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Immunomodulation in Transplantation: Promoting Tolerance and Preserving Protective Immunity

By

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Doctor of Philosophy

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B.A., University of North Carolina at Chapel Hill, 2007

B.S., University of North Carolina at Chapel Hill, 2009

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Abstract

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Transplantation is a widely accepted treatment for many end-stage organ diseases. However, current post-transplant immunomodulatory regimens are extremely toxic and non-specific, which limits long-term graft survival and leaves patients susceptible to opportunistic infections. Costimulation blockade (CoB) with monoclonal antibodies is a novel treatment modality aimed at preventing the activation and proliferation of alloreactive T lymphocytes. While CoB better targets graft-reactive immune responses and offers a reduced toxicity profile compared to current standard of care drugs, several barriers remain that limit the widespread adoption of CoB in transplantation. Our studies aimed to refine the balancing act of immunomodulation following transplantation, in order to promote the tolerance of a foreign organ while simultaneously maintaining protective, pathogen-specific immune responses and limiting off-target side effects.

Patients receiving belatacept, a novel CTLA4-Ig fusion protein that blocks CD28-mediated costimulation, often experience lymphoproliferative complications following primary infection with Epstein-Barr Virus. Rapamycin, an anti-fungal macrolide used for decades in transplantation to inhibit graft-specific T cell proliferation, has recently been shown to paradoxically enhance $CD8^+$ T cell responses to pathogen infection. We utilized a clinically-relevant murine model to demonstrate that rapamycin was able to enhance the quantity and quality of $CD8^+$ T cell responses to gammaherpesvirus infection in the presence of CTLA4-Ig. This augmented immune response reduced viral burden in infected animals, suggesting that this treatment may be able to improve patient outcomes and limit infectious complications.

We also investigated the use of CoB targeting the CD40-CD154 costimulatory pathway, which is involved in many highly immunogenic processes. While anti-CD154 antibodies showed great promise in preclinical studies, thromboembolic complications during clinical trials, due to interactions of the Fc portion of antibody molecules with platelet-expressed Fc receptors, stymied the translation of this therapy. We tested a novel Fc-silent anti-CD154 domain antibody, and found that this modified reagent was equally efficacious at prolonging graft survival and limiting alloreactive T cell responses compared to Fc-intact therapy.

These findings provide proof-of-concept that safe, efficacious CoB therapeutic regimens may be developed to aid in the promotion of tolerance in graft recipients and reduce morbidity and mortality in this vulnerable population.

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Chapter 1

Introduction

History of Clinical Transplantation

Since the first successful human tissue transplant in 1905, clinical transplantation has rapidly become the primary treatment modality for end-stage tissue and organ failure. Despite the relatively short history of transplantation, the idea of exchanging organs from one human has existed for thousands of years. The Chinese historian Lieh Tzu reported that Pien Ch'iao, a legendary physician who practiced medicine in the 5th century BC, had exchanged the hearts of two men, who both later recovered to live healthy lives (1). Other legends describe a miracle operation performed by saints Cosmas and Damian in the fourth century AD, in which a cadaveric leg was successfully transplanted to a living recipient (2). The veracity of these legends is doubtful, but they serve to illustrate the point that the idea of using a functioning organ from another body to replace a defective one has existed for quite some time.

However, the first major technical breakthroughs that paved the way for organ transplantation as it exists today did not occur until the early 20th century, when aseptic technique allowed for the first successful corneal transplantations (3). Fully vascularized transplants also became a possibility when Austrian physician Alexis Carrel described his technique for vascular anastomoses (4). This report, which described the process of suturing blood vessels together in order to transplant a feline kidney, would help Carrel earn the Nobel Prize for Physiology and Medicine in 1912. Interestingly, while Carrel noted that none of the organs that he had placed in experimental animals functioned for more than a few days, he never pursued the mechanisms underlying this graft failure, and thus the immunologic basis for organ rejection would remain a mystery until decades later (5).

While Carrel's surgical breakthrough overcame the major physical hurdle to clinical transplantation, when investigators began applying these techniques in animal models of transplantation during the 1930s, 40s, and 50s, the immunologic barriers to vascularized graft acceptance became apparent. It was during this time that two related findings began to shape the nascent field of transplant immunology. First, Ray Owen, a veterinary researcher at the

University of Wisconsin, described the phenomenon of hematologic chimerism in dizygotic cattle twins (6). It had previously been reported that female cattle with male twins would be sterile upon maturation, due to the exposure to male hormones through a shared circulatory system in utero. However, Owen also noted that blood antigens from the male twin would persist in these female "freemartins" to adulthood, and later studies would show that freemartins could surprisingly tolerate skin grafts from their male twin. Eight years later, Peter Medawar and colleagues Peter Billingham and Leslie Brent expanded upon this idea by performing a similar experiment in mice. Their seminal paper in Nature demonstrated that mice that received injections of splenocytes from a genetically distinct strain in utero could tolerate allogeneic skin grafts of that same strain as adults, whereas mice that did not receive such injections would all rapidly reject grafts of this type (7). Taken together, these two findings indicated that early exposure to certain antigens could "tolerize" animals and allow them to receive transplanted tissues that would otherwise be impossible. While this sort of protocol could obviously not be performed in humans, the principle of "actively acquired tolerance" had a profound impact on the direction of the field, and, most importantly, the realization that transplant rejection was an immunologic phenomenon would instruct much of the research of the next few decades.

Given that transplantation studies at this time were still quite experimental in nature, it came as quite a surprise that the first successful clinical transplantation took place only one year later. The operation involved two identical twin brothers, one of whom needed a new kidney due to chronic nephritis, and was performed by Joseph Murray on December 23rd, 1954 (8). The long-term success of this operation was predicated on the fact that the recipient was genetically identical to the donor, and thus no immunologic barrier existed to prevent the graft from being accepted. Nonetheless, this remarkable technical achievement was the first demonstration that a human kidney could be physically transplanted and continue to function in a new host – the recipient, Richard Herrick, survived for 8 years following the operation. Following this milestone, several other groups were also able to exchange organs between pairs of twins, but

transplantation remained a very risky and experimental procedure at this time. Certain groups reported varying degrees of success in unrelated organ transplantation using simultaneous bone marrow ablation and total body irradiation in order to prevent rejection (5), but the true watershed moment in transplantation would come following the discovery of several key immunosuppressive drugs.

The Origins of Immunosuppressive Therapy

The discovery of 6-mercaptopurine by Gertrude Elion and George Hitchings in 1951 represented a landmark in the field of transplantation, even though the first clinical transplant had yet to be performed. This drug was originally intended as a therapy for acute childhood leukemia, but the finding that this metabolite could inhibit cellular proliferation was soon applied to transplantation (9). A derivative of 6-MP, azathioprine, was then formulated and used successfully to prevent immune responses in both rabbits (10) and dogs (11). These early attempts at impairing the immune system achieved moderate success in both animal and human models, but still were not powerful enough to make transplantation a reliable procedure with long-lasting benefits for humans. The next breakthrough came in 1963, when a report from Thomas Starzl's group at the University of Colorado described the reversal of a case of allograft rejection by the addition of high dose prednisone to the immunosuppressive regimen (12). This strategy was remarkably successful, leading to eventual graft tolerance in some cases, and steroids remain a part of most immunosuppressive protocols today to help control perioperative inflammation. However, the considerable side effects of prolonged steroid use precluded their use as maintenance immunosuppression, and they were also unable to reliably promote long term graft survival; thus, the search continued for more effective immunosuppressive agents.

Throughout the 1960s and early 1970s, only a handful of novel agents were introduced for clinical use in transplantation. Work in animal models by Gowans and colleagues had identified lymphocytes as the cells primarily responsible for graft rejection (13, 14), and thus new

therapeutics sought to target these cells. Antilymphocyte globulin (ALG), antisera derived by the immunization of horses with human lymphoid tissue, was designed to deplete the recipient of all lymphocytes and allow for engraftment of the organ (15, 16). However, variability of ALG preparations in terms of strength and purity prevented its widespread clinical use. It was not until the landmark discovery of monoclonal antibody production by Kohler and Milstein that the true potential of antibody therapy in transplantation began to be realized (17). Monoclonal antibodies generated by hybridomas allowed for a much finer degree of specificity in targeting alloreactive lymphocytes, but were also purer and less immunogenic, and thus safer to mass-produce and use clinically. The application of this technology to clinical transplantation began with the development of OKT3, a monoclonal antibody against the CD3 portion of the T cell receptor complex, which showed efficacy in reversing kidney allograft rejection (18), and many transplant induction protocols using monoclonal antibodies are still in use. However, a truly groundbreaking discovery in transplant immunosuppression came in 1976, when Borel and colleagues first described the calcineurin inhibitor (CNI) cyclosporine A (19-21). These fungal metabolites are able to bind to calcineurin, a critical component in lymphocyte signaling downstream of the T cell receptor, and prevent it from acting on its downstream targets (22). Thus, alloreactive T cells never receive an activating signal and remain naïve and unable to mediate graft rejection. While CNIs showed a remarkable ability to prevent T cell activation and subsequent rejection in humans, and remain the main agent used in most immunosuppressive protocols, these drugs also have extremely toxic side effects, including nephrotoxicity and cardiotoxicity (23, 24). Recent reports have shown that while CNI therapy has been able to dramatically reduce the occurrence of acute rejection (within the first 12 months post-transplant) since their introduction in the late 1970s, over that same period of time, the overall half-life of grafted organs have remained relatively unchanged (25). In addition to the toxicity of these therapies, CNIs are non-specific in that they inhibit all T cell responses, and thus recipients become vulnerable to infectious disease owing to this global suppression of the adaptive immune system. Therefore, there is still an urgent need in

the field of transplantation for the discovery and implementation of new treatment modalities that are able to specifically target alloreactive responses in order to limit their toxicity as well as their negative impact on protective immunity in transplant patients.

Costimulation Blockade

In order for a T cell to become fully activated and mediate its effector functions, it must receive two signals. The first signal is the most specific, and comes in the form of a complex of a peptide and self-MHC molecule, which is recognized by the T cell receptor. The second signal that must be received in order for a T cell to be fully activated is costimulation. Costimulatory molecules are often inducibly expressed on antigen presenting cells during infection or other states of inflammation, and requiring this second, inducible signal is a way a safeguarding T cells from improper activation, since randomly generated T cell receptors may be reactive to self antigen (26). Indeed, many groups have shown that T cells that receive an activating signal thru the TCR without receiving the second costimulatory signal become anergic, or unable to respond when they see their antigen again, even if the second stimulation takes place in the presence of costimulation (27-29).

While T cells can receive costimulation thru a number of different receptor-ligand pairs, by far the best studied and perhaps most important costimulatory molecule interaction is that of T cell-expressed CD28 and antigen-presenting cell (APC)-expressed B7 molecules (CD80/CD86) (30). The ligation of CD28 on T cells by B7 leads to the transcription and stabilization of interleukin-2 mRNA, which is a vital growth factor for T cells undergoing clonal expansion (28, 31-33). In addition, CD28 costimulation lowers the activation threshold of T cells, allowing them to be more responsive to smaller amounts of antigen (34). Given that T cells receiving antigen stimulation in the absence of costimulation become anergic, it was thought that blocking CD28 signals following transplantation would anergize alloreactive cells specifically, allowing for

organ engraftment and long-term survival, and reducing the need for long-term maintenance immunosuppression.

One of the first successful costimulatory blockade agents developed was the fusion protein CTLA4-Ig. Cytotoxic T lymphocyte antigen-4 (CTLA4) is an Ig superfamily member, which was identified in T cells by screening of cytotoxic T cell cDNA libraries, and it shares a large degree of homology with CD28 (35). Early in vitro studies showed that, while not present on resting T cells, CTLA4 is upregulated following activation, and, most importantly, binds B7 molecules CD80 and CD86 with a higher affinity than CD28 (36). While multiple mechanisms by which CTLA4 regulates active immune responses have been proposed, which will be discussed later, the higher affinity of CTLA4 for B7 allows it to outcompete CD28 molecules for ligation. Therefore, by attaching an immunoglobulin tail to this molecule, Linsley and colleagues were able to create a soluble reagent that could prevent CD28-mediated costimulation and limit T cell responses in vitro and in vivo (36, 37). In rodent models of transplantation, CTLA4-Ig dramatically reduced rates of acute and chronic rejection in both rats and mice (36, 38-41). However, despite these successes in rodent models, CTLA4-Ig showed only moderate efficacy in non-human primates (42, 43). While disappointing, these results led to the discovery that CTLA4-Ig was not able to fully block B7 costimulatory molecules when administered in vivo, and thus a mutagenic approach was taken to alter the CTLA4-Ig molecule and increase its affinity for CD80 and CD86. The resulting compound, known as LEA29Y or belatacept, was able to bind with higher affinity to its targets, and it significantly prolonged kidney allograft survival in rhesus macaques (44). Clinical trials of belatacept began in the early 2000s, and early results suggested that belatacept was better able to preserve kidney function compared to the relative nephrotoxicity of calcineurin inhibitors (45). However, as the trials progressed over the next few years, it became apparent that while belatacept did offer a significant advantage in renal function compared to CNIs, patients receiving this therapy experienced markedly higher rates of acute cellular rejection (ACR), especially those receiving a more intense regimen (46). In addition to

these higher rates of ACR, there were also some safety concerns with this new therapy. Treatment with belatacept led to an increased risk of developing Epstein-Barr Virus-related post-transplant lymphoproliferative disorder, a malignant complication thought to be due to an ineffective T cell response to primary EBV infection (47, 48). This led the FDA to recommend that belatacept only be used in recipients that are already EBV-seropositive pre-transplant. Thus, while the approval of belatacept marked a great advance in the era of targeted immunosuppression, the balancing act of preventing rejection while preserving protective immunity remains a significant challenge for the field.

The exact mechanism of the increase in acute rejection seen following belatacept treatment is still somewhat unclear, but several hypotheses have been put forward. First, heterologous immunity, or the recognition of alloantigen by T cells generated by a previous pathogen infection, may be responsible for costimulation blockade resistant rejection, since memory T cells are overall less reliant on costimulation for activation than naïve T cells (49). Other groups have suggested that by binding to B7 molecules, CTLA4-Ig blocks not only CD28-mediated costimulation but also CTLA4 coinhibitory signals, which leads to ineffective blunting of the alloreactive response (50). There have been a few attempts to generate CD28-specific costimulation blockade, and various degrees of success have been achieved using CD28 Fab fragments to block costimulation in animal models (51-53). However, the dramatic failure of a clinical trial using a superagonist anti-CD28 antibody should serve as a cautionary tale when targeting this particularly potent costimulatory molecule (54). Finally, results in both mice and humans have suggested that belatacept may have a deleterious effect on regulatory T cells, since CD28 and CTLA4 signals may be important for the generation, homeostasis and function of this population (55-57). Negative impacts on the Treg population may be one mechanism underlying these early rejection events.

In conclusion, while the introduction of belatacept represents a step forward in limiting the toxicity of post-transplant immunomodulatory therapy, there is still a need for reagents that

can be used in combination with belatacept to safely and specifically target the alloreactive responses while helping to control early acute rejection.

The CD40 Costimulatory Pathway

The CD40 pathway is one of the most important costimulatory pathways involved in the generation of alloreactive responses, and represents an extremely attractive target for therapeutic intervention in transplantation. This pathway requires the interaction of CD40 molecules with its ligand, CD40L (also known as CD154). These TNF superfamily molecules are expressed on a wide range of tissues and cell types, with CD40 being constitutively expressed on APCs, including B cells, macrophages and dendritic cells, and CD154 expressed mainly on $CD4^+$ T cells following activation (58). The first descriptions of this pathway by Noelle and colleagues focused on the importance of CD40-CD154 interactions in promoting thymus-dependent humoral responses (59). However, it quickly became apparent that these interactions were also essential for generating cell-mediated immunity as well. Several studies showed that the crosslinking of CD40 on dendritic cells by CD154 on activated $CD4^+$ helper T cells led to the "licensing" of antigen presenting cells, by which these cells upregulate MHC class II molecules, adhesion molecules such as ICAM-1, and other costimulatory markers such as CD80 and CD86, and also produce proinflammatory cytokines such as IL-12 (60, 61).

The potent immunostimulatory effects of CD40-CD154 interactions make it one of the most attractive targets for immunomodulatory therapy. In 1995, the first studies demonstrating the dramatic effect of CD40-CD154 blockade showed that administration of donor lymphocytes and anti-CD154 blocking antibodies led to the long term survival of allogeneic islets (62). These findings were corroborated a year later, when Larsen and colleagues described the prolongation of mouse cardiac allografts with the use of anti-CD154 antibodies (63), and an additional report a year later detailed the remarkable synergy of combined blockade of CD28- and CD40-mediated costimulation in a mouse skin graft model (64). These findings were then extended to non-human

primates (42), and five years worth of intense research on this pathway culminated in a seminal report demonstrating the efficacy of a humanized anti-CD154 antibody in promoting long-term renal allograft survival in rhesus macaques (65). In addition to the long-term potency of these therapies, it is notable that in all of these studies, monoclonal antibodies against CD154 were used as short-term induction therapy as opposed to maintenance regimens, allowing for the inhibition of acute rejection without the long-term side effects of prolonged immunosuppression.

Given the remarkable efficacy seen in these studies, a number of clinical trials were initiated to attempt to translate this therapy to clinical use in transplantation, as well as autoimmune disease, such as systemic lupus erythematosus (SLE). Three anti-CD154 therapeutics were developed in the late 1990s and early 2000s. The first antibody developed for human use, ruplizumab, was a humanized version of the 5c8 clone used in the preclinical non-human primate studies (65). Trials in both renal transplantation and SLE were initiated, but both were halted in phase II due to thromboembolic complications, as well as episodes of acute rejection in five of the seven transplant recipients (66-70). Two other anti-CD154 therapeutics, toralizumab and ABI793, were developed and tested in clinical trials, and despite varying levels of therapeutic efficacy, thromboembolism continued to be a problem (67, 68, 71-73). Despite these setbacks, many groups remained optimistic regarding anti-CD154 therapy and saw little to no evidence of thromboembolic events in their studies (74). In addition, one group which had noted clotting events in certain monkey models demonstrated that a regimen of PGE, heparin and ketorolac, a non-selective COX inhibitor, was shown to be effective prophylaxis against these adverse events, indicating that thromboembolism could be avoided while maintaining anti-CD154 drug efficacy (75, 76). Other investigators attempted to target the CD40 molecule, rather than CD154, in order to avoid these CD154-associated complications. While some non-human primate studies showed efficacy of anti-CD40 in models of solid organ and islet transplantation (77-80), these drugs were not as effective as anti-CD154 when used as a monotherapy. In addition, concerns about B cell depletion and CMV infection tempered enthusiasm for this strategy, but

development of anti-CD40 therapeutics has continued, with clinical trials currently underway (81). However, given that targeting CD154 has been more effective, many groups sought to determine the mechanism by which anti-CD154 therapy was leading to thromboembolic events, with the hopes of designing alternative strategies to safely target this molecule during transplantation.

CD40 and CD154 are expressed on endothelial cells and activated platelets, respectively, and while CD40-CD154 interactions have been shown to play a role in thrombus formation and platelet activation, the exact nature of the required interactions is still somewhat unclear. One report describe the upregulation of CD154 following platelet activation, which can bind to CD40 expressed by endothelial cells and help activate the endothelium and initiate a proinflammatory immune response (82). Further studies showed that platelets shed soluble CD154 following activation. This soluble form then binds to platelet-associated β3 integrin molecules and induces platelet spreading and thrombus stabilization (83). Another report indicated that this soluble CD154 could bind CD40 expressed on platelets and activate them directly (84). However, clear evidence for the mechanism linking anti-CD154 therapy-related thromboembolisms to expression of CD154 on platelets was still lacking. Several in vitro studies suggested that the low affinity activating FcγRIIa was involved in antibody-mediated platelet activation (85-87). These studies argue that immune complexes of soluble CD154, which can also be shed following $CD4^+$ T cell activation, and anti-CD154 antibodies were crosslinking FcγRIIa on human platelets, leading to further platelet activation and thromboembolism. Furthermore, this Fc receptor is not expressed on murine platelets, which would explain the lack of evidence for thromboembolism in mouse studies. In fact, in a study using a humanized mouse model in which the human FcγRIIa was expressed on mouse platelets, Robles-Carrillo and colleagues demonstrated platelet activation and thrombus formation in mice following administration of immune complexes of sCD154 and anti-CD154 antibodies (88). Therefore, if anti-CD154 antibodies could be designed to avoid this Fc receptor interaction, that may prevent these clotting events from occurring, thus providing a safer alternative to the pro-thrombotic anti-CD154 therapeutics.

However, in addition to the thromboembolic complications seen in the early clinical trials, the exact mechanism by which anti-CD154 antibodies modulate donor-reactive T cell responses in animal models remains somewhat controversial. Several studies have proposed that Fc-mediated effects are actually required for the efficacy of anti-CD154 drugs. A report by Monk et al suggested that the tolerogenic effects of anti-CD154 antibodies were Fc- and complement-dependent, as anti-CD154 therapy was ineffective in FcγR and complement knockout animals (89). However, Daley and colleagues demonstrated that an aglycosylated form of anti-CD154, which exhibits reduced ability to bind Fc receptors and activate complement, was able to prolong graft survival as effectively as the glycosylated form (90). Other reports suggested that the nature of the immune challenge determine the relative requirements of Fc-mediated effector function, as aglycosylated antibodies were effective in preventing a non-human primate model of SLE, but lacked efficacy in islet and renal transplantation (91). Despite these myriad challenges, there is still hope for the translation of CD40-CD154 antagonism to clinical use, particularly in combination regimens that include other forms of costimulation blockade or immunosuppression. A recent report by our group identified a novel Fc-silent anti-CD154 antibody that showed remarkable efficacy in a murine model of transplantation (55). This antibody carried a mutation in the Fc portion that abrogated Fc binding and complement activation (92-94), yet showed the same mechanism of action as Fc-intact antibodies in preventing alloreactive responses. Furthermore, this therapy was able to synergize with CTLA4-Ig, indicating that translation of this drug for use with belatacept, a CTLA4-Ig derivative, may be possible to prolong graft survival following clinical transplantation.

The Inhibitory Fc receptors FcγRIIB

Fc receptors play a major role in both the innate and adaptive immune system, serving as a crucial link between antibody-antigen specificity and effector function. While mice and humans differ slightly in the Fc receptor genes expressed and their affinity for the constant portions of

antibody isotypes, both species have multiple activating and inhibitory Fc receptors on immune cells, allowing for both positive and negative signals to be propagated by antibody ligation (95). Activating Fc receptors are expressed by a number of innate immune cells, including granulocytes, NK cells, dendritic cells and macrophages, and ligation of these receptors by immune complexes formed by antigen and their corresponding antibody or antibody-coated cells can lead to a variety of consequences, including phagocytosis, inflammatory cytokine and chemoattractant production, oxidative burst, or modulation of antigen processing (96). The inhibitory Fc receptor, FcγRIIB, also has a widespread expression pattern and serves as a negative regulator of antibody-mediated effector functions, as the intracellular portion of this molecule contains an ITIM motif capable of recruiting phosphatases to inhibit signaling events (97).

To date, much of the literature investigating the function of FcγRIIB has focused on its critical role in B cell development, homeostasis, and germinal center control (95). FcγRIIB is the only Fc receptor expressed by B cells, and this receptor regulates only of the most important processes in humoral immune responses, affinity maturation in germinal centers. As B cells undergo somatic hypermutation in secondary lymphoid organs, simultaneous ligation of the B cell receptor and FcγRIIB lead to B cell survival, while those B cells that mutate and lose affinity for antigen and receive only FcγRIIB undergo apoptosis through a c-Abl-dependent mechanism (98). FcγRIIB can also control B cell autoimmunity at this stage as well, as conditional knockouts of FcγRIIB on class-switched B cells show increased levels of autoantibody production (99). In addition, plasma cell homeostasis also relies on FcγRIIB. Plasma cells do not express the B cell receptor, but maintain expression of FcγRIIB. Thus, the balance of pro-apoptotic FcγRIIB signaling and survival signals within the bone marrow niche combine to regulate the size and makeup of the plasma cell pool (100).

While T cells have long been thought to not express Fc receptors, a recent study investigating the consequences of repeated T cell stimulation on transcription profiles discovered that OVA-specific OT-I $CD8^+$ T cells upregulated the gene encoding FcγRIIB following repeated

infection with *Listeria monocytogenes* engineered to express the OVA protein (101). A follow-up report from the same group demonstrated that expression of this receptor on infection-generated memory $CD8^+$ T cells controlled their expansion to secondary rechallenge, and that blocking this receptor with monoclonal antibody therapy led to increased recall proliferation of these cells (102). The authors postulated that the expression of FcγRIIB on memory $CD8^+$ T cells may have evolved as a mechanism to prevent unnecessary T cell recall expansion and subsequent exhaustion when circulating IgG antibodies are able to effectively control a pathogenic challenge. While effects on FcγRIIB on B cells and APCs must be considered, if it is able to be specifically targeted, T cell-expressed FcγRIIB may represent a potential target for immunotherapy in transplantation, especially given that many transplant recipients have large populations of donor-reactive memory T cells.

The Importance of Treg for Transplant Tolerance

The goal of tolerizing transplant recipients to alloantigen in order to achieve long-term graft survival without maintenance immunosuppression has been the holy grail of transplantation immunology since Billingham, Brent and Medawar's first murine experiments (7). However, the effect seen in these studies could only be achieved in a short, prenatal window, rendering these findings inapplicable to humans. However, investigators have still attempted to apply the lessons learned from the past five decades to turn transplant tolerance from a laboratory pipe dream into a clinical reality (103).

The first attempts at inducing transplant tolerance focused on conditioning recipients to donor antigen in a non-inflammatory setting, with a similar idea to that of costimulation blockade. The principle behind this strategy is to engage the alloreactive T cell population within the recipient with antigen (i.e. transfused donor lymphocytes bearing the allogeneic MHC molecules) in a less immunologically insulting environment than a transplant, in order to attempt to anergize alloreactive T cells prior to the transplant. This strategy gained traction in the 1960s and 70s, with

the first successful donor "conditioning" reported in a model of canine kidney transplant, followed by more mechanistic descriptions of this strategy in rats (104, 105). These strategies were eventually translated to human with some success (106), and further studies combined this "conditioning" with concomitant administration of coreceptor or costimulation blockade, in order to more effectively anergize the alloreactive population (107, 108). The introduction of calcineurin inhibitors in the 1980s and their remarkable efficacy may have led donor pre-conditioning to fall out of favor, but this strategy, as well as mixed chimerism strategies to partly reconstitute the recipient immune system with donor T cells, is still being pursued by a number of groups and holds promise as one of the more feasible strategies for inducing transplant tolerance in adults (109, 110).

Regulatory T cells, or Treg, are a subset of $CD4^+$ T cells that are able to mediate suppressive immune functions, rather than the proinflammatory effector functions of other $CD4^+$ subsets, and recent reports have suggested that Treg may play an important role in the induction and maintenance of transplantation tolerance (111, 112). The idea of a T cell type that could suppress rather than enhance immune responses first arose in the 1970s, with several groups describing a phenomenon of infectious tolerance, whereby transferring populations of lymphocytes from tolerized mice to new hosts would make these hosts tolerant to the same immunizing antigen (113, 114). However, enthusiasm for these discoveries was tempered in the late 1980s when a report challenged the existence of the I-J MHC locus, which was purported to encode the soluble factors by which the suppressor cells mediated their function (115). The subsequent fallout from studies challenging the idea of suppressor cells stymied progress in this field until the mid-1990s, when Sakaguchi and colleagues published a seminal paper attributing the properties of dominant tolerance to a group of splenocytes that expressed high amounts of the IL-2 receptor alpha chain, or CD25 (116). This finding renewed interest in regulatory T cells, and ultimately the discovery of Foxp3 as the master regulator transcription factor for this lineage led to widespread acceptance of Treg as a bona fide T cell subset (117).

While initial work on suppressor T cells in the 1970s focused solely on secreted immunoregulatory factors, studies over the past decade have proposed a number of both secreted and contact-dependent mechanisms by which Treg can mediate their effects. The first proposed mechanisms stemmed from the finding that Treg express high amounts of CD25, which, along with CD122 and CD132, is one of three components of the high-affinity receptor for IL-2 (118). One study suggested that expression of CD25 acts as a "sink" for IL-2, which consumes the cytokine and leads to cytokine deprivation-induced apoptosis in effector T cell subsets (119). There are several other surface molecules implicated in Treg function, but most of these molecules are thought to act in direct contact with target cells, either APCs or effector T cells. The first, and perhaps most important, is CTLA4, which is expressed constitutively on Treg, rather than the inducible expression seen in effector subsets following activation. As mentioned before, there are several cell-extrinsic and cell-intrinsic mechanisms that have been proposed to explain the importance of CTLA4 in regulating effector T cell responses, but early reports suggested that Treg-expressed CTLA4 could be involved in the suppressive function of these cells, as the blockade of this molecule with anti-CTLA4 antibodies prevented $CD4^+CD25^+$ cells from suppressing $CD4^+CD25^-$ cell proliferation in vitro (120). Further support has come in the past decade, with studies showing that genetically ablating CTLA4 specifically on Treg populations prevented their function and led to spontaneous autoimmunity in vivo (121). CTLA4-mediated function may include both the removal of B7 costimulatory molecules from APCs (a process known as trans-endocytosis) (122) and perhaps the induction of indoleamine-2-deoxygenase (IDO) production in B7-expressing cells (123). In addition to CTLA4, other molecules suggested to play a role in Treg function are the cell-surface ectoenzymes CD39 and CD73, which can suppress effector T cells responses by generating extracellular adenosine (124, 125). In addition, other surface molecules such as Lag-3, Nrp-1, TIGIT and GITR have also been implicated in Treg function, although their roles are less clear (126-129).

While these surface molecules undoubtedly play a large role in the suppressive function of Treg, secreted molecules have also been shown to be important for modulating immune responses. Perhaps most associated with Treg function is the anti-inflammatory cytokine IL-10. Neutralization of IL-10 was shown to prevent Treg from establishing alloantigen-specific tolerance (130), and Treg-specific ablation of the IL-10 gene led to inflammation in the lungs and colon (131). The role of TGF-β, another cytokine that has been closely associated with Treg function, is less clear. While some reports showed that this factor is required to prevent autoimmune immunopathology (132), others have argued that Treg are able to mediate their suppressive function in the absence of this protein (133). There may be some redundant role of TGF-β in Treg-mediated immunosuppression, but further research is required to determine its exact function. IL-35, a recently discovered interleukin heterodimer consisting of Epstein-Barr-virus-induced gene 3 (Ebi3) and IL-12 alpha subunit, has also been shown to be a mechanism of suppression, as Tregs deficient in either subunit are functionally impaired in a model of T cell-dependent colitis (134). Finally, Granzyme A and B, cytokines most often associated with cytotoxic potential in $CD8^+$ T cells, can also be expressed by human Treg and lead to perforin-dependent killing of effector T cells (135).

These results demonstrate Treg mediate their suppressive functions through various mechanisms, there is experimental evidence that these cells play an important role in preventing both acute and chronic allograft rejection. In many animal studies, alloantigen-specific Treg that had been generated in vitro and then transferred to transplanted mice showed that these cells could promote long-term tolerance, through both CTLA4- and IL-10-dependent mechanisms (130, 136-140). Another study demonstrated the ability of human Treg to prevent transplant arteriosclerosis, a chronic condition that leads to hardening and thickening of arteries within the organ and eventual graft failure, in a humanized murine model (141). While these studies showed that Treg are able to prevent rejection when present in great enough numbers, the numbers of Treg present in unmanipulated hosts is usually insufficient to allow for graft tolerance. Therefore,

current work in regulatory T cell therapy in transplantation is focused on either expanding existing Treg or adoptive immunotherapy of ex vivo generated Treg in order to curb alloreactivity.

However, while the absolute numbers of Treg certainly matter in determining the outcome of an alloreactive response, it is currently unclear if Treg derived from natural, or thymically-derived Treg (nTreg), or Treg induced in the periphery (iTreg) are better for curbing alloreactivity. One study determined that the pool of allospecific Treg that arise following a tolerance induction protocol came from both the pre-existing nTreg pool as well as the conversion of naïve non-Foxp3$^+$ CD4$^+$ cells into Foxp3$^+$ iTreg (142). However, other models have suggested that in some settings the conversion of naïve Treg into antigen-specific iTreg is critical for the establishment of long-term graft tolerance. These studies, which were performed in the presence of a tolerizing regimen of donor-specific transfusion and anti-CD154 antibodies, demonstrated that naïve CD4$^+$ T cells are converted to antigen-specific iTreg that then migrate to the graft to mediate their suppressive effects (143, 144).

Finally, as mentioned above, one of the major drawbacks of treatment with the costimulation blockade agent belatacept is its deleterious effect on Treg. Given that CD28 signaling has long been associated with Treg homeostasis and function (145, 146), it was not surprising that an agent designed to block this signals may have off target effects on the Treg population. Indeed, in a MHC Class II mismatch mouse model of transplantation, where graft survival is dependent on Treg, CTLA4-Ig treatment actually led to accelerated rejection due to a loss of Treg populations in the periphery (57). However, recent data from our group has shown that while treatment with CTLA4-Ig led to a decrease in endogenous Treg populations, alloantigen-specific Treg induced by anti-CD154 treatment were maintained during combined treatment with CTLA4-Ig (55). Interestingly, previous studies had indicated that a fraction of Treg populations are constantly turning over in steady-state conditions, and that while this maintenance proliferation was associated with an increase in expression of CTLA4, blocking the CD28 pathway could prevent this proliferation (147). These results would indicate that the cell-

intrinsic properties of antigen-specific iTreg and endogenous nTreg are different, and that perhaps some other signal, either through the iTreg TCR or cytokine receptors, may allow these cells to preferentially survive in the face of CTLA4-Ig treatment.

Regulatory T cells are an integral part of the adaptive immune system's ability to control inflammatory responses (148), and by harnessing the potential of this population, transplant immunologists may be able to more effectively and naturally prevent alloreactivity while limiting the use of toxic immunosuppressant therapy.

Heterologous Immunity and Alloantigen-Specific Memory T Cells

Most of the experimental animal studies investigating the immunologic mechanisms underlying alloreactive responses and transplant tolerance have been performed in relatively pathogen-free environments. However, throughout their lifetimes, humans are constantly exposed to infectious agents that lead to the generation of immunologic memory in the form of memory $CD4^+$ and $CD8^+$ T cells and long-lived antibody responses. While these responses are often able to protect an individual against re-infection, they may be a barrier to the induction of transplant tolerance through a phenomenon known as heterologous immunity (149). The T cell receptor recognizes short peptide sequences in the context of an MHC molecule, and thus is able to crossreact with distinct peptide epitopes that do not share complete sequence homology (150). Studies of human cells have shown that virus-specific T cells clones are capable of cross-reacting with peptide-MHC complexes expressed by allogeneic cells (151). In fact, the dual recognition of alloepitopes by pathogen-induced cells is quite common in human T cell populations. One study measured the HLA reactivity of a number of virus-specific clones from healthy individuals, with 45% of isolated T cell clones reacting against at least one allogeneic HLA molecule (152). In addition, EBV-specific T cells from HLA-B*07 healthy controls were shown to proliferate in a mixed lymphocyte reaction when exposed to a common alloantigen, HLA-B*44 (153). Therefore, memory T cells generated by a viral infection that crossreact with alloantigen may be able to

mediate graft rejection. Several experimental models in mice have implicate these cells in graft rejection (154-156), and human studies have shown that pathogen-generated allospecific memory cells are present in the circulation of organ recipients at the time of transplantation (157, 158).

These potentially alloreactive memory cells are significant in transplantation immunotherapy, since memory cells possess a much lower activation threshold than naïve cells and are able to more rapidly proliferate and mediate their effector functions (159, 160). In addition, memory cells are less reliant on costimulation in order to achieve full activation (161). This independence from costimulation of memory cells means that these cells may not be as sensitive to many of the tolerance-inducing costimulation blockade regimens that work so well in animal models (162, 163). In addition, the generation of memory cells crossreactive to alloantigen can increases the precursor frequency of graft-specific cells. Tolerance induction protocols in non-human primates were shown to be ineffective when animals harbored a larger number of donor-reactive memory cells (164), and specifically targeting memory cells with an anti-CD2 antibody promoted graft survival in another NHP model (165). Furthermore, a study of human kidney recipients showed that higher numbers of donor-reactive, IFN-γ-producing lymphocytes pre-transplant correlated with a risk of acute rejection episodes (166). Therefore, memory T cells generated by environmental exposure to pathogens that crossreact with donor antigens may present a barrier to the induction of transplant tolerance.

Post-Transplant Opportunistic Infections and Morbidity

While the primary goal of transplant immunosuppression is the inhibition of alloreactive responses, global immunosuppression can leave patients vulnerable to a wide range of infectious complications resulting from opportunistic bacterial, fungal and viral infections. Immediately following transplantation, patients are the most susceptible to complications due not only to the surgical wounds and subsequent inflammation, but also due to the fact that the immediate post-operative stage is when patients are subjected to the highest doses of immunosuppressants to

avoid acute rejection. Bacterial infections, including nosocomial-acquired methicillin-resistant *Staphylococcus* and vancomycin-resistant *Enterococcus* species, are of particular concern at this stage (167). Several experimental models have investigated the effect of infection at the time of transplantation, and shown that bacterial or viral infections can actually prevent the induction of tolerance (168, 169). However, as detailed below, the most common post-transplant infections tend to occur between 1 and 12 months post-transplantation, with viral complications being particular ly problematic for transplant patients.

Some of the most common infections include de novo infection with and reactivation of latent and persistent viruses, which are a major source of morbidity and mortality post-transplant (170). Cytomegalovirus, a beta herpesvirus, is the most common infection following kidney transplantation (167). In addition to the risk that this virus poses to an immunosuppressed individual, CMV infection can also invade the allograft and lead to chronic nephropathy and allograft rejection (171). For this reason, most transplant patients are either treated prophylactically or preemptively (once CMV replication is detected) to avoid CMV complications, and anti-viral agents such as valgancyclovir are commonly used to treat this disease (172). Other viral infections common to kidney recipients and major causes of graft failure are polyoma viruses, include BK and JC virus. These viruses are extremely common, as BK seroprevalence is over 85% in the general population. Polyoma-virus associated nephropathy (PVAN) is the major complication arising post-transplant, which can result in graft failure in up to 50% of cases (173). The main mode of treatment is reduction of immunosuppression, with several anti-viral therapies undergoing clinical evaluation for use to control this disease (174). Finally, Epstein-Barr Virus (EBV) is another common pathogen that can lead to severe pathology following transplantation, and, as described below, these complications are strongly associated with the use of CD28-directed costimulation blockade.

Epstein-Barr Virus is a double-stranded DNA virus of the γ herpesvirus family. Approximately 90-95% of adults in the developed world are seropositive for this virus, but

pathology is normally effectively controlled by adaptive T cell responses, and the virus persists mostly in a latent state in its reservoir, memory B cells (175). However, many transplant recipients, especially pediatric patients, are still EBV seronegative at the time of transplantation, and exposure to EBV while subject to immunosuppressive regimen can lead to the development of EBV-associated post-transplant lymphoproliferative disorder (PTLD) (176, 177). PTLD is a term that denotes a spectrum of disorders that all share the feature of uncontrolled lymphocyte proliferation. This disease state had a range of severity, and several known risk factors include the age of the recipient, the type of organ transplanted, the intensity and makeup of the immunosuppression regimen, and EBV serostatus prior to transplant (178). Since T cell responses are a major factor in controlling the lytic phase of primary gamma herpesvirus infections (175, 179), impairment of T cell responses due to immunosuppression can lead to development of PTLD. As evidence of this, the magnitude of virus-specific $CD8^+$ T cell responses have been shown to be a correlate of protection against PTLD development (180), and numerous animal models of gammaherpesvirus infections also rely on cytotoxic $CD8^+$ T cells for viral control (181). Of particular interest to our group, the initial clinical trials of belatacept showed that patients treated with this drug were at an increased risk of developing EBV-associated PTLD (47). The FDA accordingly issued a black-box warning, indicating that belatacept should not be used in EBV seronegative transplant recipients. However, since many pediatric patients stand to benefit the most from the long-term functional benefits of belatacept over calcineurin inhibitors, strategies to improve EBV-specific responses are of particular interest to this population.

Current treatments for PTLD take two general approaches, targeting the virus itself or attempting to boost the adaptive immune response either by reducing the level of immunosuppression or selectively enhancing virus-specific responses. One case study claimed that antiviral treatment with acyclovir was effective in treating PTLD (182), but this treatment has not been formally evaluated in a large-scale trial. Interferon treatment has also shown a limited amounts of success (183), but a lack of mechanistic data and concerns about off-target effects on

transplanted organs has limited its widespread acceptance as a viable treatment. Currently, secondary to the reduction of immunosuppression, the use of rituximab, a chimeric antibody against the B cell antigen CD20, has also shown promise in the treatment of PTLD by directly targeting and depleting the proliferating B cells, with response rates as high as 50% in some studies (184, 185). However, the most effective treatment for PTLD remains the reduction of immunosuppression, with the goal of allowing the endogenous T cell response to recover and respond to the proliferating B cells. In addition, other treatments that have focused on enhancing EBV-specific immune responses have shown some promise in preventing and treating PTLD. While adoptive cellular therapy with T cells expanded and primed to EBV antigens ex vivo has been effective in small studies in hematopoietic (186) as well as solid organ transplantation (187), this is a time-consuming and expensive process, and unlikely to become common clinical practice. Another potential strategy may be the administration of therapeutic agents that can adjuvant EBV-specific T cell responses. Surprisingly, one candidate therapy that may be able to achieve this goal is rapamycin, a drug that has been used for decades in the context of transplantation and has recently been shown in a number of studies to not only inhibit viral replication, but may, as detailed below, paradoxically enhance pathogen-specific immune responses.

mTOR Inhibition in Transplantation

Rapamycin is a macrolide produced by the bacteria Streptomyces hygroscopicus, and was discovered in soil samples from Easter Island in the 1970s (188). Intracellularly, rapamycin acts by binding to FK506/rapamycin-binding protein 12 (FKBP12), and then binding and inhibiting the kinase activity of the mechanistic target of rapamycin, or mTOR (189, 190). The mTOR kinase is responsible for integrating signals from the extracellular environment, including the growth factors, amino acids, glucose, and stress (191). mTOR is actually a subunit of two distinct protein complexes known as mTORC1 and mTORC2. These complexes differ in both their downstream signaling targets as well as their sensitivity to inhibition with rapamycin. mTORC1

signals through S6K1 and 4E-BP1, which are intimately involved in the control of mRNA translation and protein synthesis (192), is sensitive to inhibition with rapamycin. mTORC2 acts on a different set of substrates, including Akt, PKC, and SGK, and is critical in controlling actin reorganization and cell polarization (193). While some data suggest that prolonged treatment with rapamycin may lead to inhibition of mTORC2 (194), this complex is thought to be generally insensitive to inhibition with this therapy, due to a lack of binding partner for the rapamycin/FKBP12 complex.

Due to its ubiquity in mammalian cells, it is not surprising that mTOR signaling plays an extremely important role in both innate and adaptive immune responses. Rapamycin has a variety of effects on both the maturation of dendritic cells and their ability to uptake and present antigen to the adaptive immune system. Studies have shown that treatment with rapamycin can limit the maturation and function of DCs, specifically the upregulation of costimulatory molecules to stimulate T cell responses (195). In addition, human dendritic cells differentiated in the presence of rapamycin were defective in their ability to endocytose extracellular antigen (196). These findings, along with a larger body of data, suggest that rapamycin has an overall tolerogenic effect on dendritic cell biology. In addition to the effects on antigen presenting cells, rapamycin has dramatic effects on both $CD4^+$ and $CD8^+$ T cells. Costimulatory signals through CD28 are required for full T cell activation, and mTOR is a key component of this intracellular signaling cascade (197). In addition, recent $CD4^+$ T cells integrate a number of environmental signals, such as cytokines and growth factors, to differentiate into a variety of lineages with distinct effector functions, including Th1, Th2, Th17, Tfh, and Treg subsets (198). Extensive work by Powell and colleagues have demonstrated that either genetic disruption of mTORC1 or mTORC2, as well as treatment with rapamycin, can influence $CD4^+$ T cell differentiation and alter cell fate (199, 200). As a result, several studies have used rapamycin to promote Treg expansion following transplantation in both murine and non-human primate models (201-203).

While originally intended as an anti-fungal therapy, it was noted that rapamycin possessed potent anti-proliferative properties and thus was considered an ideal candidate for use following transplantation, in order to limit the proliferation of alloreactive lymphocytes (204). A number of animal models of transplantation provided evidence that rapamycin could prevent allograft rejection at therapeutic levels ranging from 5-30ng/mL, including rodent, rabbit, dog, pig and baboon (205). In 1999, the FDA approved rapamycin for use in human renal transplantation, and over the last 15 years rapamycin has become widely used clinically. However, in the last few years, one of the most surprising properties of rapamycin was identified – the paradoxical enhancement of $CD8^+$ T cell responses to certain pathogen infections.

Rapamycin-Induced Augmented T Cell Immunity

While immunosuppression following transplantation can lead to serious infectious complications due to impaired immune responses, one interesting finding during the early clinical experience with rapamycin was that patients treated with this drug paradoxically experienced fewer infectious and malignant complications, specifically in terms of CMV infection and reactivation (206-208). Given that the mechanism of action of rapamycin is the inhibition of the intracellular kinase mTOR, whose downstream signaling targets include molecules intimately involved in mRNA translation including S6K1 and 4E-BP1 (193), the direct anti-viral effects of rapamycin were thought to be largely responsible for the improvement in infection-related outcomes in treated patients. Indeed, several studies have shown that rapamycin is able to inhibit viral replication in a number of models, including CMV, EBV, and HIV (209-212).

However, a seminal study in 2009 demonstrated that mTOR inhibition with rapamycin could paradoxically enhance cytotoxic $CD8^+$ T cell memory generation in a model of acute infection (213). These findings were then extended to non-human primates in a study in which sirolimus treatment during prime-boost vaccination with modified Vaccinia Ankara (MVA) led to an increased number of virus-specific IFN-γ producing $CD8^+$ T cells (214). In addition to the

immunostimulatory effects of rapamycin on $CD8^+$ T cell responses to pathogen infection, rapamycin is also able to enhance tumor-specific responses. Rapamycin treatment enhanced tumor protection in a homeostatic-proliferation induced memory model (215), and also enhanced protection following vaccination with tumor antigen (216). Given that rapamycin had historically been utilized as an immunosuppressant and anti-proliferative, these results came as a surprise to the transplant community. A study by Ferrer and colleagues used a transgenic mouse system to compare the effects of rapamycin in the setting of a pathogen infection to its effects following transplantation, using a modified *Listeria monocytogenes* modified to express chicken ovalbumin (OVA), as well as transgenic mOVA mice that constitutively express OVA under the B-actin promoter. This allowed the investigators to compare the effects of rapamycin on a target population of OT-I T cells engineered to express a TCR specific for OVA. By directly comparison of the effects of rapamycin in these different inflammatory settings while controlling for differences in antigen or TCR affinity, this study demonstrated that while rapamycin enhanced the quantity and quality of $CD8^+$ T cell responses to pathogen infection, it failed to do so in the setting of a graft (217), indicating that the context in which rapamycin is administered can determine its effects on T cell responses.

The mechanism by which mTOR inhibition is able to augment pathogen- and tumor-specific responses can be explained through the lens of $CD8^+$ T cell differentiation. mTOR signaling affects various aspects of $CD8^+$ T cell biology, especially the expression of homing receptors such as CD62L and CCR7, as well as transcription factors Tbet and Eomesodermin. Hypotheses of exactly how $CD8^+$ T cells differentiate from naïve cells to effector to memory differ, but most models suggest that this process occurs on a gradient and is dependent on cumulative signals received from TCR, costimulation, and cytokines, especially IL-2, and that increasing strength or duration of these signals leads cells to lose memory potential and adopt a more “terminal effector” phenotype with a limited ability to survive long-term (218, 219). Rapamycin may function to reduce the overall amount of differentiation signaling that occurs

within these cells, allowing them to maintain a more memory-like phenotype, with increased proliferative and survival capacity (220, 221), and the transcription factors Tbet and Eomesodermin play a critical role in this process. These molecules cooperate during early T cell activation to induce key gene expression for T cell migration and effector function, including CXCR3, CXCR4, granzyme B, and perforin (222, 223). Eomesodermin in particular has been shown to be directly responsible for transcription of CD122, responsible for responsiveness to IL-15, a key cytokine in $CD8^+$ T cell memory (224). Studies investigating the effect of rapamycin on the expression of these markers have shown that treatment with rapamycin enhances the ratio of Eomesodermin to Tbet, which enhances the generation of memory rather than effector lineages (225). In terms of migratory ability, naïve $CD8^+$ T cells express high amounts of CD62L and CCR7, which allows them to home to secondary lymphoid organs, where their ligands are highly expressed and increases the likelihood of encountering cognate antigen presenting by dendritic cells (226). mTOR signaling following activation, through costimulatory molecules and other receptors, induces a downregulation of these markers, which allows these activated effectors to migrate out of lymphoid organs to peripheral sites. Rapamycin treatment allows $CD8^+$ T cells to retain expression of CD62L and CCR7 and home to these secondary lymphoid organs, which may help in generating high quality central memory-like T cells (227). It is hypothesized that antigen persistence or differences in inflammatory milieu are responsible for the differential effect of rapamycin on pathogen versus graft specific responses; however, further studies are needed to fully delineate this dichotomous phenomenon.

$CD8^+$ T Cell Metabolism and Cell Fate

Another finding to emerge from studies of the pro-memory effects of mTOR inhibition on $CD8^+$ T cell responses to pathogen infection was the integral role of T cell metabolism on these responses. Naïve $CD8^+$ T cells are a metabolically quiescent population, relying mainly on oxidative phosphorylation to generate ATP. However, when activated, T cells must rapidly adjust

their metabolism to meet the bioenergetic demands required by the extensive proliferation the takes place during the expansion phase of the immune response. This switch to mainly glycolytic ATP production, also known as the Warburg effect (228), is not as efficient as oxidative phosphorylation, but is necessary to produce biochemical intermediates necessary for growing and dividing cells. Following the peak of an infection, T cells undergo a massive contraction phase, wherein 90-95% of the expanded effector T cell population undergoes apoptosis, and a small fraction of this population survives this phase and forms a stable, long-lived memory population (218). However, in order to maintain itself, this memory population must again adjust metabolically back to predominantly oxidative phosphorylation, as the glycolytic phenotype is not sustainable (229).

One of the first studies to address the link between metabolism and T cell memory demonstrated that by promoting fatty acid oxidation, either through the use of rapamycin or metformin, could potentiate memory formation (230). Follow up studies further delineated the metabolic differences in various steps in T cell differentiation. While naïve and memory T cells were generally more oxidative and effector T cells were more glycolytic, the most notable difference was the enhanced spare respiratory capacity (SRC) of memory T cells compared to both naïve and effector cells (231). SRC in this setting is defined as the difference between baseline oxygen consumption and this same measure following uncoupling of ATP production from electron transport chain with FCCP. An increase in SRC indicates that memory cells have a greater mitochondrial potential, which allows cells to cope with situations of environmental stress and may be important for long-term survival of this cell population (232). While one of the mechanisms by which rapamycin may enhance memory generation is through increasing the spare respiratory capacity of $CD8^+$ T cells, this has yet to be demonstrated experimentally. However, several reports have demonstrated that rapamycin does dramatically affect metabolism by mimicking signals similar to amino acid deprivation and promoting fatty acid oxidation (233-235), and thus these effects may be partly responsible for rapamycin's immunostimulatory effects.

Conclusions

Immunomodulation strategies have advanced significantly since the dawn of clinical transplantation, and graft and patient survival are at all time highs. However, significant challenges remain in extending the long-term survival of allografts and balancing immunosuppression to preserve protective immunity. Mechanistic study of the underlying immunologic processes involved will allow for more targeted immunotherapy and better patient outcomes following organ transplantation.

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Chapter 2

An Anti-CD154 Domain Antibody Prolongs Graft Survival and Induces FoxP3$^+$ iTreg in the Absence and Presence of CTLA-4 Ig

Abstract

The use of monoclonal antibodies targeting the CD154 molecule remains one of the most effective means of promoting graft tolerance in animal models, but thromboembolic complications during early clinical trials have precluded their use in humans. Furthermore, the role of Fc-mediated deletion of CD154-expressing cells in the observed efficacy of these reagents remains controversial. Therefore, determining the requirements for anti-CD154-induced tolerance will instruct the development of safer but equally efficacious treatments. To investigate the mechanisms of action of anti-CD154 therapy, two alternative means of targeting the CD40-CD154 pathway were used: a non-agonistic anti-CD40 antibody, and an Fc-silent anti-CD154 domain antibody. We compared these therapies to an Fc-intact anti-CD154 antibody in both a fully allogeneic model and a surrogate minor antigen model in which the fate of alloreactive cells could be tracked. Results indicated that anti-CD40 mAbs as well as Fc-silent anti-CD154 domain antibodies were equivalent to Fc-intact anti-CD154 mAbs in their ability to inhibit alloreactive T cell expansion, attenuate cytokine production of antigen-specific T cells, and promote the conversion of $Foxp3^{+}$ iTreg. Importantly, iTreg conversion observed with Fc-silent anti-CD154 domain antibodies was preserved in the presence of CTLA4-Ig, suggesting this therapy is a promising candidate for translation to clinical use.

Introduction

Blockade of CD40-CD154 interactions during T cell priming has shown to be a highly effective means of inducing long-term survival of allografts and transplantation tolerance in both murine and non-human primate models (1, 2). However, the clinical potential of this therapy has yet to be realized due to the thromboembolic complications observed during clinical trials of an anti-CD154 monoclonal antibody (3). Thus, determination of the requirements needed to achieve this salutary effect would guide development of novel therapeutics targeting this pathway with improved safety profiles and potential for translation for clinical use.

The mechanisms by which anti-CD154 antibodies induce profound immunomodulation of donor-reactive T cell responses in animal models, resulting in long-term graft survival and in some cases tolerance, is still controversial. A previous report suggested that the tolerogenic effects of anti-CD154 antibodies were Fc- and complement-dependent (4). However, Daley and colleagues demonstrated that an aglycosylated form of anti-CD154, which exhibits reduced ability to bind Fc receptors and activate complement, was able to prolong graft survival as effectively as the glycosylated form (5). In addition, antibodies that target the CD40 molecule have also been shown to be efficacious in preventing alloreactivity in both mouse and non-human primates (6, 7). Therefore, our study aimed to directly compare the effects of an Fc-intact anti-CD154 antibody to both a non-agonistic anti-CD40 antibody as well as a clinically translatable Fc-silent anti-CD154 domain antibody (dAb). This novel reagent was generated by fusing a human anti-mouse CD154 Vκ domain antibody to a mutated mouse IgG1 Fc (D265A), to abrogate FcgR interactions (8-10).

Accumulating evidence suggests that the presence of $CD4^+$ $CD25^+$ regulatory T cells (Treg) may be critical for the induction of graft tolerance (11, 12). Previous work by our group has shown that perturbation of the CD40-CD154 pathway with an Fc-intact anti-CD154 mAb antibody led to the generation of antigen-specific induced Treg (iTreg) (13). However, whether this conversion requires the deletion of alloreactive effector T cells or simply blockade of the

CD40-CD154 interaction remained unknown. Furthermore, previous studies demonstrated that CTLA4-Ig and Fc-intact anti-CD154 synergize to promote graft survival (2, 14), despite the known negative effect of CTLA4-Ig on Treg (15, 16). Therefore, we sought to determine if iTreg generation was preserved when CTLA4-Ig was given in combination with a potentially clinically translatable anti-CD154 dAb.

To address these issues, we employed both a fully allogeneic model of skin transplantation as well as a transgenic system of minor antigen disparity in which the non-self antigen ovalbumin (OVA) is constitutively expressed on donor-derived cells. We demonstrate that, relative to an Fc-intact anti-CD154 antibody, both anti-CD40 as well as Fc-silent anti-CD154 dAbs were able to comparably prolong graft survival, attenuate alloreactive cytokine production and promote robust iTreg conversion. Importantly, the iTreg conversion observed following anti-CD154 dAb treatment was preserved in the presence of CTLA4-Ig treatment, highlighting the potential synergy of these therapies. Taken together, these results suggest that blockade of the CD40-CD154 pathway, rather than Fc-mediated deletion of alloreactive cells, mechanistically underlies the attenuation of donor-reactive $CD8^+$ T cell responses, induction of $Foxp3^+$ iTreg, and prolongation in graft survival observed following CD154 antagonism. These studies provide proof-of-concept that development of Fc-silent anti-CD154 domain antibodies may be translatable for use in clinical transplantation.

Materials and Methods

Mice

B6-Ly5.2/Cr (H2-K^b, CD45.1), C57BL/6 (H2-K^b, CD45.2), and BALB/c (H-2K^d) mice were obtained from NCI (Frederick, MD). OT-I and OT-II transgenic mice, purchased from Taconic Farms (Germantown, NY), were bred to Thy1.1^+ background at Emory University. mOVA mice (17) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained in accordance with Emory University IACUC guidelines (Atlanta, GA). All animals were housed in pathogen-free animal facilities at Emory University.

Donor Specific Transfusion and Adoptive Transfers

For DST administration, 10^7 BALB/c bone marrow cells or mOVA splenocytes were given prior to transplantation. For adoptive transfers, spleen and mesenteric LNs of OT-I and OT-II mice were processed and $1.5x10^6$ of each CD45.2^+ or Thy1.1^+ OT-I and OT-II were injected intravenously.

Skin Transplantation and Antibody Treatment

Full thickness tail, ear, or trunk skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages. Where indicated, mice were treated with 250μg CTLA4-Ig (Bristol Myers Squibb), 250μg hamster monoclonal anti-mouse CD154 (MR-1, BioExpress, West Lebanon, NJ), rat anti-mouse CD40 (6) (7E1-G2b, Bristol Myers Squibb) or human anti-mouse CD154 Vκ domain antibody fused to a mutated mouse IgG1 Fc (D265A) to abrogate FcgR interactions (Figure S1) (8-10) (Bristol Myers Squibb), intraperitoneally on days 0, 2, 4, and 6 post-transplantation.

Surface Stains and Flow Cytometry

Spleens or draining axillary and brachial LN were stained for CD4, CD8, Thy1.1, and CD44 (Biolegend). Samples were analyzed using an LSRII FACS machine (BD Biosciences). Data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Intracellular Cytokine Staining

Where indicated, responder splenocytes were stimulated with BALB/c splenocytes or 10nM $OVA_{257-264}$ (Genscript, Inc.) in the presence of 10 μg/mL Brefeldin A for 4-5 hours. Intracellular staining kit was used to detect TNF, IFN-γ (Biolegend), and IL-2 (BD Biosciences) according to manufacturer's instructions.

T_{reg} Staining

Splenocytes and draining lymph nodes were processed and stained with antibodies to CD4, CD8, Thy1.1 and CD25 (Biolegend). Intracellular staining with anti-Foxp3 was performed using intranuclear staining kit (eBiosciences), according to manufacturer's instructions.

Surface Plasmon Resonance

Surface Plasmon resonance (SPR) studies were performed on a Biacore T100 instrument (Biacore, GE Healthcare) at 25C, and data analysis was performed using BiacoreT100 evaluation software V2.0.2. Sensor surfaces were prepared by immobilizing 20 ug/ml Fab fragment from a murine anti-6XHis antibody (Bristol Myers Squibb) in 10 mM sodium acetate pH 4.5, on flow cells 1 and 2 of a CM5 sensor chip to a density of 3200 RU. 7 ug/ml of murine Fc receptors mCD64 (R&D Systems #2074-FC), mCD32b (R&D #1460-CD), mCD16 (R&D #1960-FC) and mCD16-2 (R&D #1974-CD) were captured on flow cell 2 using a contact time of 30 s at 10 ul/min, resulting in capture levels of ~550-900 RU. The binding of Fc-modified anti-CD154 dAb was evaluated in comparison to a positive control 97 kDa protein containing a wild type murine IgG2

Fc domain (protein-mIgG2A). Each protein was tested at 1 uM concentration using an association time of 120 s and a dissociation time of 180 s at 30 ul/min, and the surfaces were regenerated between cycles using 2 pulses of 10 mM glycine pH 2.5 for 15 s at 30 ul/min.

Statistical Analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For analysis of T cell accumulation, one-way ANOVA tests were performed, followed by Tukey post-test. Analyses were done using GraphPad Prism software (GraphPad Software Inc).

Results

Anti-CD154 domain antibodies are functionally equivalent to Fc-intact anti-CD154 mAbs

We sought to investigate the requirements for CD40-CD154 immunotherapy-mediated survival by comparing the effects of an anti-CD40 mAb (7E1-G2b) vs. an Fc-modified anti-CD154 dAb that is unable to mediate any effector function (Figure S1) (10) vs. the traditional Fc-intact anti-CD154 mAb. We applied these therapies in a stringent BALB/c → B6 model of murine skin transplantation. Control animals treated with CTLA4-Ig alone rapidly rejected their grafts with an MST of 15.5 d. In contrast, the addition of each reagent targeting the CD40-CD154 pathway significantly prolonged graft survival, with anti-CD154, anti-CD40 and anti-CD154 dAb extending MST to 33, 37 and 31 days, respectively (Fig 1A, B). Therefore, blockade of CD40-CD154 interactions with novel anti-CD154 dAbs for only the first 6 days post-transplantation is sufficient to prolong graft survival to a similar extent as Fc-intact anti-CD154 treatment.

Activation and cytokine production of alloreactive T cells are diminished in the presence of anti-CD154 dAbs

We next sought to obtain a more granular assessment of the impact of each therapeutic strategy on the alloreactive $CD4^+$ and $CD8^+$ T cell response following transplantation. B6 mice received BALB/c skin grafts and were treated with anti-CD154, anti-CD40 or Fc-silent anti-CD154 dAbs. At day 14 post-transplant, splenocytes were harvested and analyzed for expression of activation markers. Untreated mice developed a robust allogeneic response, as evidenced by the higher proportion of CD44-expressing $CD4^+$ and $CD8^+$ T cells (26.3±0.8% and 34.7±2.2%), whereas mice treated with anti-CD154, anti-CD40 and Fc-silent anti-CD154 dAbs all showed significant reductions in these populations (Figure 2A, B). A large percentage of $CD8^+$ T cells from untreated animals were able to make both interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) following ex vivo restimulation with BALB/c cells, whereas the cytokine production in

animals treated with anti-CD154, anti-CD40 or Fc-silent anti-CD154 dAbs was significantly blunted (Figure 2C, D).

Anti-CD154 dAbs inhibit donor-reactive T cell expansion in response to a minor antigen

Using this fully allogeneic model, we were unable to determine if alloreactive cells were no longer present in these animals, or if they were present but simply unable to produce cytokines. In order to more fully dissect the mechanism by which these therapies act, we employed a system in which naïve OVA-specific congenically labeled CD45.2$^+$ OT-I and Thy1.1$^+$ OT-II T cells were transferred to naïve mice that then received OVA-expressing skin grafts (Figure 3A). This model allows us to track the effect of CD40-CD154 blockade on the fate of antigen-specific cells following a skin graft (13, 18). We chose to focus on the expansion of donor-reactive CD8$^+$ T cells, as CD8$^+$ cells have been shown to be the primary mediators of costimulation blockade-resistant rejection (19). As expected, untreated mice showed robust expansion of CD45.2$^+$ CD8$^+$ antigen-specific T cells in both the draining lymph nodes and spleen at day 11 post-transplant. Donor specific transfusion (DST) treatment alone only moderately attenuated this expansion. However, perturbation of the CD40 pathway resulted in a significant reduction in both the percentage and absolute numbers of donor-reactive CD8$^+$ T cells in anti-CD154, anti-CD40, and Fc-silent anti-CD154 dAb-treated animals, and all treatments inhibited donor-reactive CD8$^+$ T cell expansion to equivalent levels. Similar findings were also observed in the donor-reactive effector CD4$^+$ Foxp3$^-$ T cell compartment in these animals (Figure 3B, C). These antigen-specific cells express a high level of CD44, indicating that they have undergone activation following encounter with antigen. Longitudinal studies have shown that the majority of these cells eventually undergo apoptosis (13), and that those remaining are subject to peripheral tolerance mechanisms including anergy and regulation (20). These results indicate that blocking CD40-CD154 interactions, rather than selective depletion of activated donor-reactive CD154$^+$ T cells, is sufficient to prevent antigen-specific T cell expansion following transplantation.

Anti-CD154 dAbs reduce numbers of functional graft-specific $CD8^+$ T cells and prevent differentiation into multi-cytokine producers

In order to determine whether donor-reactive $CD8^+$ T cell functionality was similarly impacted by the three modalities of CD40 pathway antagonism, we examined donor-reactive $CD8^+$ T cell compartments from treated animals following ex vivo stimulation with cognate antigen. Results showed that greater than 50% of donor-reactive $CD8^+$ T cells in untreated animals produced IFN-γ following restimulation, and more than 30% produced both TNF and IFN-γ (Figure 4A, B). DST treatment alone resulted in a modest reduction in the ability of donor-reactive $CD8^+$ T cells to produce cytokines, resulting in fewer double-cytokine producing cells. However, DST treatment combined with any of the three modalities of CD40 pathway antagonism further blunted this response. Each of the three modalities of CD40 pathway antagonism resulted in a decrease in the frequency of donor-reactive $CD8^+$ IFN-γ^+ T cells, and also to a decrease in donor-reactive $CD8^+$ multifunctional IFN-γ^+ TNF^+ T cells (Figure 4C), which have been shown to be highly potent mediators of graft rejection (21). Thus, in addition to reducing overall expansion of alloreactive cells, an Fc-silent anti-CD154 dAb functionally impairs the remaining cells, further diminishing their potential to mediate graft injury.

Administration of Fc-silent anti-CD154 dAbs results in antigen-specific iTreg generation

Previous results from our group and others have shown that treatment with DST and anti-CD154 leads to the peripheral conversion of donor-reactive $CD4^+$ T cells into $Foxp3^+$ iTreg, which may be important for the establishment of stable graft tolerance (11, 13). Therefore, we interrogated the ability of alternative CD40-CD154 blocking therapies to mediate this effect. We determined the frequency of $Thy1.1^+$ $CD4^+$ donor-reactive cells that converted from naïve $CD4^+$ cells into $CD4^+$ $CD25^+$ $Foxp3^+$ Treg. Untreated animals showed a low frequency of donor-reactive $Thy1.1^+$

$CD4^+$ $Foxp3^+$ iTreg in the draining lymph nodes by day 11, and DST treatment alone had no effect on the number of $Thy1.1^+$ $CD4^+$ $Foxp3^+$ iTreg. As we have shown previously, treatment with Fc-intact anti-CD154 resulted in a significant peripheral conversion of donor-reactive $Thy1.1^+$ $CD4^+$ T cell precursors into $Foxp3^+CD25^+$ iTreg. Notably, similar frequencies of $Thy1.1^+$ $CD4^+$ $Foxp3^+$ iTreg were observed in animals treated with anti-CD40 antibodies and Fc-silent anti-CD154 dAbs (Figure 5A, B). In addition, the ratio of Treg cells to effectors has been shown to be an important parameter in determining the overall suppressive capacity of Treg populations (22). Treatment with all three CD40 pathway antagonists increased the Treg:effector cell ratios for both $CD4^+$ and $CD8^+$ cells (Figure 5C). Taken together, these results indicate that alternative CD40-CD154 blockade in the presence of antigen can result in donor-reactive $Foxp3^+$ iTreg conversion and establishment of dominant regulatory mechanisms, which may be critical for the establishment of transplant tolerance.

Anti-CD154 dAbs results in iTreg generation even in the presence of CTLA4-Ig

Given the potential for clinical translation of Fc-silent anti-CD154 therapy and long-standing interest in combination therapy with CD28 costimulation blockade, we sought to determine the effect of CD28 blockade on CD154 antagonism-induced iTreg generation. This question was especially pertinent due to the known negative impact of CTLA4-Ig costimulation blockade on the frequency of Treg (15, 16). We found that the frequencies of endogenous $CD4^+$ $CD25^+$ $Foxp3^+$ nTreg were decreased when CTLA4-Ig was administered either alone or in combination with anti-CD154 dAbs (Figure 6A, B). Importantly, however, while CTLA4-Ig treatment alone showed no effect on iTreg conversion, anti-CD154 dAb-induced donor-reactive $Thy1.1^+$ $Foxp3^+$ iTreg conversion was preserved in the presence of CTLA4-Ig (Figure 6A, B). This preserved induction of antigen-specific $Foxp3^+$ iTreg in the presence of CTLA4-Ig was similar to that observed when animals were treated with Fc-intact CD154 in the presence of CTLA4-Ig (data not shown).

We further investigated the relative expression levels of the transcription factor Helios within donor-reactive Thy1.1^{+} Foxp3^{+} iTreg generated following CD154 antagonism with anti-CD154 dAb. Early reports suggested that Helios was a marker of thymic-derived natural Treg (nTreg) (23), but subsequent studies have provided evidence to the contrary (24). Here, we observed high levels of Helios expression on both endogenous nTreg (Figure 6C, left panel) and donor-reactive Thy1.1^{+} Foxp3^{+} iTreg compartments (Figure 6C, right panels), supporting recent work suggesting that Helios is a marker of recent antigen encounter rather than a marker of nTregs (25). Helios expression was not affected by the addition of CTLA-4-Ig in the presence of anti-CD154 dAbs (Figure 6C).

Discussion

In this study, we demonstrated that blockade of CD40-CD154 interactions, as opposed to FcR-dependent deletion of activated CD154-expressing alloreactive effectors, is sufficient to prevent the expansion of alloreactive $CD8^+$ T cells and promote the conversion of antigen-specific iTreg. This study therefore provides proof-of-concept that an Fc-silent anti-CD154 domain antibody may provide a clinically translatable treatment with comparable efficacy to an Fc-intact therapy. In addition, the results presented here are consistent with previous data showing that aglycosylated anti-CD154 antibodies recapitulated the effects of Fc-intact anti-CD154 reagents. However, generation of these reagents required a complicated "sewing PCR" technique, and large scale production of these reagents for clinical use may prove challenging. (5).

While our data suggest that it is blockade of CD40-CD154 interactions that is responsible for the attenuation of alloreactive T cell responses, the precise cellular interactions most critically impacted by CD40-CD154 blockade remain to be elucidated. While the most likely interaction inhibited by anti-CD154 antibody treatment is that between CD154 expressed on activated $CD4^+$ T cells and CD40 expressed on dendritic cells or other APC, emerging data suggests that CD40-CD154 interactions between other cell types may be affected as well. Bhadra and colleagues have shown that CD40 expression on $CD8^+$ T cells is potentially important in preventing their exhaustion (26). In addition, work from our group has indicated that CD40 expression on $CD8^+$ T cells can prevent iTreg induction (27). A recent report also revealed that CD154 can be expressed on dendritic cells and is important for $CD8^+$ T cell responses to certain viral infections (28). However, it is unknown if these interactions are relevant during alloreactive responses, and further studies will help clarify the individual cellular and molecular interactions inhibited by this antibody therapy.

Our data also revealed an Fc-independent ability of CD154 antagonism to result in the conversion of antigen-specific $CD4^+$ T cells into $Foxp3^+$ iTreg. The phenomenon of antigen-specific iTreg generation following CD154 antagonism has been noted in previous studies (13,

29), but the mechanisms underlying this effect remain to be fully elucidated. Our results demonstrate for the first time that Fc-independent blockade of the CD40-CD154 pathway is sufficient to generate high frequencies of antigen-specific $CD4^+$ iTreg in the context of an alloreactive response. Further research is needed to compare the relative suppressive and migratory capacities of $Foxp3^+$ Treg generated under these conditions.

Because we observed that one of the major effects of CD154 antagonism is to induce a population of $Foxp3^+$ iTreg, we investigated the effect of CD28 blockade using CTLA4-Ig on these cells. We found that CD28 blockade had no effect on the induction of antigen-specific iTreg, which was somewhat surprising given the well-appreciated role of CD28 in the generation and maintenance of $Foxp3^+$ nTreg, and the fact that CTLA4-Ig administration profoundly diminishes nTreg populations (15, 16). These data are potentially clinically important in light of the recent FDA approval of belatacept, a second-generation CTLA4-Ig fusion protein (30). While this drug provides a significant benefit over standard calcineurin-inhibitor based regimens due to its reduced cardiovascular and nephrotoxicity profile, patients treated with belatacept experienced higher rates of acute rejection (31). As such, there may be an opportunity to reduce the frequency of early rejection events in belatacept-treated patients by coupling it with other low toxicity T cell-directed immunotherapies. Indeed, numerous published reports over the last two decades have demonstrated in both mouse and non-human primate trials that anti-CD154 antibodies can synergize with CTLA4-Ig in attenuating alloreactivity and promoting graft survival (2, 14), Our data demonstrating antigen-specific $CD4^+$ $Foxp3^+$ iTreg generation in animals treated with both Fc-silent anti-CD154 dAbs and CTLA4-Ig provide further evidence that combinatorial use of these costimulation blockers may be beneficial in attenuating the alloreactive T cell response, and highlight the potential for these therapies to be used together to prolong graft survival following clinical transplantation.

Figure 2.1 Anti-CD154 domain antibodies are functionally equivalent to Fc-intact anti-CD154 mAbs.

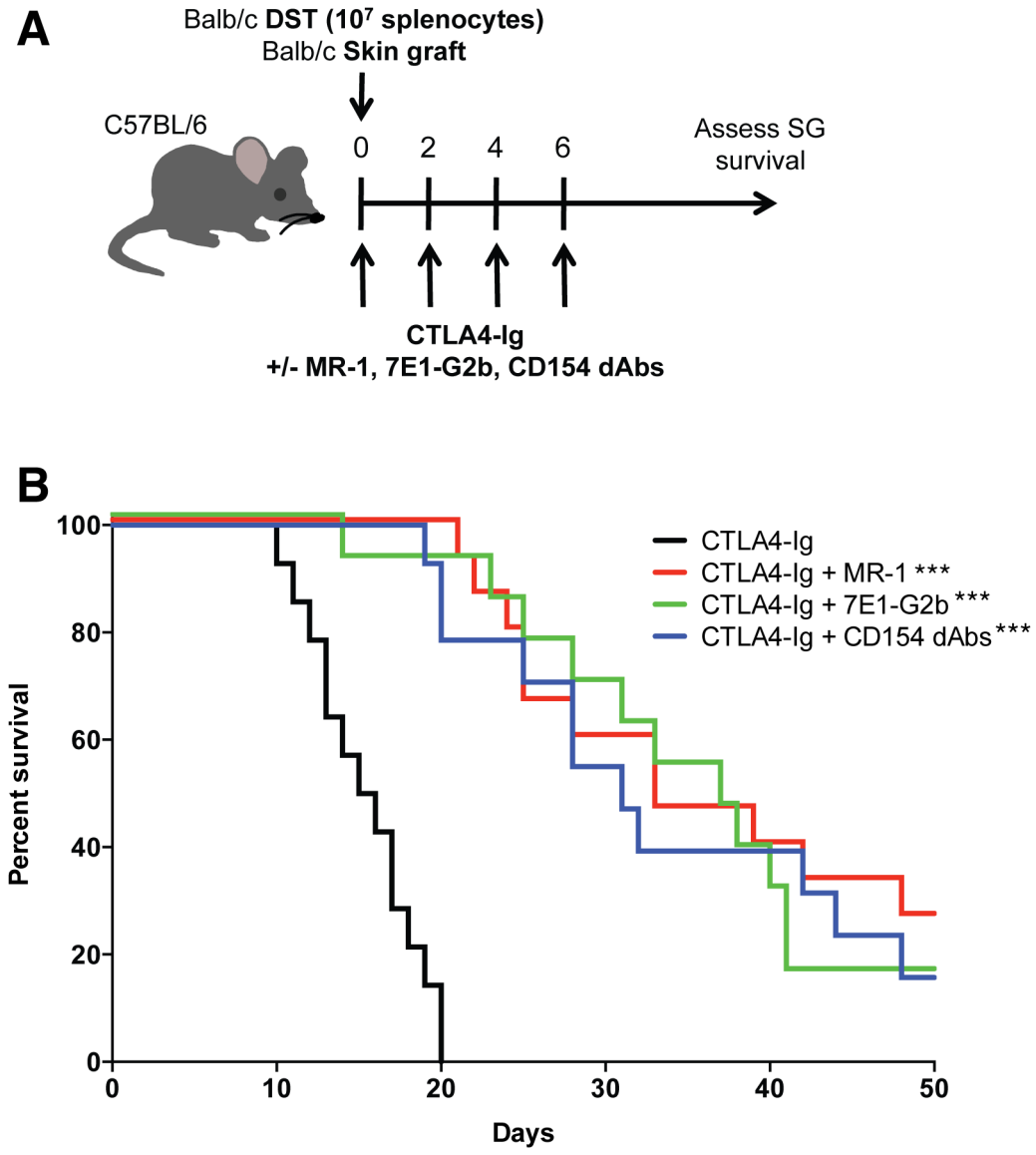


Figure 2.1 Anti-CD154 domain antibodies are functionally equivalent to Fc-intact anti-CD154 mAbs. (A) On day 0, B6 mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/or 250 μg of CTLA4-Ig, MR-1, 7E1-G2b or CD154 dAbs where indicated. (B) Mice treated with CTLA4-Ig alone rejected skin grafts with an MST of 15.5 d. Addition of MR-1 prolonged MST to 33 days, whereas addition of 7E1-G2b or CD154 dAbs led to an MST of 37 and 31 days, respectively. Data are cumulative of three independent experiments with a total of fourteen to fifteen mice per group. *** $p < 0.001$ compared to CTLA4-Ig alone. Comparisons between MR-1, 7E1-G2b and CD154 dAbs were not statistically significant.

Figure 2.2. Activation and cytokine production of alloreactive T cells are diminished in the presence of anti-CD154 dAbs.

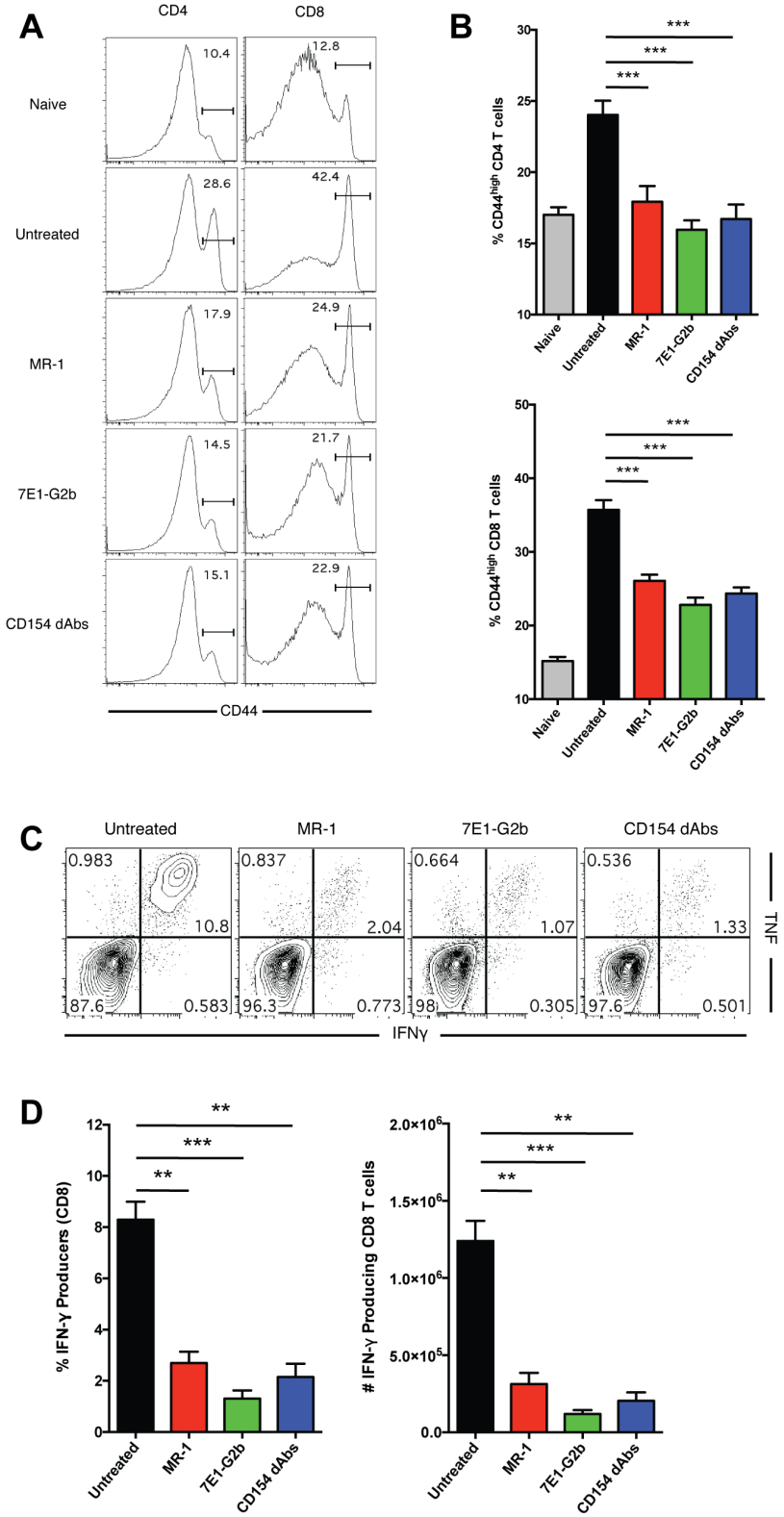


Figure 2.2. Activation and cytokine production of alloreactive T cells are diminished in the presence of anti-CD154 dAbs. (A) Representative histograms of CD44 expression on splenic $CD4^+$ and $CD8^+$ T cells isolated from grafted mice at day 14. (B) Frequencies of $CD44^{hi}$ cells among $CD4^+$ and $CD8^+$ T cell populations. Treatment with MR-1, 7E1-G2b or CD154 dAbs results in the reduction of CD44 expression on both $CD4^+$ and $CD8^+$ cells. (C) Representative flow plots of intracellular cytokine staining of recipient splenocytes, gated on $CD8^+$ T cells. 10^6 recipient splenocytes were incubated with $2x10^6$ BALB/c stimulator splenocytes for 5 hours and then fixed and permeabilized before being stained for FACS analysis. (D) Frequencies and absolute numbers of cytokine-producing $CD8^+$ T cells. Treatment with MR-1, 7E1-G2b or CD154 dAbs results in the reduction of percentage of multicytokine producing $CD8^+$ T cells. Data are cumulative of two separate experiments with a total of 8 to 10 mice per group. ** $p < 0.01$, *** $p < 0.001$.

Figure 2.3. Anti-CD154 dAbs inhibit donor-reactive T cell expansion in response to a minor antigen.

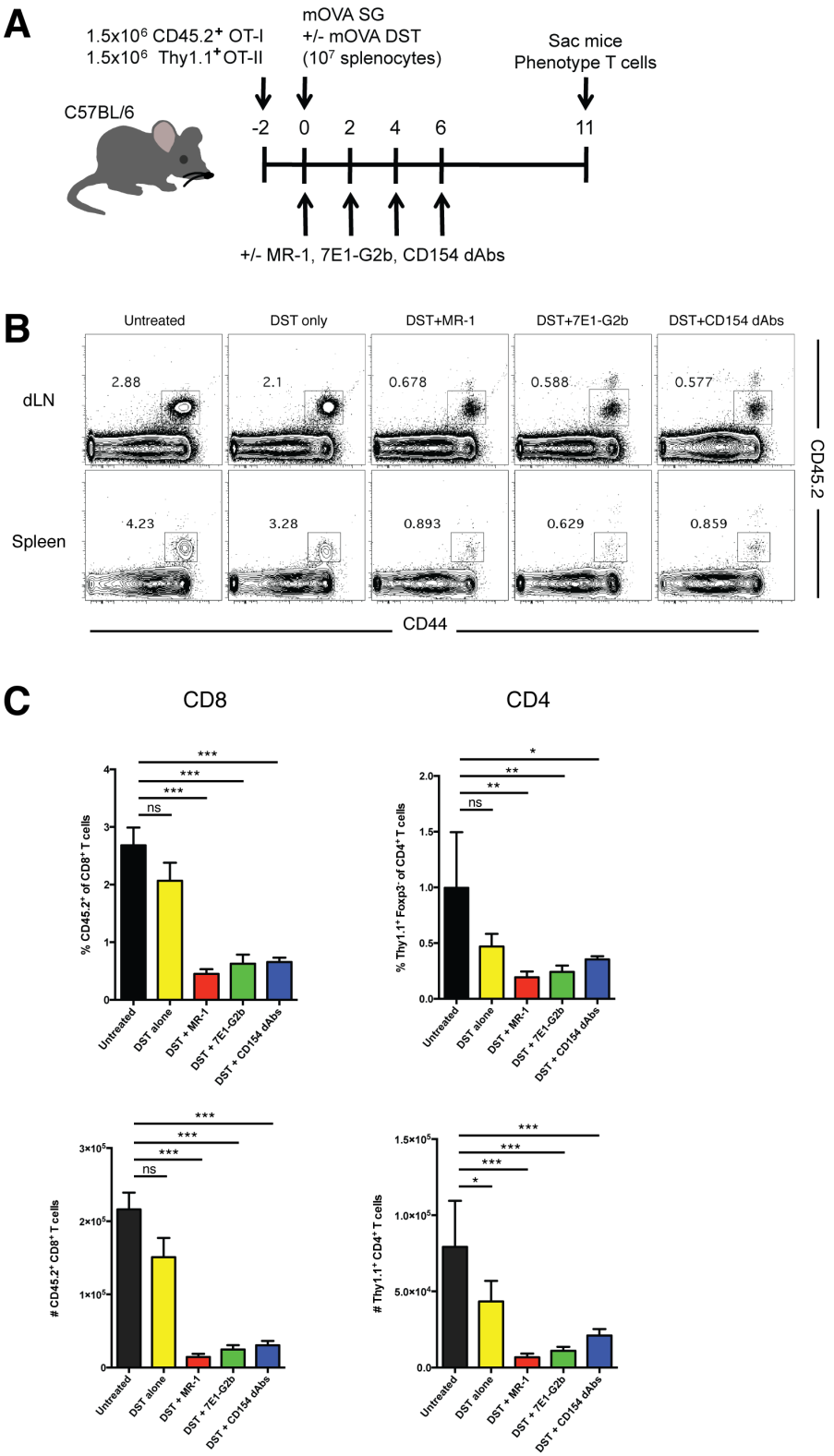


Figure 2.3. Anti-CD154 dAbs inhibit donor-reactive T cell expansion in response to a minor antigen. (A) Mice were adoptively transferred with $1.5x10^6$ $CD45.2^+$ $CD8^+$ OT-I and $1.5x10^6$ $Thy1.1^+$ $CD4^+$ OT-II T cells 2 days before transplantation. On day 0, mice were transplanted with mOVA SG and were treated with 10^7 mOVA DST and/or 250 μg of MR-1, 7E1-G2b or CD154 dAbs where indicated. Mice were sacrificed at day 11. (B-D) Representative flow plots of $CD8^+$ T cells (B) and the frequencies and absolute numbers of antigen-specific $CD8^+$ and effector $CD4^+$ T cells at day 11 (C). DST treatment alone moderately diminished the frequency of antigen-specific $CD8^+$ T cells, but the addition of MR-1, 7E1-G2b or the CD154 dAbs resulted in a significant reduction in the frequencies of these cells. Data are representative of four independent experiments with a total of fourteen to sixteen mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2.4. Anti-CD154 dAbs reduce numbers of functional antigen-specific cells and prevent differentiation into multiple cytokine producers.

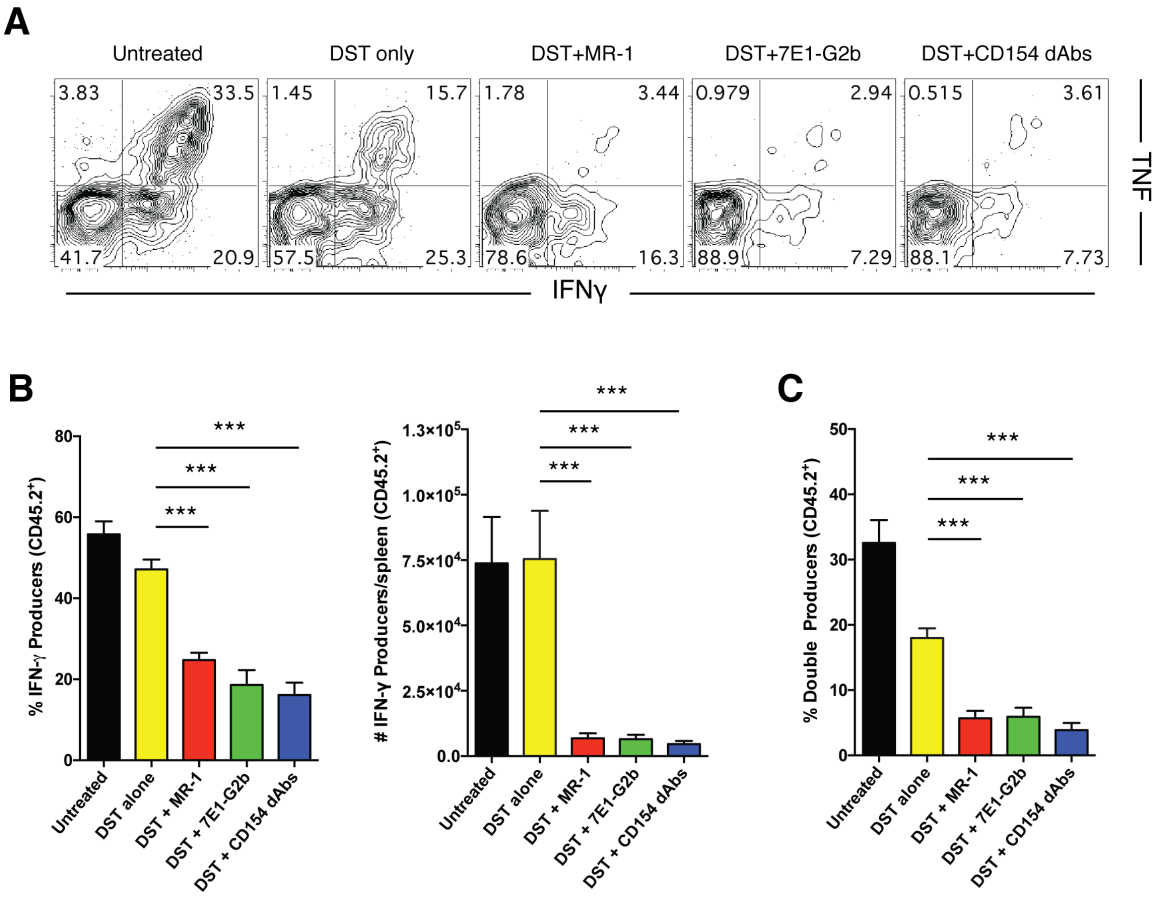


Figure 2.4. Anti-CD154 dAbs reduce numbers of functional antigen-specific cells and prevent differentiation into multiple cytokine producers. (A) Representative flow plots of intracellular cytokine staining of splenic antigen-specific $CD8^+$ T cells harvested at day 11 and stimulated for 4 hours in vitro with SIINFEKL peptide. (B) Percentages and absolute numbers of IFN-γ producing antigen-specific $CD8^+$ T cells. Compared to DST alone, treatment with MR-1, 7E1-G2b or CD154 dAbs significantly reduced the frequency and absolute numbers of IFN-γ-producing antigen-specific $CD8^+$ T cells in the spleen. (C) Frequencies of double cytokine producing antigen-specific $CD8^+$ T cells within each treatment group. Treatment with MR-1, 7E1-G2b or CD154 dAbs resulted in the reduction of percentage of multicytokine producing antigen-specific $CD8^+$ T cells. Data are representative of three independent experiments with a total of eleven to twelve mice per group. *** $p < 0.001$.

Figure 2.5. Administration of Fc-silent anti-CD154 dAbs results in antigen-specific iTreg conversion.

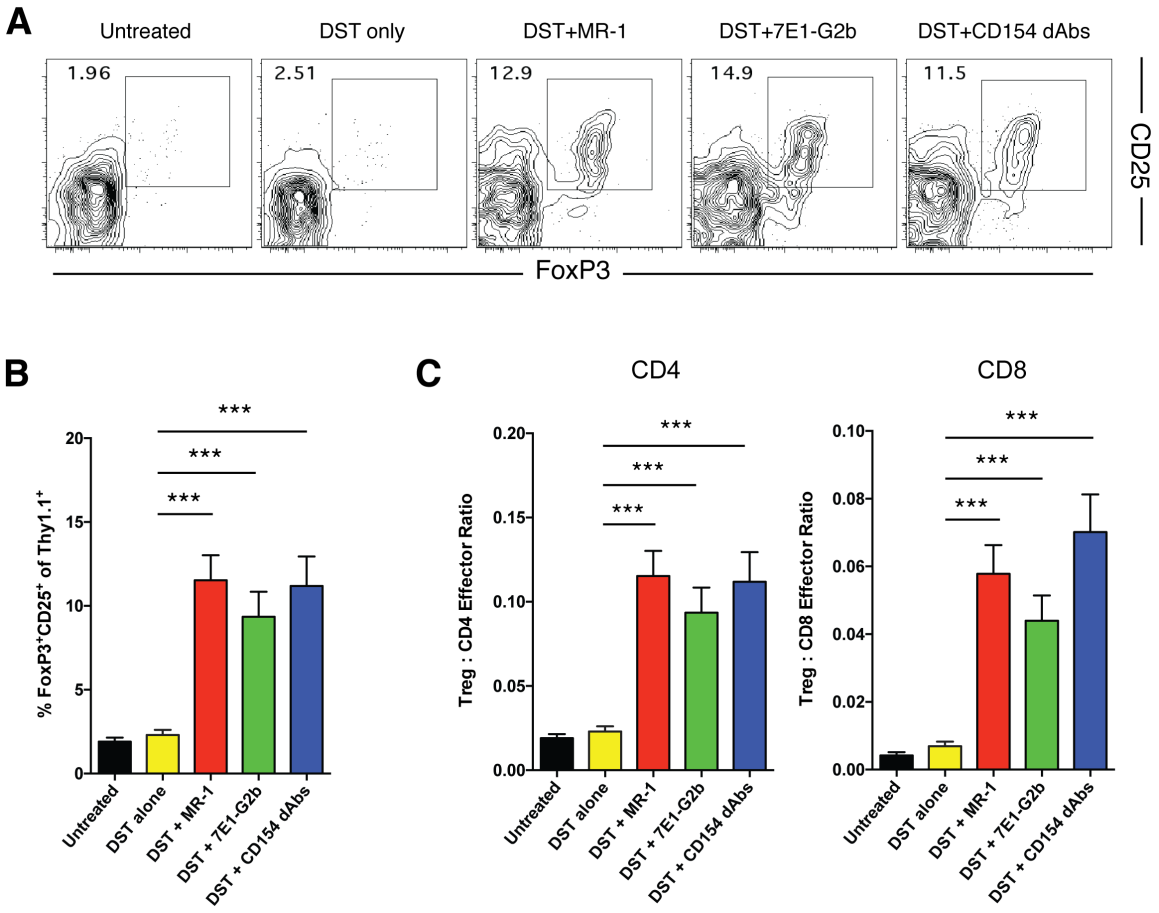


Figure 2.5. Administration of Fc-silent anti-CD154 dAbs results in antigen-specific iTreg conversion. (A) Representative flow plots of $CD25^+$ $Foxp3^+$ iTreg among antigen-specific $Thy1.1^+$ $CD4^+$ T cells in draining lymph nodes at day 11. (B) Summary data of percentages of $CD25^+$ $Foxp3^+$ cells within the antigen-specific $CD4^+$ T cell compartment. Treatment with MR-1, 7E1-G2b or CD154 dAbs resulted in a significant increase in the percentage of antigen-specific iTregs. (C) Treg:Effector cell ratio for antigen-specific $CD4^+$ and $CD8^+$ T cells in each treatment group. Treatment with MR-1, 7E1-G2b or CD154 dAbs results in a significant increases in the ratio of Tregs to both $CD4^+$ and $CD8^+$ antigen-specific effector cells. Data are cumulative of two separate experiments with a total of eight to nine mice per group. *** $p < 0.001$.

Figure 2.6. Fc-Silent Anti-CD154 dAbs results in iTreg generation even in the presence of CTLA4-Ig.

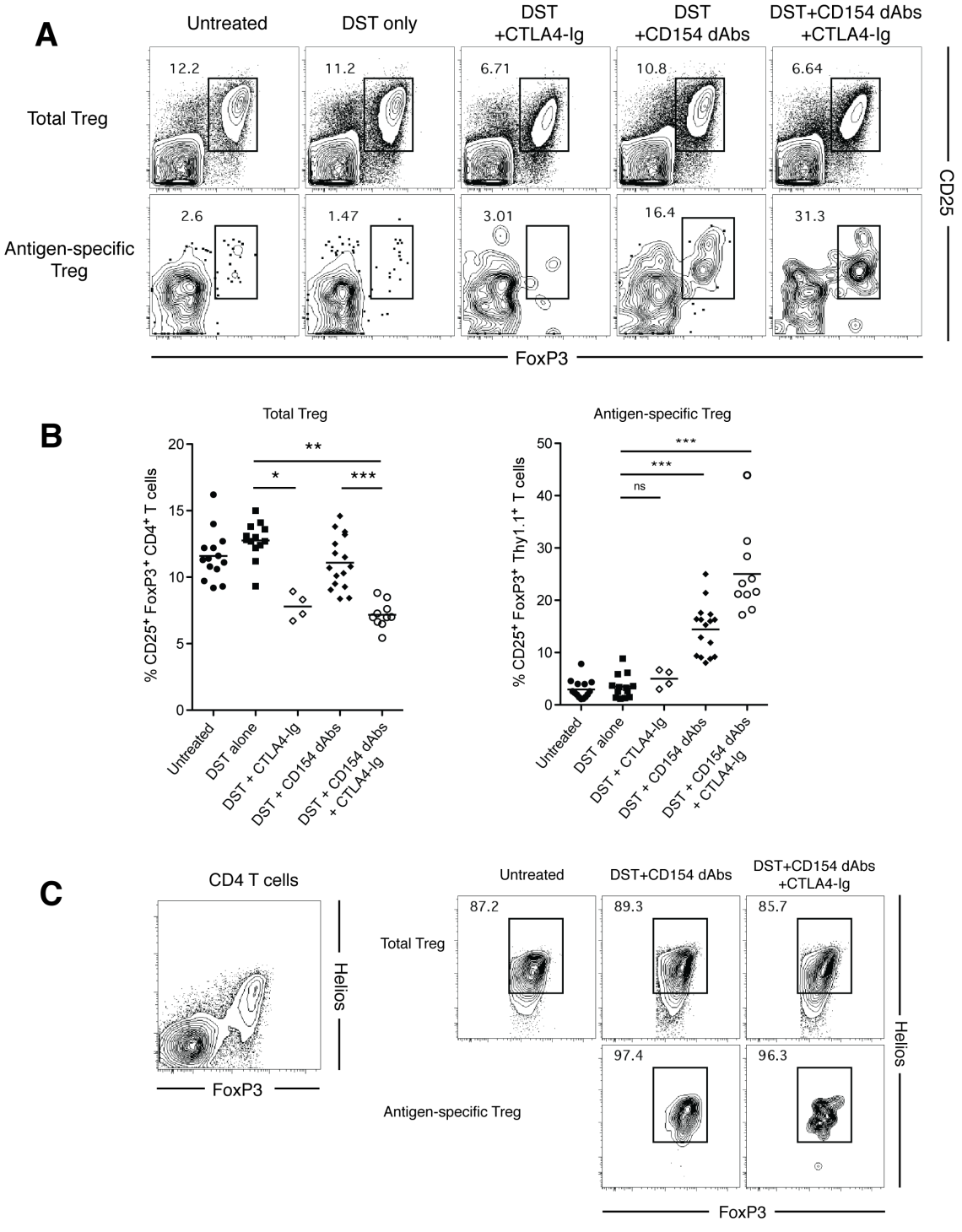
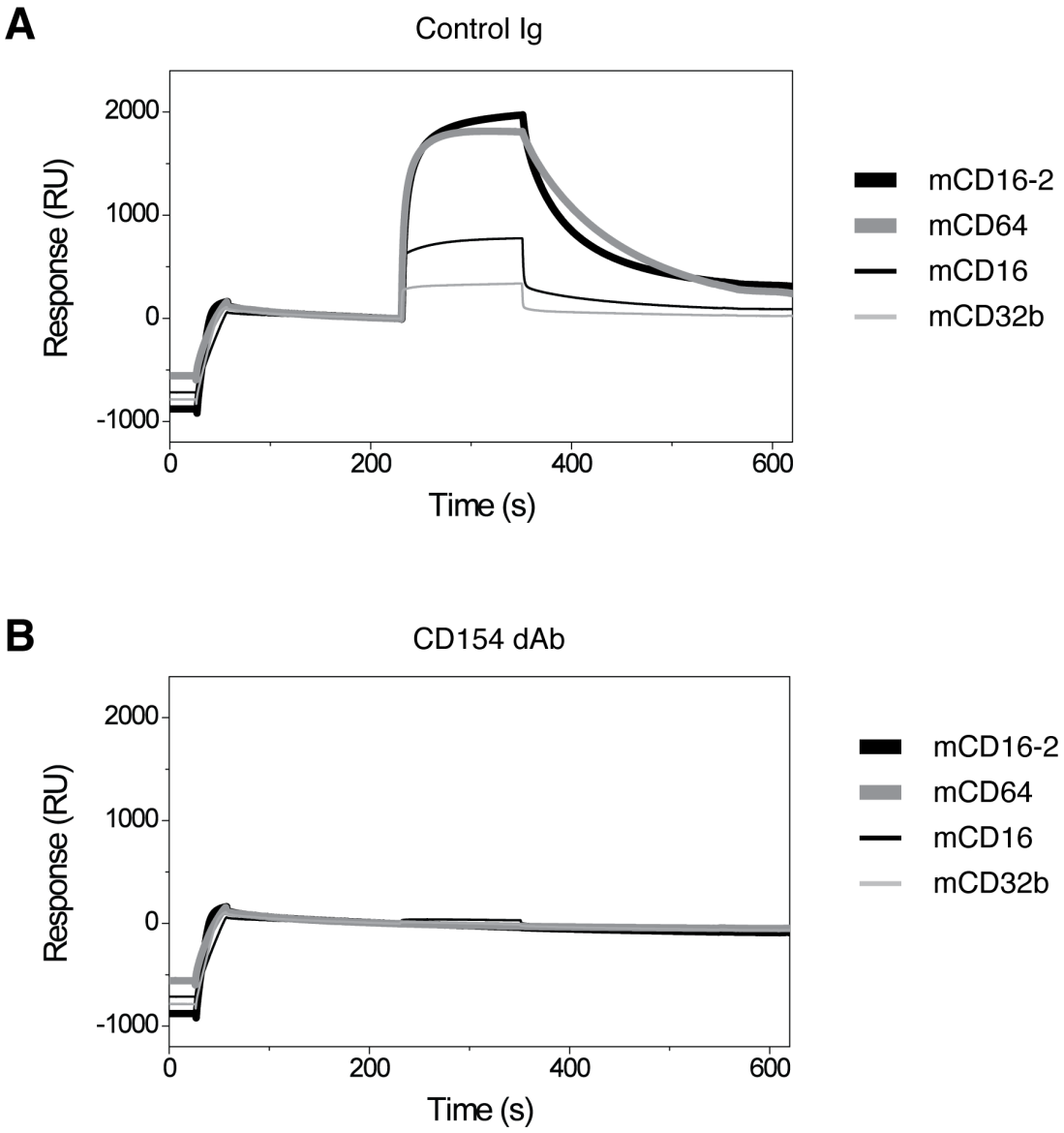


Figure 2.6. Fc-Silent Anti-CD154 dAbs results in iTreg generation even in the presence of CTLA4-Ig. (A) Representative flow plots of total $CD4^+$ T cells (top panels) and antigen-specific $CD4^+$ ($Thy1.1^+$) T cells (bottom panels) in draining lymph nodes at day 11, with gates on $CD25^+$ $Foxp3^+$ cells. (B) Percentages of $CD25^+$ $Foxp3^+$ cells within the total $CD4^+$ T cell compartment and the antigen-specific $CD4^+$ T cell compartment. (C) Representative flow plots of Helios expression on total $CD4^+$ T cells (left panel), as well as on total endogenous $CD4^+$ $CD25^+$ $Foxp3^+$ Treg (upper right panels) and antigen-specific $Thy1.1^+$ iTreg (lower right panels). Data are cumulative of four separate experiments with a total of ten to fourteen mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplemental Figure 2.1. Fc-modified anti-CD154 dAb is deficient in Fc receptor binding.



Supplemental Figure 2.1. Fc-modified anti-CD154 dAb is deficient in Fc receptor binding. (A and B) Binding of antibodies to immobilized Fc receptors was measured by surface plasmon resonance. Antibodies were injected through flow cells containing the indicated immobilized murine Fc receptors. A 97 kDa positive control protein fused to a wild type mIgG2A Fc domain demonstrates efficient binding to his-tagged mCD64, mCD32b, mCD16, and mCD16-2, captured on an anti-his Fab surface (A), whereas no Fc receptor binding is observed for the Fc-modified anti-CD154 dAb (B).

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Chapter 3

The Inhibitory FcγRIIB Functionally Inhibits Donor-Reactive Memory CD8$^+$ T Cell Responses Following Transplantation

Abstract

Fc receptors are a critical component of the adaptive immune system, binding to the constant portion of immunoglobulin molecules and mediating important effector mechanisms. T cells have long been thought to not express these receptors, but recent data suggests that memory T cells may express the inhibitor Fc receptor FcγRIIB. We observed the upregulation of FcγRIIB on both $CD4^+$ and $CD8^+$ T cells following a graft in both fully allogeneic and minor mismatch models of transplantation. Interestingly, most highly functional alloreactive cells capable of making TNF and IFNγ in response to ex vivo restimulation also coexpressed FcγRIIB, suggesting that this molecule may have evolved to regulate $CD8^+$ T cell expansion and immunopathology. In addition, we observed that systemic blockade of FcγRIIB during an allograft rechallenge response enhanced memory $CD8^+$ T cell recall expansion. We also observed that memory $CD8^+$ T cells generated by different pathogen infections expressed varying amounts of FcγRIIB, and that this expression correlated with the resistance of these cell populations to combined costimulation and integrin blockade during a memory response. Taken together, these results indicate that FcγRIIB may be a novel coinhibitory pathway that regulates alloreactive T cell responses, and further work will determine whether or not FcγRIIB is functioning to intrinsically inhibit T cell responses in the context of primary and memory responses to allograft.

Introduction

The adaptive immune system has evolved as a critical component of protection against pathogen infection. However, due to the random nature of T and B cell receptor generation, several mechanisms exist to prevent unwanted immune responses directed against self-antigens. As detailed previously, central and peripheral tolerance to self is maintained in part by the so-called “two signal" model for T cell activation in which both TCR ligation and costimulatory molecule engagement are required to achieve full activation (1). However, costimulation is not simply a binary switch to activate or inhibit T cell activation; rather, the phenotype of a T cell response is determined by the overall balance of both signals received through both costimulatory molecules, such as CD28 and CD40, as well as coinhibitory molecules, such as CTLA4 and PD-1 (1).

T cells can mediate both acute and chronic allograft rejection, and the discovery of calcineurin inhibitors, a class of drugs that inhibit signaling downstream of the T cell receptor, has led to marked reduction in acute rejection and subsequent improvements in short term outcomes for solid organ recipients (2). However, the many off-target side effects, as well as the non-specific nature of this therapy, can result in significant morbidity and mortality in transplant recipients (3). Immunotherapeutic reagents targeting the CD28 costimulatory pathway have shown great promise as a less toxic means of preventing T cell responses (4-6), but costimulation blockade is less effective at inhibiting memory T cells, as these cells have a much lower activation threshold and require fewer costimulatory signals (7). Over a lifetime of exposure to various pathogens, humans develop large memory T cell populations, and studies have shown that these memory cells can often crossreact with alloantigens during transplantation (8, 9). Landmark work by Adams and colleagues showed that while combined blockade of the CD28 and CD40 costimulatory pathways could induce skin graft tolerance in naïve mice, these therapeutic regimens were ineffective in recipients that had previously been exposed to pathogen infection, and that pathogen-induced memory T cells were responsible for mediating this graft

rejection (10). A more recent study found that adding integrin blockade to a costimulation blockade regimen was able to effectively control crossreactive memory T cell-mediated rejection (11), but integrin antagonists are often not tolerated well by patients (12, 13). Thus, the identification of molecules and pathways that regulate memory T cells responses remains an important goal for immunotherapy in clinical transplantation.

Fc receptors are a heterogeneous group of transmembrane immunoglobulin family molecules that bind to antibody molecules and represent the effector arm of the humoral immune system. These receptors mediate a wide range of processes, including antibody dependent cellular cytotoxicity, endocytosis of antigen-antibody immune complexes, cytokine and chemokine production, and B cell development and homeostasis (14). Much like T cell costimulatory and coinhibitory molecules, the balance of activating and inhibitory Fc receptor signaling determines the outcome of antibody engagement of these receptors. FcγRIIB is the only inhibitory Fc receptor and is conserved in both mice and humans. This receptor can inhibit intracellular signaling pathways, and this is dependent on the ITIM motif located in the cytoplasmic region. Following phosphorylation of this ITIM by the Src-family kinase LYN, the inhibitory phosphatase SHIP is recruited, which is responsible for downmodulating signaling pathways, including those mediated by the B cell receptor or activating Fc receptors (15, 16). FcγRIIB is expressed by many immune cell types, including B cells, dendritic cells, macrophages and granulocytes (15). While early immunology literature speculated that T cells may express some form of Fc receptor (17-19), the general consensus for the past few decades has been that T cells do not express this class of receptor (14).

Previous studies in our lab indicated that FcγRIIB$^{-/-}$ mice reject primary skin grafts faster than their wild type counterparts in the presence of CTLA4-Ig (20). Here, we sought to define the mechanisms underlying this accelerated rejection. While there are numerous reports indicating that FcγRIIB deficient mice make abnormal humoral immune responses and demonstrate altered antigen presentation (21-23), our data reveal that CTLA4-Ig effectively inhibited graft-specific

antibody formation in both WT and FcγRIIB$^{-/-}$ animals. Instead, we found that FcγRIIB was upregulated on memory T cells in these animals following transplantation, corroborating recently published data showing that antigen-specific CD8^{+} T cells responding to both bacterial and viral infection expressed this molecule on the cell surface, and this expression was maintained at a high level in memory T cells (24, 25). Our data suggest a functional role for FcγRIIB on CD8^{+} T cells during transplantation, and implicate FcγRIIB as a novel coinhibitory pathway that regulates alloreactive T cell responses.

Materials and Methods

Mice

B6-Ly5.2/Cr (H2-K^b, CD45.1), C57BL/6 (H2-K^b, CD45.2), and BALB/c (H-2K^d) mice were obtained from NCI (Frederick, MD). FcγRIIB$^{-/-}$ mice were obtained from Jackson Laboratories (Bay Harbor, ME). OT-I and OT-II transgenic mice, purchased from Jackson Laboratories, were bred to Thy1.1$^+$ background at Emory University. mOVA mice (26) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained in accordance with Emory University IACUC guidelines (Atlanta, GA). All animals were housed in pathogen-free animal facilities at Emory University.

Anti-OVA ELISA

Alloantibody formation to OVA was determined using soluble OVA antigen (Sigma-Aldrich, St. Louis, MO). Wells of Immulon 4HBX plates (Thermo Scientific, Waltham, MA) were coated with 50ug/mL OVA antigen overnight, and then incubated with serial dilutions of serum. Anti-OVA IgG or IgM was detected using biotin-conjugated Anti-IgG or Anti-IgM (Biolegend, San Diego, CA), Avidin-HRP (Life Technologies, Carlsbad, CA), and TMB Substrate Kit (Thermo Scientific). Reactions were stopped using 2N H_2SO_4, and plates were read at 450nm using a SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA).

Adoptive Transfers, Infections, and Skin Transplantation

For adoptive transfers prior to infection, spleens of OT-I mice processed and 10^4 Thy1.1$^+$ OT-I were injected intravenously. Forty-eight hours later, mice were infected with 10^4 Listeria-monocytogenes (LM)-OVA, $2x10^3$ pfu murine gammaherpesvirus 68 (gHV)-OVA, or 10^5 pfu murine polyoma virus (PyV)-OVA. LM and gHV were administed intraperitoneally, PyV was given via footpad injection. For adoptive transfers prior to grafting, spleens of OT-I and OT-II mice were processed and 10^6 each of Thy1.1$^+$ OT-I and OT-II were injected intravenously. Forty-

eight hours later, full thickness tail, ear, or trunk skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages.

Antibody Treatment

Where indicated, mice were treated with 250μg CTLA4-Ig (Bristol Myers Squibb), 250μg hamster monoclonal anti-mouse CD154 (MR-1, BioXCell, West Lebanon, NJ), 250ug rat anti-mouse CD49d (VLA-4, BioXCell) or 400ug anti-mouse CD16/32 (2.4G2, BioXCell), intraperitoneally on days 0, 2, 4, and 6 post-transplantation.

Surface Stains and Flow Cytometry

Peripheral blood, spleen or draining axillary and brachial LN were stained for CD4, CD8, Thy1.1, FcγRIIB, CD62L and CD44 (Biolegend; Invitrogen; BD Biosciences, Franklin Lakes, NJ). For intracellular staining, responder splenocytes were stimulated with a 2:1 ratio of BALB/c splenocytes in the presence of 10 μg/mL Brefeldin A for 5 hours. Intracellular staining kit was used to detect TNF and IFN-γ (Biolegend), according to manufacturer's instructions. Samples were analyzed using an LSRII FACS machine (BD Biosciences). Data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Statistical Analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For two comparisons, one-way students T test was performed. For three or more comparisons, one-way ANOVA with Tukey's post-test was performed. Analyses were done using GraphPad Prism software (GraphPad Software Inc).

Results

FcγRIIB deficient mice demonstrate accelerated graft rejection in the presence of costimulation blockade

FcγRIIB is known to play an important role in several aspects of adaptive immune responses, and we first sought to determine the impact of this receptor in alloreactive responses to transplantation using a minor mismatch OVA model of skin transplantation (Figure 1A). Due to the rapid rejection of skin grafts in this model in untreated animals, we also treated both WT and FcγRIIB$^{-/-}$ mice with CTLA4-Ig, in order to reveal any differences in rejection kinetics. While untreated WT mice rejected their organs rapidly, with a median survival time (MST) of 18 days, CTLA4-Ig treatment significantly extended MST to 31.5 days and led to long-term graft survival in approximately 40% of WT recipients (Figure 1B). However, treated FcγRIIB$^{-/-}$ mice all rapidly rejected their grafts, with an MST of 20.5 days, and no animals in this group displayed long-term survival (Figure 1B). Given the important role that FcγRIIB plays in regulating humoral responses, we assessed the formation of graft-specific antibodies in these mice at day 14 post-transplantation. While WT mice generated a robust anti-OVA IgG response, CTLA4-Ig was able to effectively inhibit alloantibody responses in both WT and FcγRIIB$^{-/-}$ mice (Figure 1C). Importantly, none of the recipients had detectable IgM titers at day 14 (data not shown), indicating an overall lack of antibody production in treated animals, rather than defective class-switching. Thus, we concluded that the accelerated rejection seen in FcγRIIB$^{-/-}$ animals was not the result of augmented humoral immunity.

FcγRIIB$^{-/-}$ mice have normal baseline memory T cell compartments

Given the accelerated rejection we observed in FcγRIIB$^{-/-}$ mice, and the fact that memory cells are an important mediator of costimulation blockade-resistant rejection (10, 11), we first assessed the baseline memory phenotype of these mice compared to WT controls. Memory-like populations of CD44-expressing CD4^{+} and CD8^{+} T cells are often seen in mice maintained in non-germ free

colonies, and approximately 8-10% of both $CD4^+$ and $CD8^+$ T cells expressed high levels of CD44 in our WT controls (Figure 2A, B). Importantly, T cells in $Fc\gamma RIIB^{-/-}$ mice showed similar levels of CD44 expression, and the percentages and absolute numbers of naïve ($CD44^{lo}$), central memory-like ($CD44^{hi}$ $CD62L^{hi}$) and effector memory-like ($CD44^{hi}$ $CD62L^{lo}$) T cells. Thus, we concluded that baseline differences in memory-like cells were likely not responsible for the rejection phenotype observed. Interestingly, while naïve and $Fc\gamma RIIB^{-/-}$ T cells did not express the inhibitory Fc receptor FcγRIIB, we were able to detect expression of this receptor on effector-memory-like $CD44^{hi}$ $CD62L^{lo}$ populations in WT mice (Figure 2C). This surprising result suggested that this receptor may play a cell-intrinsic role in the regulation of T cell alloreactivity, and we sought to further investigate this hypothesis.

Polyclonal allograft-specific $CD8^+$ T cells upregulate FcγRIIB following transplantation

We next investigated the expression of FcγRIIB on $CD8^+$ T cell populations in the context of transplantation. Compared to naïve B6 mice, mice receiving a fully allogeneic BALB/c skin graft upregulated FcγRIIB on $CD8^+$ T cell populations (Figure 3A-C). Splenocytes from graft recipients were restimulated ex vivo with BALB/c antigen to induce cytokine production, in order to phenotypically characterize the FcγRIIB-expressing T cell population. Interestingly, while very few non-cytokine producing cells express FcγRIIB, approximately 60-70% of alloreactive cells capable of making proinflammatory cytokines IFNγ and TNF co-expressed the inhibitory Fc receptor (Figure 3D, E). Taken together, this suggests that FcγRIIB may be a novel coinhibitory pathway capable of regulating T cell proliferation and expansion, similar to other coinhibitory molecules such as CTLA4 and PD-1 that are upregulated following T cell activation (1).

Therapeutic blockade of FcγRIIB leads to enhanced memory recall responses to a secondary graft rechallenge

We hypothesized that FcγRIIB may be able to inhibit the primary or recall expansion of $CD8^+$ T

cells in the setting of transplantation. In order to be able to specifically track alloreactive cells responding to a graft, rather than relying on cytokine production to identify these populations, we next performed experiments using the transgenic OVA system. Congenically labeled OT-I and OT-II T cells, genetically engineered $CD8^+$ and $CD4^+$ T cells specific for the chicken ovalbumin protein, were adoptively transferred to WT mice, which then received an OVA-expressing skin graft (mOVA) (Figure 4A). While naïve OT-I and OT-II cells did not express FcγRIIB, both of these graft-specific T cell populations upregulated this receptor over time following transplantation, in all compartments in which these cells could be detected (Figure 4B, C). Since expression increases over time and peaks on memory T cell populations, we next investigated the role of this receptor at memory time points. Mice received OT-I and OT-II cells and a primary mOVA graft and allowed to reject these grafts in the absence of treatment (Figure 4D). These animals were then regrafted at day 30 and either left untreated or treated with the FcγRIIB-blocking 2.4G2 monoclonal antibody, and memory T cell responses were detected 5 days later in the draining lymph node. 2.4G2 was able to completely block FcγRIIB on $CD8^+$ T cells (Figure 4G, H), and this treatment led to a greater expansion of alloreactive $CD8^+$ T cells at this timepoint (Figure 4E, 4F). These data suggest that FcγRIIB may play an important role in modulating secondary recall responses to an allograft.

FcγRIIB expression on memory $CD8^+$ T cells inversely correlates with resistance to combined costimulation and integrin blockade

Memory T cells are a major mediator of costimulation blockade-resistant rejection, and recent work from our group showed that prior infection with different pathogens can lead to varying efficacy of combined costimulation and integrin blockade during a graft rechallenge (27). We further extended these studies to interrogate the expression of FcγRIIB on memory $CD8^+$ T cell populations and the role it may play in altering therapeutic efficacy of costimulation blockade. In order to normalize the cell population responding to these infections, we again used the OVA

transgenic system. $CD8^+$ OT-I T cells were adoptively transferred to B6 mice, and these mice were then infected with one of three pathogens engineered to express the OVA protein – Listeria monocytogenes (LM-OVA), an acute bacterial infection; murine gammaherpesvirus 68 (gHV-OVA), a latent viral infection; and Polyoma virus (PyV-OVA), a persistent viral infection. Mice were bled at a memory timepoint, and we confirmed that the percentage and anatomical distribution of memory OT-I T cells was equal in all three groups (Figure 5B, data not shown). However, these pathogens induced different levels of expression of FcγRIIB on antigen-specific $CD8^+$ T cells (Figure 5C). When challenged with an mOVA skin graft and treated with a combination of CTLA4-Ig, anti-CD154 and anti-VLA4, animals previously infected with LM-OVA demonstrated long-term graft survival, with an MST of over 100 days. However, animals infected with gHV-OVA rejected their grafts much more quickly in the presence of combination therapy, with an MST of 20 days, and the antibody regimen was completely ineffective at prolonging graft survival in PyV-OVA infected animals (Figure 5D). Interestingly, the level of expression of FcγRIIB correlated with the MST of graft survival (Figure 5E), and importantly, these antigen-specific $CD8^+$ T cells were the mediators of graft rejection in this model, as depletion of these cells using an anti-Thy1.1 antibody prior to graft challenge allowed for almost 100% long-term graft survival following combination treatment (data not shown). Therefore, these data suggest that FcγRIIB represents a potential therapeutic target to control memory T cell populations and dramatically improve the efficacy of costimulation blockade during transplantation.

Discussion

In this study, we have shown that allospecific T cells responding to transplantation express the inhibitory Fc receptor FcγRIIB, and that therapeutic blockade of this receptor can enhance recall responses to transplantation. While many reports in the immunology literature have suggested that T cells do not express Fc receptors (14), a recent study identified the upregulation of FcγRIIB on memory T cells (25), and our work has corroborated these studies by demonstrating the expression of FcγRIIB on both allospecific $CD4^+$ and $CD8^+$ T cells. Blockade of this receptor during memory recall enhanced $CD8^+$ T cell recall to a graft challenge, and interestingly, we have shown that expression of FcγRIIB on memory T cell populations correlates with their susceptibility to combined costimulation and integrin blockade, a finding that has important implications for clinical transplantation.

Inhibitory signaling by FcγRIIB is critical in B cells to regulate antibody responses and prevent autoimmunity, and numerous studies have attempted to dissect the complicated FcγRIIB inhibitory signaling mechanisms in these cells. FcγRIIB ligation can modulate different signaling pathways depending on whether or not the B cell receptor is engaged at the same time. When the BCR and FcγRIIB are ligated simultaneously, the ITIM domain located within the cytoplasmic region of FcγRIIB recruits the inositol polyphosphate 5'-phosphotase SHIP, which is able to hydrolyze intermediate messenger molecules such as PIP_3 and thus prevent calcium flux (16, 28, 29). However, when FcγRIIB is crosslinked in the absence of B cell signaling, a distinct, ITIM-independent, pro-apoptotic signaling pathway is initiated by phosphorylation of FcγRIIB by the tyrosine protein kinase ABL (30). In addition, there are multiple isoforms of FcγRIIB. The FcγRIIB-1 isoform expressed by B cells is responsible for inhibitory signaling pathways mentioned above, whereas ligation of the FcγRIIB-2 isoform, which is expressed on DCs, macrophages, and other myeloid cell types, results in endocytosis of antibody-bound material (14). Our studies have not yet addressed whether or not FcγRIIB is acting in a cell-intrinsic manner to inhibit $CD8^+$ T cell expansion, but we hypothesize that the mechanism of action of

FcγRIIB in T cells may be similar to the SHIP-mediated hydrolysis of PIP3, as there is significant overlap between the signaling pathways downstream of the T cell and B cell receptor (31, 32). Detailed molecular analysis of the isoform expressed by T cells as well as signaling studies similar to those performed in B cells will be required to confirm this hypothesis.

The effect of immunoglobulin in the serum on alloreactive T cells responses and memory formation also remains to be addressed. The previous report that identified FcγRIIB expression on $CD8^+$ T cells in a model of viral infection suggested that antigen-specific antibody is required for FcγRIIB-mediated inhibition of memory recall responses (25). The authors hypothesized that expression of this inhibitory receptor evolved to prevent $CD8^+$ T cell exhaustion when humoral memory to the infecting pathogen already exists. This idea also relates to the signaling pathways induced by this receptor in T cells, and whether or not simultaneous ligation of FcγRIIB and the TCR is required for this receptor to inhibit T cell responses. For example, in individuals with preexisting humoral and T cell memory to alloantigen received a transplant expressing those antigens, the donor-specific antibodies may in fact dampen the cellular alloreactivity by colligation of FcγRIIB on graft-reactive T cells. Whether or not target cells must be coated in allospecific antibodies in order to inhibit T cell alloimmunity, or if a certain level of immunoglobulin present in the serum is sufficient to prevent these responses, remains to be determined.

The expression of FcγRIIB on memory $CD8^+$ T cells and the correlation with susceptibility to costimulation and integrin blockade raise the exciting possibility that this pathway may offer a potential target for therapeutic intervention during transplantation. Intravenous immunoglobulin (IVIg) has been used for decades in the treatment of various diseases, including immunothrombocytopenia (ITP) and other autoimmune disorders (33). While initial studies hypothesized that ligation of FcγRIIB directly by immunoglobulin may have been responsible for the anti-inflammatory effect (34), later reports indicated the therapeutic efficacy of IVIg was independent of the signaling molecules associated with FcγRIIB (35). Detailed

studies of the mechanism of action of IVIg have shown that the certain carbohydrate moieties present in the polyclonal Ig infusion bind to type II Fc receptors, including SIGN-R1 in mice and its human ortholog DC-SIGN (36), and induce the production of IL-33 and, subsequently, IL-4 y (37). The elaboration of these cytokines then induces the upregulation of FcγRIIB on macrophage populations, which is also required for IVIg efficacy. However, the effect of this therapy on the expression of FcγRIIB on T cells and the contribution of this population to the efficacy of IVIg therapy has yet to be determined. Interestingly, one study performed in steroid-resistant rejecting transplant patients showed that IVIg treatment was able to reverse rejection at a comparable rate to OKT3, an anti-CD3 depleting antibody (38), demonstrating the potential of this therapy to be used in a clinical transplant setting.

In addition to this established treatment modality, recent work has also identified alternative binding partners for FcγRIIB, including the acute phase reactants SAP and CRP (39, 40). While these are pleiotropic molecules with a number of immunologic effects, and the feasibility of their use as in vivo treatment are unknown, it does raise a possibility that small molecules inhibitors other than monoclonal antibodies may be able to be designed to specifically target FcγRIIB. Likewise, other investigators have utilized novel genetic engineering to design bi-specific antibodies, known as dual-affinity retargeting molecule (DART) (41). These DART molecules were able to specifically target FcγRIIB on B cells, and could easily be engineered to target T cells expressing this molecule in order to inhibit their secondary expansion.

While many recent studies have focused on the abrogation of Fc interactions with therapeutic antibodies in order to avoid unwanted complications, these results suggest that perhaps the interaction of Fc-containing reagents with FcγRIIB may in fact inhibit cellular alloimmunity and provide some benefit to patients. Further work will examine the different roles that this receptor plays in both cell mediated and humoral immunity, with the goal of designing safe and effective therapies for transplant recipients.

Figure 3.1. FcγRIIB deficient mice demonstrate accelerated graft rejection in the presence of costimulation blockade.

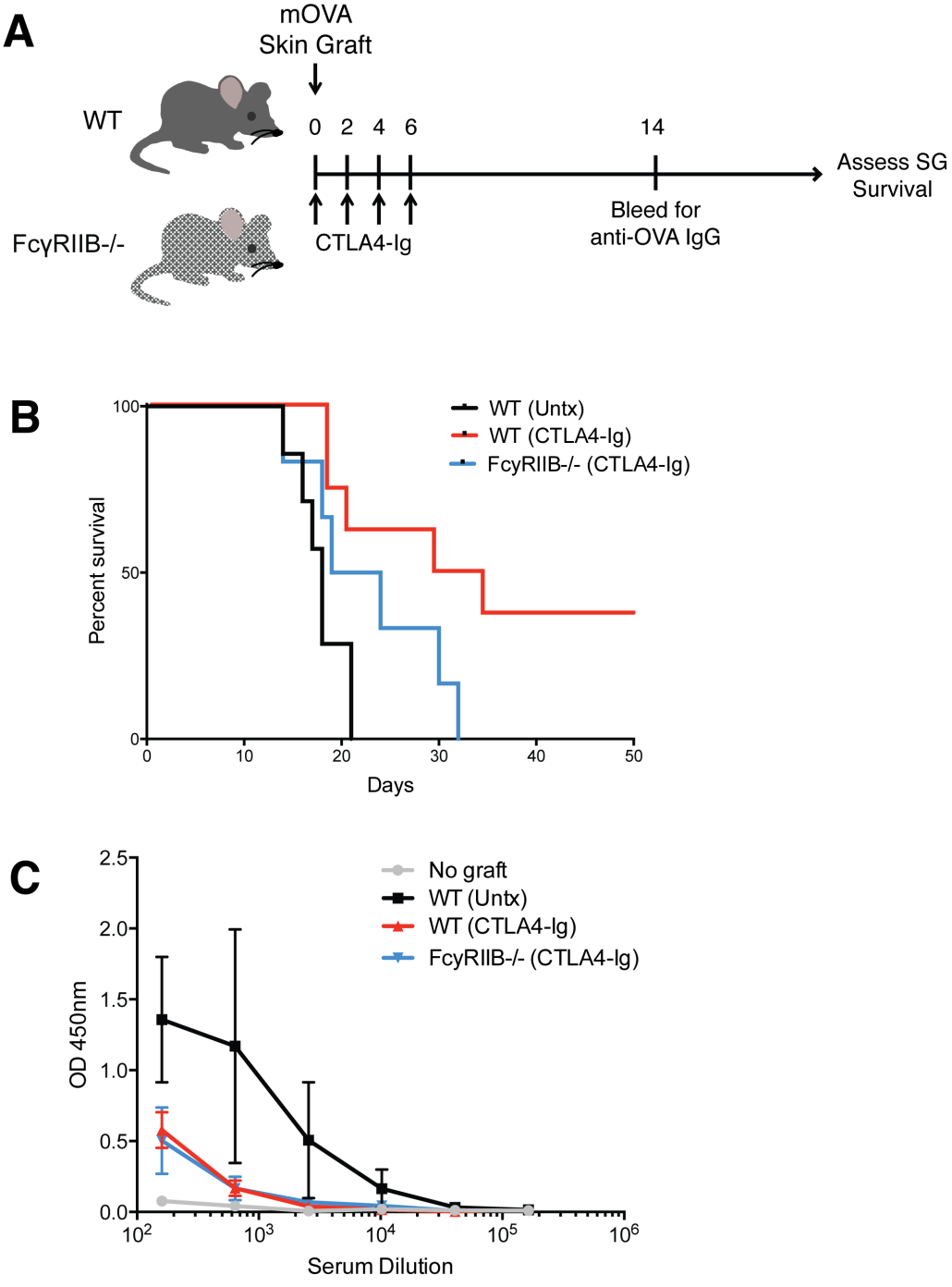

Figure 3.1. FcγRIIB deficient mice demonstrate accelerated graft rejection in the presence of costimulation blockade. (A) On Day 0, B6 or FcγRIIB$^{-/-}$ mice were grafted with mOVA skin, and treated i.p. with either saline or 250ug of CTLA4-Ig on day 0, 2, 4, and 6. Mice were bled at Day 14 to collect serum. (B) Graft survival among untreated WT mice and CTLA4-Ig treated WT and FcγRIIB$^{-/-}$ mice. (C) Anti-OVA IgG ELISA performed on serum from Day 14 post-transplantation. Naïve ungrafted B6 mice served as a negative control.

Figure 3.2. FcγRIIB deficiency does not alter baseline memory T cell compartments.

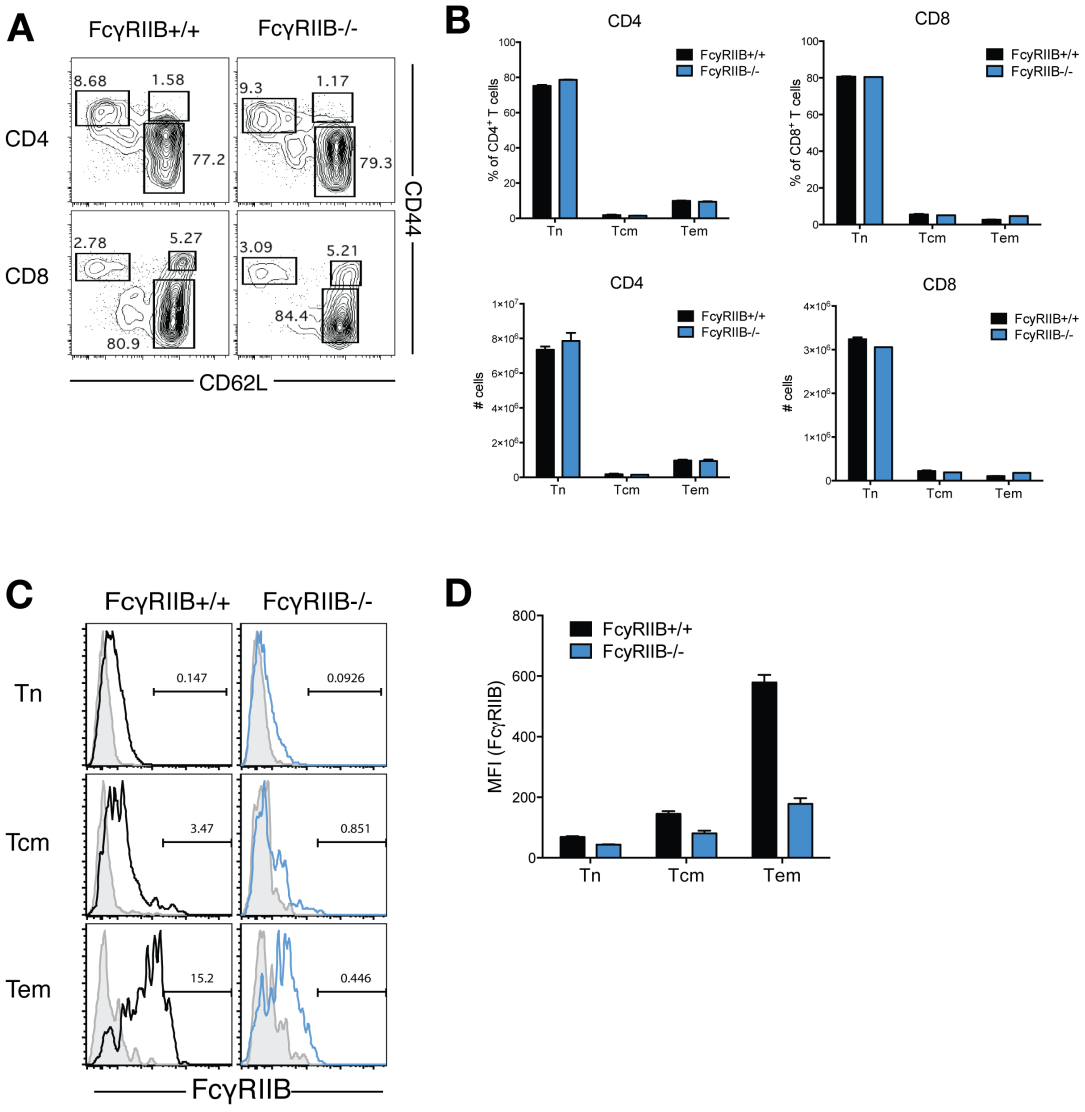

Figure 3.2. FcγRIIB deficiency does not alter baseline memory T cell compartments. (A) Representative flow plots of $CD4^+$ and $CD8^+$ splenocytes of WT and $Fc\gamma RIIB^{-/-}$ mice, subdivided into naïve ($CD62L^{hi}$ $CD44^{lo}$), central memory ($CD62L^{hi}$ $CD44^{hi}$) and effector memory ($CD62L^{lo}$ $CD44^{hi}$) compartments. (B) Frequencies of splenic $CD4^+$ and $CD8^+$ T cell subpopulations. (C) Representative histograms of FcγRIIB expression, gated on $CD8^+$ T cell memory compartments.

Figure 3.3. Polyclonal allograft-specific CD8$^+$ T cells upregulate FcγRIIB following transplantation.

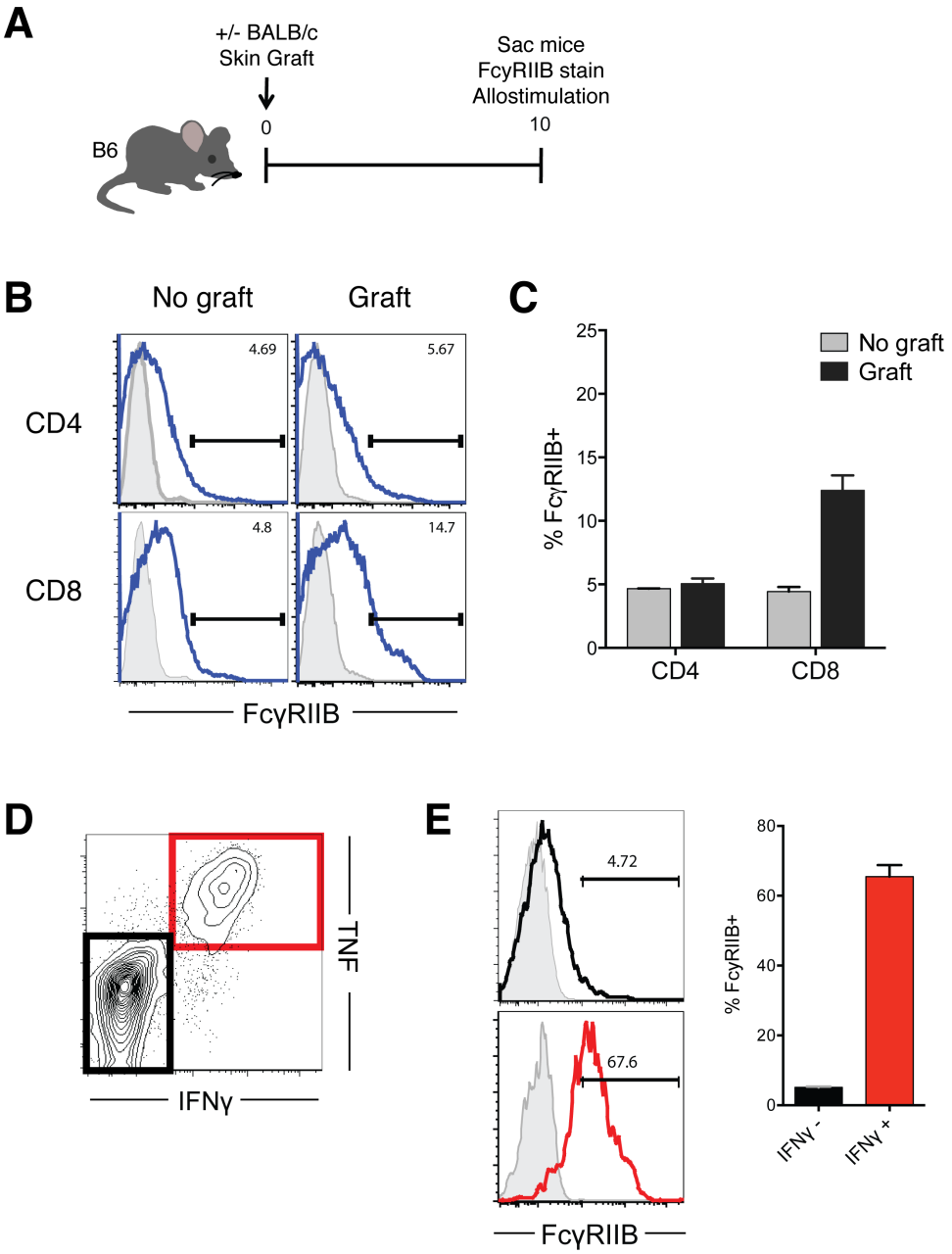

Figure 3.3. Polyclonal allograft-specific $CD8^+$ T cells upregulate FcγRIIB following transplantation. (A) B6 mice were grafted with fully allogeneic BALB/c skin and sacrificed at Day 10 post-transplant. (B) Representative histograms of FcγRIIB on splenic $CD4^+$ and $CD8^+$ T cells in naïve and grafted mice. (C) Frequencies of $FcγRIIB^+$ $CD4^+$ and $CD8^+$ T cells in naïve and grafted mice. (D) Splenocytes from graft recipients were incubated with a 2:1 ratio of BALB/c "stimulator" cells (splenocytes), and stained for production of IFNγ and TNF. (E) Frequencies of FcγRIIB+ cells among IFNγ positive and negative CD8+ T cell populations.

Figure 3.4. Therapeutic blockade of FcγRIIB leads to enhanced memory recall responses to a secondary graft rechallenge.

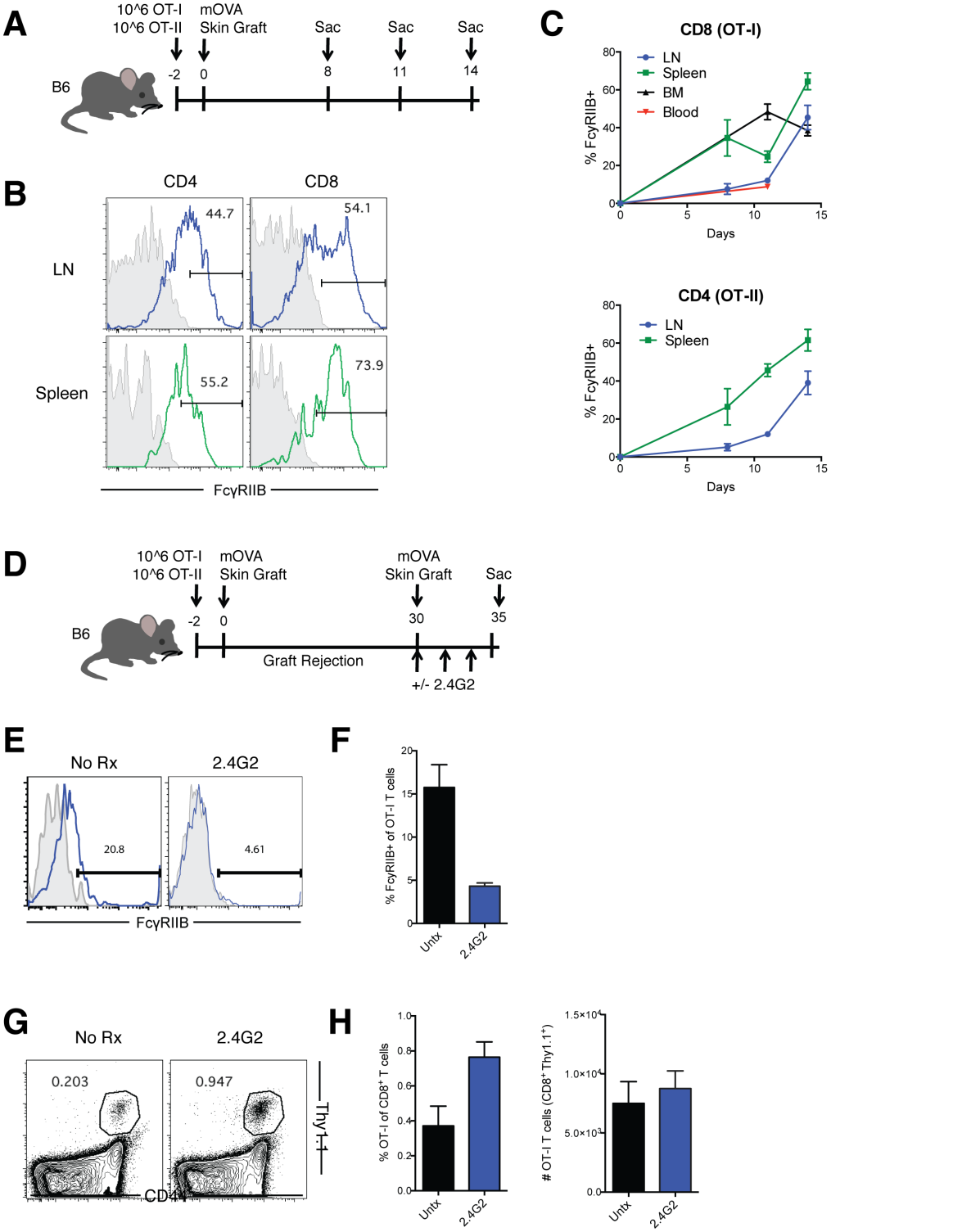

Figure 3.4. Therapeutic blockade of FcγRIIB leads to enhanced memory recall responses to a secondary graft rechallenge. (A) B6 received 10^6 OT-I and 10^6 OT-II T cells, and two days later received an mOVA skin graft. Groups of mice were sacrificed at Day 8, 11, and 14. (B) Representative histograms of FcγRIIB expression on Thy1.1$^+$ OT-I (CD8) and OT-II (CD4) T cell populations at Day 14 post-transplant in the draining lymph nodes and spleen. (C) Frequencies of FcγRIIB$^+$ cells among Thy1.1$^+$ OT-I (CD8) and OT-II (CD4) T cell populations in the bone marrow, spleen, lymph node and peripheral blood. (D) B6 received 10^6 OT-I and 10^6 OT-II T cells, and two days later received an mOVA skin graft. Following graft rejection, mice received a second graft at Day 30, and treated with either saline or 400ug 2.4G2 i.p. at Day 0, 1, and 3 post-transplant. (E and F) Representative histograms and frequencies of FcγRIIB$^+$ Thy1.1$^+$ OT-I T cells in the draining lymph node at Day 5 post-regraft. (G and H) Representative flow plots, frequencies and absolute numbers of Thy1.1$^+$ OT-I T cells in the draining lymph node at Day 5 post-regraft.

Figure 3.5. FcγRIIB expression on memory CD8$^+$ T cells inversely correlates with resistance to combined costimulation and integrin blockade.

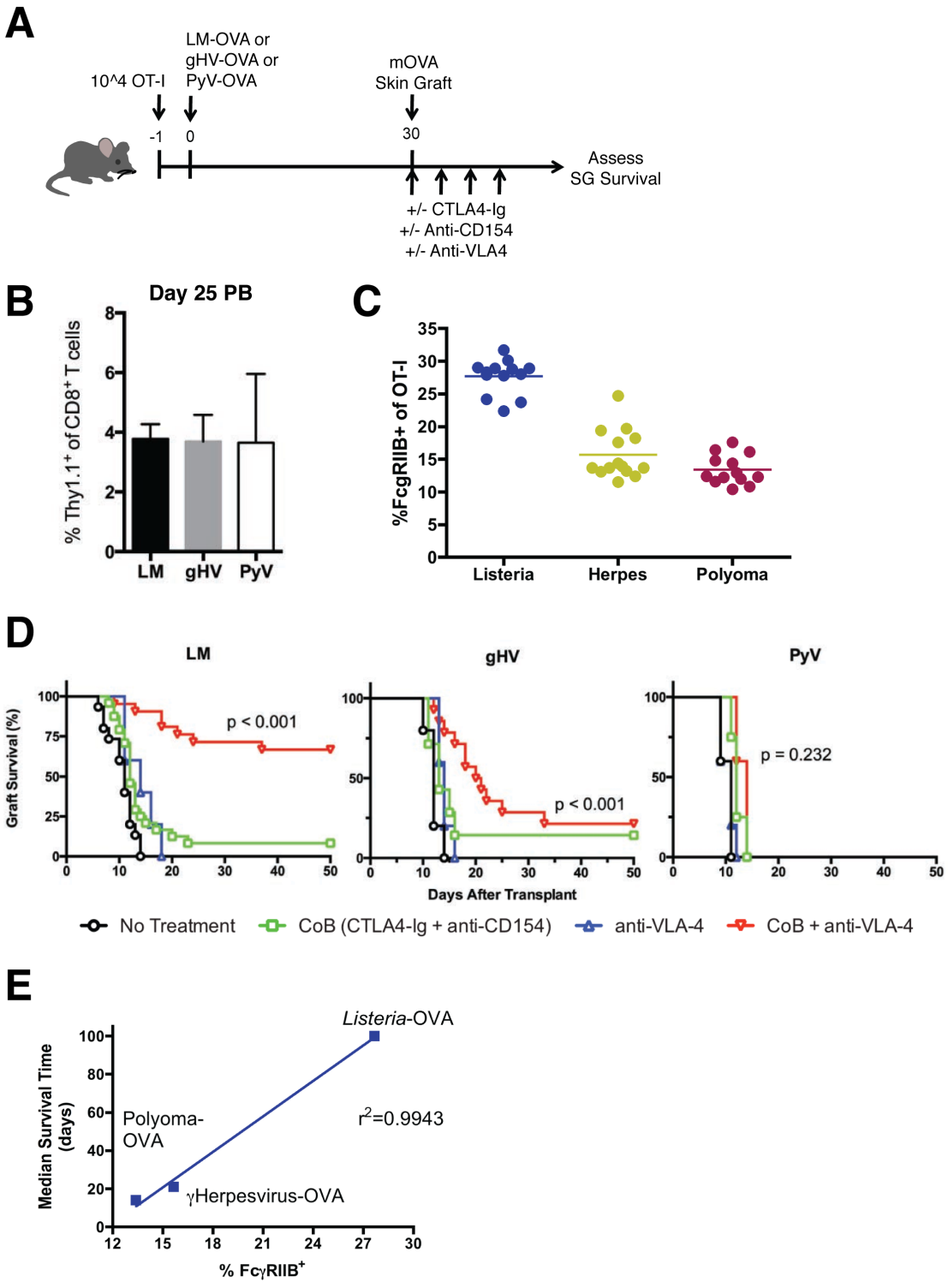

Figure 3.5. FcγRIIB expression on memory CD8$^+$ T cells inversely correlates with resistance to combined costimulation and integrin blockade. (A) B6 mice received 10^4 OT-I T cells and one day later were infected with either LM-OVA, gHV-OVA or PyV-OVA. (B) Frequencies of Thy1.1+ OT-I T cells in the peripheral blood at Day 25 post-infection. (C) Frequencies of FcγRIIB expression on Thy1.1+ OT-I T cells in the peripheral blood at Day 25 post-infection. (D) At Day 30, mice were grafted with mOVA skin, and treated with either saline, CoB (anti-CD154 and CTLA4-Ig), anti-VLA4, or all three drugs in combination at days 0, 2, 4, and 6 post-transplant, and graft survival was measured. (E) Correlation of mean survival time with mean FcγRIIB expression at Day 25 post-infection.

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Chapter 4

Rapamycin Ameliorates the CTLA4-Ig-Mediated Defect in CD8$^+$ T Cell Immunity During Gammaherpesvirus Infection

Abstract

Latent viral infections are a major concern among immunosuppressed transplant patients. During clinical trials with belatacept, a CTLA4-Ig fusion protein, patients showed an increased risk of Epstein-Barr Virus-associated post-transplant lymphoproliferative disorder, thought to be due to a deficient primary $CD8^+$ T cell response to the virus. Using a murine model of latent viral infection, we observed that rapamycin treatment alone led to a significant increase in virus-specific $CD8^+$ T cells, as well as increased functionality of these cells, including the ability to make multiple cytokines, while CTLA4-Ig treatment alone significantly dampened the response and inhibited the generation of polyfunctional antigen-specific $CD8^+$ T cells. However, the addition of rapamycin to the CTLA4-Ig regimen was able to quantitatively and qualitatively restore the antigen-specific $CD8^+$ T cell response to the virus. This improvement was physiologically relevant, in that CTLA4-Ig treated animals exhibited a greater viral burden following infection that was reduced to levels observed in untreated immunocompetent animals by the addition of rapamycin. These results reveal that modulation of T cell differentiation though inhibition of mTOR signaling can restore virus-specific immune competence even in the absence of CD28 costimulation, and have implications for improving protective immunity in transplant recipients.

Introduction

Latent viral infections and subsequent complications are a major source of morbidity and mortality among immunosuppressed transplant patients (1). Epstein-Barr Virus (EBV), a double-stranded DNA virus that infects approximately 90% of adults in the developed world (2), is normally effectively controlled by adaptive T cell responses. However, in the immunosuppressed period following transplantation, patients are at increased risk of developing EBV-associated post-transplant lymphoproliferative disorder (PTLD), a common malignancy characterized by transformed B lymphocytes, the host cell type for latent EBV infection (3).

Belatacept, a second-generation CTLA4-Ig derivative, has recently been shown in clinical trials to be an effective immunomodulatory agent for use following renal transplantation (4). This therapy has the added benefit of a much-improved toxicity profile compared to the current standard of care, calcineurin inhibitors (CNIs) (5). However, one drawback of this therapy noted in early trials was an increased incidence of EBV-associated PTLD, which led the FDA to issue a warning that prevents belatacept from being administered to EBV-seronegative organ recipients (6). Unfortunately, many pediatric patients that would benefit most from the reduced toxicity and subsequent long-term functional advantages of belatacept are EBV-seronegative. Therefore, treatment strategies that could improve primary EBV responses in the presence of belatacept may reduce the risk of these complications and allow belatacept to be used in this high-risk pediatric cohort.

Rapamycin is an anti-fungal macrolide that has been used clinically for decades to attenuate alloreactive T cell proliferation following transplantation (7). According to the 2011 UNOS SRTR report, mTOR inhibitors are used in 0.5-8.7% of regimens at the time of transplantation, and 5.8-10.4% of regimens at one year post-transplant, depending on the type of organ transplanted (8). Side effects of rapamycin include hyperlipidemia, wound healing issues, stomatitis, and anemia, but these complications may be offset by improvements in renal function and lower rates of malignancy compared to CNIs (9). Surprisingly, despite its well known

immunosuppressive properties, administration of rapamycin (sirolimus or everolimus) following transplantation has also been shown to lower the risk of infectious complications, particularly those associated with cytomegalovirus (CMV) (10-13). One study compared low-dose sirolimus treatment with either standard or low-dose CNIs, and found that while patients receiving sirolimus had a higher frequency of some adverse events, their risk of developing a CMV infection was approximately 50% lower than the patients receiving CNIs (10). These promising results were not only specific to CMV, as one case study noted that chronic HCV viremia spontaneously resolved in two liver recipients following conversion to sirolimus (14), and another report suggested that regimens containing sirolimus alone or in combination with prednisone could provide protection against the development of BK-virus nephropathy (15).

While these studies suggested a clinical benefit of sirolimus, little mechanistic data existed to explain the improvement of viral outcomes in these patients. Several groups had hypothesized that rapamycin could directly abrogate viral replication, given the central role of mTOR signaling in transcription. In one study, rapamycin was shown to inhibit viral particle formation during Kaposi-Sarcoma Herpes Virus infection, a gammaherpes virus related to EBV (16). Conversely, in vitro studies of the CMV life cycle in human cells showed that the infection-induced activation of PI3-Kinase and the subsequent upregulation of transcriptional machinery required for viral replication was unperturbed by rapamycin treatment (17). Therefore, the virostatic effects of rapamycin may depend on the particular life cycle of a given virus. However, these studies did not investigate the impact of the immune response to the virus, and recent reports using animal models suggested that mTOR inhibition with low-dose rapamycin treatment could actually enhance $CD8^+$ T cell responses, and in particular long-term memory generation, following pathogen infection (18). Furthermore, our group demonstrated that the immunological context in which rapamycin is given determines its effect, and that rapamycin can augment pathogen-reactive, but not graft-reactive, $CD8^+$ T cell responses (19). These findings suggest that rapamycin could be used in the setting of transplantation to improve protective immune responses

without simultaneously augmenting harmful alloreactive responses. Given the complications seen following EBV infection in belatacept-treated patients, rapamycin may be a potential option to use along with belatacept to enhance pathogen-specific T cell responses in these vulnerable patients.

We have established a murine model of latent gammaherpesvirus infection in which CTLA4-Ig treatment leads to a defective $CD8^+$ T cell response, resulting in increased viral burden. Murine gammaherpesvirus 68 (MHV68) is a rodent gammaherpesvirus that shares a large degree of both functional and genetic homology with EBV (20). We sought to determine if the addition of rapamycin could ameliorate MHV68-specific $CD8^+$ T cell responses in the presence of CD28 blockade, and thus restore viral control in these animals. Using MHC Class I tetramers to track the $CD8^+$ T cell response in the peripheral blood and ex vivo viral peptide stimulation to measure $CD8^+$ T cell functionality, we observed that rapamycin is able to augment the primary response to MHV68. While CTLA4-Ig treatment inhibited both the expansion of MHV68-specific $CD8^+$ T cells and their ability to produce cytokines, the addition of rapamycin to a CTLA4-Ig regimen was able to restore the $CD8^+$ T cell response to levels seen in untreated animals, and, importantly, this also translated to an ability to improve overall viral burden. These results indicate that rapamycin may be a candidate for synergistic therapy during treatment with belatacept in order to improve anti-viral responses and outcomes in transplant patients.

Materials and Methods

Mice and Viral Infections

B6-Ly5.2/Cr (H2-K^b) mice were obtained from the National Cancer Institute (Frederick, MD). Virus stocks were prepared as previously described (21). Mice were infected with $2x10^3$ MHV68-OVA intraperitoneally. All animals were housed in BSL2 animal facilities at Emory University and maintained in accordance with Emory University Institutional Animal Case and Use Committee guidelines (Atlanta, GA).

Surface Stains and Flow Cytometry

Peripheral blood and spleens were processed and stained for CD8 and CD127 (Biolegend, San Diego, CA), as well as fluorochrome conjugated p56 (H-2K^b, AGPHNDMEI) and p79 tetramers (H-2K^d, TSINFVKI) (NIH Tetramer Core, Atlanta, GA). Samples were analyzed using a multicolor LSRII FACS machine (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Peptide Stimulation and Intracellular Cytokine Staining

For p56 and p79 peptide stimulation, responder splenocytes were stimulated with 10μM p56 (ORF6, AGPHNDMEI), or 10μM p79 (ORF61, TSINFVKI) peptides (Genscript, Inc., Piscataway, NJ) in the presence of 10μg/mL Brefeldin A (Golgi Plug, BD Biosciences) for 4 hours. Intracellular staining kit (BD Biosciences) was used to detect TNF and IFN-γ (Biolegend), and IL-2 (BD Biosciences) according to manufacturer's instructions.

Skin Transplantation and Allostimulation Assay

Full thickness tail, ear, or trunk skins were transplanted onto dorsal thorax of recipient $CD45.1^+$ B6 mice and secured with adhesive bandages. Where indicated, mice were treated i.p. with saline, 1.5μg rapamycin (every day) or 250μg CTLA4-Ig (Bristol Myers Squibb, every other day) for 20

days. Animals were then sacrificed, and recipient splenocytes were stimulated with fresh $CD45.2^+$ BALB/c splenocytes in the presence of 10 μg/mL Brefeldin A for 5 hours followed by intracellular staining for cytokine production.

LD-PCR

Limiting dilution PCR to determine the frequency of viral genomes was performed as previously described (21, 22). Briefly, splenocytes were counted, serial diluted, and proteinase K digested prior to a two-step nested PCR reaction for the MHV68 gene ORF50. Replicate wells were scored for genome positive PCR amplification. Frequency was then determined by applying Poisson distribution.

Statistical Analysis

For single comparisons, students unpaired t test was performed. For multiple comparisons, one-way ANOVA tests were performed, followed by Tukey post-test on significant results. All analyses were done using GraphPad Prism software (GraphPad Software Inc, San Diego, CA).

Results

Rapamycin enhances the primary CD8$^+$ T cell response to a gammaherpesvirus infection

Given the benefits observed following rapamycin treatment in other models of infection (18, 19), we first sought to investigate the effect of rapamycin treatment alone on the CD8$^+$ T cell response to MHV68 infection. To track the polyclonal response to this infection in the periphery, we serially bled mice during the course of primary infection and used MHC Class I tetramers to identify CD8$^+$ T cells specific for the immunodominant lytic phase epitopes of MHV68, p56 and p79 (23) (Figure 1A). While differences in untreated and rapamycin-treated animals were not significant prior to the peak of the response, we observed that by day 20 in the peripheral blood there were significantly greater numbers of MHV68-specific CD8$^+$ T cells in rapamycin-treated animals (Figure 1B-D). Interrogation of the MHV68-specific response in the spleen at day 20 revealed that rapamycin treatment led to both greater percentages and absolute numbers of CD8$^+$ T cells specific for the virus (Figure 1E, F). The increased number of tetramer positive cells were also functional, as rapamycin treated animals had greater percentages and numbers of CD8$^+$ T cells able to produce the anti-viral cytokine IFN-γ following ex vivo restimulation with p56 and p79 peptide (Figure 1G, H). It is worth noting that while rapamycin increases the frequency and number of IFN-γ positive cells, a comparison of the number of tetramer positive cells with the number of cytokine secreting cells revealed that rapamycin does not increase the proportion of tetramer positive cells able to produce IFN-γ, but rather increased the overall size of this antigen-specific population (data not shown). These results are in line with previous studies in TCR transgenic populations where the number, but not frequency, of antigen-specific IFN-γ producers was increased following treatment with rapamycin (19). Furthermore, the beneficial effect of rapamycin on antigen-specific CD8$^+$ T cell responses was durable, as these animals maintained higher frequencies of tetramer-positive cells in the peripheral blood as late as 70 days post-infection, even when rapamycin was only administered for the first 20 days (Figure S1). Taken

together, these results reveal that mTOR inhibition with rapamycin is able to enhance the number of $CD8^+$ T cells responding to a gammaherpesvirus infection.

CTLA4-Ig inhibits the primary $CD8^+$ T cell response to a gammaherpesvirus infection

Due to the EBV-specific signature seen during clinical trials of belatacept, we sought to determine if blockade of the B7-CD28 pathway in our murine model had a detrimental effect on the primary $CD8^+$ T cell response to gammaherpesvirus infection. Following the same infection protocol, we observed that blockade of CD28 costimulatory signals via treatment with CTLA4-Ig dramatically dampened the peripheral response to MHV68 over the first three weeks of infection (Figure 2B, C). CTLA4-Ig treatment also resulted in much lower percentages and numbers of MHV68-specific $CD8^+$ T cells in the spleen (Figure 2D, E) and significantly impaired the ability of $CD8^+$ T cells cells to make IFN-γ in response to MHV68 peptides (Figure 2F, G). These results indicate that lack of CD28-mediated costimulation leads to a defective primary response to an MHV68 infection, which may in part explain the increased incidence of EBV-associated complications following clinical costimulation blockade.

The addition of rapamycin to a CTLA4-Ig regimen restores $CD8^+$ T cell expansion and cytokine function

Given the improvements seen in MHV68-specific $CD8^+$ T cell responses when treating with rapamycin, we next sought to determine if rapamycin was able to augment this response in the presence of CTLA4-Ig. Animals were infected and treated with both rapamycin and CTLA4-Ig during the primary infection. While the magnitude of the antigen-specific $CD8^+$ T cell response in CTLA4-Ig plus rapa treated animals was initially similar to animals that received CTLA4-Ig alone, the frequency of p56- and p79-specific $CD8^+$ T cells in dual-treated animals reached the frequency observed in untreated animals by day 20 post-infection (Figure 3A, B), and the increased number of cells was maintained in dual-treated animals at 70 days post-infection

(Figure S1). Similarly, the addition of rapamycin to the CTLA4-Ig treatment regimen was able to restore both percentages and absolute numbers of MHV68-specific $CD8^+$ T cells in the spleen to levels similar to those observed in untreated animals (Figure 3C, D). Most importantly, adding rapamycin to CTLA4-Ig treatment rescued the ability of $CD8^+$ T cells to make IFN-γ in response to ex vivo restimulation with viral peptides (Figure 3E, F). These results demonstrate that the salutary effect of mTOR inhibition on MHV68-specific $CD8^+$ T cell responses is independent of the B7-CD28 pathway, and thus is retained in the presence of CTLA4-Ig treatment.

CTLA4-Ig-induced defects in memory generation and multicytokine production are ameliorated by rapamycin

Previous reports indicated that rapamycin improves the generation of high-quality memory $CD8^+$ T cells (18), and expression of the IL-7R alpha chain, or CD127, during the effector phase of primary infection is associated with cells that go on to survive as long-lived memory $CD8^+$ T cells (24, 25). While blockade of CD28 costimulatory signals via CTLA4-Ig treatment did not significantly alter the expression of CD127 on MHV68-specific effector cells, mTOR inhibition via rapamycin, either alone or in combination with CTLA4-Ig, led to a significant increase in expression of CD127 on MHV68-specific $CD8^+$ T cells (Figure 4A, B). These data suggest that one mechanism by which mTOR inhibition augments $CD8^+$ T cell responses in the absence of CD28-mediated costimulation is through enhanced generation of high-quality memory $CD8^+$ T cells.

Next we further investigated the nature of CTLA4-Ig-mediated defects in $CD8^+$ T cell cytokine production. Production of proinflammatory cytokines is one of the most important mechanisms by which $CD8^+$ T cells mediate their effector function, and studies have suggested that cells able to make multiple cytokines may be more potent effectors and better able to control viral infection (26-28). While rapamycin alone slightly improved the percentage of polyfunctional $CD8^+$ T cells able to produce a combination of IFN-γ, TNF and IL-2, treating mice with CTLA4-Ig led to a

significant reduction in cells able to produce two or three cytokines (Figure 4C, D). However, the addition of rapamycin was able to enhance multicytokine production in MHV68-specific $CD8^+$ T cells at both acute (day 20) and memory (day 75) timepoints, which may contribute to improved viral control in dual-treated animals (Figure 4C, D; Figure S1). Furthermore, we confirmed that the immune enhancing properties of mTOR inhibition were confined to the context of a pathogen infection, as rapamycin treatment did not exhibit an immunostimulatory effect in the absence or presence of CTLA4-Ig in response to a fully allogeneic skin graft (Figure 5).

CD28 costimulation blockade results in increased viral burden that is diminished by the addition of mTOR inhibition

Finally, we sought to determine the physiological effect that treatment and the resulting $CD8^+$ T cell response had on viral burden experienced by infected animals. We monitored the levels of MHV68-infected cells by performing a limiting-dilution PCR assay on splenocytes at day 20 post-infection. While rapamycin treatment alone did not significantly alter viral burden, inhibition of CD28 costimulatory signals via CTLA4-Ig led to an approximately 3-fold increase in the percentage of MHV68-infected cells in the spleen (Figure 6A, B, D). However, the addition of rapamycin to the CTLA4-Ig treatment regimen resulted in significantly improved control of MHV68 infection, such that the frequencies of infected cells in spleens of animals treated with CTLA4-Ig and rapamycin were similar to those observed in untreated animals (Figure 6C, D). Taken together, our results indicate that the restoration of MHV68-specific $CD8^+$ T cell responses by the addition of rapamycin leads to a physiologically significant improvement in protective immunity compared to animals that receive CTLA4-Ig alone.

Discussion

In this study, we demonstrate that low-dose treatment with rapamycin functions to enhance the primary $CD8^+$ T cell response to a latent viral infection. Previous studies of the immunostimulatory effect of rapamycin utilized LCMV Armstrong (18) and *Listeria monocytogenes* (19), acute infections that are cleared within 5-8 days post-infection (29, 30). Because antigen persistence is a key component of programming $CD8^+$ T cell differentiation (31, 32), we sought to determine if the benefit of rapamycin is observed during gammaherpesvirus infection, in which antigen persists for longer periods of time. Most virologic studies report that the lytic phase of this virus lasts for approximately 14-16 days, and some lytic phase antigens may be expressed periodically following reactivation events during latency (20). Our study now suggests that mTOR inhibition through treatment with rapamycin can enhance $CD8^+$ T cell responses in the setting of prolonged antigen exposure.

Furthermore, while CD28 blockade with CTLA4-Ig leads to a defective $CD8^+$ T cell response and higher viral burden, concurrent mTOR inhibition is able to restore functionality to the response, suggesting that this may be an adjunct therapy to consider in transplant patients suffering from EBV-associated complications following belatacept treatment. The importance of costimulation in $CD8^+$ T cell programmed differentiation is well documented. Antigenic stimulation in the absence of costimulation, specifically CD28-mediated signals, results in T cell anergy and apoptosis (33, 34), due in part to ineffective IL-2 production and a failure to upregulate the pro-survival molecule Bcl-xl (35, 36). In addition, the effect of mTOR signaling on T cell differentiation and memory generation has also been well investigated. Specifically, mTOR inhibition with low-dose rapamycin has been shown to enhance the quality and quantity of memory $CD8^+$ T cells generated in response to viral infection (18), and one of the mechanisms by which this is achieved is the attenuation of T cell contraction following the peak of the anti-viral response (37). Thus, by partially blocking mTOR signaling, T cells undergo less terminal effector cell differentiation, staying in a more memory-like, long-lived state. By demonstrating that

rapamycin can enhance $CD8^+$ T cell responses even in the presence of CTLA4-Ig, we have gained greater insight into the molecular mechanisms underlying these processes, in that the enhancement of memory generation through mTOR inhibition is independent of CD28 signaling.

We observed that mTOR inhibition through rapamycin results in increased expression of CD127 (IL-7R alpha chain) on virus-specific $CD8^+$ T cells both in the absence and presence of CTLA4-Ig. Naïve $CD8^+$ T cells express high amounts of CD127, and this expression is downregulated following strong antigen and cytokine stimulation (38). However, a subset of effector cells retains expression of CD127 during the response, and these cells go on to form long-lived memory populations (25, 39). Previous work has also shown that a greater proportion of antigen-specific effector cells retained expression of CD127 during treatment with rapamycin following LCMV infection (18). Interestingly, we did not observe altered expression of T cell coinhibitory molecules PD-1, LAG-3, TIM-3, or 2B4 following mTOR inhibition either in the presence or absence of CTLA4-Ig (Figure S2), suggesting that rescuing cells from T cell exhaustion is not the mechanism by which rapamycin leads to enhanced virus-specific $CD8^+$ T cell responses in this model. Thus, based on our observations that rapamycin functions to increase expression of CD127 on virus-specific $CD8^+$ T cells both in the absence and presence of CTLA4-Ig (Figure 4), we conclude that a likely mechanism underlying the observed increase in mHV-specific memory T cells is increased differentiation of $CD127^{hi}$ long-lived memory precursors in the setting of mTOR inhibition. This interpretation is also consistent with the observed kinetics of the response, insofar as rapamycin did not result in an increase in virus-specific T cells at the peak of the response, but instead functioned to augment the frequency of virus-specific T cells during the contraction phase of the response and out into memory (Figure 1 and S1). The signaling networks and gene expression changes following mTOR inhibition that lead to the increased expression of CD127 are an area of intense investigation, Furthermore, while cells in animals treated with CTLA4-Ig alone downregulated CD127 to the same degree as those left untreated, those treated with rapamycin and CTLA4-Ig exhibited an increased frequency of

CD127-expressing antigen-specific $CD8^+$ T cells. These novel findings indicate that CD28 costimulation does not play a role in CD127 downregulation following T cell activation, allowing the beneficial effect of mTOR inhibition to be maintained during CD28 blockade.

This study also establishes an important murine model in which CD28 blockade leads to an ineffective primary immune response and loss of control of gammaherpesvirus infection, which may provide valuable insight into EBV-associated complications in belatacept-treated patients. Despite the fact that EBV-related PTLD affects 1-20% of transplant recipients (40), the effect of the primary immune response against the virus on the etiology of this disorder is still not completely clear. The major risk factors for developing PTLD include a high EBV viral load and the age of the recipient, with pediatric recipients being the most at risk. The typical treatment strategy for PTLD is to reduce the overall level of immunosuppression, which has resulted in variable levels of success in controlling lymphoproliferation, and also leaves patients vulnerable to acute rejection episodes (41). One report showed that patients with a high EBV viral load were more likely to demonstrate an "exhausted" EBV-specific $CD8^+$ T cell profile ($PD\text{-}1^+$ $CD127^-$), which also suggests that the quality of the immune response to the virus is important for controlling the virus. In addition, advanced studies of human $CD8^+$ T cell subsets specific for influenza, CMV, or EBV showed distinct patterns of cytokine and surface marker expression depending on the virus by which they were elicited (42). Therefore, some specific defect induced by CD28 blockade may result in the EBV-specific infectious signature seen in belatacept-treated patients. Our model may represent a useful tool for investigating defects in $CD8^+$ T cell responses that lead to the loss of viral control in the setting of CD28 blockade-based immunosuppression.

Similar to work in the acutely cleared LCMV Armstrong model (18), we found that the impact of concurrent mTOR inhibition in augmenting the $CD8^+$ T cell response to MHV68 was observed following the peak of the response, during the T cell contraction phase. Thus, it is possible that mTOR inhibition functions during the contraction phase of the response to limit $CD8^+$ T cell programmed cell death; alternatively, mTOR inhibition could be functioning early

during the response to induce a distinct differentiation program that results in reduced contraction during the later phases of the response. This was addressed by Araki et al (18), which demonstrated that rapamycin improved memory $CD8^+$ T cell generation when given only during the expansion phase (days 1 to 8), but not when only administered following the peak of the response (days 8 to 30). Therefore, while the immunostimulatory effect of rapamycin materializes during the contraction phase, it would seem that mTOR inhibition during the initial programming of these cells is responsible for their improved long-term survival. This may have implications for the timing of therapeutic interventions with rapamycin, in that it may be more effective in modulating the programming of primary responses to pathogens rather than preventing antigen-specific $CD8^+$ T cell attrition. In addition, while it appears that the $CD8^+$ T cell response in CTLA4-Ig treated animals does not contract substantially following the peak of infection at day 16, we hypothesize that this is due to the impaired functionality of the MHV68-specific cells and their inability to control the virus, rather than increased survival of $CD8^+$ T cells in animals treated with dual therapy, suggested by the increased expression of CD127 in these populations.

In conclusion, while treatment with belatacept is associated with a distinct renal functional benefit compared to CNIs, the risk of developing EBV-associated PTLD has led to a contraindication being issued for EBV-seronegative recipients (6, 43). Our study has demonstrated that the salutary effects of rapamycin on the response to a gammaherpesvirus infection are maintained even in the presence of CTLA4-Ig, and that combining costimulation blockade with mTOR inhibition may be a feasible clinical immunosuppression strategy. Further studies are warranted to dissect the exact time frames in which the benefit of rapamycin treatment can be maximized, as well as the effect of these treatments on existing EBV-specific memory responses in seropositive recipients.

Figure 4.1. Rapamycin enhances the CD8$^+$ T cell response to gammaherpesvirus infection.

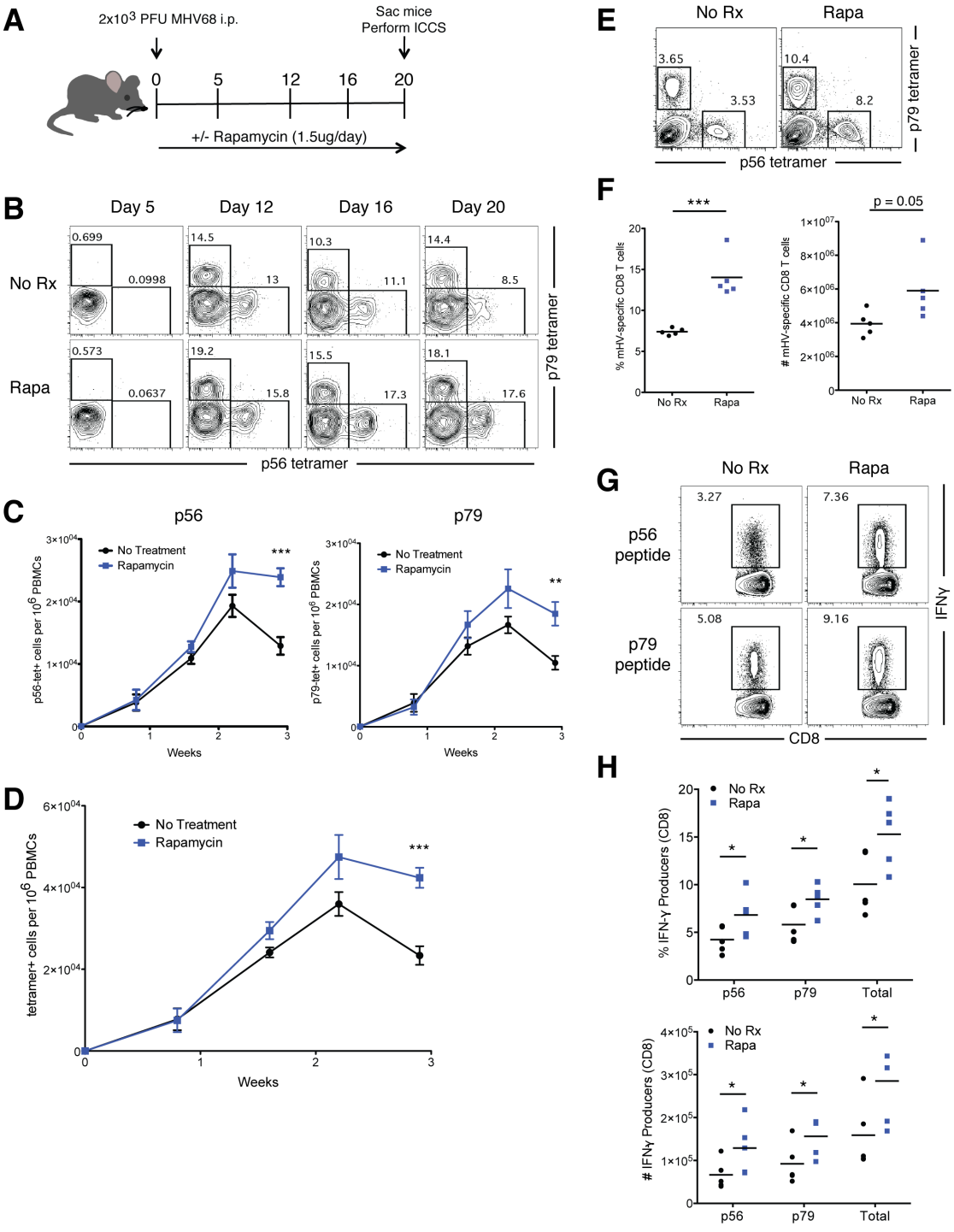


Figure 4.1. Rapamycin enhances the CD8$^+$ T cell response to gammaherpesvirus infection. (A) On Day 0, B6 mice were infected with 2x10^3 pfu of mHV68 i.p., and treated i.p. with either saline or 1.5ug of rapamycin daily. (B-D) Mice were bled at serial time points, and the expansion of total p56- and p79-tetramer positive CD8$^+$ T cells was monitored in the peripheral blood. Combined tetramer positive cells are shown in D. (E and F) Percentages and absolute numbers of mHV-tetramer positive CD8$^+$ T cells in spleens of infected mice at day 20 post-infection. (G) Splenocytes were harvested at Day 20 and stimulated ex vivo with p56 and p79 peptide, and then stained for cytokine production. (H) Percentages and absolute numbers of IFN-γ-producing CD8$^+$ T cells. Data shown are from one experiment with 4-5 mice per group; results are representative of 5 independent experiments with a total of 20-25 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.2. CTLA4-Ig inhibits the primary CD8$^+$ T cell response to a gammaherpesvirus infection.

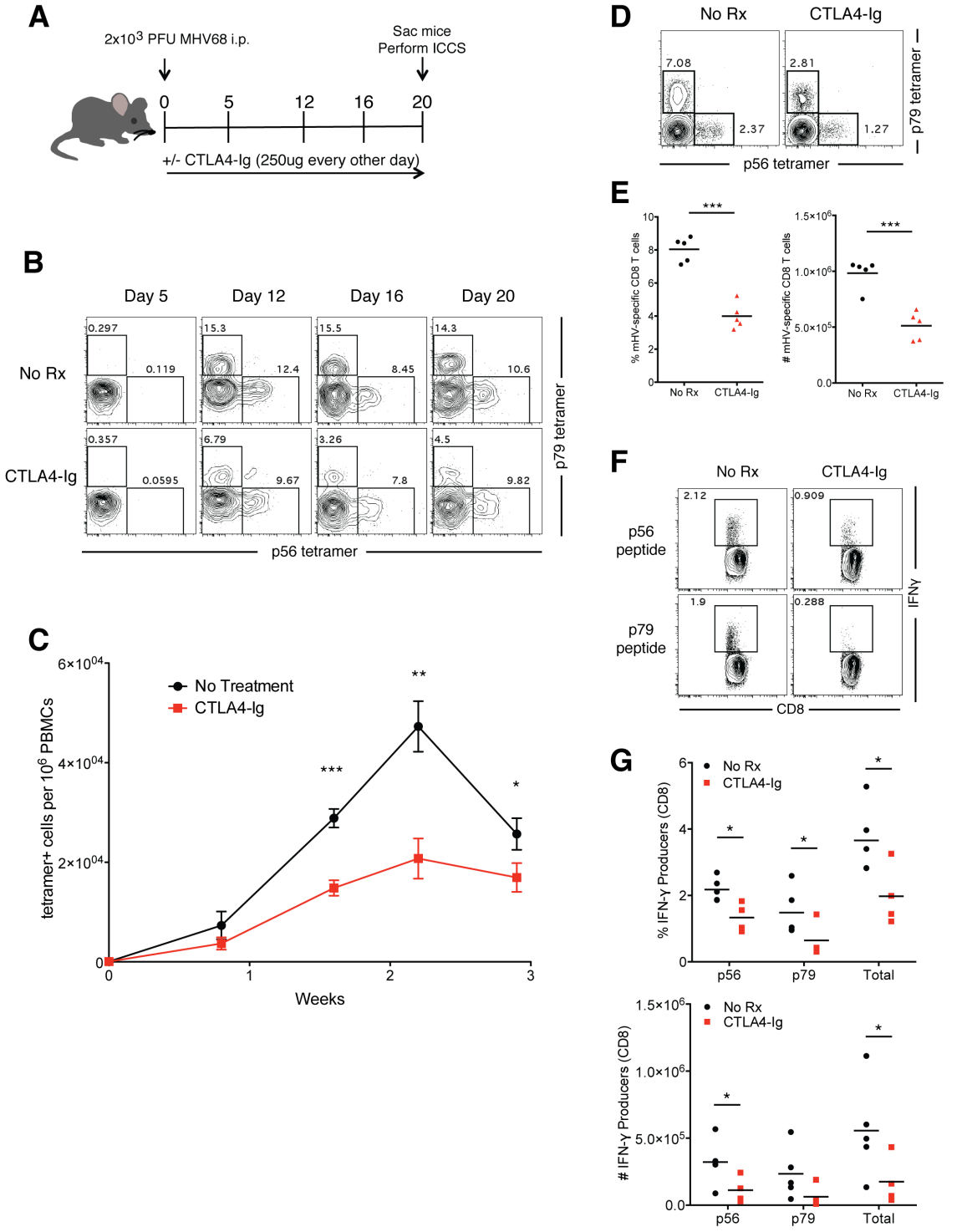


Figure 4.2. CTLA4-Ig inhibits the primary $CD8^+$ T cell response to a gammaherpesvirus infection. (A) On Day 0, B6 mice were infected with $2x10^3$ pfu of mHV68 i.p., and treated i.p. with either saline or 250ug of CTLA4-Ig every other day. (B and C) Mice were bled at serial time points, and the expansion of total mHV-tetramer positive $CD8^+$ T cells was monitored in the peripheral blood. (D and E) Percentages and absolute numbers of mHV-tetramer positive $CD8^+$ T cells in spleens of infected mice at Day 20 post-infection. (F) Splenocytes were harvested at Day 20 and stimulated ex vivo with p56 and p79 peptide, and then stained for cytokine production. (G) Percentages and absolute numbers of IFN-γ-producing $CD8^+$ T cells. Data shown are from one experiment with 4-5 mice per group; results are representative of 5 independent experiments with a total of 20-25 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.3. The addition of rapamycin to a CTLA4-Ig regimen restores CD8$^+$ T cell expansion and cytokine function.

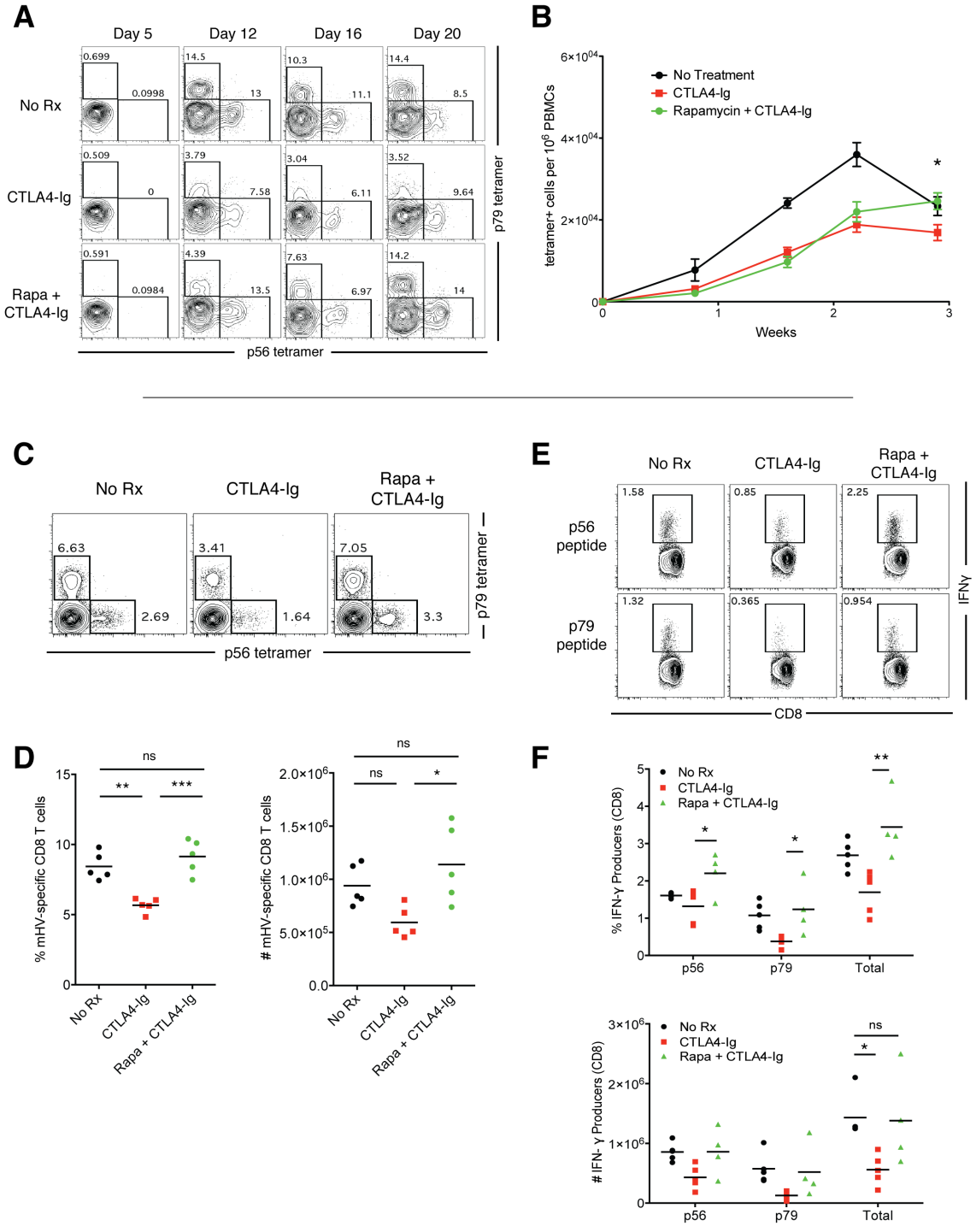


Figure 4.3. The addition of rapamycin to a CTLA4-Ig regimen restores $CD8^+$ T cell expansion and cytokine function. (A and B) On Day 0, B6 mice were infected with $2x10^3$ pfu of mHV68 i.p, and treated i.p. with either saline, 1.5ug of rapamycin daily, or 250ug of CTLA4-Ig every other day. Mice were bled at serial time points, and the expansion of total mHV-tetramer positive $CD8^+$ T cells was monitored in the peripheral blood. (C and D) Percentages and absolute numbers of mHV-tetramer positive $CD8^+$ T cells in spleens of infected mice at Day 20 post-infection. (E) Splenocytes were harvested at Day 20 and stimulated ex vivo with p56 and p79 peptide, and then stained for cytokine production. (F) Percentages and absolute numbers of IFN-γ-producing CD8+ T cells. Data shown are from one experiment with 4-5 mice per group; results are representative of 5 independent experiments with a total of 20-25 mice per group. * $p < 0.05$, ** $p < 0.01$.

Figure 4.4. Rapamycin treatment rescues the CTLA4-Ig-mediated defects in memory generation and multicytokine production.

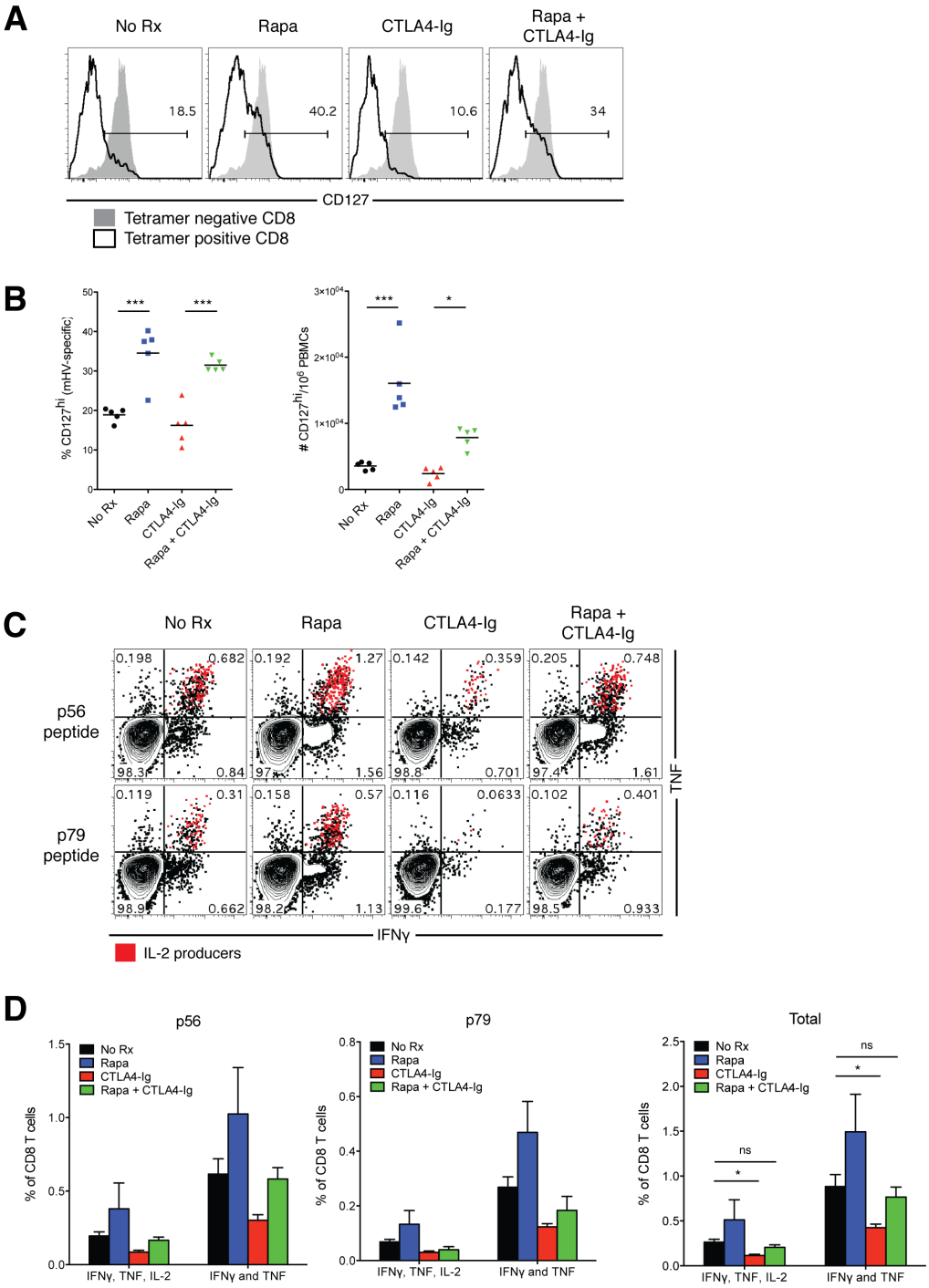


Figure 4.4. Rapamycin treatment rescues the CTLA4-Ig-mediated defects in memory generation and multicytokine production. (A) Expression of CD127 on $CD8^+$ T cells in the peripheral blood at Day 20 post-infection. Gray – tetramer negative $CD8^+$ T cells, Black line – tetramer positive $CD8^+$ T cells. (B) Percentages and absolute numbers of $CD127^{hi}$ tetramer positive $CD8^+$ T cells in the peripheral blood at Day 20. (C) Splenocytes were harvest at Day 20 and stimulated ex vivo with p56 and p79 peptide, and then stained for cytokine production. (D) Percentages and absolute numbers of multicytokine-producing $CD8^+$ T cells. Data shown are from one experiment with 4-5 mice per group; results are representative of 5 independent experiments with a total of 20-25 mice per group. * $p < 0.05$, *** $p < 0.001$.

Figure 4.5. Rapamycin does not augment the CD8$^+$ T cell response to allograft in the presence or absence of CTLA4-Ig.

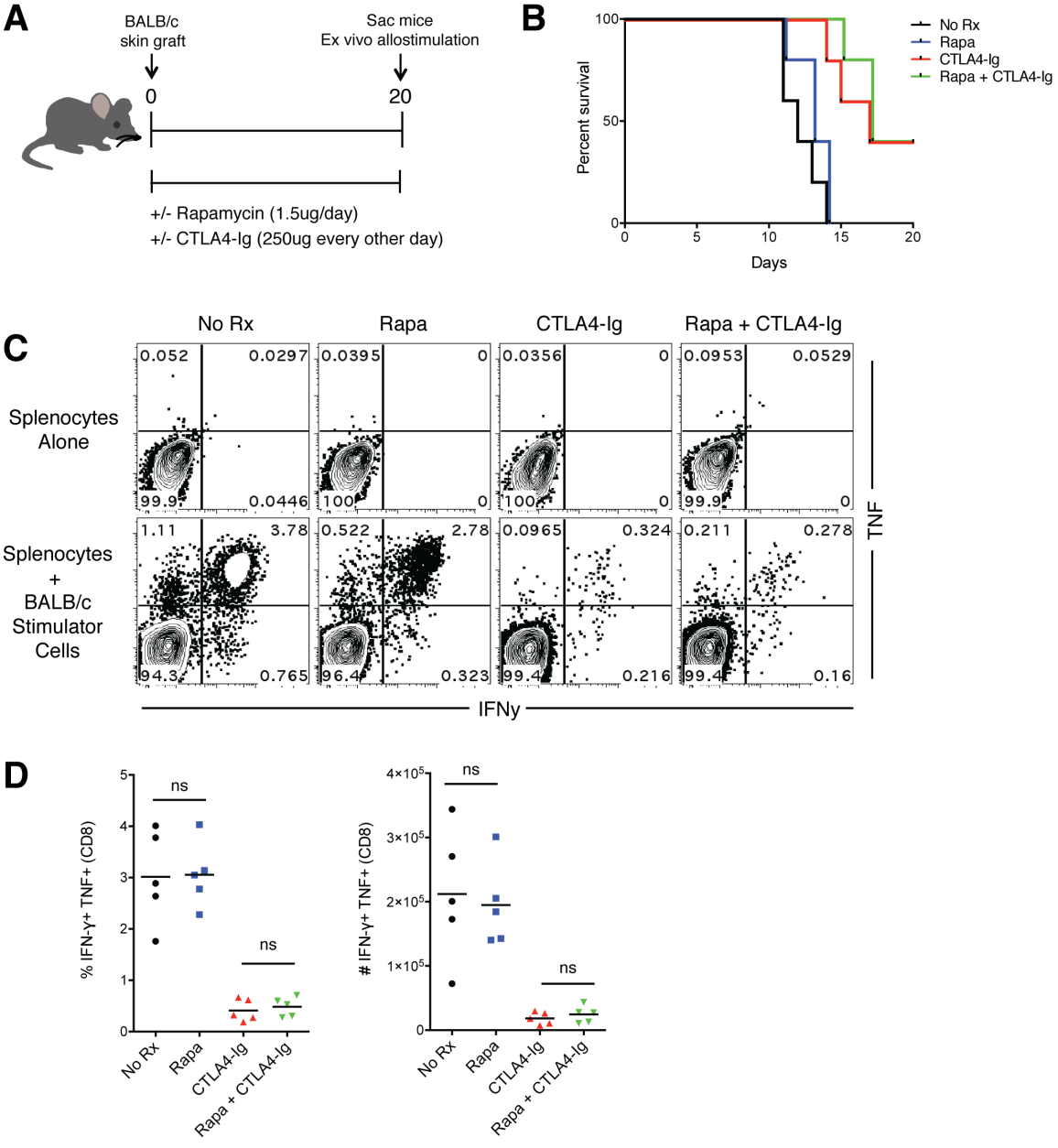


Figure 4.5. Rapamycin does not augment the CD8$^+$ T cell response to allograft in the presence or absence of CTLA4-Ig. (A) On Day 0, CD45.1$^+$ B6 mice were grafted with fully allogeneic BALB/c skin, and treated i.p. with either saline, 1.5ug of rapamycin daily, 250ug of CTLA4-Ig every other day, or both drugs in combination. Splenocytes were harvested at Day 20 and stimulated ex vivo with fresh CD45.2$^+$ BALB/c stimulator cells, and then stained intracellularly for cytokine production. (B) Skin graft survival of transplanted animals. CTLA4-Ig treated animals show modest improvement in graft survival, and rapamycin treatment does not accelerate graft rejection. (C) Representative flow plots of unstimulated and allostimulated splenocytes, gated on CD45.1$^+$ CD8$^+$ T cells. (D) Percentages and absolute numbers of IFN-γ and TNF "double producing" CD8$^+$ T cells. Data shown are from one experiment with 5 mice per group.

Figure 4.6. CTLA4-Ig leads to an increased viral burden that is ameliorated by the addition of rapamycin.

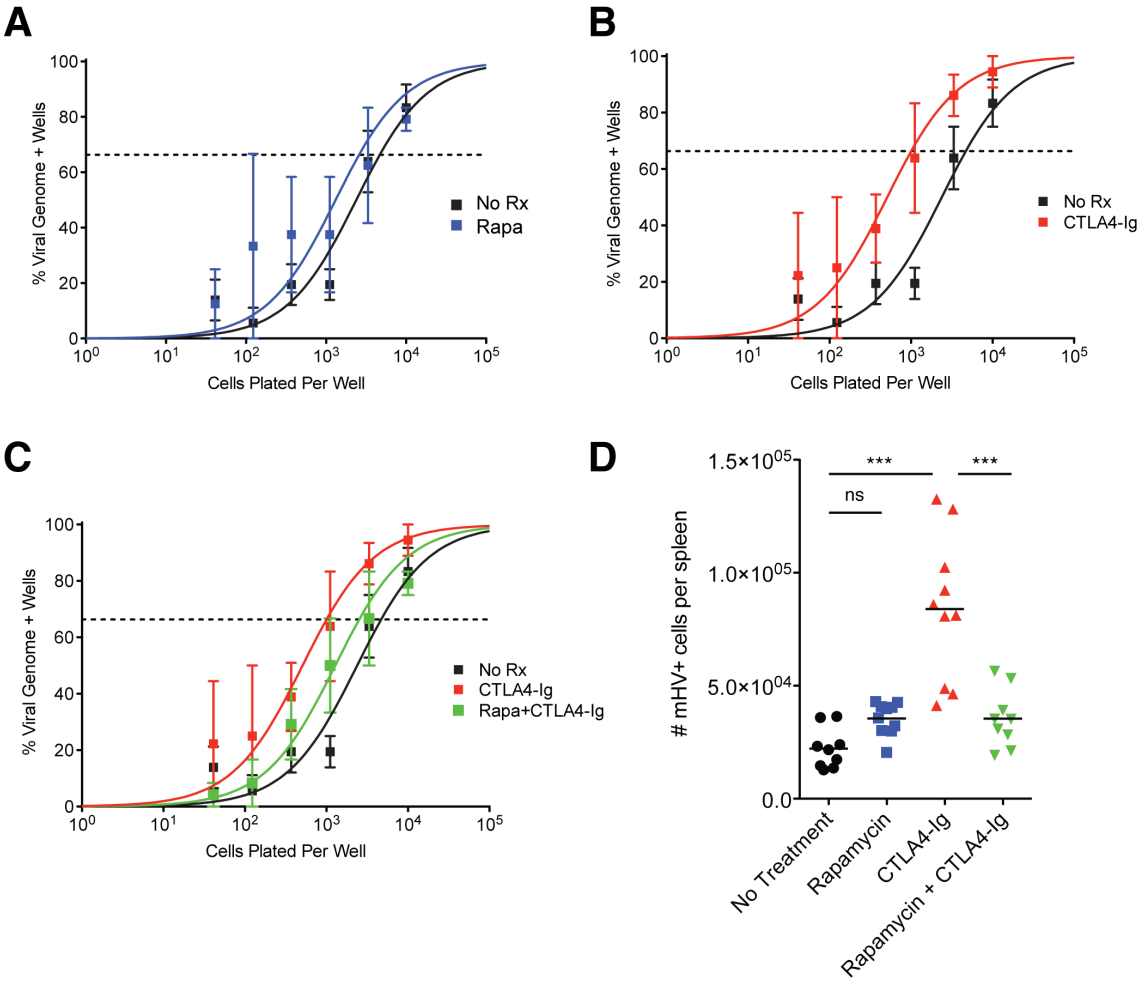
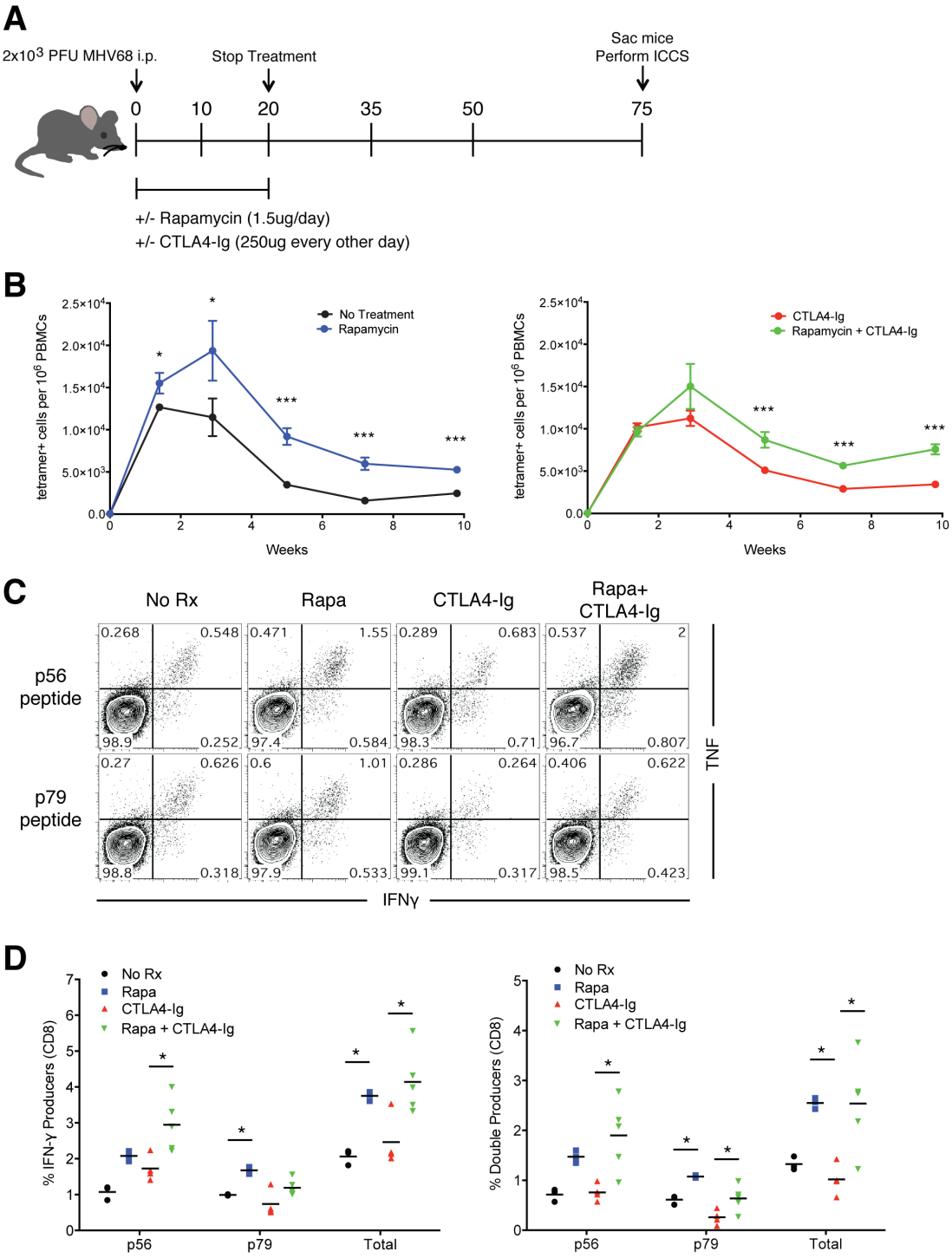


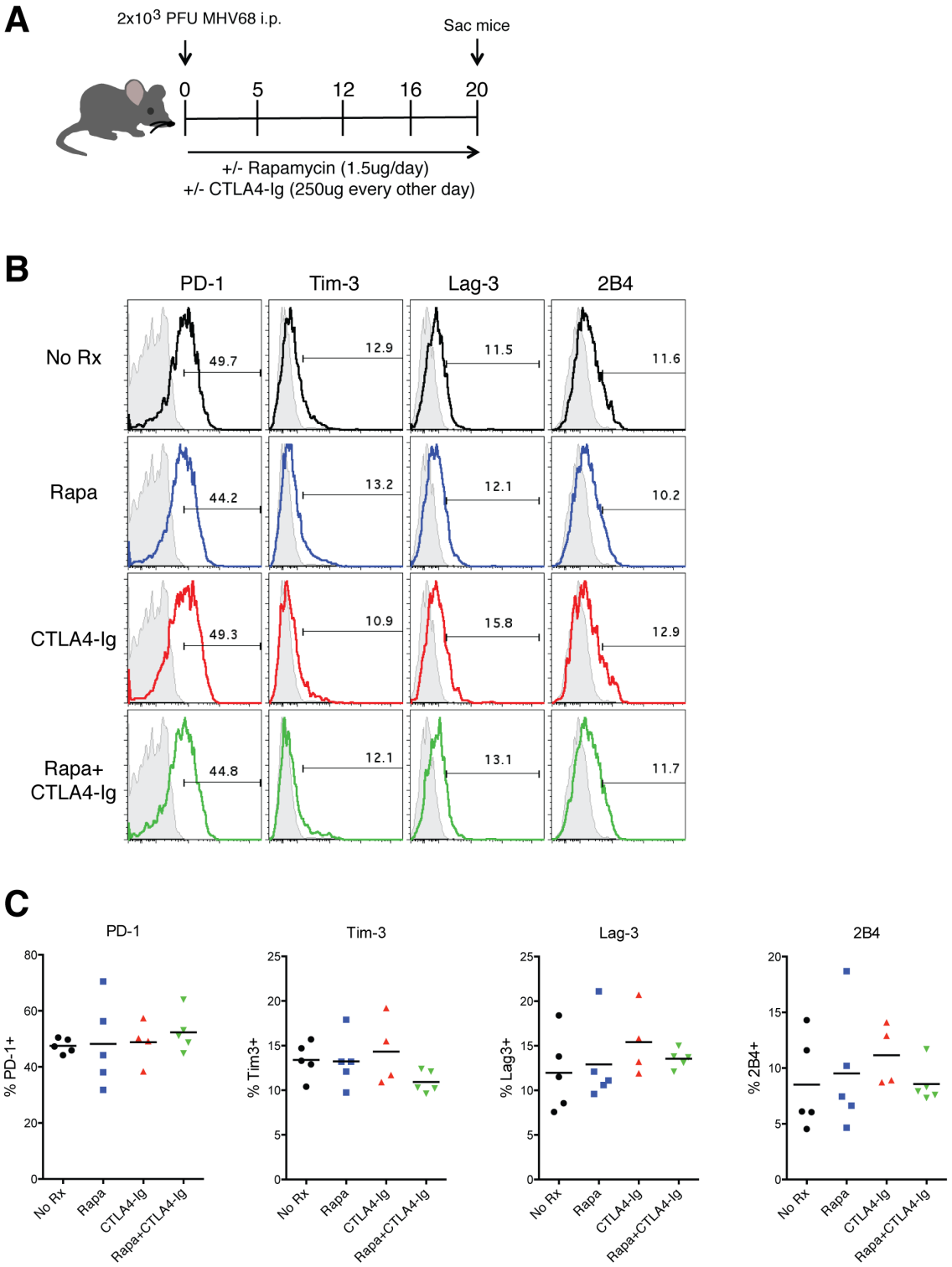
Figure 4.6. CTLA4-Ig leads to an increased viral burden that is ameliorated by the addition of rapamycin. (A-C) LD-PCR detection of genome positive cells. Infected splenocytes were isolated at Day 20 and plated in serial dilutions. Nested PCR with single-copy sensitivity was performed and the percent genome positive wells were plotted. Twelve PCRs were performed for each dilution and six dilutions were performed for each sample. (D) Frequencies of genome positive cells were multiplied by total number of splenocytes to determine approximate number of infected cells per spleen. Data shown are from two independent experiments with a total of 9-10 mice per group. *** $p < 0.001$.

Supplemental Figure 4.1. The beneficial effects of rapamycin are durable even after the cessation of treatment.



Supplemental Figure 4.1. The beneficial effects of rapamycin are durable even after the cessation of treatment. (A) On Day 0, B6 mice were infected with $2x10^3$ pfu of mHV68 i.p., and treated i.p. with either saline, 1.5ug of rapamycin daily, 250ug of CTLA4-Ig every other day, or both drugs in combination. (B) Mice were bled at serial time points, and the expansion of total mHV-tetramer positive $CD8^+$ T cells was monitored in the peripheral blood. (C) Splenocytes were harvested at Day 75 and stimulated ex vivo with p56 and p79 peptide, and then stained for cytokine production. (D) Percentages of IFN-γ-producing $CD8^+$ T cells, and percentages of IFN-γ and TNF "double producing" $CD8^+$ T cells. Data shown are from one experiment with 3-5 mice per group; results are representative of 2 independent experiments with a total of 6-10 mice per group. * $p < 0.05$, *** $p < 0.001$.

Supplemental Figure 4.2. CD28 blockade does not lead to an upregulation of coinhibitory markers on virus-specific CD8$^+$ T cells.



Supplemental Figure 4.2. CD28 blockade does not lead to an upregulation of coinhibitory markers on virus-specific $CD8^+$ T cells. (A) On Day 0, B6 mice were infected with $2x10^3$ pfu of mHV68 i.p., and treated i.p. with either saline, 1.5ug of rapamycin daily, 250ug of CTLA4-Ig every other day, or both drugs in combination. (B) Mice were sacrificed at Day 20 and splenocytes were stained for tetramer binding and surface expression of coinhibitory markers. Flow plots are gated on tetramer positive cells, and gray lines indicate isotype control staining, with colored lines showing the mHV-specific population. (C) Percentages of tetramer positive cells staining positive for PD-1, Tim-3, Lag-3 and 2B4.

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Chapter 5

Discussion

Balancing immunomodulatory therapy to specifically target graft-reactive responses and preserve protective immunity

Patient outcomes have improved dramatically since the early days of transplantation, due to the introduction of various immunosuppressive agents and the refinement of treatment regimens to optimize graft acceptance and limit inflammation. Our studies aimed to better understand the mechanisms by which adaptive immune responses to an allograft could be specifically targeted with low toxicity therapy, such as monoclonal antibodies targeting T cell costimulation, thus promoting graft tolerance while sparing protective immunity against pathogens. In addition, we also sought to identify common scenarios in which specific pathogens present serious challenges to immunosuppressed individuals, and investigate therapeutic interventions to improve infectious outcomes without a concomitant increase in alloreactivity. This balancing act represents the great dilemma facing physicians in managing patients post-transplant, and through careful mechanistic work, we hope to have shed light on two important pathways targeted by immunotherapeutic agents following transplantation.

CD40-CD154 costimulation blockade: specifically targeting allograft responses

Fc receptors and anti-CD154 therapy

Since the mid-1990s, the CD40-CD154 pathway has been recognized to play a critical role in the generation of alloreactive T and B cell responses, due to its involvement in a number of highly immunogenic processes (Figure 1). Due to the potential involvement of FcγRIIA expressed on human platelets in the etiology of thromboembolisms experienced by patients receiving anti-CD154 therapy in clinical trials (Figure 1E), we compared Fc-silent anti-CD154 domain antibody and showed that they were as effective as Fc-intact antibodies at prolonging graft survival (1). Indeed, since our study was published, several other groups have reported that Fc-modified antibodies directed at CD154 show no sign of inducing thromboembolism and are

efficacious in models of transplantation and autoimmunity. A similar version of the CD154 domain antibody fused to a modified Fc region has shown remarkable potency in prolonging kidney survival in rhesus macaques (A. Adams, personal communication). In addition, a pegylated Fab fragment against CD154, CDP7657, did not activate human platelets in vitro and showed a similarly low rate of pulmonary thromboembolism comparable to saline-treated controls in rhesus monkeys, as opposed to higher rates seen in animals treated with Fc-intact anti-CD154 (2), and a follow up phase I trial in humans showed no signs of treatment-related thromboembolism (3).

While these studies provided evidence that clinical translation of Fc-silent anti-CD154 therapeutics is feasible, these results are at odds with previously published data that showed a lack of efficacy of anti-CD154 when used in animals deficient for the Fc receptor common γ chain (4). The authors of this study used this finding to support their hypothesis that Fc-mediated depletion of CD154-expressing cells was necessary for therapeutic efficacy of anti-CD154. However, given our results that Fc-silent therapy was equally efficacious, we sought to explore alternative hypotheses for the findings in Fc γ chain deficient animals. Interestingly, unpublished observations from our lab have suggested that Fcerg1$^{-/-}$ mice make a more robust CD8^{+} T cell response following a fully allogeneic BALB/c skin graft, demonstrating enhanced proinflammatory cytokine production and accelerated graft rejection. This phenomenon occurs even in the presence of immunosuppression, suggesting that some intrinsic property of the T cell response in these animals, rather than a lack of Fc-mediated depletion, is responsible for the failure of anti-CD154 therapy observed in the Monk study (4). In addition, other investigators have found that following infection with the chronic virus LCMV-clone 13, virus-specific CD8^{+} T cells expand more in Fcerg1$^{-/-}$ mice than in WT mice (G. Punkosdy, R. Ahmed, personal communication). Taken together, these results implicate these animals have enhanced T cell responses to multiple stimuli, and do not support the conclusion that the failure of tolerance in

Fcerg1$^{-/-}$ mice treated with anti-CD154 was due to a specific requirement for Fc-mediated depletion of alloreactive cells.

One potential explanation for the aberrant immune responses seen in these animals is the possibility that the Fc receptor γ chain could negatively regulate CD8^{+} T cell responses. This molecule has been shown to be able to compete with TCR zeta chain for binding to the alpha and beta subunits of the TCR (5-7). These novel TCR complexes are functionally active and able to induce cytotoxic activity, but not IL-2 production; therefore, the association of the Fc γ chain with the TCR may be able to lower the threshold for T cell activation. An alternative explanation may be differences in their intrinsic genetic background. These knockout mice were generated in the 129 embryonic stem cells, and then backcrossed to the C57BL/6 strain (8). However, there are genetic remnants in close proximity to the *Fcerg1* gene that are still of 129 origin and may affect the expression of closely related molecules. Therefore, while knockout mice are a valuable tool to investigate the role of certain genes and gene products in immune responses, epigenetic effects resulting from gene interactions from the original background strain may cloud the interpretation of any findings. Currently, these Fc γ chain knockouts are being generated on a B6 background, in order to circumvent these issues (G. Punkosdy, personal communication). Therefore, careful examination of the genetic makeup of targeted knockouts, as well as additional studies performed in strain-matched knockouts, must be done in order to definitively implicate certain pathways in immune processes. In addition, novel technologies, including CRISPR-Cas9 methods, may be an alternative to generate better gene knockout models. This method uses an RNA template to introduce double-stranded breaks in DNA at selected locations in the genome, which are then repaired by error-prone polymerases, leading to functional inactivation of the target gene (9). This would provide a faster and more reliable method of targeting genes in vivo compared to the currently used homologous recombination methods in embryonic stem cells, and could avoid the potential gene interaction issues described above.

The Role of Fc Receptors in Therapeutic Antibody Efficacy

Monoclonal antibody therapy, particularly targeting T cell costimulatory molecules, has shown great promise in the modulation of alloreactive T cell responses. However, potentially harmful interactions of Fc receptors with therapeutic antibodies, including cytokine storm and thromboembolism (10-12), have prompted the development of next-generation costimulation blockade, which uses Fc-devoid reagents to avoid these complications (13, 14). However, while some Fc interactions can lead to harmful adverse events, other Fc-mediated effects may in fact be beneficial in terms of limiting alloreactive responses. While we have confirmed that Fc interactions were not required for the efficacy of anti-CD154 therapy in our models, it remains a possibility that in certain scenarios, Fc-mediated depletion or Fc-aided receptor crosslinking may actually enhance the efficacy of some therapies. For example, in a model of tumor immunotherapy, anti-CTLA4 antibody efficacy relied on the depletion of intra-graft regulatory T cells by FcγRIV-expressing macrophages (15). While this mechanism is certainly of use in cancer immunotherapy, where the aim is to target and kill certain cell types, the interaction of antibodies with the inhibitory Fc receptor, FcγRIIB, is of much greater interest to us as transplant immunologists. Our studies presented in Chapter 3 have shown that T cells express this inhibitory receptor, and that it may be able to functionally inhibit memory $CD8^+$ T cell responses. Interestingly, expression of FcγRIIB on antigen-specific memory $CD8^+$ T cell populations correlated with increasing resistance to costimulation and integrin blockade therapy. In this scenario, all three antibodies contained active Fc regions, albeit of a different species (rat, human, and hamster). While we do not know the affinity of these antibodies for murine Fc receptors, it is possible that some interaction of these isotypes may contribute to the inhibition of graft-specific recall responses. Therefore, treatment with Fc-intact antibodies, or those modified to enhance affinity for FcγRIIB, may in fact provide added immunomodulation in certain transplant settings.

Finally, while the contribution of the interaction of monoclonal antibodies with Fc receptors to the control of alloimmunity remains somewhat unclear, treatment with polyclonal

antibody preparations, also known as intravenous immunoglobulin (IVIg), is a well-accepted therapy that has been used for years as an immunomodulatory strategy, and recent work has helped clarify its mechanism of action. Since IVIg therapy is ineffective in FcγRIIB-deficient animals (16), it was initially hypothesized that the immunoglobulin may have been interacting directly with this inhibitory receptor. In contrast, later studies showed that the interaction of sialylated Fc regions with a type II Fc receptor, known as DC-SIGN in humans and SIGN-R1 in mice, is responsible for mediating the anti-inflammatory effects of IVIg treatment by inducing the production of Th2 cytokines IL-4 and IL-33 (17-19). However, these studies still show a definitive role for FcγRIIB, as the upregulation of this receptor on macrophages is required for the anti-inflammatory effect (19). The role of B cells in the anti-inflammatory effects of IVIg is less clear, and some reports have suggested that these cells may in fact be dispensable for the salutary effects of treatment. In one model of murine autoimmune disease, the deletion of SHIP, SHP-1 or Btk, all molecules known to be involved in the downstream signaling pathways of FcγRIIB in B cells, had no effect on the IVIg-induced amelioration of disease (20). However, more recent work has suggested that CD23, a type II Fc receptor expressed by B cells, may be preferentially ligated by sialylated Fc portions, which are known to be the active agent present in IVIg preparations responsible for the beneficial effects of treatment (21). Human studies have indicated that patients with chronic inflammatory demyelination polyneuropathy had lower expression of FcγRIIB on B cells, but that clinically effective IVIg treatment led to an increase of this receptor on these cell populations (22). This may be partially responsible for the beneficial effects of IVIg by raising the threshold for B cell activation, but additional work remains to be done to elucidate the different pathways responsible.

The effect of Th2 cytokines on T cell-expressed FcγRIIB has yet to be determined, but could potentially play a role in the mechanism of action of IVIg. In terms of the feasibility of using this approach to treat transplant patients, a clinical trial in 2001 showed that IVIg treatment was as effective as the anti-CD3 antibody OKT3 in treating steroid-resistant rejection (23). Since

physicians are already very familiar with this treatment, and it is well tolerated by patients, this may be an effective tool in preventing transplant rejection and limiting treatment-related morbidity associated with steroids and calcineurin inhibitors. However, one drawback of IVIg therapy is the cost of harvesting immunoglobulin from human donors. A more cost-effective solution may be to identify the active receptors responsible for the beneficial effects of this therapy, possible including T cell-expressed FcγRIIB, and generate recombinant molecules able to bind these targets. A more specific alternative to targeting FcγRIIB on T cells may be the use of dual affinity retargeting molecules (DARTs), which are dual-specificity antibodies able to bind simultaneously to two distinct targets (24). Further work is necessary to determine the mechanism of action of IVIg in preventing alloreactive responses, as well as the contribution of T cell-expressed FcγRIIB to treatment efficacy in this model.

Anti-CD40 or Anti-CD154: Which molecule to target?

While most strategies to block CD40-CD154 interactions have targeted the inducibly-expressed CD154 ligand, many groups, including our own, have also attempted to instead target the CD40 molecule expressed on APC. While CD154 is an attractive target, due to the fact that it does not have any intrinsic signaling capability (25), and thus the risk of crosslinking and activating a costimulatory pathway is lower, results in several anti-CD40 preclinical models have been encouraging, and clinical trials are currently underway to test the efficacy of this strategy in humans.

One of the first anti-CD40 clones tested in non-human primate models of transplantation was Chi220, a chimeric mouse anti-human CD40 antibody. In a preliminary study, transient therapy with Chi220 alone was able to prolong renal allograft survival, and prevented the production of donor-specific antibodies when given in combination with CTLA4-Ig (26). However, prolonged Chi220 exposure impaired primary anti-viral responses to CMV infection, and this treatment also led to the peripheral depletion of $CD20^+$ B cells (26). Therefore, it was

unclear if the blockade of CD40-CD154 interactions or simply the general depletion of APC was more important for the mechanism of action of Chi220, or if both actions contributed to the overall efficacy. Later studies of Chi220 showed that this antibody was partially agonistic, and that a short course of Chi220 combined with belatacept significantly prolonged islet allograft survival without impacting pre-existing anti-viral immunity (27). Chi220 was also effective in promoting the engraftment of neonatal porcine islets in non-human primates, despite the high barrier posed by the potent xenogeneic response in recipient animals (28).

A number of other anti-CD40 antibodies have been developed since the introduction of Chi220. 3A8 is another partially agonistic antibody, but does not deplete B cells in vivo. Despite its inability to block the binding of soluble CD154 to CD40 and the fact that it induces CD80 and CD86 expression on APC in vitro, treatment with 3A8 was able to significantly prolong islet allograft survival and prevent alloantibody formation in rhesus macaques (29, 30). 2C10, a fully human antibody to CD40, binds to a distinct epitope of the CD40 molecule from 3A8 and Chi220, does not activate or deplete B cells, and prolonged allogeneic islet survival in a non-human primate model (31). However, the anti-CD40 therapeutic that is furthest along in the clinical translation pipeline is 4D11, also known as ASKP1240. Initially described in both renal and islet transplantation models in cynomolgus monkeys, 4D11 showed minimal efficacy when given as induction therapy, and also caused significant depletion of peripheral B cells. However, maintenance treatment led to long-term kidney allograft survival, as well as islet allograft survival as a monotherapy, and also prevented donor-specific antibody formation (32-34). These results led to the initiation of clinical trials, and phase I studies in humans indicated that this therapy was well-tolerated and effective at achieving CD40 receptor occupancy at doses similar to those used in non-human primates, suggesting that this is a promising avenue for use in human autoimmune disease and transplantation (35).

However, while abrogation of CD40-CD154 interactions have been shown to be remarkably effective an inhibit alloimmune responses, several studies have investigated the

potential of CD154 to interact to molecules distinct from its classical interaction with CD40. One group in particular has demonstrated the ability of CD154 to bind Mac-1 (CD11b) on monocytes and macrophages (36, 37). These investigates showed that the expression of CD154 on activated endothelium allowed for the recruitment and migration of Mac-1-expressing leukocytes into atherosclerotic lesions (Figure 1F), which could be inhibited by the systemic injection of a peptide that blocked the specific binding motif used by CD154 to interact with Mac-1 (36). These results highlight the myriad roles that CD154 plays in both immune and inflammatory conditions and the potential consequences of global inhibition, as well as demonstrate the potential for the development of strategies that prevent specific CD154 interactions while preserving others. Therefore, careful consideration of the myriad interactions of CD154 and CD40, not only with each other but also any alternative binding partners, is critical for the development of safe and effective therapeutics for clinical use.

Prominent role of T cell-derived CD40 signals during alloimmunity

Another CD40-CD154 interaction that may play a role in modulating graft-reactive responses is the interaction of CD154 on activated $CD4^+$ T cells and CD40 expressed by activated $CD8^+$ T cells (Figure 1C). While some studies have suggested that this arm of CD40 signaling is dispensable for mounting effective cytotoxic responses, the contribution of this form of "T cell help" to the generation and maintenance of $CD8^+$ T cell populations remained somewhat controversial, and the potential requirement for these interactions in responses to transplant were unknown.

While resting $CD8^+$ T cells do not express CD40, a percentage of $CD8^+$ T cells upregulate this molecule following activation (38, 39). An initial report from Bourgeois et al showed that in an adoptive transfer model in which $CD8^+$ T cells were responding to the male histocompatibility antigen H-Y, CD40 was not required on $CD8^+$ T cells for initial response but was necessary to generate populations capable of secondary recall responses (40). Conversely,

other studies indicated that CD40 expressed by $CD8^+$ T cells is not required for optimal cytotoxic responses or memory formation in response to either a bacterial or viral infection (41, 42). However, a critical difference in these studies is the nature of the immune challenge presented. CD40 on $CD8^+$ T cells appeared to be required for an effective response to the tissue antigen H-Y, whereas in infectious models, microbial components may induce potent inflammatory responses that bypass the requirement for CD40-CD154 signaling.

Our lab recently investigated the role of this interaction in the generation of graft-reactive $CD8^+$ T cell responses by again taking advantage of the transgenic ovalbumin system (39). By using defined populations of alloantigen-specific $CD4^+$ and $CD8^+$ T cells, this system allowed for the specific genetic ablation of CD40 on a number of different cell subsets in isolation, including alloreactive $CD8^+$ T cells, donor-derived antigen-presenting cells, or recipient-derived APC. CD40 deficiency on only $CD8^+$ T cells was sufficient to prolong graft rejection and significantly reduce the expansion and cytokine production of this population. However, the origin of the CD154 signals that help generate these $CD8^+$ responses is still unclear, as they may be provided by $CD4^+$ or APC populations. Nonetheless, we confirmed that $CD8^+$ T cell-intrinsic CD40 signals are not required in an infectious model, as $CD40^{-/-}$ OT-I T cells responded as well as WT OT-Is to a bacterial challenge (39). Therefore, it would appear that the requirement for CD40 signaling on $CD8^+$ T cells depends on the inflammatory microenvironment during T cell priming. While the source of CD154 signals cannot be determined from these studies, the ovalbumin model may provide a template for future studies to tease apart the exact interactions required for these effects.

These studies highlight the unique nature of the CD40 system and why we believe that it is such an attractive pathway to target in the setting of transplantation. Specifically, the fact that CD40-CD154 interactions are required for alloreactive responses, but dispensable in some cases for protective immunity, makes therapeutic blockade of this pathway it a perfect candidate for use in the clinical arena. However, these treatments are still in the early stages of clinical development, and for patients currently receiving transplants, there are few true alternatives to

calcineurin inhibitor-based therapy. While belatacept has shown remarkable promise as a less-toxic means of preventing acute rejection, there are several notable drawbacks to this therapy. In addition to the increased rates of acute rejection seen in patients treated with this drug, there are also specific infectious complications associated with belatacept treatment, namely that primary infection with Epstein-Barr Virus increases the risk of developing post-transplant lymphoproliferative disorder (PTLD). Therefore, the second aim of our work was to investigate potential treatments that could mitigate the risk of these complications and allow patients to benefit from the reduced toxicity profile of costimulation blockade.

Promoting protective immune responses post-transplantation with mTOR inhibitors

The divergent effect of rapamycin on graft- and pathogen-specific responses

Rapamycin is an immunosuppressive drug that has been used for decades in transplantation to prevent T cell-mediated alloreactivity, but surprisingly, patients treated with this therapy experienced fewer infectious complications (43-45). While studies have indicated that rapamycin can indeed inhibit viral replication in a number of models (46-48), additional mechanistic work done by Araki and colleagues discovered that treatment with low doses of rapamycin can also paradoxically enhanced cytotoxic $CD8^+$ T cell responses to viral infection (49). These findings were also extended when our group demonstrated that rapamycin is able to enhance pathogen specific, but not graft-specific, $CD8^+$ responses even when the antigen and responding T cell population were identical (50). However, the precise mechanisms that underlie the dichotomous effect of rapamycin are still somewhat unclear. Initially, it was hypothesized that perhaps the presence of pathogen-associated molecular patterns (PAMPs) during viral or bacterial infection may have influenced the ability of rapamycin to enhance pathogen-specific but not graft-specific responses. However, when grafted mice were simultaneously infected with a non-specific bacterial infection, rapamycin did not enhance the expansion of graft-specific $CD8^+$ T cells (50), suggesting that TLR ligation or other pathogen-associated signaling alone was

insufficient to promote rapamycin-induced augmentation of cytotoxic responses. Several alternate mechanisms may be responsible for the differences seen in pathogen and graft specific T cell responses. First, while the most of the infectious models used in these studies, such as the bacteria *Listeria monocytogenes*, are rapidly cleared by the immune system, graft-associated antigen may persist in immune microenvironments for longer times and at difference levels. Several studies have shown that duration of antigen exposure (51), as well as the amount of antigen (52), can dramatically affect the outcome of a T cell response, and thus this may play some role in the dichotomous effect of rapamycin. In addition, while the type of inflammation and the ligation of TLR have been shown to not be fully responsible for rapamycin-induced enhancement of $CD8^+$ T cell responses (50), the duration of inflammation may play a significant role. T cell differentiation and memory formation are intrinsically linked to mTOR signaling (53), and the signal strength, determined by a combination of TCR affinity, costimulation, and proinflammatory cytokines, that is detected by T cell populations dictates the overall outcome of a T cell response (54). In addition to systemic inflammation, potential differences in the type of antigen presenting cell may alter the effect of rapamycin. While an intracellular bacteria such as *Listeria monocytogenes* prime T cells mainly through professional antigen presenting cells such as macrophages and DCs (55, 56), graft-specific immune responses may be initiated by cells from the graft itself and presenting directly to alloreactive T cells in the recipient (57), and these graft-migrating cells may not be of the same subset or activation status as those participating in priming during an infection. Subtle differences in either the subset of antigen presenting cells, the requirements for $CD4^+$ T cell help, or the timing of presentation may all contribute to the differences seen in the effect of rapamycin on $CD8^+$ T cells. Finally, recent work has begun to uncover a wealth of data surrounding T cell metabolism and the control that it exerts on T cell responses and memory fate. Specifically, we now know that naïve T cells utilize primarily oxidative phosphorylation (OXPHOS) to generate energy, but, upon activation, switch to a more glycolytic phenotype, and that in order to form stable memory, a metabolic switch back to the more efficient OXPHOS

pathway is required (58). In addition, mTOR, the target of rapamycin treatment, is a key regulator of metabolism in T cells, and signaling from this complex controls the uptake and processing of glucose, lipids, and amino acids (53). In vitro studies have recently shown that rapamycin treatment in vitro is able to enhance both OXPHOS and glycolysis in activated T cells, and when transferred to infected mice, rapamycin-treated cells were able to form much larger populations of long-lived memory cells (59). Furthermore, autophagy, a metabolic process by which cells break down cytosolic materials to maintain homeostasis, has recently been shown to be required for $CD8^+$ T cell memory formation following an infection (60). The authors of this study suggested that since mTOR signaling is known to directly downregulate autophagy, the rapamycin-induced augmentation of $CD8^+$ T cell memory formation following an infection may have been due in part to increased rates of autophagy. Since metabolism is a key determinant of T cell fate, further studies should attempt to determine any potential differences in the baseline metabolic changes during T cell activation following a pathogen infection or a graft, as this may have important implications effect of mTOR inhibition on T cells responding to these types of immunological stimulus.

Heterologous immunity and the clinical use of rapamycin

While pathogen-specific T cell responses provide important protection against infection, they also may be capable of crossreacting to foreign allogeneic antigens, in a process known as heterologous immunity (61). In fact, one study showed that as many as 45% of virus-specific human T cell clones were able to crossreact to panel of human leukocyte antigens (HLA) (62). Therefore, when one considers the clinical use of rapamycin to enhance pathogen-specific immunity in the context of transplantation, the augmentation of pathogen-specific T cell responses that may then crossreact with a graft is a potential concern. Our group performed an experiment where T cell memory was generated by infection with LM-OVA, and then at a memory timepoint, these cells were restimulated with either a graft or a second infection, and

animals were treated with rapamycin. Importantly, despite the initial infectious stimulus, only cells that were responding to the secondary infection were augmented by mTOR inhibition (Figure 2). These data suggest that the effect of rapamycin on a particular primary or secondary T cell response depends on the immune environment at the time of recall, rather than the initial stimulus. In addition, our studies in Chapter 4 showed that simultaneous infection with gHV during a fully allogeneic skin graft did not result in rapamycin-induced augmentation of graft reactivity when animals were treated with a combination of rapamycin and CTLA4-Ig. Therefore, these results indicate that the use of rapamycin as an adjuvant therapy to promote pathogen-specific immunity during costimulation blockade treatment can be a safe and effective way to limit infection-associated morbidity in transplant recipients.

Conclusions and Perspectives

While the past few decades have seen dramatic improvement in post-transplant immunosuppressive therapies, several barriers remain that limit the long term health and survival of grafted organs and transplant recipients. Costimulation blockade with monoclonal antibodies is a promising therapeutic modality, but infectious and thromboembolic complications have prevented the widespread adoption of these treatments. In this thesis, we have demonstrated the efficacy of a novel Fc-silent anti-CD154 costimulation blockade therapy, paving the way for clinical development of a safer and equally efficacious treatment. In addition, our data suggesting that rapamycin is able to enhance virus-specific $CD8^+$ T cell responses in the context of CD28 costimulation blockade addresses a serious clinical dilemma of EBV-associated lymphoma during belatacept treatment. These findings will hopefully provide a framework for future studies to improve post-transplant immunomodulation, with the goal of providing patients with effective, low-toxicity therapies that improves the quality of life of this population.

Figure 5.1. Complexities of CD40-CD154 Interactions in the Immune System

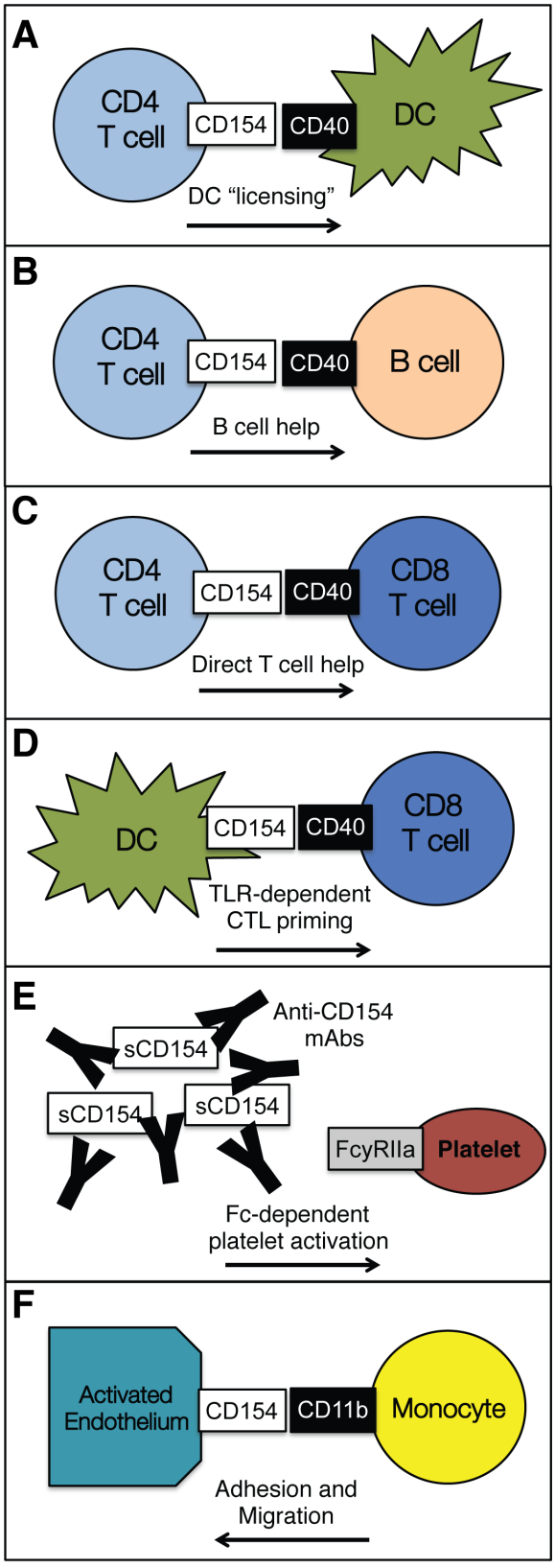
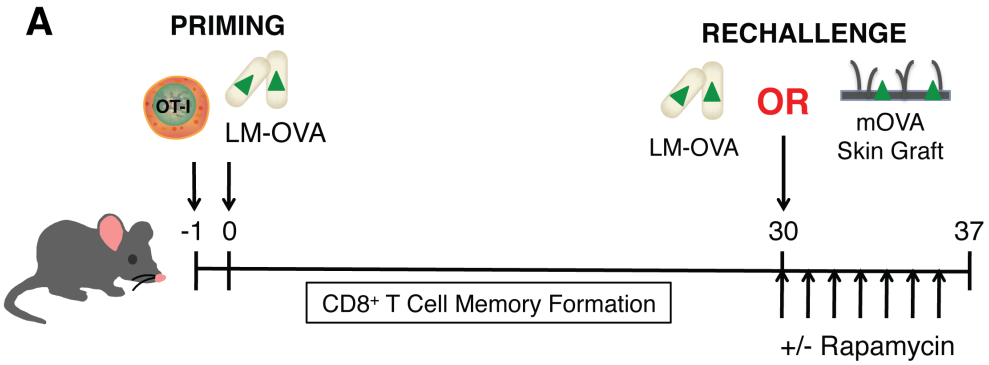


Figure 5.1. Complexities of CD40-CD154 Interactions in the Immune System

The TNF-family member CD40 and its ligand, CD40L (CD154), are expressed by a wide variety of cell types and the signaling induced by this binding leads to a range of potent immunostimulatory events. (A) Activated $CD4^+$ T cells upregulate CD154, which binds to the CD40 expressed by dendritic cells. This leads to DC activation, which includes the production of proinflammatory cytokines such as IL-12. (B) $CD4^+$ T cells and B cells undergo linked recognition, wherein T cells recognize their cognate peptide:MHC Class II complex expressed by B cells. T cells then provide costimulatory signals through B cell-expressed CD40, and this "T cell help" is critical for generating effective humoral responses. (C) $CD4^+$ T cell-expressed CD154 can also provide direct help to $CD8^+$ T cells through CD40, which has been suggested to play a role in the formation of $CD8^+$ T cell memory. (D) CD154 is also inducibly expressed on DC following toll-like receptor ligation, and the interaction with CD40 expressed on $CD8^+$ T cells can help generate potent pathogen-reactive $CD8^+$ T cell responses. (E) A hypothesis for anti-CD154-associated thromboembolic complications centers on the expression of the activating Fc receptor FcγRIIa on human platelets, which can be crosslinked by immune complexes of soluble CD154 and anti-CD154 antibodies, leading to further platelet activation and undesired clotting events. (F) An alternate ligand for CD154 has also been described. CD154 expressed on activated endothelial cells may induce the arrest and migration of monocytes into inflamed tissues through its interaction with Mac-1, also known as CD11b.

Figure 5.2. The Timing of Rapamycin Treatment Dictates its Effect on Pathogen- and Graft-Specific CD8$^+$ T Cell Responses



B

Rechallenge Type	No Rx	Rapa
LM-OVA (Infection)	+	+++
mOVA (Graft)	+	+

Figure 5.2. The Timing of Rapamycin Treatment Dictates its Effect on Pathogen- and Graft-Specific Memory CD8$^+$ T Cell Responses

(A) 10^4 OT-I transgenic T cells were adoptively transferred to B6 mice that were then infected with 10^5 cfu Listeria monocytogenes-OVA. At a memory timepoint 30 days post-infection, mice were rechallenged with either another LM-OVA infection or an OVA-expressing skin graft. Both groups were subdivided into mice that were then treated daily with either saline or 1.5ug rapamycin intraperitoneally. (B) At day 7 post-rechallenge, mice were sacrificed and the expansion of OVA-specific cells in the spleen was enumerated. Rapamycin treatment enhanced the recall response to LM-OVA, but not to the mOVA skin graft, despite the fact that both memory populations responding to the secondary rechallenge were generated by pathogen infection on day 0. These results indicate that the stimulus at the time of rapamycin treatment, rather than the initial memory-forming stimulus, dictates the effect of rapamycin on memory CD8$^+$ T cell responses.

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