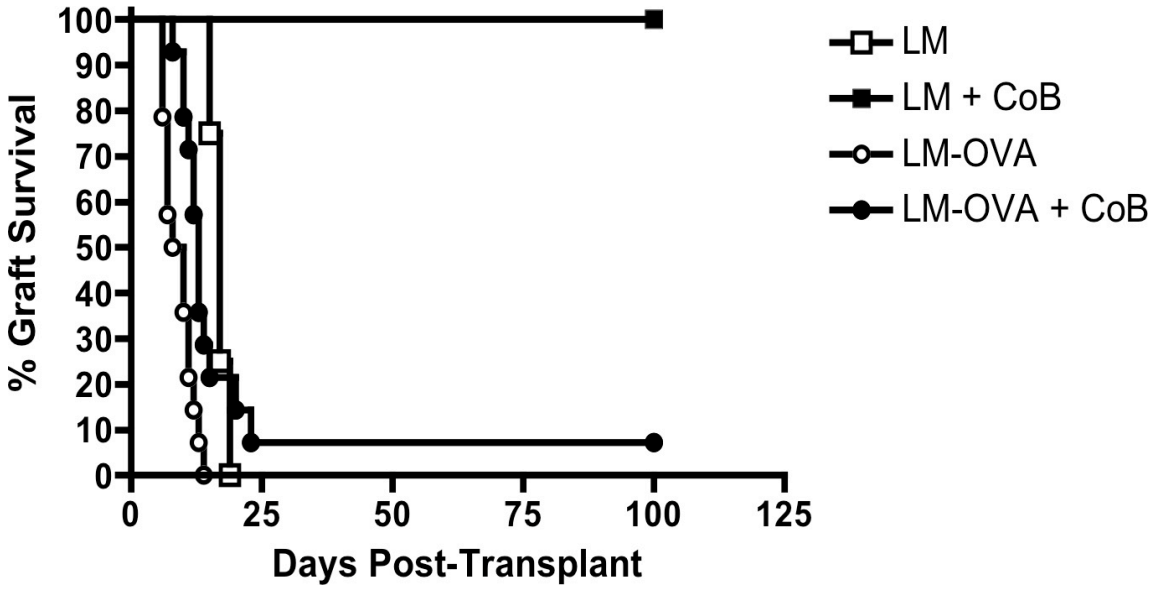

Figure 3: **Memory OT-I cells are resistant to CoB and reject mOVA skin grafts**. Survival curves of mOVA skin grafts in C57BL/6 recipients adoptively transferred with naïve OT-I T cells and then infected with either wild-type *Listeria* or LM-OVA. The recipients were either treated with costimulatory blockade or left untreated. Combined results from two independent experiments (4-14 mice/group) are shown.

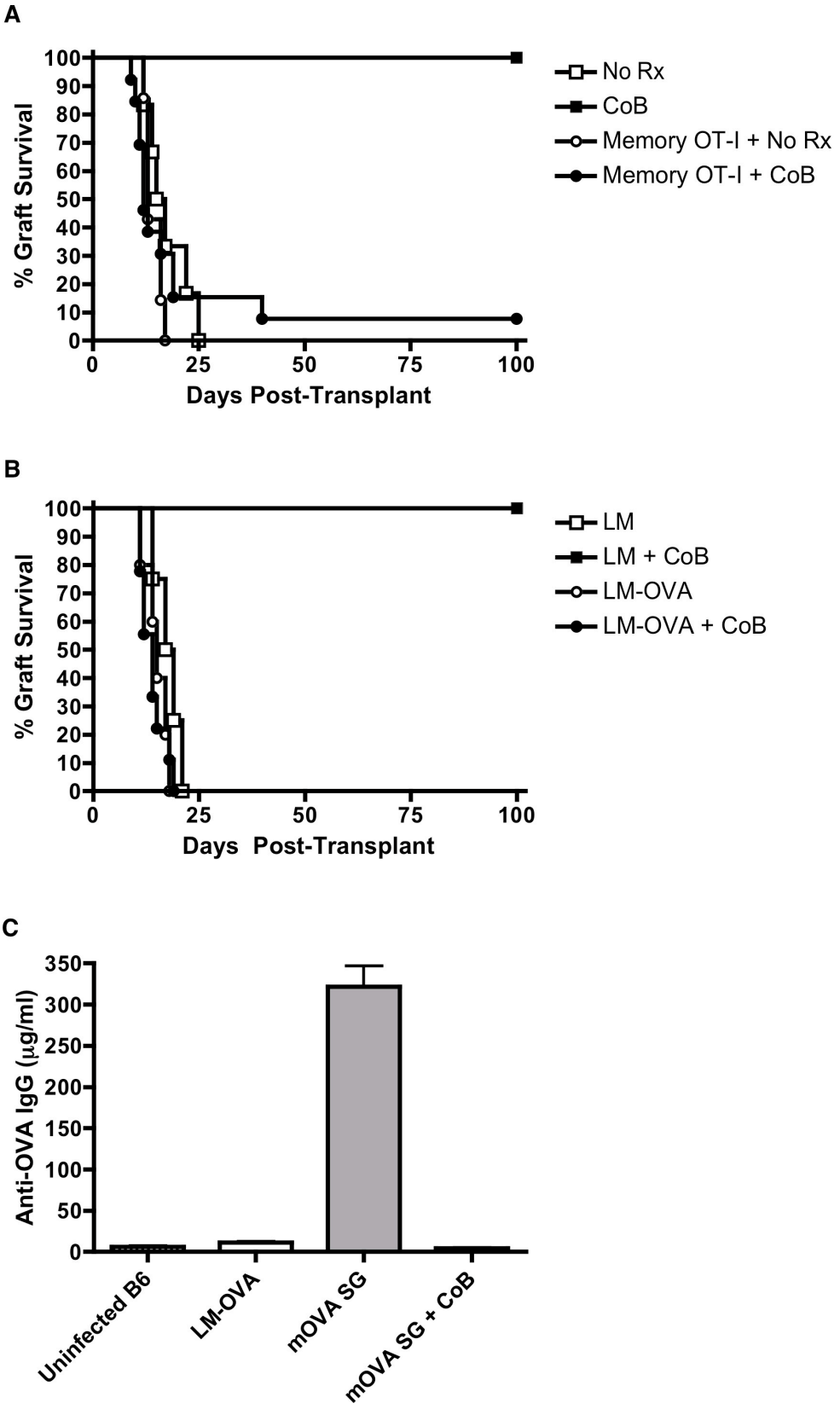

Figure 4 (next page for caption)

Figure 4: **$CD8^+$ memory T cells are sufficient to mediate costimulatory blockade-resistant transplant rejection**. (A) Survival curves of mOVA skin grafts in naïve C57BL/6 mice after adoptive transfer of 10^6 memory OT-I that were MACS-sorted from B6.OT-I^{Memory} mice. Graft recipients were either treated with costimulatory blockade or left untreated. Combined results from two independents (6-13 mice/group) are shown. (B) Naïve μMT mice lacking B-cells or serum immunoglobulin were adoptively transferred with OT-I T cells and infected with either wild-type *Listeria* (LM) or ovalbumin-expressing *Listeria* (LM-OVA). After waiting 30 days, the mice were transplanted with mOVA skin grafts and then left untreated or else treated with CoB. Combined results from 2 independent experiments (4-5 mice/group) are shown. (C) ELISA for anti-OVA IgG in naïve uninfected C57BL/6 mice, LM-OVA infected mice, naïve mice receiving an mOVA skin graft one month previously, and naïve mice receiving an mOVA skin graft that also received peri-operative CoB (n= 3 mice/group). Error bars represent the mean $\pm$ SEM.

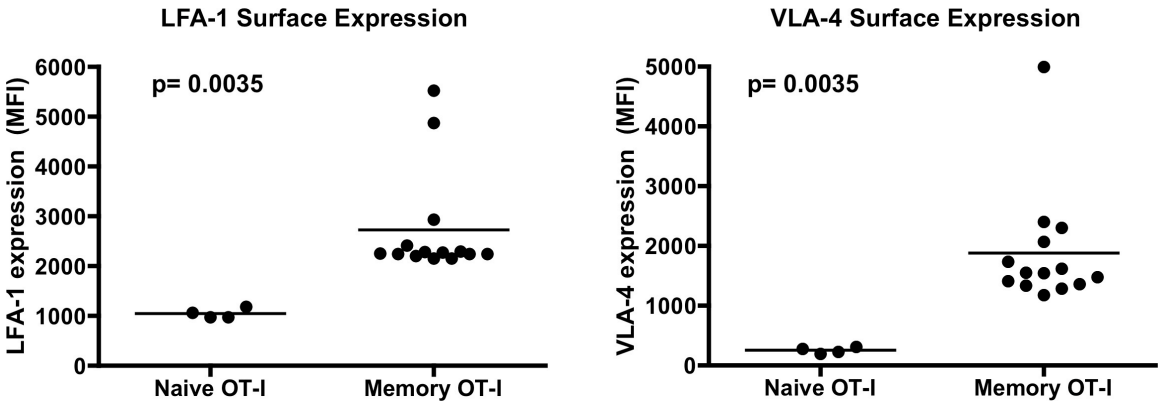

Figure 5: Integrin upregulation on memory T cells.

Surface expression of VLA-4 and LFA-1 in naïve OT-I T cells and in memory OT-I T cells (30 days after LM-OVA infection) as assessed by flow cytometry of $CD8^{+}Thy1.1^{+}$ OT-I cells obtained from the peripheral blood. Integrin surface expression is expressed as mean fluorescent intensity. Combined results from two independent experiments are shown.

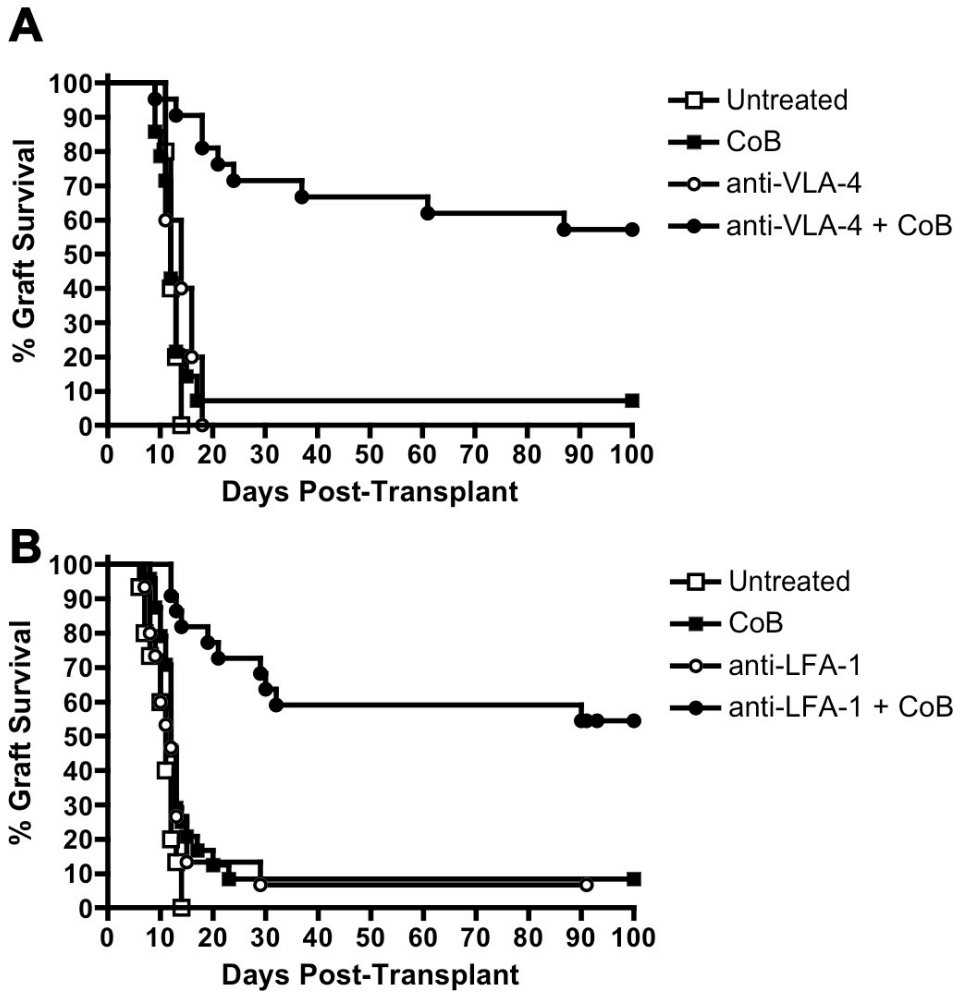

Figure 6: Dual costimulatory/integrin blockade prolongs graft survival.

(A) Survival curves of mOVA skin grafts in C57BL/6 recipients in which memory OT-I T cells were induced by LM-OVA infection. Recipients were either left untreated (n=15) or treated on POD#0,2,4 and 6 with costimulatory blockade alone (CTLA-4Ig + anti-CD154, n=24), anti-VLA-4 alone (n=5) or combined CoB + anti-VLA-4 (n=22). (B) Survival curves of mOVA skin grafts in C57BL/6 recipients in which memory OT-I T cells were induced by LM-OVA infection. Recipients were either left untreated or treated on POD#0,2,4 and 6 with costimulatory blockade alone (CTLA-4Ig + anti-CD154), anti-LFA-1 alone (n=15) or combined CoB + anti-LFA-1 (n=22). Combined results from four independent experiments are shown. All error bars represent the mean $\pm$ SEM.

Treatment	Median Survival Time (in days)
Untreated	12
CoB alone	12
αVLA-4 alone	14
αLFA-1 alone	12
αVLA-4 + CoB	**115[A]**
αLFA-1 + CoB	**101[A]**

Table 1: Combined costimulatory/integrin blockade prolongs graft survival even against a donor-specific CD8$^+$ T cell response. mOVA skin grafts were transplanted onto C57BL/6 recipients in which memory OT-I T cells were induced by LM-OVA infection. Recipients were either left untreated (n=15) or treated on POD#0,2,4 and 6 with costimulatory blockade alone (CTLA-4Ig + anti-CD154, n=24), anti-VLA-4 alone (n=5), anti-LFA-1 alone (n=15) or combined CoB + anti-VLA-4 (n=22). [A] $p < 0.0001$ vs. untreated recipients.

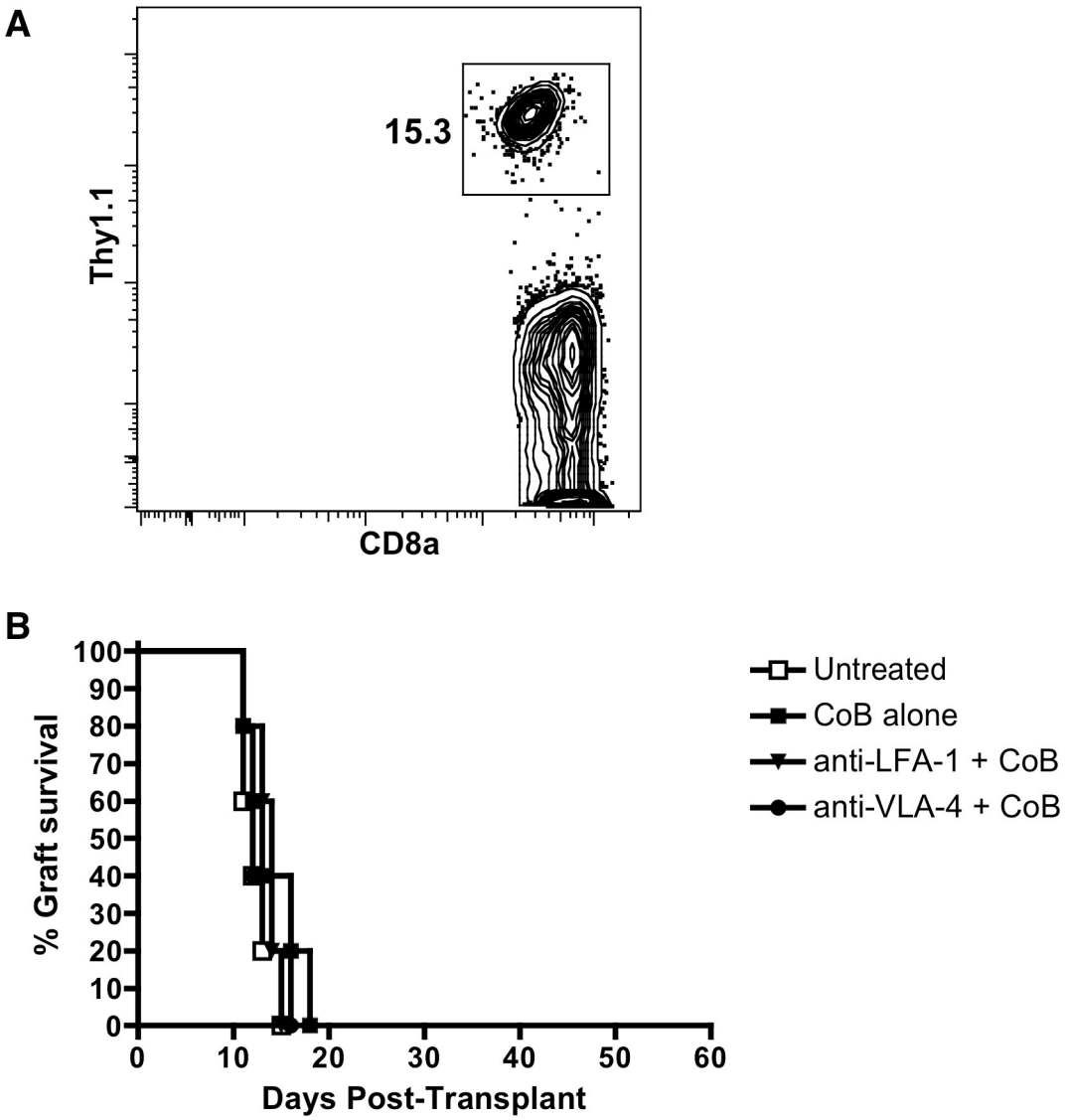

Figure 7: High memory OT-I frequency after serial LM-OVA infection prevents graft survival prolongation with combined integrin/costimulatory blockade.

Naïve C57BL/6 mice were adoptively transferred with OT-I cells and then serially infected with LM-OVA three times, each separated by a month. (A) Representative flow plot of OT-I frequency after serial LM-OVA infection. (B) Combined integrin and costimulatory blockade regimens fail to prolong mOVA skin graft survival in recipients with high frequencies of memory OT-I T cells after serial LM-OVA infection.

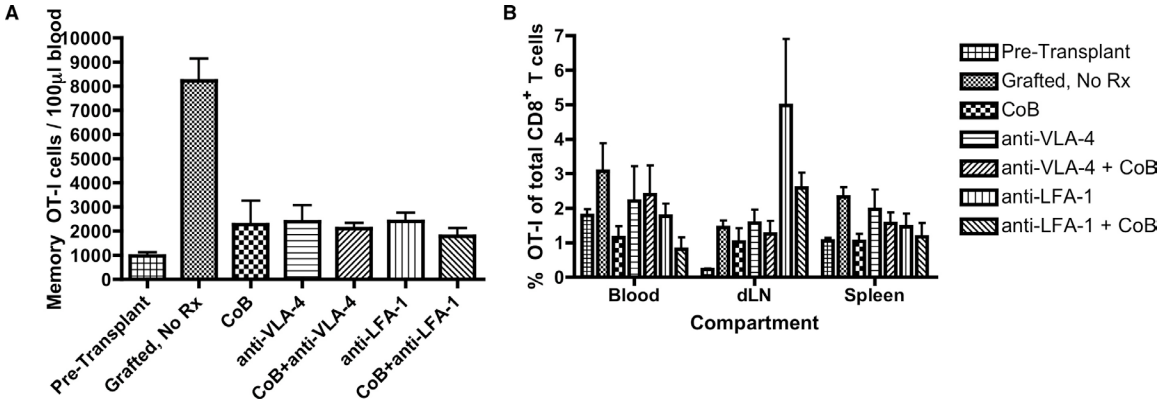

Figure 8: Dual blockade does not inhibit donor-reactive memory CD8+ T cell expansion and accumulation compared to CoB alone.

Skin grafts from mOVA donors were transplanted onto C57BL/6 recipients in which memory OT-I cells were generated by prior LM-OVA infection. The transplant recipients were left untreated or were treated on POD#0, 2, 4 and 6 with costimulatory blockade alone, integrin antagonist alone, or a dual blockade regimen. (A) On POD#7, the absolute number of memory OT-I T cells in the peripheral blood was quantified by flow cytometry. (B) Frequency of memory OT-I T cells as a percentage of total $CD8^+$ T cells on POD#7 is shown for the blood, draining lymph node and spleen compartments amongst the recipients treated with different immunosuppression regimens. Cumulative data from two independent experiments (3-6 mice/group) are shown. Error bars represent the mean $\pm$ SEM.

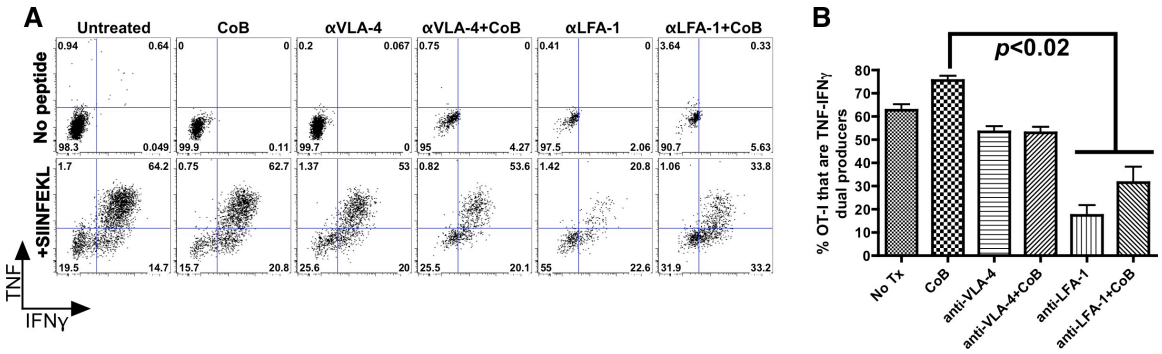

Figure 9: LFA-1 blockade attenuates CD8$^+$ memory T cell cytokine response.

(A) Intracellular cytokine staining of memory OT-I T cells harvested from C57BL/6 recipients on POD#7 after mOVA skin graft placement. Splenocytes were either left unstimulated or stimulated for four hours with $OVA_{257-264}$ peptide (SIINFEKL). (B) LFA-1 blockade inhibits the ability of memory OT-I T cells to become dual-producers of TNF and IFN-γ upon *ex vivo* restimulation. Results show (A) representative data or (B) summary of combined data from three independent experiments (5-6 mice/group). All error bars represent the mean ± SEM.

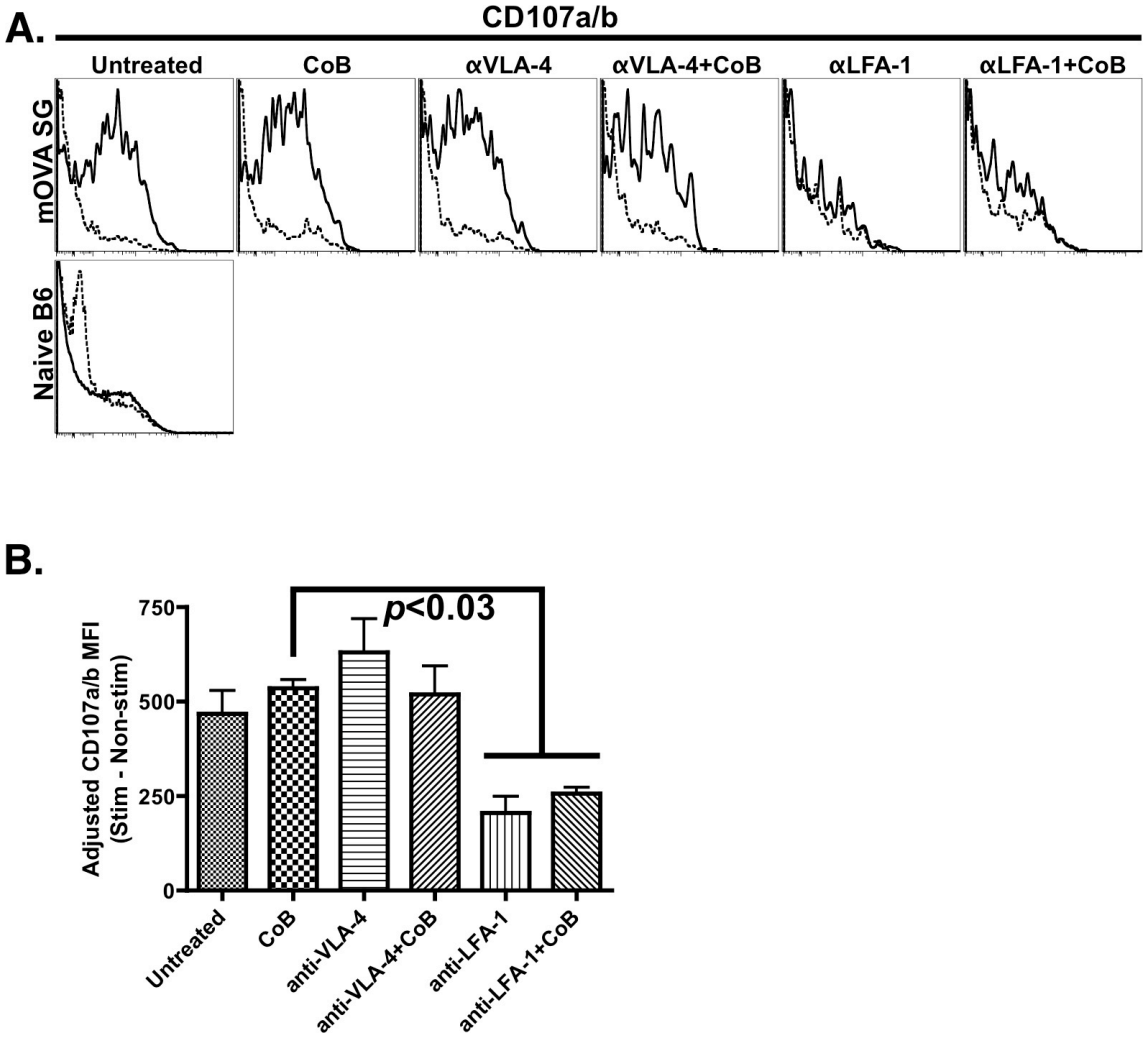

Figure 10: LFA-1 blockade attenuates CD8$^+$ memory T cell degranulation. (A) CD107-based degranulation assay of memory OT-I T cells harvested from C57BL/6 recipients on POD#7 after mOVA skin graft placement. Splenocytes from these recipients were either left unstimulated (dashed line) or stimulated for four hours with SIINFEKL peptide (solid line). (B) Adjusted MFI of CD107a/b surface localization of memory OT-I T cells. Results show (A) representative data or (B) summary of combined data from two independent experiments (3-6 mice/group). All error bars represent the mean $\pm$ SEM.

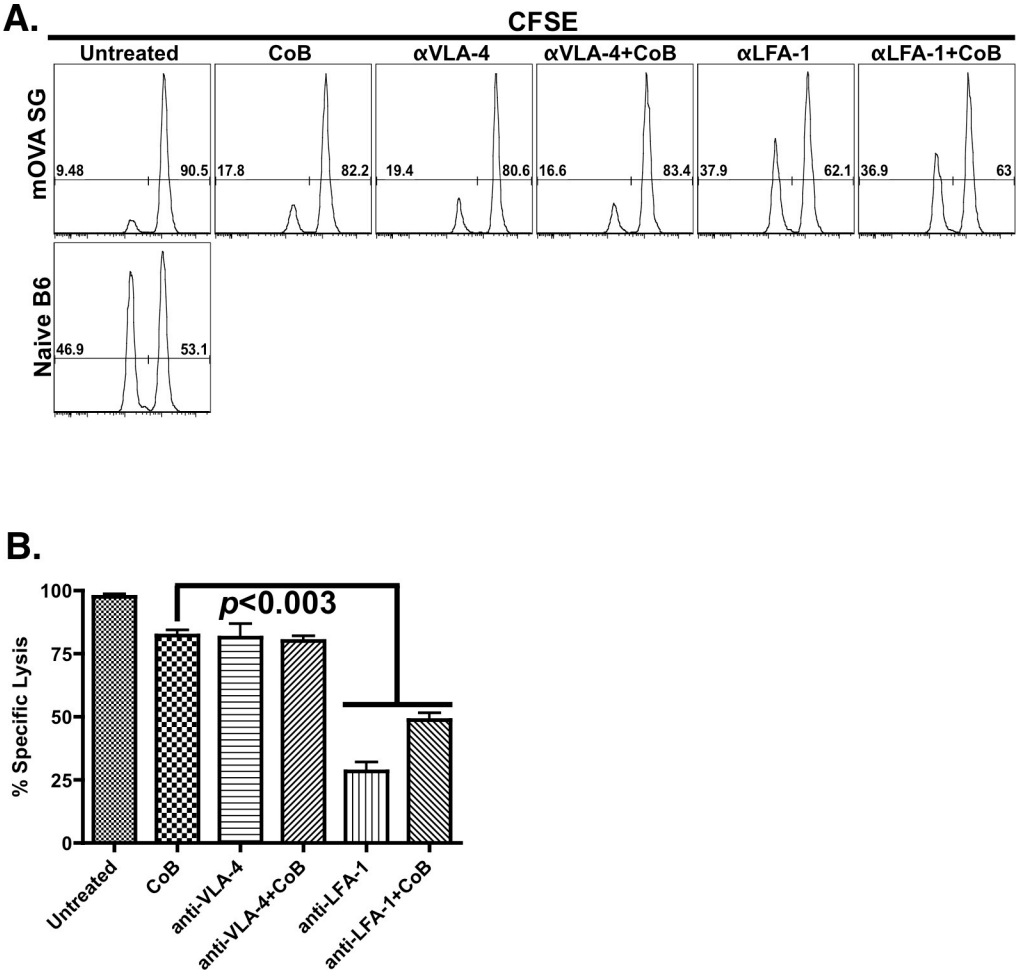

Figure 11: LFA-1 blockade attenuates CD8$^+$ memory T cell cytotoxicity. (A and B) *In vivo* CTL of memory OT-I T cells in recipients of mOVA skin grafts to assess impact of different immunosuppression regimens on memory T cell cytotoxicity. Results show (A) representative data or (B) summary of combined data from five independent experiments (4-10 mice/group). All error bars represent the mean $\pm$ SEM.

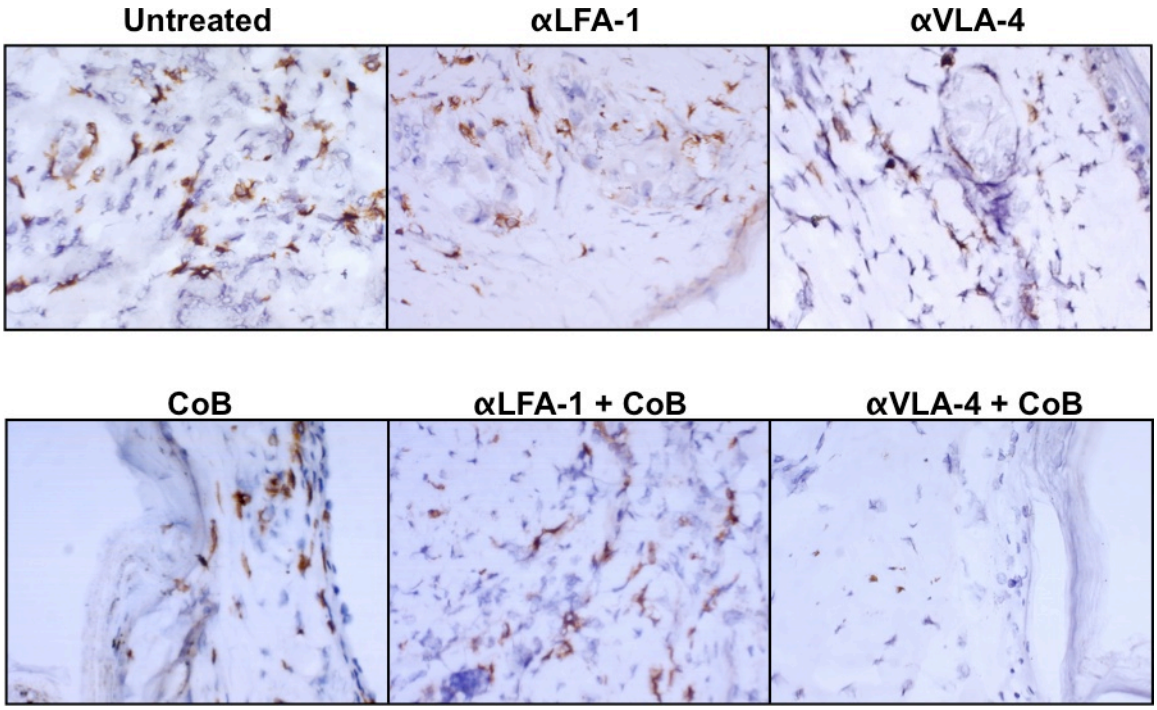

Figure 12: VLA-4 blockade inhibits donor-specific CD8$^+$ memory T cell trafficking to the graft. Immunohistochemistry analysis of POD#7 explanted mOVA skin grafts from recipients with memory OT-I T cells, staining for Thy1.1 marker found only on memory OT-I T cells (shown 40x magnification). Representative images over two independent experiments (3 mice/group) are shown.

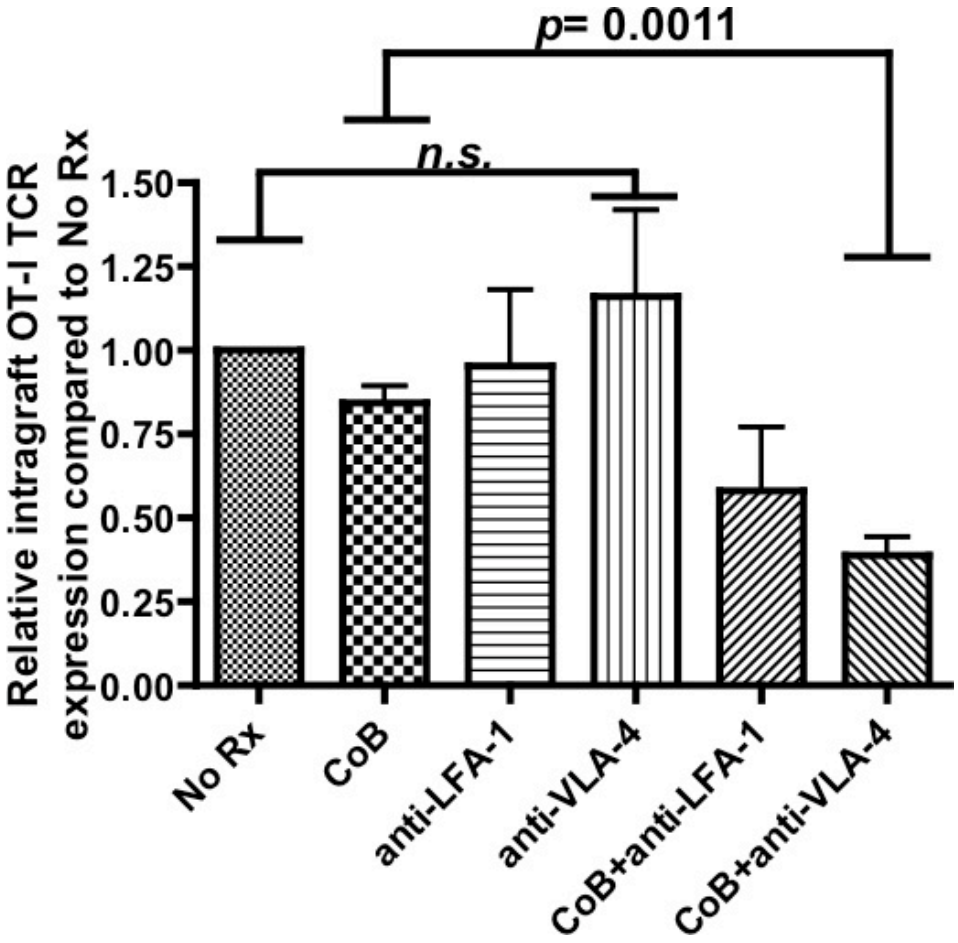

Figure 13: VLA-4 blockade inhibits donor-specific CD8$^+$ memory T cell trafficking to the graft. Quantitative real-time PCR assay for OT-I TCR expression using cDNA generated from mOVA skin grafts explanted on POD#7. Combined results from two independent experiments (3-6 mice/group) are shown. All error bars represent the mean $\pm$ SEM.

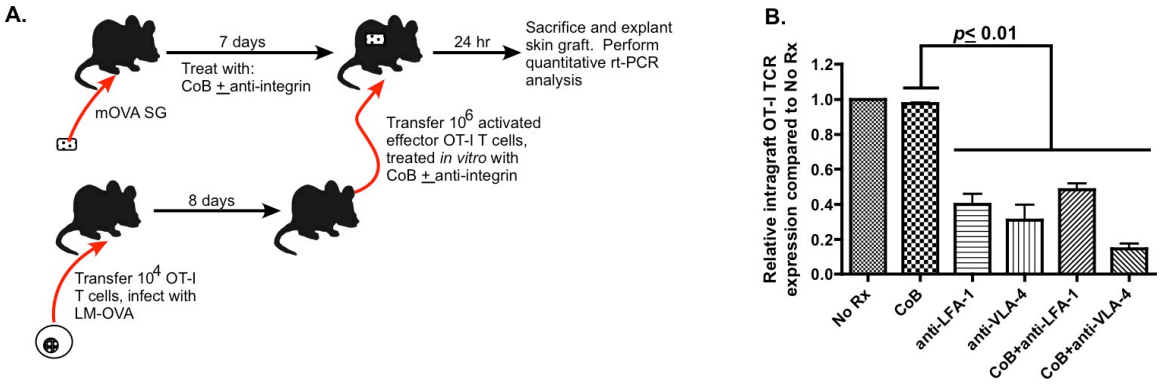

Figure 14: VLA-4 blockade inhibits donor-specific CD8$^+$ memory T cell trafficking to the graft. (A) Experimental design for assay to determine the effect of integrin blockade on donor-specific effector T cell trafficking to transplanted skin grafts. (B) VLA-4 blockade inhibits trafficking of adoptively transferred OT-I to mOVA skin grafts. Combined results from three independent experiments (8-10 mice/group) are shown. All error bars represent the mean ± SEM.

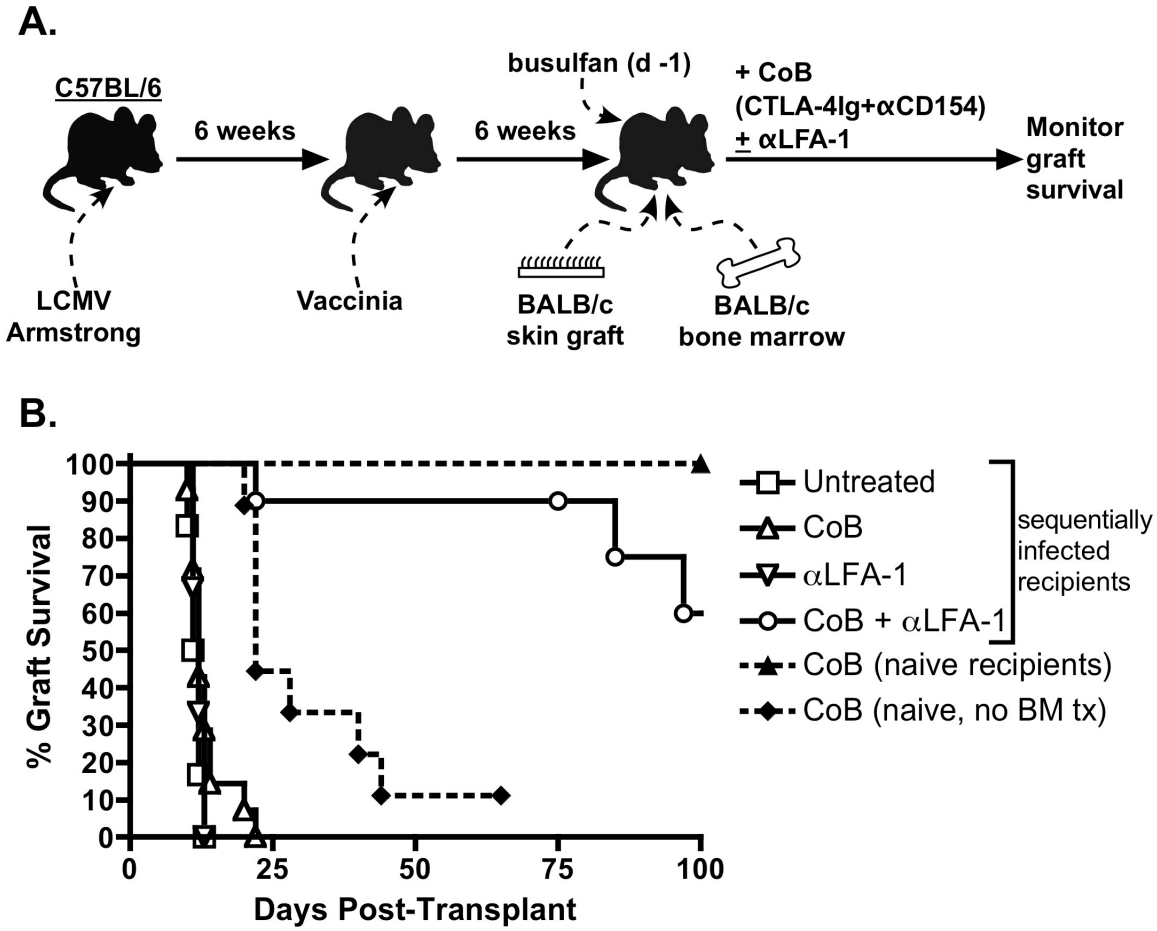

Figure 15: **Combined costimulatory and LFA-1 blockade prolongs graft survival against a heterologous immune response**. (A) Experimental model of transplant rejection mediated by a heterologous immune response. (B) Survival curves of BALB/c skin grafts transplanted onto C57BL/6 recipients that had been previously infected with LCMV and Vaccinia to generate an anti-donor heterologous immune response. These sequentially-infected recipients were either untreated (n= 6, MST=11.5 days) or treated with CoB alone (n=14, MST=12 days), anti-LFA-1 alone (n=3, MST=12 days) or CoB + anti-LFA-1 (n=10, MST>100 days, $p<0.0001$). Uninfected C57BL/6 mice treated with CoB in the absence (n=3, MST=3) or presence of BALB/c bone marrow transplant (n=5, MST>100 days) were also grafted.

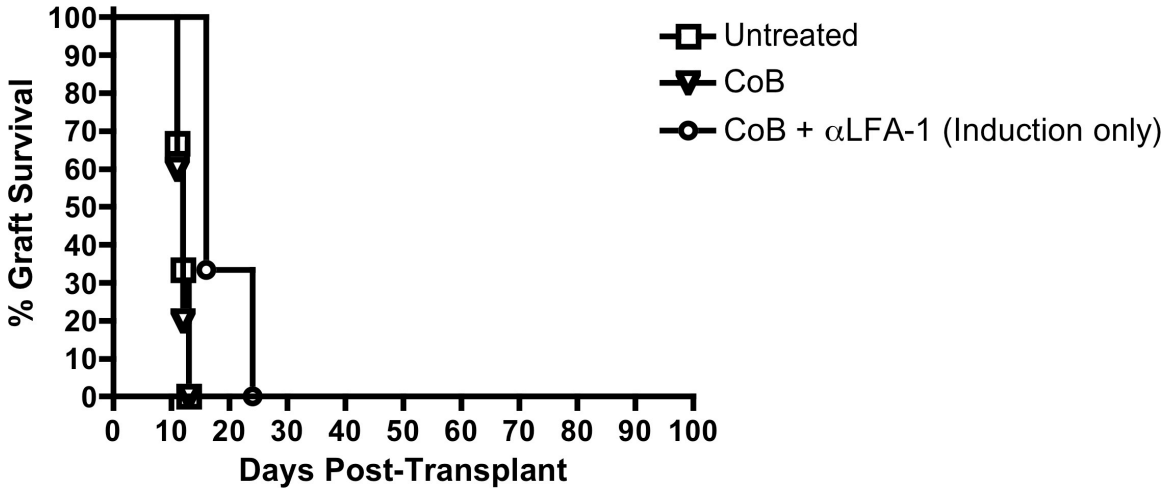

Figure 16: **Short-course induction anti-LFA-1 fails to prolong skin graft survival.** Survival curves of BALB/c skin grafts transplanted onto C57BL/6 recipients that had been previously infected with LCMV and Vaccinia to generate an anti-donor heterologous immune response. These sequentially-infected recipients were either untreated (n=3, MST= 12 days) or treated with an induction course (on POD#0, 2, 4 and 6) of CoB (n=5, MST= 12 days) or an induction course of combined CoB and anti-LFA-1 with no further maintenance anti-LFA-1 (n=3, MST= 16 days).

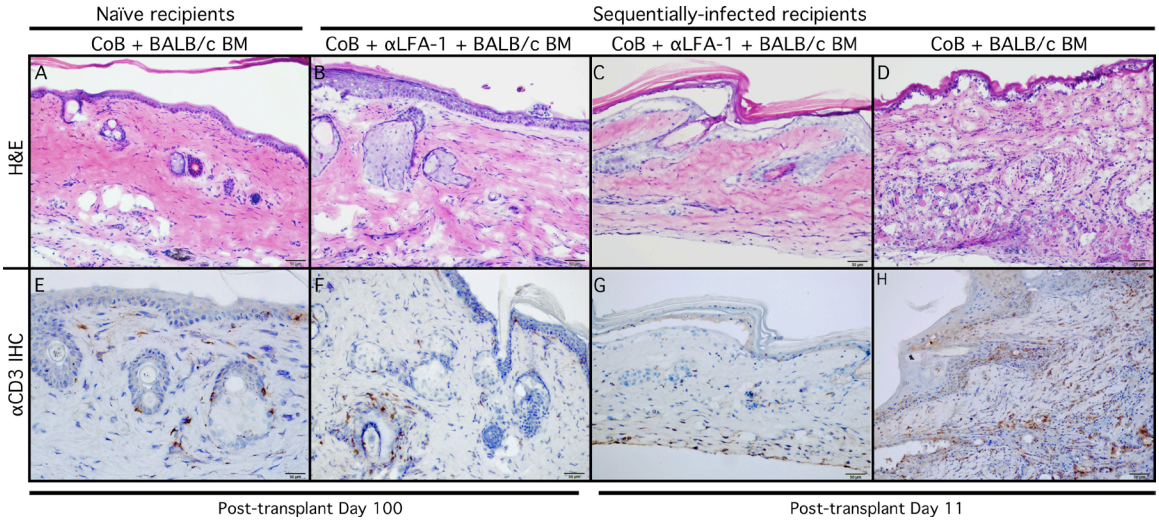

Figure 17: **Combined costimulatory and LFA-1 blockade prevents graft infiltration**. Representative H&E stains (panels A-D) and anti-CD3 immunohistochemistry stains (panels E-H) of explanted skin grafts are shown at 20x magnification. Grafts were explanted from naïve recipients (panels A and E) or sequentially-infected recipients (panels D and H) treated with CoB and BALB/c BM transplant. Other grafts were explanted from sequentially-infected recipients treated with CoB + anti-LFA-1 + BALB/c BM transplant (panels B, C, F and G). Grafts were harvested on POD#11 or POD#100, as indicated.

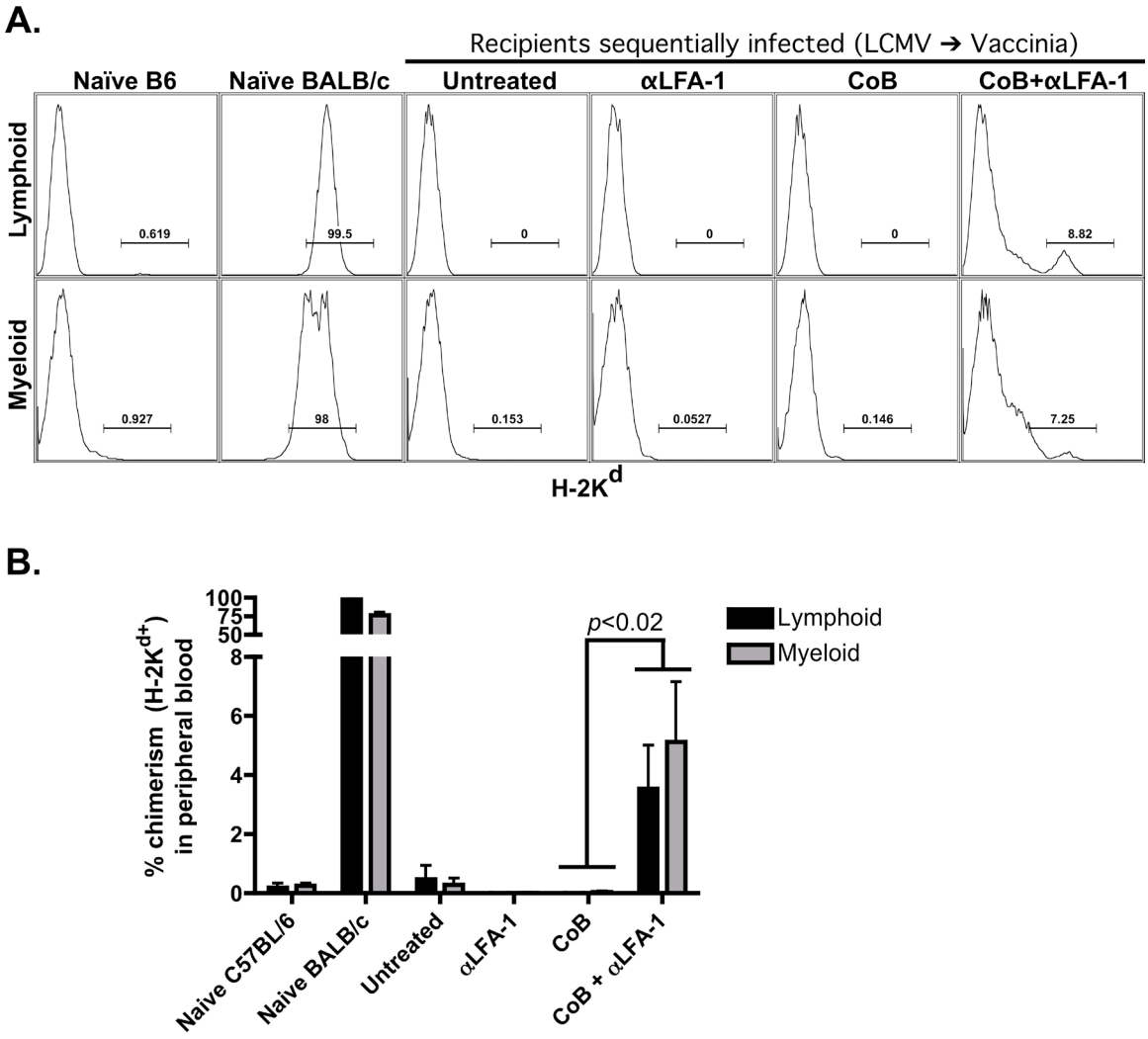

Figure 18: **Combined costimulatory and LFA-1 blockade enables long-term donor chimerism despite an anti-donor heterologous immune response**. (A) Representative flow cytometry demonstrating lymphoid ($CD3^+CD4^+$) and myeloid ($CD11b^+$) chimerism in peripheral blood as assessed by H-$2K^d$ surface expression measured 60 days following transplantation with BALB/c skin and bone marrow (n= 3-5 mice/group). (B) Summary of lymphoid and myeloid H-$2K^d$ chimerism in transplant recipients treated with different immunosuppression regimens. All error bars represent the mean $\pm$ SEM.

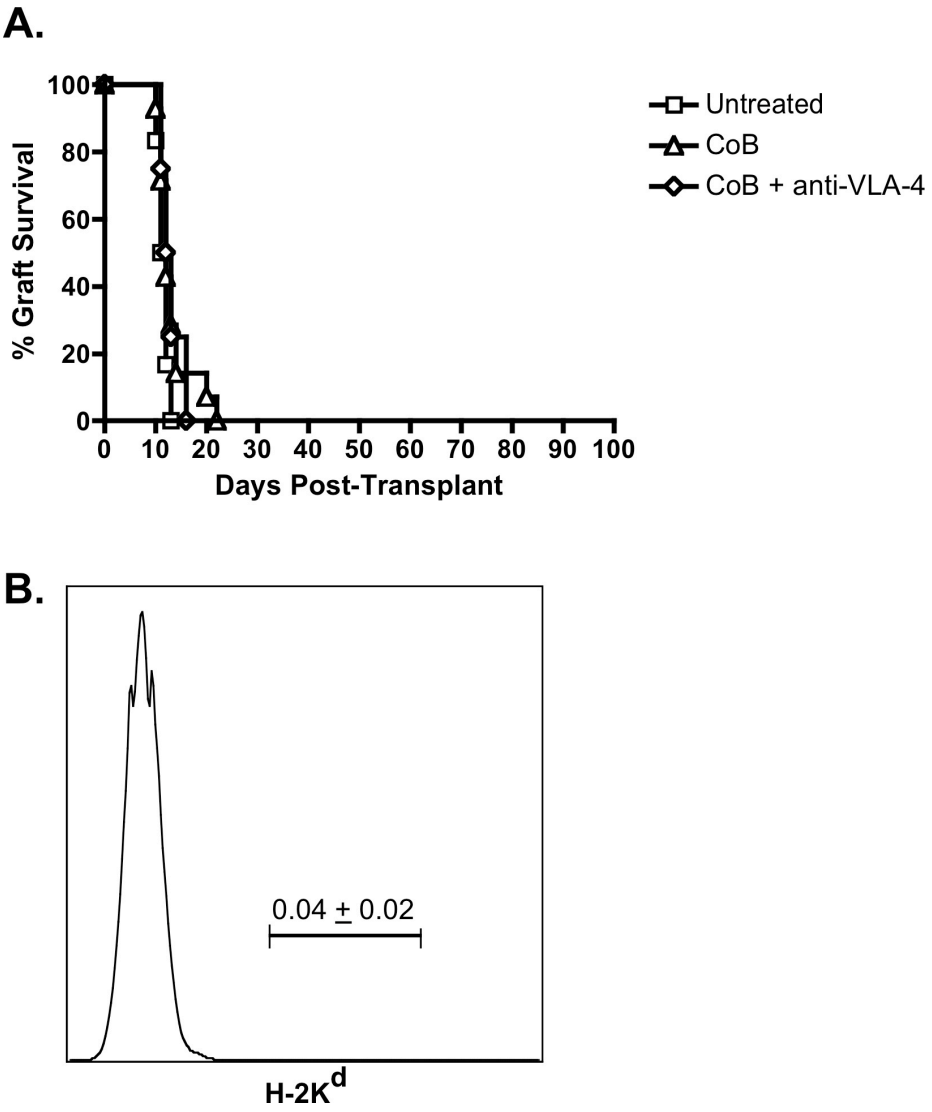

Figure 19: **Combined costimulatory and VLA-4 blockade did not prolong graft survival against a robust anti-donor heterologous immune response**. (A) Survival curves of BALB/c skin grafts transplanted onto C57BL/6 recipients that had been previously infected with LCMV and Vaccinia to generate an anti-donor heterologous immune response. These sequentially-infected recipients were either untreated (n= 6) or treated with CoB alone (n=14), or CoB + anti-VLA-4 (n=4). (B) Flow plot of lymphoid chimerism (as assessed by H-2K^d) expression on POD#60 after BALB/c bone marrow transplant in heterologous immune recipients treated with CoB + anti-VLA-4.

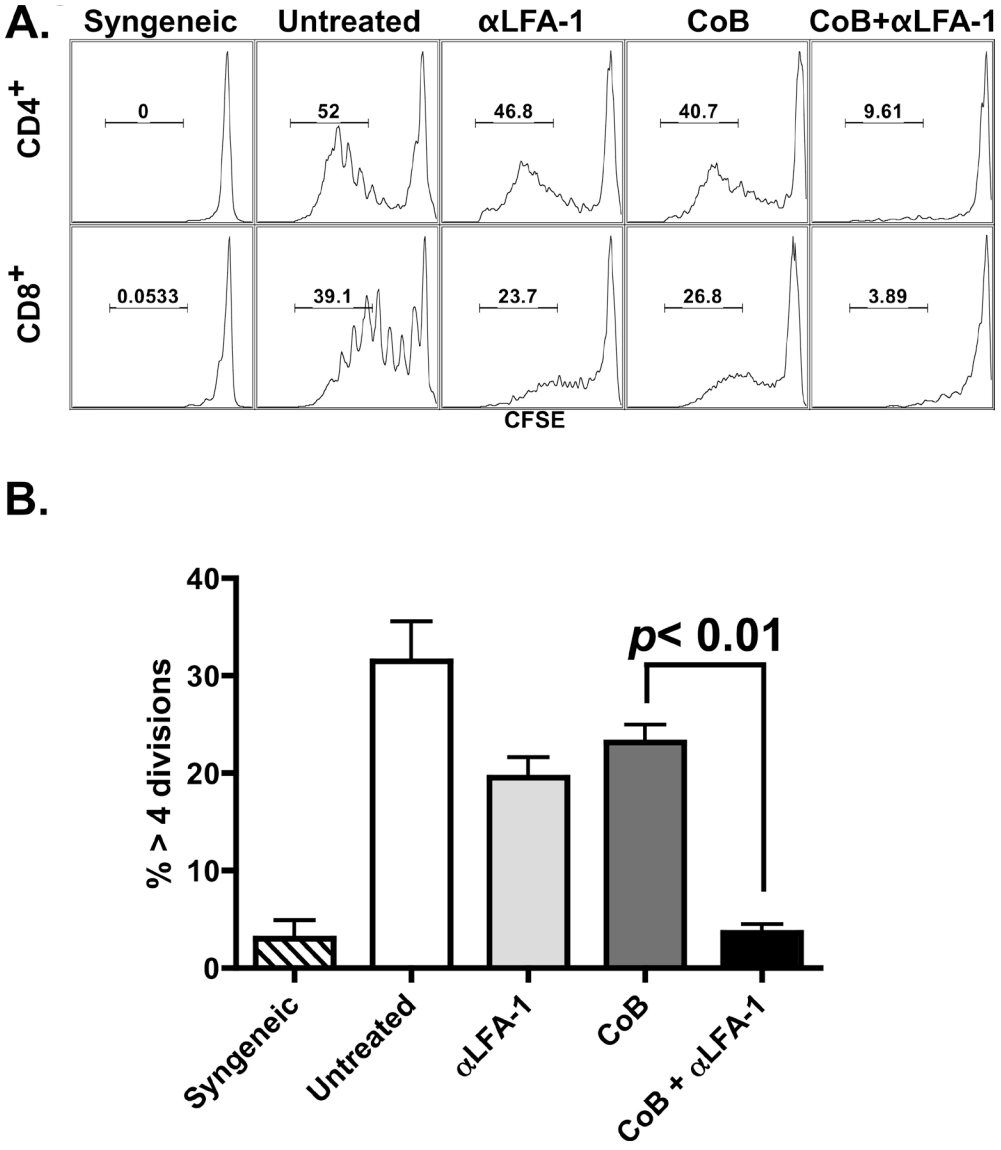

Figure 20: **Combined costimulatory and LFA-1 blockade suppresses alloreactive T cell proliferation in recipients with a robust anti-donor heterologous immune response.** (A) Representative histograms of CFSE dilution for $CD4^+$ and $CD8^+$ T cells after *in vivo* mixed lymphocyte reaction in presence of no treatment, CoB alone, anti-LFA-1 alone, or combined blockade (n= 3-8 mice/group). (B) Summary of $CD8^+$ T cell proliferation during *in vivo* MLR as measured by percentage of total $CD8^+$ T cells that had undergone >4 divisions after 72 hours. All error bars represent the mean $\pm$ SEM.

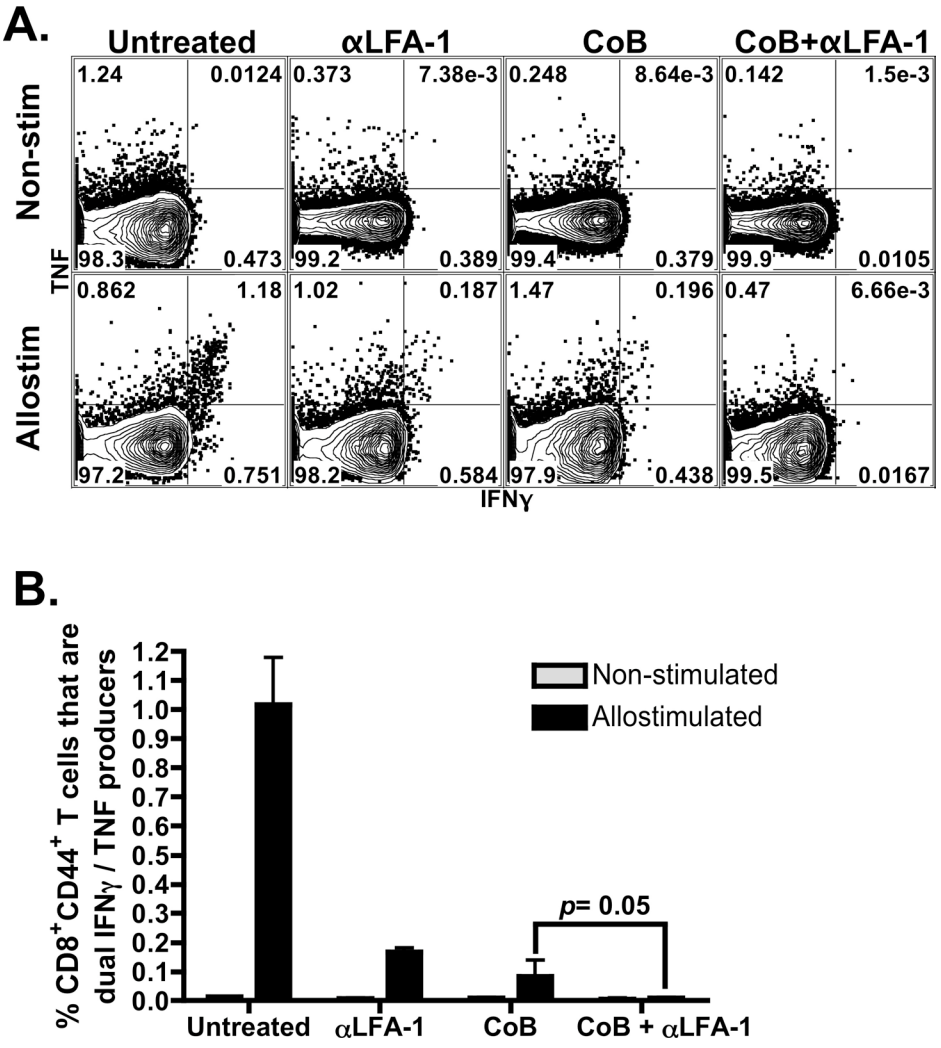

Figure 21: **Combined costimulatory and LFA-1 blockade suppresses alloreactive T cell cytokine production in recipients with a robust anti-donor heterologous immune response.** (A) Representative flow plots of intracellular cytokine staining for IFN-γ and TNF in $CD8^+CD44^+$ splenocytes harvested on POD#60 from skin graft recipients treated with the specified immunosuppression regimen (n=3 mice/group). (B) Summary of the impact of different immunosuppression regimens on the percentage of $CD8^+CD44^+$ splenocytes that were highly activated dual producers of IFN-γ and TNF. All error bars represent the mean ± SEM.

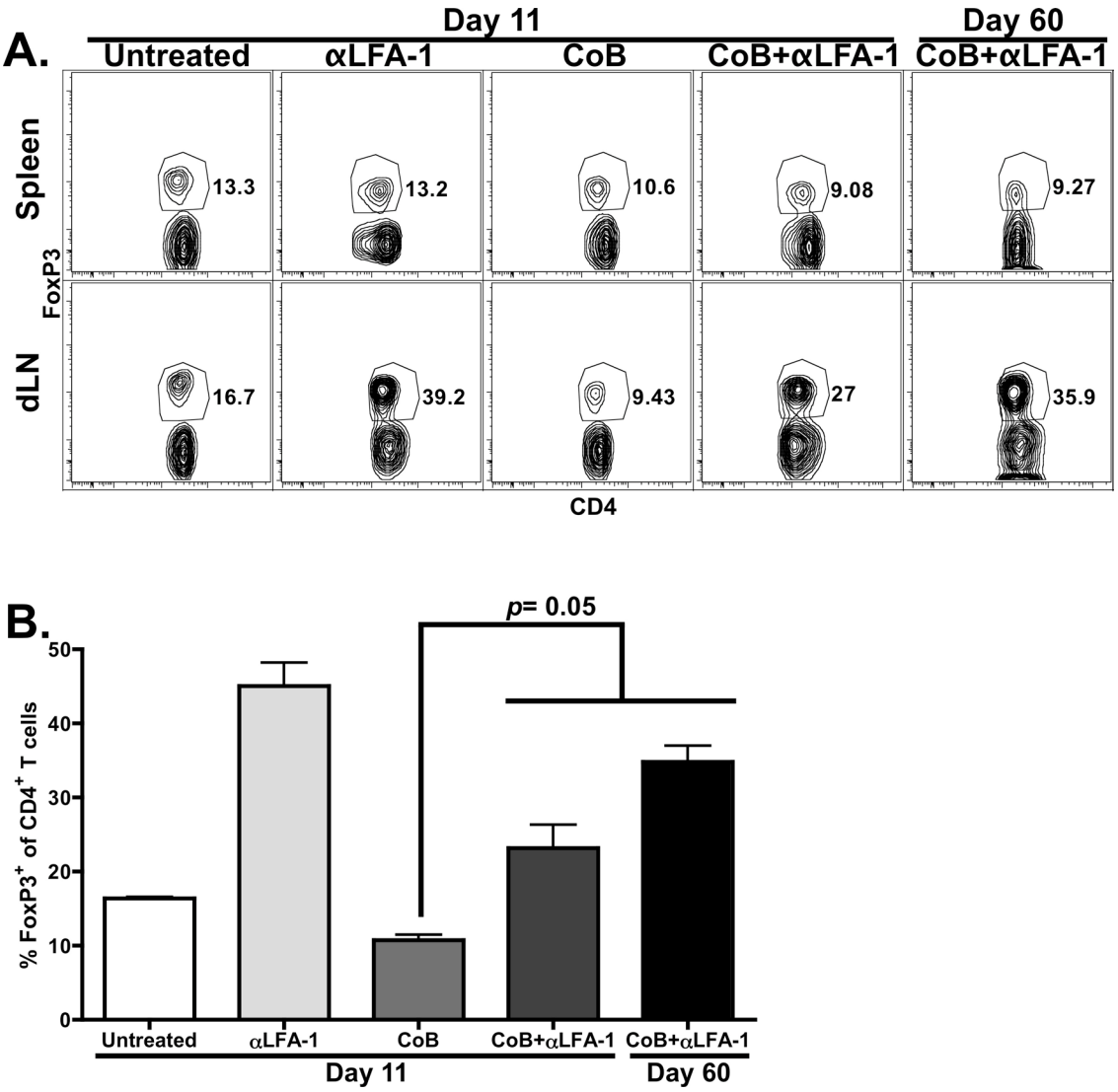

Figure 22: **Combined costimulatory and LFA-1 blockade promotes allospecific T cell accumulation in dLN**. (A) Representative plots of the frequency of FoxP3$^+$ Tregs in the spleen and draining lymph nodes of skin graft recipients treated with the specified immunosuppression regimen (n=3 mice/group). (B) Summary of how different immunosuppression regimens impact the accumulation of FoxP3$^+$ Tregs in the draining lymph nodes on POD#11 or POD#60 after a BALB/c skin graft. All error bars represent the mean $\pm$ SEM.

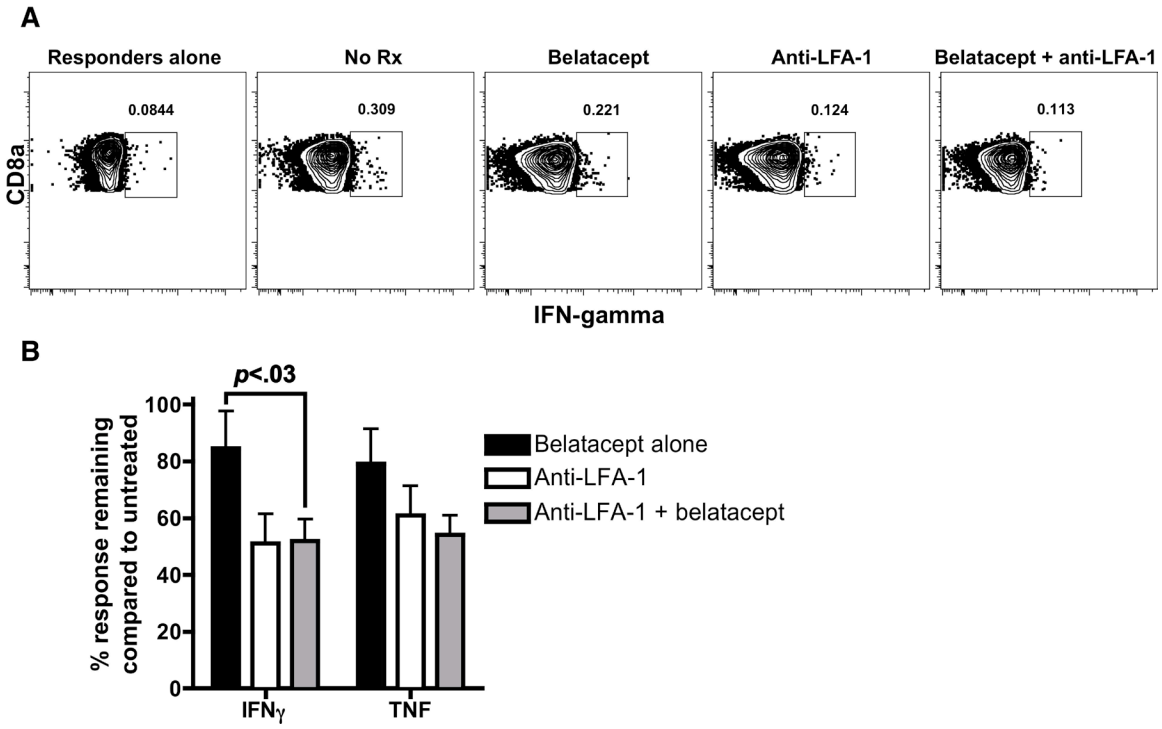

Figure 23: **LFA-1 blockade (but not costimulation blockade) inhibits human CD8$^+$ memory T cell effector responses**. Allostimulation assays performed with human PBMCs that were either untreated or incubated in the presence of belatacept alone, anti-LFA-1 alone, or combined belatacept and anti-LFA-1. Intracellular cytokine staining assessed IFN-γ and TNF production by CD45RA$^-$ alloreactive memory CD8$^+$ T cells. Results show (A) representative flow plots of IFN-γ production by responder cells or (B) summary figure of data normalized against untreated controls (four replicate experiments). All error bars represent the mean $\pm$ SEM.

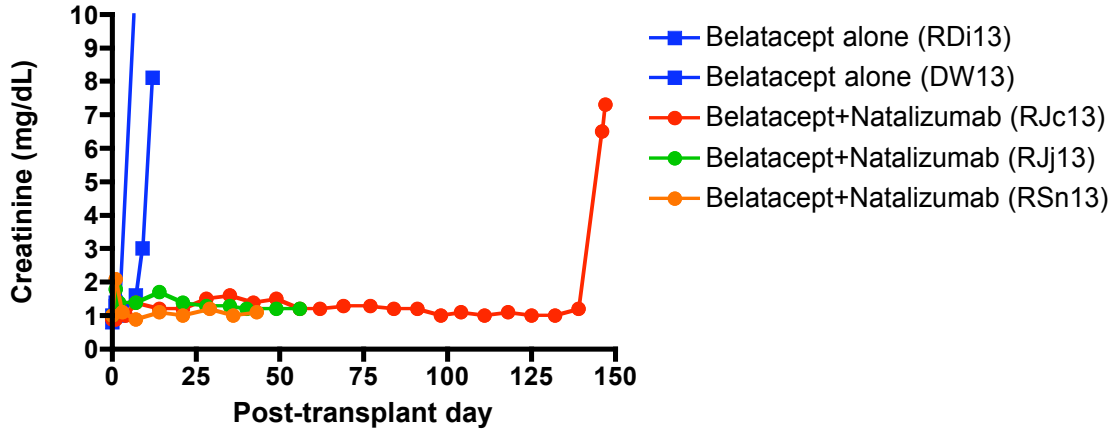

Figure 24: **Combined belatacept and natalizumab significantly prolong kidney allograft survival in rhesus macaques**. Fully MHC-mismatched kidney allografts were transplanted into rhesus macaques treated with belatacept monotherapy (rejected on day 7 and 8) or combined belatacept and natalizumab (rejected on day 147, >66 and >60). Recipients were treated with belatacept on POD 0, 4, 7, 14, 28, 42, 56, 70 and 84. Those receiving natalizumab were treated on POD 0, 4, 7, and weekly thereafter until POD 42.

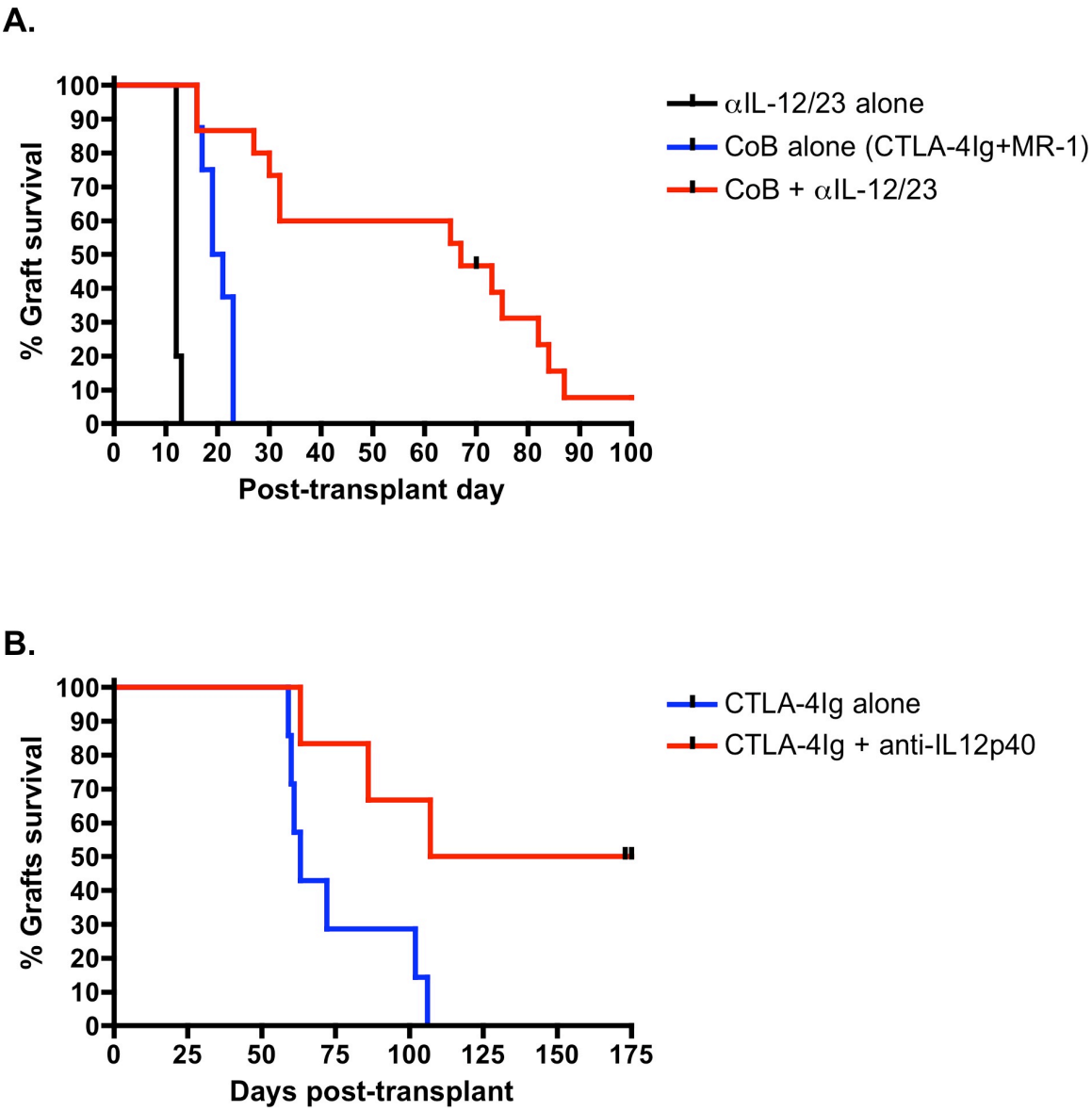

Figure 25: **CoB + anti-IL-12/23 significantly prolongs skin and cardiac allograft survival**. (A) BALB/c => C57BL/6 skin grafts were performed and recipients were treated with anti-IL-12/23 alone (median survival time= 11 days), CoB alone (MST= 20 days) or CoB + anti-IL-12/23 (MST= 67 days). (B) Heterotopic BALB/c => C57BL/6 cardiac grafts were performed and recipients were treated with CTLA-4Ig alone (MST= 63 days) or combined CTLA-4Ig + anti-IL-12/23 (MST= 141 days).

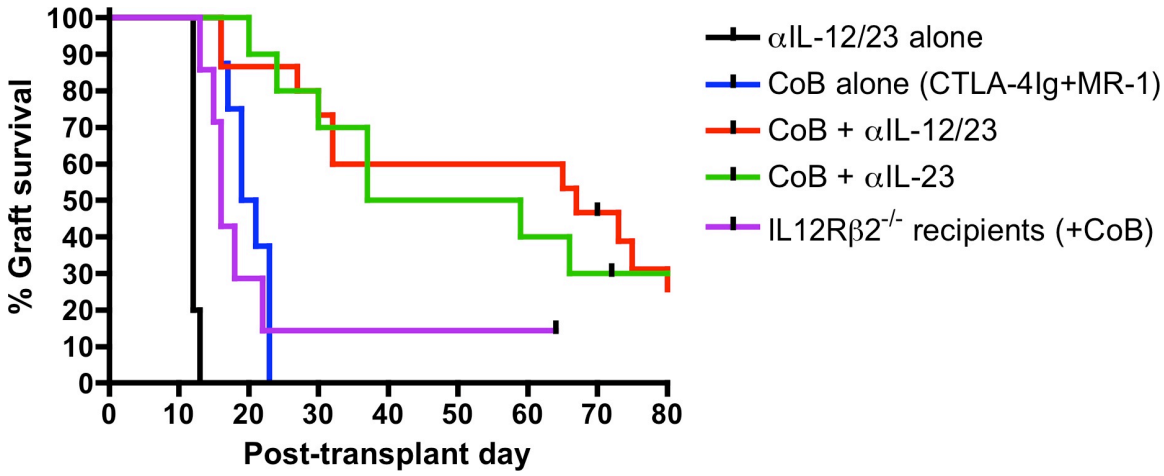

Figure 26: **Prolonged graft survival with CoB + anti-IL-12/23 is mediated predominantly by IL-23 blockade**. To model costimulatory and selective IL-12 blockade, BALB/c skin grafts were performed using B6.IL-12Rβ2$^{-/-}$ recipients, which were then treated with CoB alone. To model costimulatory and selective IL-23 blockade, BALB/c => C57BL/6 skin grafts were performed and the recipients were treated with polyclonal anti-IL-23p19 antibody.

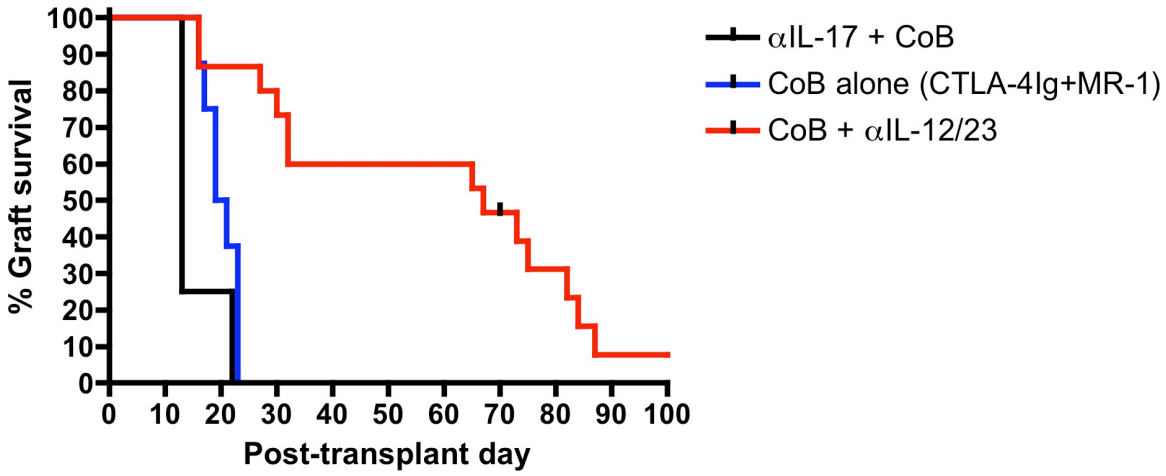

Figure 27: **Combined costimulatory and anti-IL-17A fails to prolong allograft survival**. BALB/c => C57BL/6 skin grafts were performed, and recipients were treated with either CoB alone, CoB + anti-IL-12/23, or CoB + anti-IL-17A (4-12 mice/group).

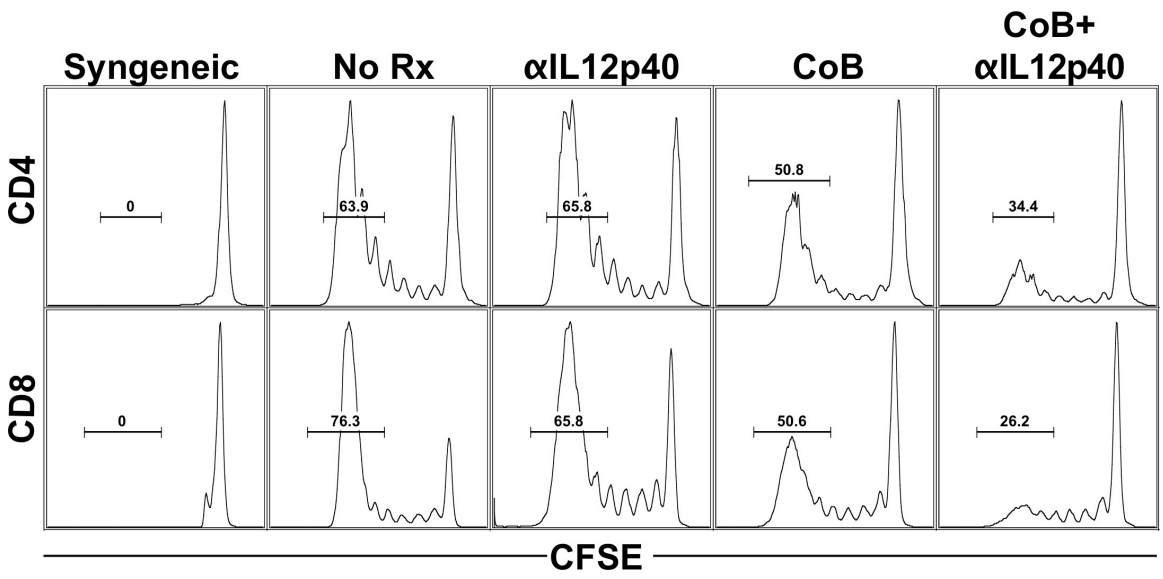

Figure 28: **Combined costimulatory and anti-IL-12/23 blockade potently suppresses alloreactive T cell proliferation**. CFSE-labeled C57BL/6$^{CD45.1+}$responder cells were adoptively transferred into sub-lethally irradiated BALB/c mice, which were sacrificed after 72 hours. Representative histograms of CFSE dilution for $CD4^+$ and $CD8^+$ responder T cells after *in vivo* mixed lymphocyte reaction in presence of no treatment, anti-IL-12/23 alone, CoB alone, or combined CoB + anti-IL-12/23 (4 mice/group, repeated in 2 independent experiments).

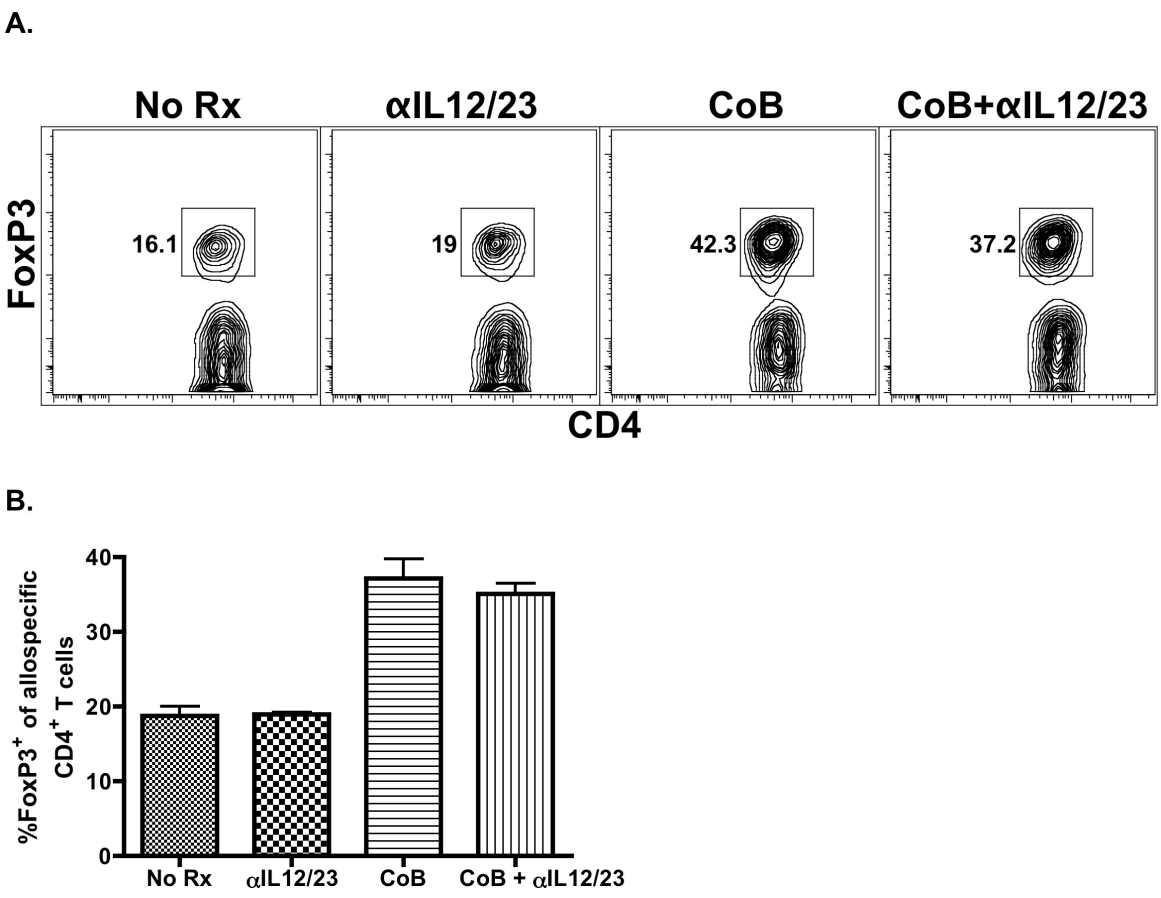

Figure 29: **Treatment with CoB or CoB+anti-IL-12/23 enables accumulation of allospecific FoxP3$^+$ Tregs**. (A) Representative plots of the frequency of allospecific (undergoing >4 divisions) FoxP3$^+$ Tregs in the spleen following an *in vivo* mixed lymphocyte reaction after treatment with the specified immunosuppression regimen (n=4 mice/group). (B) Summary of how different immunosuppression regimens impact the accumulation of allospecific FoxP3$^+$ Tregs during an *in vivo* mixed lymphocyte reaction. All error bars represent the mean $\pm$ SEM.

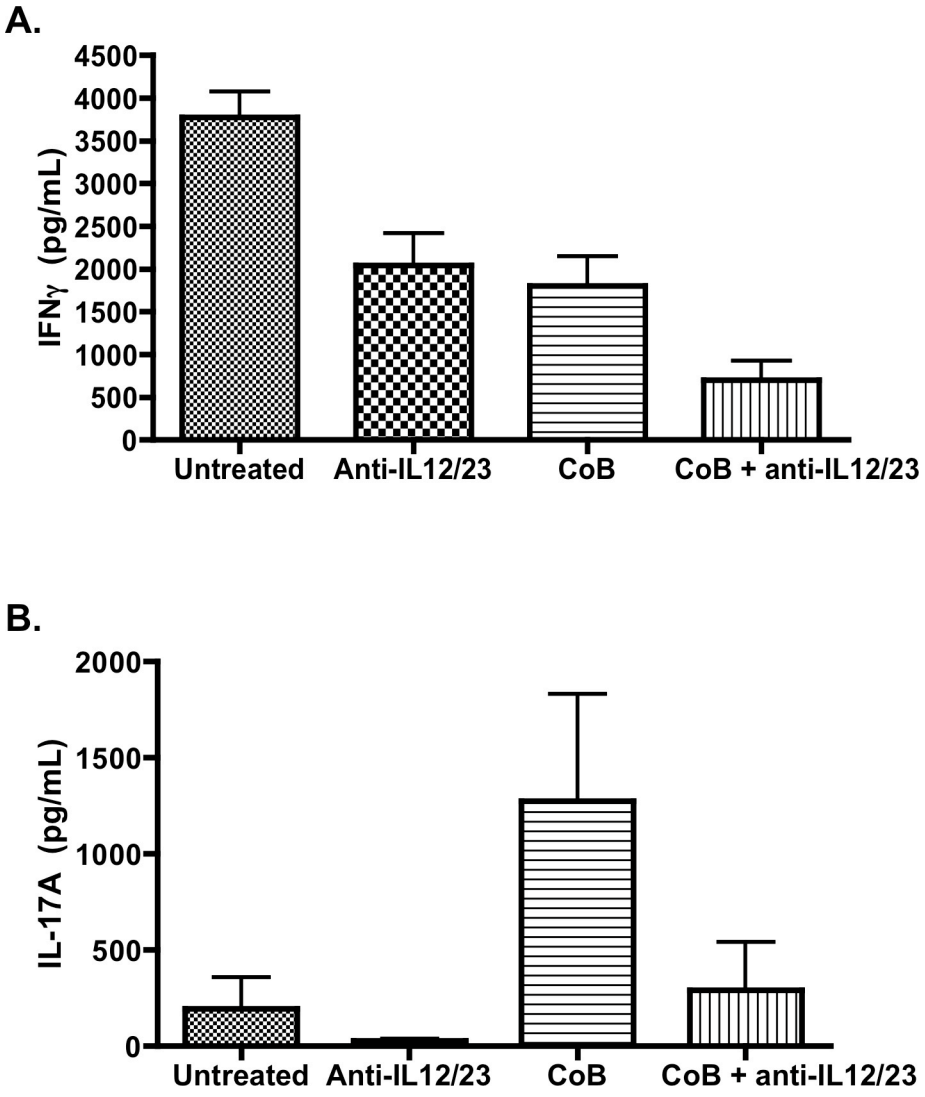

Figure 30: **Treatment with costimulatory blockade induces higher levels of IL-17 by alloreactive dLN cells compared to untreated skin graft recipients**. BALB/c skin grafts were transplanted onto C57BL/6 recipients, which were either untreated or received anti-IL-12/23 alone, CoB alone or CoB + anti-IL-12/23. On post-transplant day 24, dLN cells were harvested and cultured with fresh BALB/c responders for 48 hours, after which supernatants were harvested for cytometric bead array analysis of (A) IFN-γ or (B) IL-17A. 4 mice per group, in 2 independent experiments. Error bars represent mean $\pm$ standard error of mean.

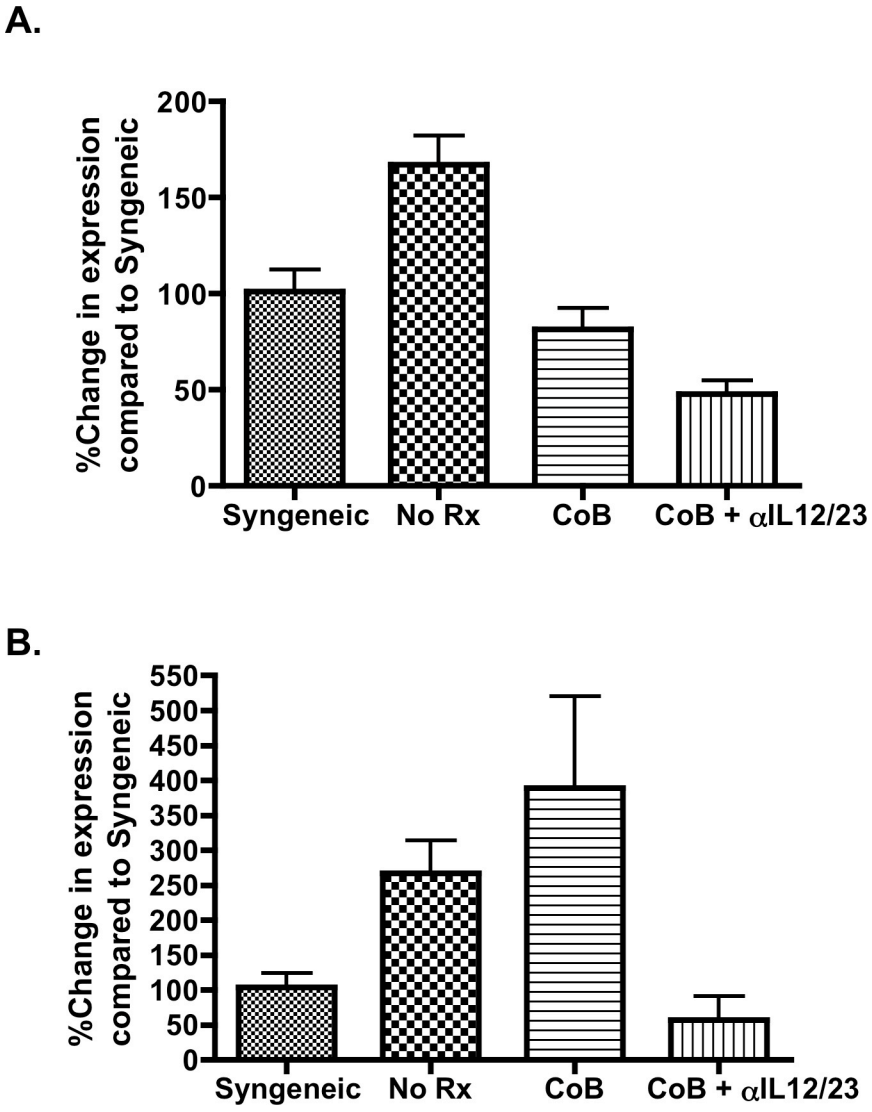

Figure 31: **Treatment with costimulatory blockade induces higher levels of IL-17 transcripts by alloreactive dLN cells compared to untreated skin graft recipients**. BALB/c skin grafts were transplanted onto either BALB/c syngeneic recipients or C57BL/6 recipients, which were either untreated, CoB-treated or treated with CoB + anti-IL-12/23. On post-transplant day 24, dLN cells were harvested and quantitative RT-PCR was performed using Taqman primers and probes specific for (A) IFN-γ or (B) IL-17A. Expression levels were standardized against syngeneic controls.

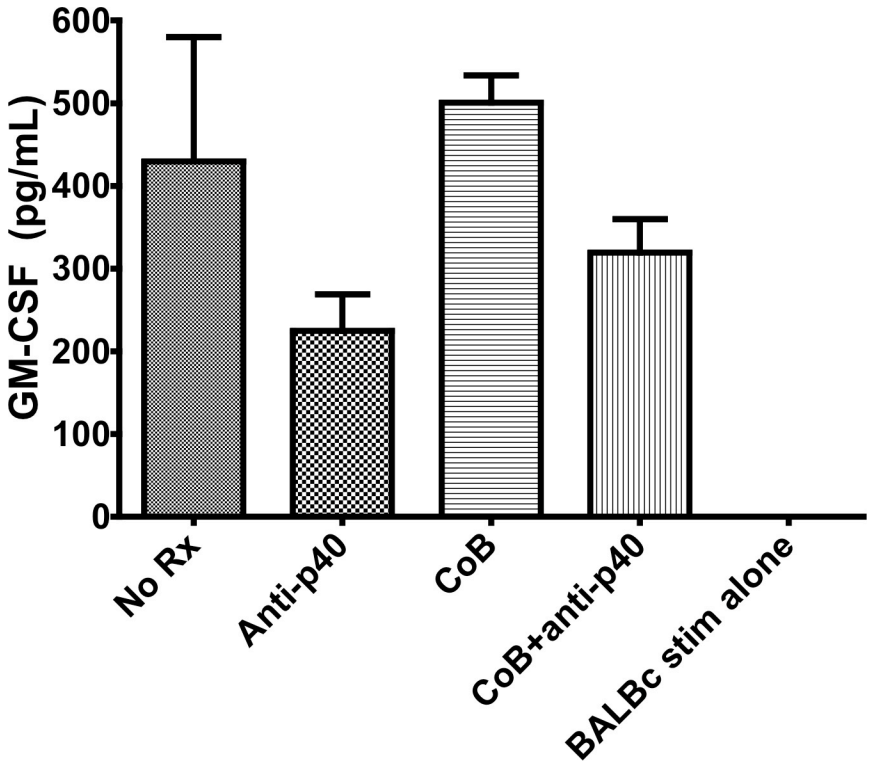

Figure 32: **Anti-IL-12/23 suppresses the allospecific production of GM-CSF**. BALB/c skin grafts were transplanted onto C57BL/6 recipients, which were either untreated or received anti-IL-12/23 alone, CoB alone or CoB + anti-IL-12/23. Untreated syngeneic grafts were also performed as controls. On post-transplant day 15, dLN cells were harvested and cultured with fresh BALB/c responders for 48 hours, after which supernatants were harvested for cytometric bead array analysis of GM-CSF. 4-5 mice per group, in 2 independent experiments. Error bars represent mean $\pm$ standard error of mean.

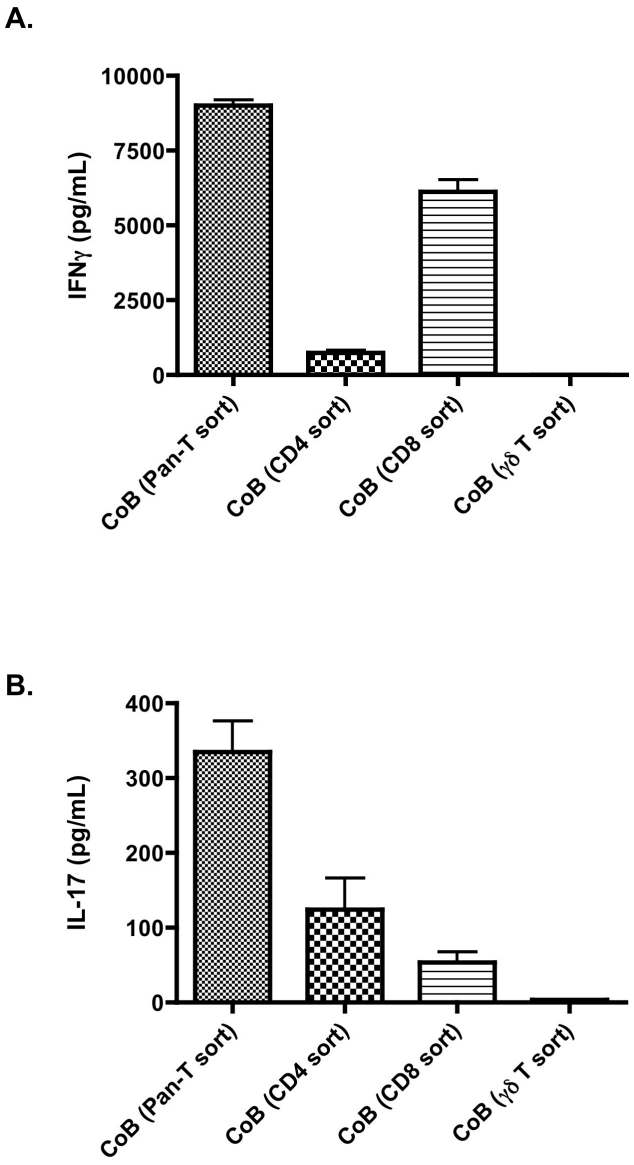

Figure 33: **Allostimulation-induced IL-17A is produced predominantly by CD4$^+$ T cells**. BALB/c skin grafts were transplanted onto C57BL/6 recipients, which were treated with CoB. On post-transplant day 15, dLN cells were harvested and sorted with magnetic-assisted cell sorting into total T cells, CD4$^+$, CD8$^+$ or γδ T cells. Sorted populations were stimulated with BALB/c splenocytes for 48 hrs, after which supernatants were harvested for cytometric bead array analysis of (A) IFN-γ or (B) IL-17A. Error bars represent mean $\pm$ standard error of mean.

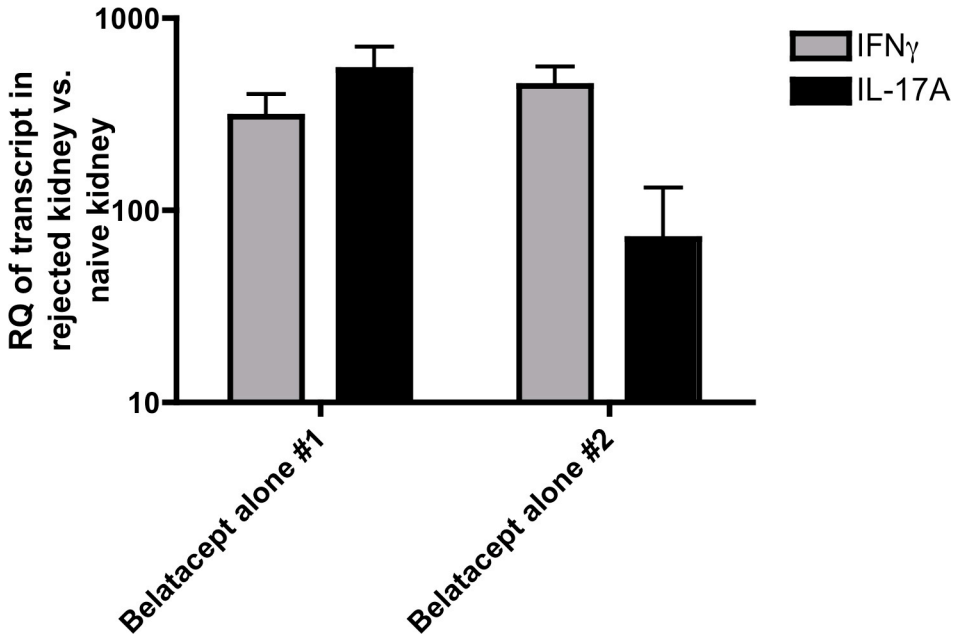

Figure 34: **Characterization of kidney allograft rejection in belatacept-treated non-human primates**. Kidney transplants were performed in two rhesus macaques, which were treated with belatacept. At the time of allograft rejection, RNA was extracted from the kidney allograft and converted to cDNA. Taqman RT-PCR was performed using primer/probe pairs specific for IFN-γ or IL-17A, which were standardized against cDNA obtained from a naïve untransplanted rhesus kidney. Relative expression quotient (RQ) of these cytokines reveals upregulated IFN-γ and IL-17A in these allografts undergoing belatacept-resistant rejection.

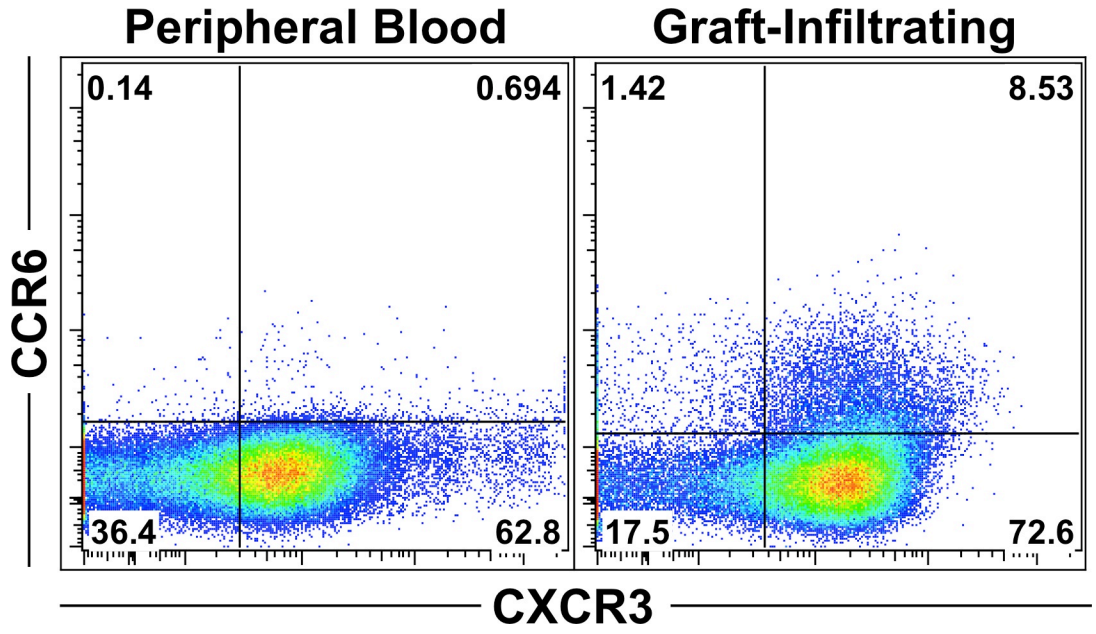

Figure 35: **Chemokine receptor profile of graft-infiltrating lymphocytes from primate kidney allografts undergoing belatacept-resistant rejection is consistent with Th17 cells**. Representative flow cytometry depicting surface expression of CCR6 and CXCR3 on graft-infiltrating lymphocytes extracted from belatacept-treated rhesus macaque kidney allografts undergoing acute rejection. Graft-infiltrating lymphocytes were obtained by mechanical disruption of the kidney allograft, followed by purification in a Ficoll gradient.

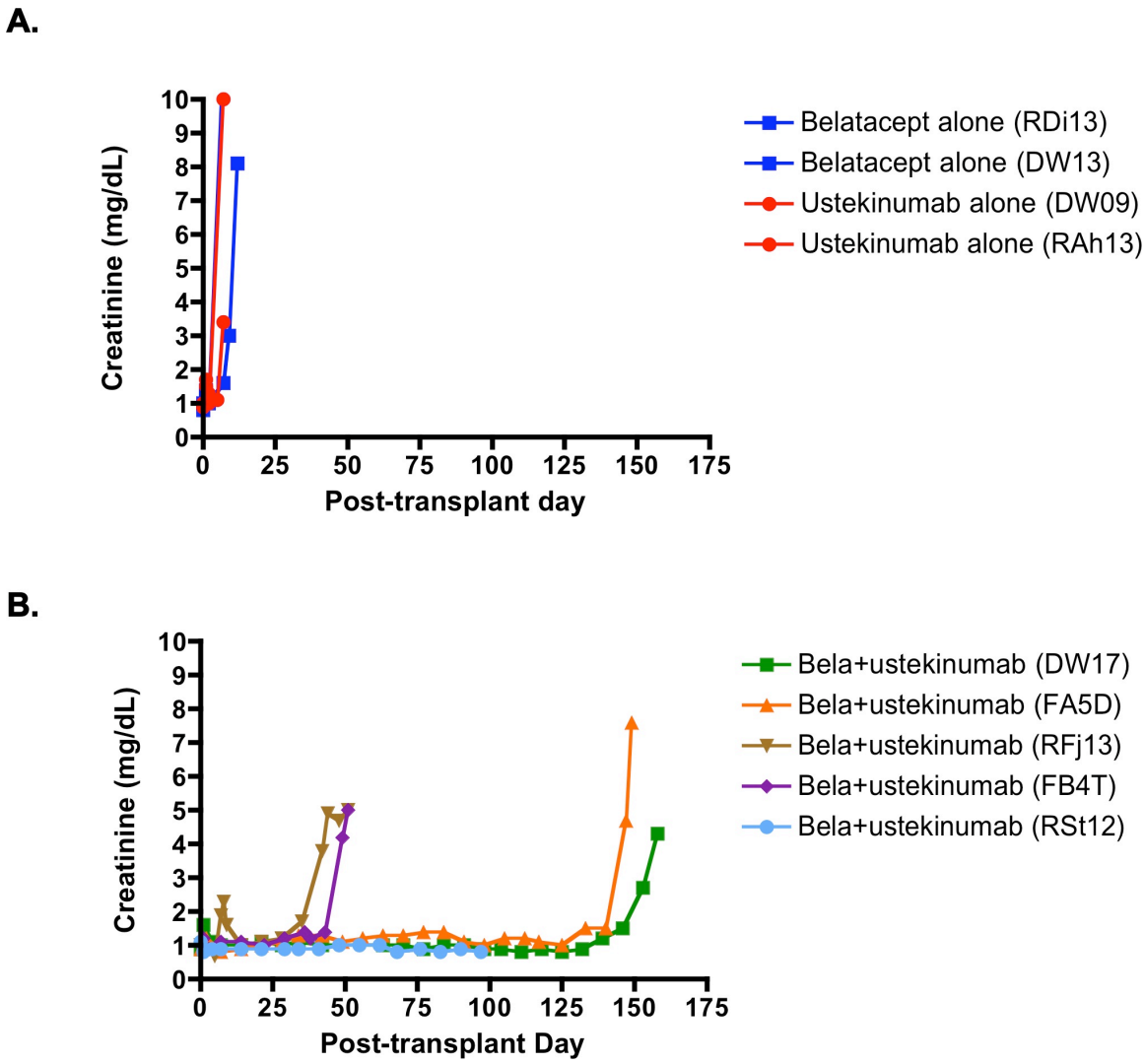

Figure 36: **Combined belatacept and ustekinumab significantly prolongs non-human primate kidney allograft survival**. Kidney transplants were performed in maximally-mismatched rhesus macaques. Recipients were treated with either (A) belatacept or ustekinumab monotherapy or (B) combined belatacept + ustekinumab. Serum creatinine was monitored at least weekly after transplant.

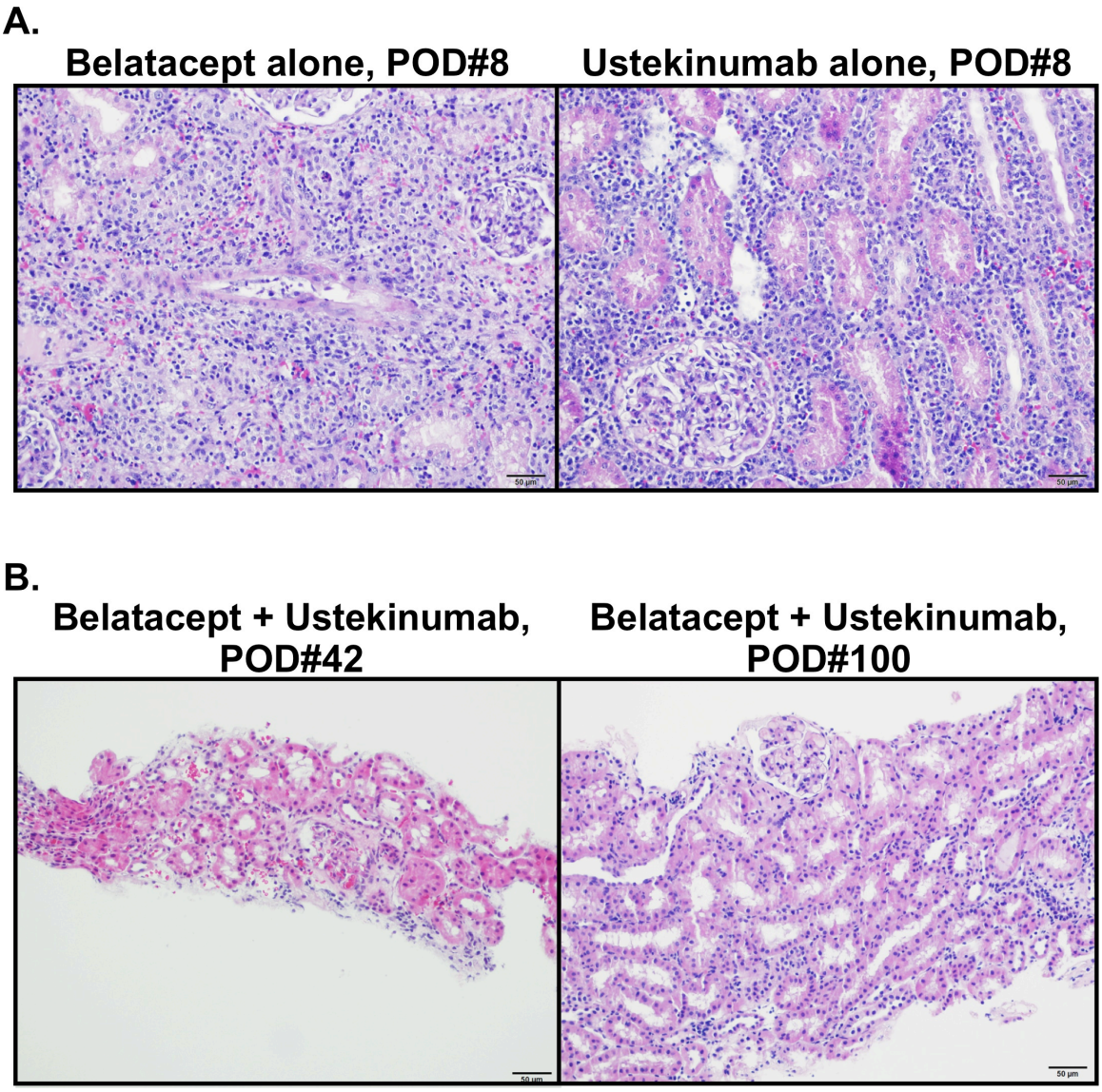

Figure 37: **Combined belatacept and ustekinumab prevents graft-infiltration of kidney allografts in non-human primates.** H&E staining of kidney biopsies from (A) kidney allografts in rhesus macaques treated with either belatacept or ustekinumab monotherapy or (B) kidney allografts in recipients treated with combined belatacept and ustekinumab. Histographs shown magnified 20x.

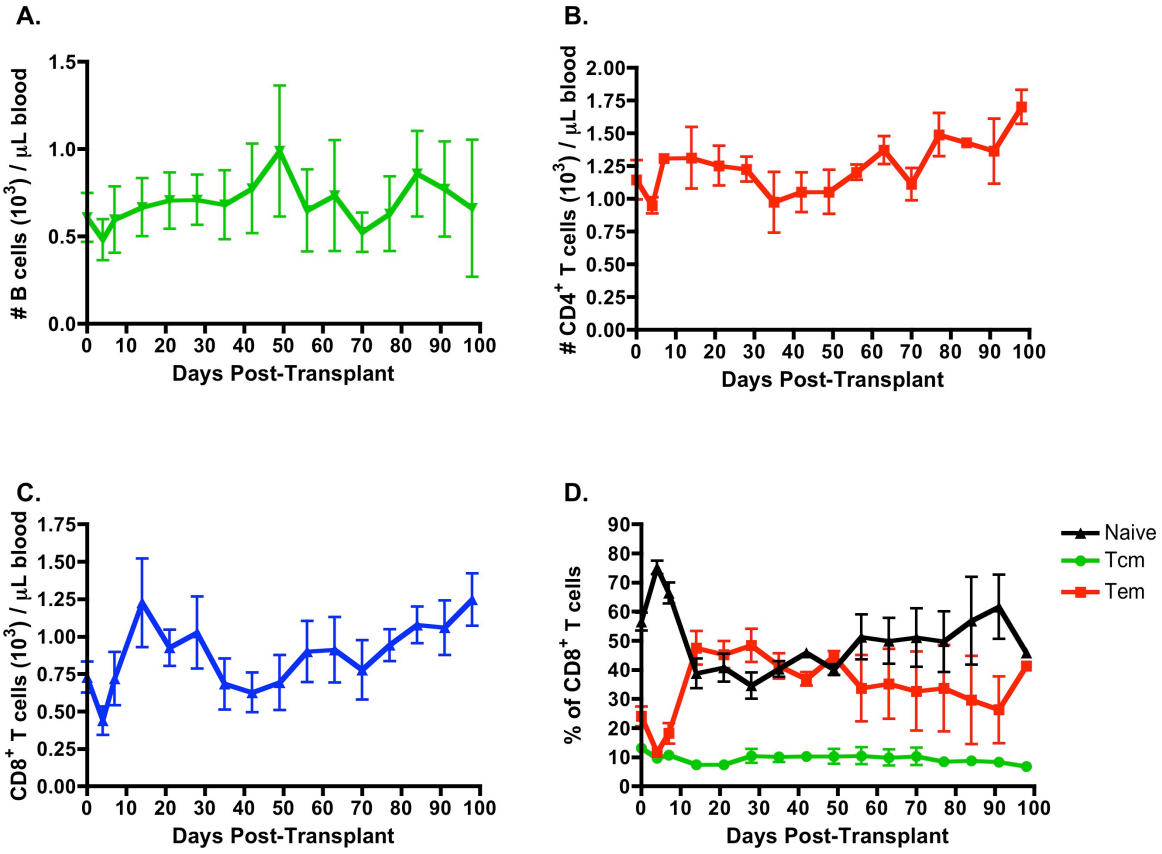

Figure 38: **Combined belatacept and ustekinumab does not deplete B cell or T cell populations**. Kidney transplants were performed in rhesus macaques, which were treated with combined belatacept + ustekinumab. Weekly flow cytometry was performed to assess absolute numbers of (A) B cells ($CD20^+CD3^-$), (B) $CD4^+CD3^+$ T cells and (C) $CD8^+CD3^+$ T cells. (D) Flow cytometry was also performed to examine memory subsets of $CD8^+$ T cells to evaluate the fraction of Naïve ($CD28^+CD95^{lo}$), central memory ($CD28^+CD95^{hi}$), and effector memory ($CD28^-CD95^{hi}$) cells.

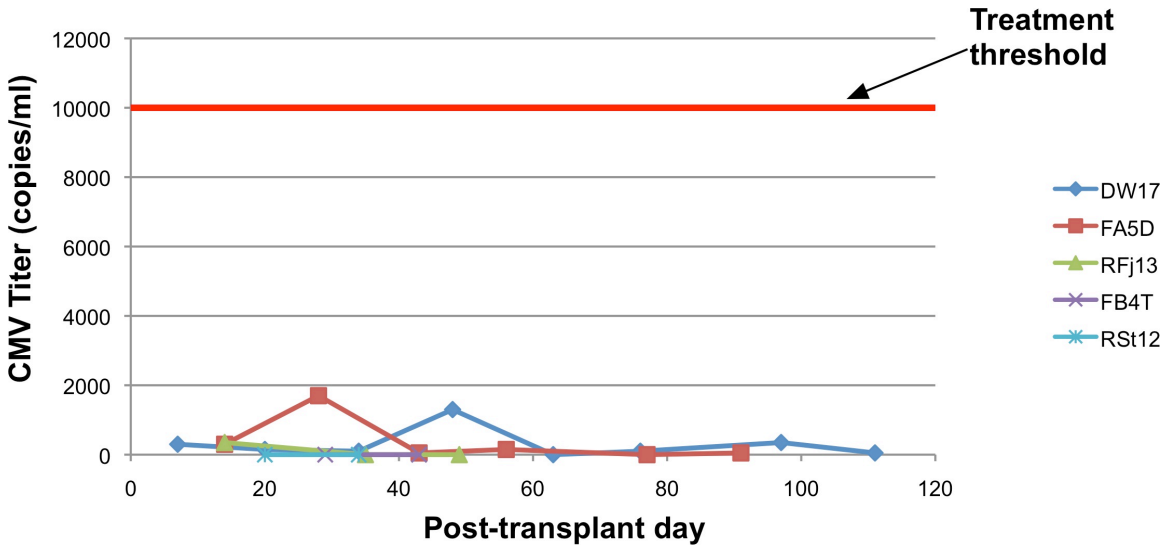

Figure 39: **Combined belatacept and ustekinumab treatment does not cause reactivation of CMV viremia**. Kidney transplants were performed in rhesus macaques, which were treated with combined belatacept + ustekinumab. CMV viral titers in whole blood were determined bi-weekly by RT-PCR.

Treatment Regimen	Effect on Th1 Alloresponses	Effect on Th17 Alloresponses	Effect on Tregs
No Treatment	+++	+	
CoB alone	+	+++	
CoB + anti-IL-12/23	+	+	+++

Figure 40: **Integrated model of the effects of combined costimulatory and IL-12/23 blockade on alloresponses**. In untreated acute transplant rejection, Th1 alloresponses dominate. Treatment with CoB alone potently inhibits Th1 cytokines such as IFN-γ. Because these Th1 cytokines normally suppress the emergence of Th17 alloresponses, CoB enables a latent Th17 alloresponse to be unveiled. Treatment with a combined regimen of CoB + anti-IL-12/23 not only inhibits Th1 cytokines (through the effects of dual costimulatory and IL-12 blockade), but also suppresses Th17 immune responses, which are dependent on IL-23. In the absence of Th1 and Th17 alloresponses, graft-protecting regulatory T cells are allowed to dominate.

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