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Immunomodulation in Transplantation:

Promoting Tolerance and Protective Immunity

By

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BS. University of North Carolina—Chapel Hill, 2004

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An abstract of a dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in the Graduate Division of Biological and Biomedical Sciences

in Immunology and Molecular Pathogenesis

2011

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By Ivana Rublescki Ferrer

Transplantation recipients encounter two significant problems with a lifetime regimen of immunosuppression. The first issue is that the standard of care includes the use of such reagents as calcineurin inhibitors that lead to hypertension, dyslipidemia, type II diabetes, along with significant nephrotoxic side effects, leading to kidney failure after prolonged treatment. The second is that the same immunosuppression regimens may lead to recurrent infections of typically controlled infectious agents in healthy individuals.

Therefore, we interrogated ways to prevent allograft rejection while avoiding systemic immunosuppression. In Chapters 2-4, we investigated the effects of transient treatment with anti-CD154 costimulation blockade on innate immune cell recruitment and donor-specific $CD4^+$ and $CD8^+$ T cell activation and differentiation. Costimulation blockade significantly protects the graft from cellular infiltration, which is associated with injury to the structural matrix and cellular structure, leading to accelerate rejection. Furthermore, anti-CD154 leads to a delayed differentiation of T cells and promotes T cell conversion into a regulatory phenotype. In Chapters 5-6, we investigated the effects of mTOR inhibition with rapamycin on $CD8^+$ T cell differentiation in response to a pathogen or to an allograft. In these studies, we determined that rapamycin differentially affects T cell differentiation depending on the context of T cell activation. Treatment with rapamycin significantly augments pathogen-specific T cell responses while graft-reactive T cell responses are either maintained or diminished with this treatment.

These two studies demonstrate the potential for future treatments in transplantation. By blocking costimulatory signals, we know we can specifically target graft-reactive and damaging responses while inducing a regulatory population to maintain a quiescent state. Furthermore, the transient treatment with rapamycin shows us that certain therapies can be used to stimulate protective immune responses against pathogens, while still protecting the transplant from rejection. Although both of these treatments have their drawbacks, the following studies demonstrate that both protection of the graft and protection against infections are achievable goals post-transplantation.

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Acknowledgements

I would like to thank my advisors, Mandy Ford and Chris Larsen, for providing me with the guidance and support I needed to succeed as a PhD graduate student. A special thanks goes to Mandy, whose encouragement and dedication to fostering my scientific development have been invaluable.

I would also like to thank my family and friends who have been there for me at the times I needed them the most. I am particularly thankful to my parents who are always available to give me advice or encouraging words whether they receive a call at two in the afternoon or two in the morning, after a long day in lab. Finally, I would especially like to thank Konrad, who, even as we went through qualifying exams and dissertation defenses within days of each other, has been incredibly supportive every step of the way, with just the right dose of distraction.

I could not have accomplished this without all of you and will be forever grateful!

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Chapter 1

Introduction

Data from Organ Procurement and Transplantation Network reported that nearly 29,000 transplants were performed last year, bringing the ten-year total of transplanted organs to nearly 250 thousand procedures. While transplantation of solid organs has become a reality for end-stage organ failure, transplantation comes with several complications. The introduction of a foreign organ induces the activation of immune responses that target the transplanted graft, which subsequently induces rejection of the organ. Over the last several decades, the field of transplantation has aimed to prevent allograft rejection responses through the use of systemic immunosuppression, such as cyclosporine. However, since these drugs can cause many more complications than is tolerable for long-term use; including, but not limited to, renal toxicity, hypertension, and dyslipidemia; many research groups have sought to explore new ways to directly target the graft-reactive immune responses, but without compromising protective immunity against pathogens.

BRIEF HISTORY IN TRANSPLANTATION

Across the ages, different populations over many cultures have attempted to prolong life through the transplantation of organs or limbs. Dating back to approximately 600BC, an Indian surgeon Susrata performed reconstructive surgery using skin flaps of patients who had their noses cut-off as form of punishment. In ca. 255BC, the Chinese surgeon Pien Ch'iao supposedly exchanged the hearts in two soldiers by in order to restore a strong will and a strong spirit into each man, respectively (1). One of the most famous accounts of early transplantation dates

back to the third century AD. In this account, the two Syrian doctors, Cosmas and Damien, who later became saints, replaced the gangrenous leg of a sick man with that of a dead black man (2). These legends demonstrate the early interests in using transplantation to improve lifestyle and aid survival, but not until the advent of modern medicine do we see reliable reports of new attempts and successes in transplantation medicine.

In the 1940s, Sir Peter Medawar performed skin transplantations on burn victims during World War II. He observed that when a patient received an autograft, or one from his/herself, they fully engrafted. On the other hand, when receiving a homograft (from another individual), the patient fully rejected the transplanted skin (3). In animal studies of skin transplantation, Medawar observed that outbred rabbits transplanted with an autograft (commonly known as a "syngeneic" graft) fully accepted the transplants. On the other hand, recipients of homografts (commonly known as an "allogeneic" graft) showed rapid tissue degeneration followed by necrosis of the graft. Furthermore, after showing signs of rejection of the initial allograft, the recipients were able to reject a second skin allograft from the same donor at a much-accelerated rate. This observation led to the hypothesis that an "actively acquired immune reaction" was contributing to the rejection of the grafts based on the antigenic relationship of the donor-recipient pair (4, 5).

In the early 1950s, in a study of lymphosarcoma, Mitchison identified that the actively acquire immunity described by Medawar was a result of responses present in lymph nodes of primed animals. When the lymph nodes from a previously

primed animal were transferred to a naïve animal, the new animal was able to rapidly clear the tumor (6). Because the heightened responses to transplanted grafts and tumors were of immunological background, Medawar led pivotal studies in clarifying the process of immunological tolerance. Billingham, Brent and Medawar discovered that induced tolerance was initiated in animals *in utero*. In these experiments, CBA mouse fetuses were challenged with cells from another mouse strain (A-line). After birth, the animals were challenged with a skin graft of the same donor (A-line), which were subsequently accepted and "resembled autografts in every respect except their donor-specific albinism" (7). These results confirmed previous studies by Owen *et. al.* in dizigotic cattle twins, where placental anastemoses allowed for the free transfer of red blood cells from one calf to the other *in utero*, and induced future transplantation tolerance when the calves were grafted with each other's skin (8).

Following the discovery of acquired tolerance, Dr. Joseph Murray performed the first successful kidney transplant between identical twins (9). Since the donor and the recipient were genetically identical, no immunosuppression was needed to prevent rejection. It would be several more years before the development of successful immunosuppressive agents to prevent transplantation rejection.

INNATE IMMUNE SURVEILLANCE AND LEUKOCYTE MIGRATION

The innate immune system is an evolutionarily ancient system shared by many organisms ranging from plants to invertebrates to vertebrates (10). Innate immune cells are the first level of host defense against foreign antigens, including

transplants. Tissue macrophages and dendritic cells reside in the peripheral tissues and continuously survey the site for infection or foreign antigens through endocytosis and macropinocytosis (11). In the case of tissue injury or infection, tissue resident dendritic cells transmigrate out of the tissues to the draining lymph nodes where they can activate antigen-specific T cells. Conversely, tissue resident dendritic cells and macrophages as well as parenchymal and stromal cells secrete chemokines to recruit leukocytes to the site of injury (12-14). Chemokines are bound by sugars on the endothelial cells which helps create a chemokine gradient originating at and attracting leukocytes to the site of injury (15, 16). The secretion of IL-1, IL-6, and TNF-α by tissue macrophages induces localized changes to the endothelium to increase vascular permeability. The migration of leukocytes into injured or transplanted tissue involves several steps. The first interaction between circulating leukocytes and endothelial cells involves the tethering of leukocyte mucins to E- and P-selectins on the endothelium, which in the presence of the shear of the blood flow induces rolling of the leukocytes along the vessel wall (17). Leukocytes cease rolling upon tighter interactions with α4 integrins on the surface of the vessel walls (18-20), which leads to extravasation of the leukocyte into the injured tissue.

As previously mentioned, the recruitment of innate cells to the site of injury is triggered by a chemokine gradient, starting at the site of injury. Some chemokines secreted are very specific to the cells recruited to the site of injury; while others are induce a more pleotropic response. For example, the secretion of CXCL1 (KC) and CXCL2 (MIP-2) by activated mononuclear cells induce neutrophil migration to the

affected tissue (21, 22). Furthermore, the release of CCL3 (Macrophage Inflammatory Protein 1α—MIP-1α) and CCL4 (MIP-1β) by neutrophils infiltrating the tissues recruit macrophages as its name suggests (23), however both molecules have also been shown to attract other immune cells, including dendritic cells, natural killer cells, eosinophils and T cells (24-29). Another chemokine, RANTES (regulated on activation normal T expressed and secreted, or CCL5), was originally isolated from genetic screens of activated T cell lines (30), but has also been shown to attract numerous cell types during inflammation and tissue injury (24, 27, 29, 31-33). Although it is accepted that increased cellular infiltration into transplanted grafts increases tissue injury, it is not fully understood how the expression of certain cytokines may affect the trafficking patterns of innate and adaptive immune cells into the transplant and how immunosuppressant regimens may alter this response. In both fully- and minor-mismatched skin transplantation models, it has been described that RANTES gene expression is delayed during graft rejection (34, 35). The increased expression of RANTES in allografts has also been associated with increased $CD8^+$ T cell infiltration during acute rejection (36).

Moreover, during instances of tissue injury and inflammation, platelets are also activated and recruited to the site of injury. In recent years, platelets have been shown to express high levels of CD154 on their surface (37). The surface expressed CD154 on platelets interact with CD40 on injured endothelial cells, which leads to increased expression of inflammatory adhesion receptors, such as E-selectin, ICAM-1 and VCAM-1 (37). Furthermore, in *in vitro* cultures of endothelial cells with activated platelets led to the increased production of leukocyte attracting

chemokines such as IL-8 (37). In contrast, the inhibition of adhesion molecules prevented the secretion of the inflammatory chemokines MIP-1α, MCP-1 and IL-8 (38). Because of the immunostimulatory effect of CD154-expressing platelets on endothelial cells, we hypothesized that blockade of this pathway could inhibit chemokine production and alter cellular infiltration into the transplanted graft, thereby diminishing tissue injury. In chapter 2, we briefly discuss the effect of treatment with donor specific transfusion (DST) and/or CD40/CD154 pathway blockade on both the chemoattractants being produced within the allograft and their recruitment of immune cells into the transplanted graft.

ACTIVATION OF T CELL RESPONSES

Chronic transplantation rejection occurs through the activation of donor-reactive T cells targeting the allograft. In order to initiate an immune response, a T cell and an antigen presenting cell (APC) must interact in the spleen or lymph node. In this microenvironment, the antigen specific T cell encounters its cognate antigen, as the three-dimensional structure of a specific peptide within the groove of the major histocompatibility complex (MHC) on the APC, with the T cell receptor (TCR). There are two types of MHC: MHC class I and MHC class II. The first is expressed on all cell types in an organism and is used by cytotoxic $CD8^+$ T cells to survey the body for the presence of foreign antigens. The other molecule, MHC class II, is primarily expressed on professional antigen presenting cells—dendritic cells (DCs), macrophages and B cells—and presents short peptides of proteins that are externally taken up from the surrounding environment. Thus, MHC-peptide

complexes provide T cells with a sampling of antigens within the cell and in the surrounding environment. Because of the specificity of the TCR, T cells will typically only respond when they recognize their specific cognate antigen presented in the groove of the antigen presenting cell.

The process of T cell activation requires the transmission of three signals. "Signal one" involves antigen peptide presentation and recognition on self-MHC on the APC, interacting with the antigen-specific TCR on a T cell (39). This signal triggers the initial activation of T cells leading to the rearrangement of surface molecules forming a molecular complex at the interface of the two cells, which creates a tighter interaction and further signaling into the cells (40, 41). "Signal two" is comprised of the interaction of costimulatory molecules. The best-studied costimulatory interactions include B7 molecules on the APC binding to CD28 on the T cells, although several other costimulatory molecule pairs have been studied such as CD40/CD154, ICAM/LFA-1, 4-1BB/4-1BBL, and ICOS/ICOSL (42-47). These interactions provide the basis for intracellular signaling molecules to interact with the intracellular cytoplasmic domain of the costimulatory molecules and enhance signaling into the antigen-specific T cell. Finally, "signal three" is sometimes considered a separate signal necessary for the activation of naïve or memory $CD8^+$ T cells. These are associated with soluble factors, such as IL-2, which are released by activated APCs and are required for the activation and proliferation of naïve antigen-specific $CD8^+$ T cells and acquisition of full effector function (48, 49).

$CD4^+$ T cell signals are often necessary for the induction of naïve $CD8^+$ T cells activation and differentiation. This process, known as "licensing," occurs when a

$CD4^+$ T cell initiates the activation of the antigen presenting cell prior to $CD8^+$ T cell activation. In 1998, several groups simultaneously described the process of $CD4^+$ T cell induced APC activation prior to a cytotoxic T cell response. From these studies a novel idea was born, where the $CD4^+$ T cell, the APC, and the cytotoxic $CD8^+$ T cell needed not be in the same place at the same time for full activation. In fact, the antigen presenting cell acted as a "temporal bridge," receiving activation signals from the $CD4^+$ T cell, and was primed to activate naïve antigen-specific $CD8^+$ T cells that subsequently migrated through the lymph node (50-52). These reports described that $CD4^+$ T cells, upon recognizing their cognate antigen on the surface of the APC, could provide CD40-mediated signals to activate the APC. This CD40/CD154 interaction leads to downstream signals in the antigen-presenting cell to produce IL-12, which is important for T cell differentiation (53). Along with production of IL-12, signaling through the CD40 molecule induces APCs to decrease macropinocytosis and to upregulate MHC expression along with other costimulatory molecules, such as B7, on their surface (54). These surface changes subsequently contribute to increased T cell recognition of cognate antigens and followed by increased T cell priming. For example, because 95% of naïve $CD4^+$ T cells also express CD28 on their surface, this molecule interacts with the upregulated B7 molecules on primed APCs. CD28 signaling has been shown to promote T cell proliferation through the increased transcription and stabilization of IL-2 mRNA (57, 58), and by leading the cell into the G_1 phase of the cell cycle (55, 56), all the while promoting their survival (59, 60).

PREVENTING REJECTION WITH SYSTEMIC IMMUNOSUPPRESSION

In the 1960s, Gertrude Elion and George Hitchings developed the first immunosuppressive agent to be used in the prevention of transplantation rejection. Azathioprine, a derivative from 6-mercaptopurine, was developed as an anti-mitotic agent, thought to act by interfering with DNA synthesis (61). However, the inhibition of all cellular proliferation comes with numerous serious side effects including increased susceptibility to infection, and the need for a new therapy was evident. In 1976, Borel and colleagues first described the fungal compound Cyclosporine A (CSA), which possessed potent immunosuppressive effects (62). CSA is an immunophillin that specifically targets calcineurin, an important molecule in the activation signal cascade of T cells, and prevents their activation. By the 1990s, FK506 (tacrolimus) was identified as another calcineurin inhibitor (CNI) acting through similar mechanisms as CSA, albeit with better efficacy (63, 64). However, the use of CNIs at higher doses may not only render a patient susceptible to opportunistic infections, it may also lead them to develop nephrotoxicity, systemic hypertension, hyperlipidemia, hypercholesterolemia, neurologic complications, and diabetes mellitus, amongst other side effects (65). Despite these toxic side effects, CNIs have become the accepted clinical standard in the treatment and prevention of transplantation rejection.

Another class of immunosuppressant drugs includes the target-of-rapamycin inhibitors, including rapamycin and the second-generation drug, everolimus. These agents bind to FKBP12 and inhibit of mTOR complex I (66). The latter is a protein kinase involved in the activation of innate and adaptive immune responses allowing

cells to enter the cell cycle. The inhibition of mTOR with rapamycin also comes with several toxic side effects, including, hyperlipidemia, thrombocytopenia, reduced testosterone levels (67), mouth ulcers, impaired wound healing, and skin lesions. On the other hand, there have been several accounts reporting potentially favorable effects of rapamycin in reducing cytomegalovirus (CMV) infection in patients (68-71) and promoting immune responses against viral and bacterial infections in animal models (72-74). Rapamycin is further introduced later in this chapter (page 17) and its paradoxically favorable effects on immune responses against infectious pathogens are further discussed in chapters 5-7.

Since the advent of these systemic immunosuppressants, several other immunosuppressive regimens have been developed in the hopes of avoiding the toxicities and specifically targeting immune responses. These drugs involve the antibody depletion of immune cells that could potentially contribute to graft rejection. Antibody depletion regimens targeting whole lymphocyte populations (antithymocyte globulin (75) and off-label use of alemtuzumab), T cells (OKT3 (76, 77)) or B cells (off-label use of rituximab) have been reportedly used to treat rejection episodes or as induction agents. Not only is the efficacy of some of these therapies in question, but also the systemic depletion of immune cells may pose a significant risk of prolonged and severe immunodeficiency (especially in the case of antithymocyte globulin).

Furthermore, the protection of the graft from rejection is dependent on continuous administration of these immunosuppressive drugs, as these drugs do not provide graft specific tolerance. Therefore, there remains a need for new therapies

that can specifically target graft-reactive cells and provide sustained graft survival without systemic immunosuppression.

PREVENTING REJECTION WITH COSTIMULATION BLOCKADE

In order to circumvent the well-established complications of prolonged systemic immunosuppression, research focus has shifted to targeting allograft-specific T cell immune responses in order to better transplantation survival. The development of costimulation blockade has led the field of transplantation towards specifically inhibiting allograft rejection while minimizing off-target side effects. As state above, alloreactive T cells require not only the recognition of alloantigen on a self-MHC molecule on APCs, but also the interaction between costimulatory molecules in order to become fully activated. However, when a T cell recognizes antigen in the absence of costimulation signals, the T cell becomes anergized, a state in which cells become non-responsive to either direct TCR stimulation or future costimulatory signals (78). Conversely, the constitutive expression of CD28 on the surface of $CD4^+$ T cells provides the necessary survival signals, preventing anergy and promoting signal transduction into the T cell for activation (79). Because the absence of costimulatory signals causes significant detrimental effects to T cell activation and maturation, these molecules have become the target of several groups to inhibit donor-reactive T cell responses and to promote the protection of the allograft.

Early formulations of costimulation blockade agents included CLTA-4 Ig and anti-CD154. The former was constructed by fusing the external portion of the CTLA-

4 molecule with the F_C fragment of the IgG_1 antibody. The reasoning behind the development of this compound was that the CTLA-4 molecule would bind to the upregulated B7 molecules on the activated antigen presenting cells and prevent the CD28 on T cells from binding to their receptors. In order to promote long-term persistence of the biologic once injected *in vivo*, an the F_C portion of an IgG antibody was linked stabilize the CTLA-4 and prolonging its persistence for up to three weeks (80). The antibody against CD154 (MR1 clonotype, a hamster anti-rat CD154 antibody) was developed to specifically target the recently upregulated CD154 on recently primed T cells. Thus, both of these treatments were developed to specifically target and inhibit the full activation of T cells that had recently encountered their cognate antigen. Early studies by Larsen and colleagues revealed that inhibition of these second signals with CTLA-4 Ig and anti-CD154, in fact, led to synergistic effects in prolonging cardiac allograft survival in murine models (81). Further research in both murine and non-human primate models have shown that combined blockade of these two pathways prolong of renal and islet transplant survival (82-84).

Currently, CTLA-4 Ig has progressed in clinical trials, and a second-generation CTLA-4 Ig derivative is currently under FDA review for use in transplantation (85-87). In contrast, although anti-CD154 has been shown to be very effective at protecting animals against allograft rejection (81, 82, 88), it cannot be used as a therapeutic for the prevention of transplantation rejection. Unfortunately, pilot clinical studies with anti-CD154 were halted due to thromboembolic complications perhaps as a result of the expression of CD154 on

platelets (37, 89). Although the use of an agent that directly binds to CD154 is unlikely an option for use in the clinic, it is important to understand the mechanisms behind the effectiveness of anti-CD154 therapy in order to hopefully replicate these effects through other therapeutics. In chapters 2-4 of this dissertation, we have sought to elucidate the effects of CD154 blockade on the dendritic cell and antigen-specific T cell responses underlying the efficacy of this treatment in promoting graft survival.

PERIPHERAL TOLERANCE: REGULATORY T CELLS

In early studies of immunologic self-tolerance, several groups showed that populations of thymic derived $CD4^+$ T cells were responsible for both the development of autoimmunity and its suppression. After neonatal thymectomies in mice, the animals were overcome with autoimmune gastritis, thyroditis, and oophritis amongst other diseases (90, 91). In 1982, Shimon Sakaguchi and colleagues revealed that not only could the induced autoimmune disease be inhibited with a transfusion of mature $CD4^+$ thymocytes, but also that the original $CD4^+$ T cells responsible for autoimmunity could transfer the disease when adoptively transferred into a naïve animal (92, 93). These studies confirmed that there were two different populations of $CD4^+$ T cells incurring either a suppressive or activating effect, leading to either self-tolerance or autoimmunity, respectively. Because it was evident that at least two functionally different populations of $CD4^+$ T cells existed, several groups pushed towards finding distinguishing markers for these two populations (94-99). In 1995, it was identified that IL-2 receptor (CD25)-

expressing $CD4^+$ T cells possessed the ability to suppress inflammatory autoimmune responses when adoptively transferred into mice (99).

Since the discovery of high CD25 expression on regulatory T cells (T_{reg}), Foxp3 has been described as a better marker to distinguish T_{reg} from recently activated $CD4^+$ effector T cells. In 2001, a mutation in Foxp3 was identified as the causal link to the spontaneous development of systemic autoimmune disease in Scurfy mice (100). In fact, Foxp3 is a transcription factor expressed in the nucleus of thymic and peripheral regulatory T cells, essential for their development, a mutation in which was also described as the cause a systemic autoimmunity of multiple organs called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans (100-102).

Because regulatory T cells provide a dampening signal through the production and release of the immunosuppressive cytokine IL-10 (103, 104), they have also been deemed important in inducing transplantation tolerance (109, 110). Studies by Taylor *et al* showed that the addition of $CD4^+$ $CD25^+$ cells with costimulation blockade in a graft-versus-host model led to the prevention of disease development (105). Other groups have isolated and expanded naturally occurring T_{reg} *ex vivo* with the purpose of inducing transplantation tolerance (106) and enhancing the immunosuppressive effects of other immunosuppressive regimens, including costimulation blockade and mixed chimerism induction (107, 108). Moreover, the increased ratio of T_{reg} to effector T cells has been consistently associated with improved survival of grafts (111).

One of the mechanisms behind the potency of T_{reg} transfer is the competitive consumption of IL-2 in the surrounding environment, due to their increased expression of CD25 compared to effector T cells (112). The reduction of IL-2 in the microenvironment decreases the level of T cell proliferation including graft-specific alloreactive T cells, inducing the protection of the transplant. Another mechanism by which T_{reg} may induce suppression of effector cells is by inhibiting the migration of T cells into lymph nodes and preventing their interaction with antigen-loaded dendritic cells (DCs), thereby preventing effector T cell activation (113, 114).

New experimental methods had developed with the aim of inducing the development of naïve T cells into regulatory T cells. One of the methods for inducing Foxp3 in naïve T cells is through the *in vitro* stimulation of T cells in the presence of IL-2 and TGF-β (115, 116), which can then be used to suppress immune responses against alloantigens. Other groups have observed the conversion of peripheral $Foxp3^-$ precursors into $Foxp3^+$ T_{reg} following exposure to tolerogenic plasmacytoid DC (117). The specific changes to APC phenotype or function that promote T_{reg} conversion remain unknown and an important area of future investigation. However, peripheral conversion of naïve $CD4^+$ T cells into $Foxp3^+$ T_{reg} has also been observed following interruption or attenuation of TCR mediated signals. On the other hand, it has been observed that conversion of $Foxp3^-$ $CD4^+$ T cells into $Foxp3^+$ T cells occurs following exposure to low-dose antigen (118), and conversely, T_{reg} generation is inhibited by high TCR stimulation (119). Furthermore, blocking stimulatory signaling molecules, such as CD154 and CD4, has also been used with the purpose to induce prolonged immune tolerance (120) and with the

intention of developing regulatory T cells (121, 122). Interestingly, the detrimental effect of anti-thymoglobulin leading to homeostatic proliferation has also been associated with the increase in conversion of naïve T cells into Foxp3-expressing T_{reg} (123-125). In chapters 3 and 4, we address the effect of CD40/CD154 pathway blockade on T_{reg} development and protection of the graft.

mTOR COMPLEX SIGNAL INHIBITION WITH RAPAMYCIN

In the early 1970's on Easter Island, the compound now known as rapamycin was isolated from *Streptomyces hygroscopicus* and subsequently found to potently inhibit cell proliferation (66). In the 40 years since its discovery, rapamycin has been shown to have myriad effects on many different cell types in mammals. It is now known that rapamycin mediates these effects by forming a complex with FK506-binding protein 12 (FKBP-12), which binds to and inactivates mammalian target of rapamycin (mTOR) (66). mTOR is a serine/threonine protein kinase which is widely expressed among many cell types, and is an important component of several intracellular signaling pathways in naïve, effector, and regulatory T cells, involving the PI3K/ Akt pathway (126). While great strides have been made in the last few years to understand the impact of rapamycin on T cell metabolism, differentiation, and lineage commitment, our current understanding of the effects of rapamycin on T cell biology is encumbered by an unusually high number of paradoxical effects, the mechanisms underlying most of which have yet to be elucidated.

Rapamycin has long been appreciated as an inhibitor of cell growth, shown to differentially affect the different mTOR complexes (66). Inhibition of the mTOR complex further mediates immunosuppressive function through an anti-proliferative effect and by attenuating signaling through IL-2R and other cytokine receptors (the so-called signal three of T cell activation), thereby preventing full activation of the T cell (126). Studies in human cells revealed that the impact of rapamycin on T cell proliferation was dependent on both the strength of signal through the TCR as well as the degree of costimulation provided, such that in the presence of strong TCR signals and high costimulation, rapamycin failed to inhibit proliferation (127). However, even in the presence of costimulation, inhibition of mTOR signaling with rapamycin has been shown to induce anergy in antigen-specific T cells (128). More recent studies have demonstrated that the immunosuppressive properties of rapamycin are even broader, impacting the generation and expansion of regulatory T cells (T_{reg}) as well as the maturation and function of dendritic cells (DC) (126). Despite these pluripotent immunosuppressive effects, rapamycin proved to be a relatively weak inhibitor of graft rejection when used as a monotherapy. For example, in experimental murine models of fully MHC disparate skin transplantation, rapamycin treatment resulted in a very modest prolongation of graft survival of only a few days (129). Likewise, rapamycin (sirolimus) monotherapy is not often used in clinical transplantation; instead, rapamycin is frequently administered as part of multi-drug regimens (130). Specifically, it can be used in combination with mycophenolate mofetil, often with favorable outcomes in terms of graft survival in transplant recipients (130). In

addition, rapamycin has been combined with lymphodepleting agents prior to transplantation in order to prolong graft survival (131). Studies designed to elucidate the mechanisms underlying the observed synergism between rapamycin and lymphocyte depletion revealed that the addition of rapamycin attenuated the generation of effector memory T cells (T_{EM}) and increased the relative proportion of T_{reg} following depletional therapy, resulting in a more favorable T_{EM}/T_{reg} balance (129). Thus, the immunosuppressive effects of rapamycin during transplantation are well-documented; however, the ubiquitous nature of its target, mTOR, makes it difficult to pinpoint the precise pathways involved in rapamycin's salutary effect following transplantation. In chapters 5 and 6, we attempt to address the differential effects of rapamycin treatment on pathogen infection vs. a transplanted graft.

REFERENCES

1. Kahan BD. 1988. Pien Ch'iao, the legendary exchange of hearts, traditional Chinese medicine, and the modern era of cyclosporine. *Transplant Proc* 20: 2-12
2. Kahan BD. 1983. Cosmas and Damian revisited. *Transplant Proc* 15: 2211-6
3. Gibson T, Medawar PB. 1943. The fate of skin homografts in man. *J Anat* 77: 299-310 4
4. Medawar PB. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. *J Anat* 78: 176-99
5. Medawar PB. 1945. A second study of the behaviour and fate of skin homografts in rabbits: A Report to the War Wounds Committee of the Medical Research Council. *J Anat* 79: 157-76 4
6. Mitchison NA. 1953. Passive transfer of transplantation immunity. *Nature* 171: 267-8
7. Billingham RE, Brent L, Medawar PB. 1953. Actively acquired tolerance of foreign cells. *Nature* 172: 603-6
8. Owen RD. 1945. Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins. *Science* 102: 400-1
9. Merrill JP, Murray JE, Harrison JH, Guild WR. 1956. Successful homotransplantation of the human kidney between identical twins. *J Am Med Assoc* 160: 277-82

10. Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. 1999. Phylogenetic perspectives in innate immunity. *Science* 284: 1313-8

11. Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-52

12. Smiley ST, King JA, Hancock WW. 2001. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* 167: 2887-94

13. Devaney JM, Greene CM, Taggart CC, Carroll TP, O'Neill SJ, McElvaney NG. 2003. Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. *FEBS Lett* 544: 129-32

14. Biragyn A, Ruffini PA, Leifer CA, Klyushnenkova E, Shakhov A, Chertov O, Shirakawa AK, Farber JM, Segal DM, Oppenheim JJ, Kwak LW. 2002. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* 298: 1025-9

15. Rot A. 1992. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol Today* 13: 291-4

16. Tanaka Y, Adams DH, Shaw S. 1993. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* 14: 111-5

17. Lawrence MB, Springer TA. 1991. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 65: 859-73

18. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* 279: 381-4

19. Chan JR, Hyduk SJ, Cybulsky MI. 2001. Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J Exp Med* 193: 1149-58

20. Constantin G, Majeed M, Giagulli C, Piccio L, Kim JY, Butcher EC, Laudanna C. 2000. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13: 759-69

21. Schroder JM, Persoon NL, Christophers E. 1990. Lipopolysaccharide-stimulated human monocytes secrete, apart from neutrophil-activating peptide 1/interleukin 8, a second neutrophil-activating protein. NH2-terminal amino acid sequence identity with melanoma growth stimulatory activity. *J Exp Med* 171: 1091-100

22. Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M. 1990. Neutrophil-activating properties of the melanoma growth-stimulatory activity. *J Exp Med* 171: 1797-802

23. Fahey TJ, 3rd, Tracey KJ, Tekamp-Olson P, Cousens LS, Jones WG, Shires GT, Cerami A, Sherry B. 1992. Macrophage inflammatory protein 1 modulates macrophage function. *J Immunol* 148: 2764-9

24. Roth SJ, Carr MW, Springer TA. 1995. C-C chemokines, but not the C-X-C chemokines interleukin-8 and interferon-gamma inducible protein-10, stimulate transendothelial chemotaxis of T lymphocytes. *Eur J Immunol* 25: 3482-8

25. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B. 1996. Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. *J Immunol* 156: 322-7

26. Dahinden CA, Geiser T, Brunner T, von Tscharner V, Caput D, Ferrara P, Minty A, Baggiolini M. 1994. Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. *J Exp Med* 179: 751-6

27. Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. 1992. RANTES and macrophage inflammatory protein 1 alpha induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 176: 1489-95

28. Sozzani S, Sallusto F, Luini W, Zhou D, Piemonti L, Allavena P, Van Damme J, Valitutti S, Lanzavecchia A, Mantovani A. 1995. Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* 155: 3292-5

29. Taub DD, Sayers TJ, Carter CR, Ortaldo JR. 1995. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol* 155: 3877-88

30. Schall TJ, Bacon K, Toy KJ, Goeddel DV. 1990. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 347: 669-71

31. Lim KG, Wan HC, Bozza PT, Resnick MB, Wong DT, Cruikshank WW, Kornfeld H, Center DM, Weller PF. 1996. Human eosinophils elaborate the lymphocyte

chemoattractants. IL-16 (lymphocyte chemoattractant factor) and RANTES. *J Immunol* 156: 2566-70

32. Kuna P, Reddigari SR, Schall TJ, Rucinski D, Viksman MY, Kaplan AP. 1992. RANTES, a monocyte and T lymphocyte chemotactic cytokine releases histamine from human basophils. *J Immunol* 149: 636-42

33. Maghazachi AA, Al-Aoukaty A, Schall TJ. 1996. CC chemokines induce the generation of killer cells from CD56+ cells. *Eur J Immunol* 26: 315-9

34. Kondo T, Novick AC, Toma H, Fairchild RL. 1996. Induction of chemokine gene expression during allogeneic skin graft rejection. *Transplantation* 61: 1750-7

35. Watarai Y, Koga S, Paolone DR, Engeman TM, Tannenbaum C, Hamilton TA, Fairchild RL. 2000. Intraallograft chemokine RNA and protein during rejection of MHC-matched/multiple minor histocompatibility-disparate skin grafts. *J Immunol* 164: 6027-33

36. Koga S, Novick AC, Toma H, Fairchild RL. 1999. CD8+ T cells produce RANTES during acute rejection of murine allogeneic skin grafts. *Transplantation* 67: 854-64

37. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-4

38. Lukacs NW, Kunkel SL, Burdick MD, Strieter RM. 1993. The production of chemotactic cytokines in an allogeneic response. The role of intercellular

adhesion molecule-1 and lymphocyte function-associated antigen-3. *Am J Pathol* 143: 1179-88

39. Zinkernagel RM, Doherty PC. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* 248: 701-2

40. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395: 82-6

41. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221-7

42. DeBenedette MA, Wen T, Bachmann MF, Ohashi PS, Barber BH, Stocking KL, Peschon JJ, Watts TH. 1999. Analysis of 4-1BB ligand (4-1BBL)-deficient mice and of mice lacking both 4-1BBL and CD28 reveals a role for 4-1BBL in skin allograft rejection and in the cytotoxic T cell response to influenza virus. *J Immunol* 163: 4833-41

43. Watts TH, DeBenedette MA. 1999. T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol* 11: 286-93

44. Turka LA, Ledbetter JA, Lee K, June CH, Thompson CB. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3+ mature thymocytes. *J Immunol* 144: 1646-53

45. Fanslow WC, Clifford KN, Seaman M, Alderson MR, Spriggs MK, Armitage RJ, Ramsdell F. 1994. Recombinant CD40 ligand exerts potent biologic effects on T cells. *J Immunol* 152: 4262-9

46. Armitage RJ, Tough TW, Macduff BM, Fanslow WC, Spriggs MK, Ramsdell F, Alderson MR. 1993. CD40 ligand is a T cell growth factor. *Eur J Immunol* 23: 2326-31

47. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, Flavell RA. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409: 97-101

48. Curtsinger JM, Lins DC, Mescher MF. 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med* 197: 1141-51

49. Hernandez J, Aung S, Marquardt K, Sherman LA. 2002. Uncoupling of proliferative potential and gain of effector function by CD8(+) T cells responding to self-antigens. *J Exp Med* 196: 323-33

50. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478-80

51. Ridge JP, Di Rosa F, Matzinger P. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-8

52. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-3

53. Shu U, Kiniwa M, Wu CY, Maliszewski C, Vezzio N, Hakimi J, Gately M, Delespesse G. 1995. Activated T cells induce interleukin-12 production by monocytes via CD40-CD40 ligand interaction. *Eur J Immunol* 25: 1125-8

54. Ludewig B, Graf D, Gelderblom HR, Becker Y, Kroczek RA, Pauli G. 1995. Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10. *Eur J Immunol* 25: 1943-50

55. Bonnevier JL, Yarke CA, Mueller DL. 2006. Sustained B7/CD28 interactions and resultant phosphatidylinositol 3-kinase activity maintain G1-->S phase transitions at an optimal rate. *Eur J Immunol* 36: 1583-97

56. Bonnevier JL, Mueller DL. 2002. Cutting edge: B7/CD28 interactions regulate cell cycle progression independent of the strength of TCR signaling. *J Immunol* 169: 6659-63

57. Fraser JD, Irving BA, Crabtree GR, Weiss A. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* 251: 313-6

58. Gimmi CD, Freeman GJ, Gribben JG, Sugita K, Freedman AS, Morimoto C, Nadler LM. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci U S A* 88: 6575-9

59. Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity* 3: 87-98

60. Shi Y, Radvanyi LG, Sharma A, Shaw P, Green DR, Miller RG, Mills GB. 1995. CD28-mediated signaling in vivo prevents activation-induced apoptosis in the thymus and alters peripheral lymphocyte homeostasis. *J Immunol* 155: 1829-37

61. Elion GB. 1993. The George Hitchings and Gertrude Elion Lecture. The pharmacology of azathioprine. *Ann N Y Acad Sci* 685: 400-7

62. Borel JF, Feurer C, Gubler HU, Stahelin H. 1976. Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions* 6: 468-75

63. 1994. A comparison of tacrolimus (FK 506) and cyclosporine for immunosuppression in liver transplantation. The U.S. Multicenter FK506 Liver Study Group. *N Engl J Med* 331: 1110-5

64. 1994. Randomised trial comparing tacrolimus (FK506) and cyclosporin in prevention of liver allograft rejection. European FK506 Multicentre Liver Study Group. *Lancet* 344: 423-8

65. Schwarz A, Haller H, Schmitt R, Schiffer M, Koenecke C, Strassburg C, Lehner F, Gottlieb J, Bara C, Becker JU, Broecker V. 2010. Biopsy-diagnosed renal disease in patients after transplantation of other organs and tissues. *Am J Transplant* 10: 2017-25

66. Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. 2002. Two TOR complexes, only one of which is

rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* 10: 457-68

67. Fritsche L, Budde K, Dragun D, Einecke G, Diekmann F, Neumayer HH. 2004. Testosterone concentrations and sirolimus in male renal transplant patients. *Am J Transplant* 4: 130-1

68. Eisen HJ, Tuzcu EM, Dorent R, Kobashigawa J, Mancini D, Valantine-von Kaeppler HA, Starling RC, Sorensen K, Hummel M, Lind JM, Abeywickrama KH, Bernhardt P. 2003. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *N Engl J Med* 349: 847-58

69. Demopoulos L, Polinsky M, Steele G, Mines D, Blum M, Caulfield M, Adamkovic A, Liu Q, Harler MB, Hahn C, Singh A. 2008. Reduced risk of cytomegalovirus infection in solid organ transplant recipients treated with sirolimus: a pooled analysis of clinical trials. *Transplant Proc* 40: 1407-10

70. Haririan A, Morawski K, West MS, El-Amm JM, Doshi MD, Cincotta E, Alangaden GJ, Chandrasekar P, Gruber SA. 2007. Sirolimus exposure during the early post-transplant period reduces the risk of CMV infection relative to tacrolimus in renal allograft recipients. *Clin Transplant* 21: 466-71

71. Ozaki KS, Camara NO, Galante NZ, Camargo LF, Pacheco-Silva A. 2005. Decreased Cytomegalovirus infection after antilymphocyte therapy in sirolimus-treated renal transplant patients. *Int Immunopharmacol* 5: 103-6

72. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108-12

73. Ferrer IR, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP, Ford ML. 2010. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. *J Immunol* 185: 2004-8

74. Turner AP, Shaffer, V. O., Araki, K., Martens, C., Turner, P. L., Gangappa, S., Ford, M. L., Ahmed, R., Kirk, A. D., Larsen, C. P. 2011. Sirolimus Enhances the Magnitude and Quality of Viral-Specific CD8+ T cell Responses to Vaccinia Virus Vaccination in Rhesus Macaques. *Am J Transplant* In press

75. Brennan DC, Flavin K, Lowell JA, Howard TK, Shenoy S, Burgess S, Dolan S, Kano JM, Mahon M, Schnitzler MA, Woodward R, Irish W, Singer GG. 1999. A randomized, double-blinded comparison of Thymoglobulin versus Atgam for induction immunosuppressive therapy in adult renal transplant recipients. *Transplantation* 67: 1011-8

76. 1985. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. Ortho Multicenter Transplant Study Group. *N Engl J Med* 313: 337-42

77. Norman DJ, Kahana L, Stuart FP, Jr., Thistlethwaite JR, Jr., Shield CF, 3rd, Monaco A, Dehlinger J, Wu SC, Van Horn A, Haverty TP. 1993. A randomized clinical trial of induction therapy with OKT3 in kidney transplantation. *Transplantation* 55: 44-50

78. Jenkins MK, Schwartz RH. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* 165: 302-19

79. Harding FA, McArthur JG, Gross JA, Raulet DH, Allison JP. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356: 607-9

80. Janeway CA, Rosen FS. 1966. The gamma globulins. IV. Therapeutic uses of gamma globulin. *N Engl J Med* 275: 826-31

81. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381: 434-8

82. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Jr., Knechtle SJ. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* 94: 8789-94

83. Pearson TC, Trambley J, Odom K, Anderson DC, Cowan S, Bray R, Lin A, Hollenbaugh D, Aruffo A, Siadak AW, Strobert E, Hennigar R, Larsen CP. 2002. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* 74: 933-40

84. Lehnert AM, Yi S, Burgess JS, O'Connell PJ. 2000. Pancreatic islet xenograft tolerance after short-term costimulation blockade is associated with increased CD4+ T cell apoptosis but not immune deviation. *Transplantation* 69: 1176-85

85. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K,

Charpentier B. 2005. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 353: 770-81

86. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS, Peach RJ. 2005. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant* 5: 443-53

87. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, Massari P, Mondragon-Ramirez GA, Agarwal M, Di Russo G, Lin CS, Garg P, Larsen CP. 2010. A phase III study of belatacept-based immunosuppression regimens versus cyclosporine in renal transplant recipients (BENEFIT study). *Am J Transplant* 10: 535-46

88. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, Mordes JP, Rossini AA. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* 92: 9560-4

89. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114

90. Nishizuka Y, Sakakura T. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 166: 753-5

91. Kojima A, Prehn RT. 1981. Genetic susceptibility to post-thymectomy autoimmune diseases in mice. *Immunogenetics* 14: 15-27

92. Sakaguchi S, Takahashi T, Nishizuka Y. 1982. Study on cellular events in postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J Exp Med* 156: 1565-76

93. Sakaguchi S, Takahashi T, Nishizuka Y. 1982. Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis. *J Exp Med* 156: 1577-86

94. Sugihara S, Izumi Y, Yoshioka T, Yagi H, Tsujimura T, Tarutani O, Kohno Y, Murakami S, Hamaoka T, Fujiwara H. 1988. Autoimmune thyroiditis induced in mice depleted of particular T cell subsets. I. Requirement of Lyt-1 dull L3T4 bright normal T cells for the induction of thyroiditis. *J Immunol* 141: 105-13

95. Smith H, Lou YH, Lacy P, Tung KS. 1992. Tolerance mechanism in experimental ovarian and gastric autoimmune diseases. *J Immunol* 149: 2212-8

96. Morrissey PJ, Charrier K, Braddy S, Liggitt D, Watson JD. 1993. CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+ T cells. *J Exp Med* 178: 237-44

97. Powrie F, Mason D. 1990. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. *J Exp Med* 172: 1701-8

98. McKeever U, Mordes JP, Greiner DL, Appel MC, Rozing J, Handler ES, Rossini AA. 1990. Adoptive transfer of autoimmune diabetes and thyroiditis to athymic rats. *Proc Natl Acad Sci U S A* 87: 7618-22

99. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64

100. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27: 68-73

101. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27: 20-1

102. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27: 18-20

103. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. 2001. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol* 166: 3789-96

104. Hoffmann P, Ermann J, Edinger M, Fathman CG, Strober S. 2002. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med* 196: 389-99

105. Taylor PA, Noelle RJ, Blazar BR. 2001. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med* 193: 1311-8

106. Taylor PA, Lees CJ, Blazar BR. 2002. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99: 3493-9

107. Pilat N, Baranyi U, Klaus C, Jaeckel E, Mpofu N, Wrba F, Golshayan D, Muehlbacher F, Wekerle T. 2010. Treg-therapy allows mixed chimerism and transplantation tolerance without cytoreductive conditioning. *Am J Transplant* 10: 751-62

108. Gross DA, Chappert P, Leboeuf M, Monteilhet V, Van Wittenberghe L, Danos O, Davoust J. 2006. Simple conditioning with monospecific CD4+CD25+ regulatory T cells for bone marrow engraftment and tolerance to multiple gene products. *Blood* 108: 1841-8

109. Lin CY, Graca L, Cobbold SP, Waldmann H. 2002. Dominant transplantation tolerance impairs CD8+ T cell function but not expansion. *Nat Immunol* 3: 1208-13

110. van Maurik A, Herber M, Wood KJ, Jones ND. 2002. Cutting edge: CD4+CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell-mediated graft rejection: implications for anti-CD154 immunotherapy. *J Immunol* 169: 5401-4

111. Warnecke G, Bushell A, Nadig SN, Wood KJ. 2007. Regulation of transplant arteriosclerosis by CD25+CD4+ T cells generated to alloantigen in vivo. *Transplantation* 83: 1459-65

112. de la Rosa M, Rutz S, Dorninger H, Scheffold A. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol* 34: 2480-8

113. Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, Lafaille JJ, Dustin ML. 2006. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med* 203: 505-11

114. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. 2008. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A* 105: 10113-8

115. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-86

116. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells

through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172: 5149-53

117. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, Angeli V, Li Y, Boros P, Ding Y, Jessberger R, Trinchieri G, Lira SA, Randolph GJ, Bromberg JS. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 7: 652-62

118. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-27

119. Molinero LL, Miller ML, Evaristo C, Alegre ML. 2011. High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-{kappa}B-Dependent Manner. *J Immunol* 186: 4609-17

120. Pearson TC, Madsen JC, Larsen CP, Morris PJ, Wood KJ. 1992. Induction of Transplantation Tolerance in Adults Using Donor Antigen and Anti-Cd4 Monoclonal-Antibody. *Transplantation* 54: 475-83

121. Verbinnen B, Billiau AD, Vermeiren J, Galicia G, Bullens DMA, Boon L, Cadot P, Hens G, Dewolf-Peeters C, Van Gool SW, Ceuppens JL. 2008. Contribution of regulatory T cells and effector T cell deletion in tolerance induction by costimulation blockade. *Journal of Immunology* 181: 1034-42

122. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. 2004. Alloantigen-induced CD25(+)CD4(+) regulatory T cells can develop in vivo from CD25(-)CD4(+) precursors in a thymus-independent process. *Journal of Immunology* 172: 923-8

123. Minamimura K, Gao WD, Maki T. 2006. CD4(+) regulatory T cells are spared from deletion by antilymphocyte serum, a polyclonal anti-T cell antibody. *Journal of Immunology* 176: 4125-32

124. Knoechel B, Lohr J, Kahn E, Bluestone JA, Abbas AK. 2005. Sequential development of interleukin 2 dependent effector and regulatory T cells in response to endogenous systemic antigen. *Journal of Experimental Medicine* 202: 1375-86

125. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. 2004. CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J Immunol* 173: 7259-68

126. Thomson AW, Turnquist HR, Raimondi G. 2009. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 9: 324-37

127. Slavik JM, Lim DG, Burakoff SJ, Hafler DA. 2004. Rapamycin-resistant proliferation of CD8+ T cells correlates with p27kip1 down-regulation and bcl-xL induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. *J Biol Chem* 279: 910-9

128. Mondino A, Mueller DL. 2007. mTOR at the crossroads of T cell proliferation and tolerance. *Semin Immunol* 19: 162-72

129. D'Addio F, Yuan X, Habicht A, Williams J, Ruzek M, Iacomini J, Turka LA, Sayegh MH, Najafian N, Ansari MJ. 2010. A novel clinically relevant approach to tip the balance toward regulation in stringent transplant model. *Transplantation* 90: 260-9

130. Halloran PF. 2004. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 351: 2715-29

131. Swanson SJ, Hale DA, Mannon RB, Kleiner DE, Cendales LC, Chamberlain CE, Polly SM, Harlan DM, Kirk AD. 2002. Kidney transplantation with rabbit antithymocyte globulin induction and sirolimus monotherapy. *Lancet* 360: 1662-4

Chapter 2

CD154 Blockade Alters Innate Immune Cell Recruitment and Programs Alloreactive $CD8^+$ T Cells Into KLRG-1^{high} Short-Lived Effector T Cells

ABSTRACT

CD154/CD40 blockade combined with donor specific transfusion remains one of the most effective therapies in prolonging allograft survival. Despite this, the mechanisms by which these pathways synergize to prevent rejection are not completely understood. We conducted a detailed longitudinal analysis of the kinetics and magnitude of T cell expansion and differentiation in response to an allogeneic skin graft in the presence of CD154/CD40 pathway blockade. Results demonstrated that treatment with anti-CD154 vs. DST had distinct and opposing effects on activated $CD44^{high}$ $CD62L^{low}$ $CD8^{+}$ T cells in skin graft recipients. Specifically, CD154 blockade delayed alloreactive $CD8^{+}$ T cell responses, while DST accelerated them. DST inhibited the differentiation of alloreactive $CD8^{+}$ T cells into multi-cytokine producing effectors, while CD40/CD154 blockade led to the diminution of the KLRG-1^{low} long-lived memory precursor population compared with either untreated or DST treated animals. When combined, anti-CD154 and DST acted synergistically to profoundly diminish the absolute number of IFN-γ producing alloreactive $CD8^{+}$ T cells. These findings demonstrate that the previously described ability of anti-CD154 and DST to result in alloreactive T cell deletion involves both delayed kinetics of T cell expansion and differentiation and inhibited development of KLRG-1^{low} memory precursor cells.

INTRODUCTION

Current immunosuppressive regimens in organ transplantation require life-long administration and result in off-target toxicities such as nephrotoxicity and cardiovascular and metabolic complications (1). Considering these significant co-morbidities, much work over the years has focused on the development of novel modes of immunosuppression. The development of costimulation blocking molecules has been the basis for research by several groups to specifically target and inhibit the full activation of alloantigen-specific T cells at the time of transplantation. One of the most effective pathways for therapeutic intervention is the CD154/CD40 pathway, blockade of which results in profound inhibition of graft rejection (2-4). However, translation of therapeutic blockade of this pathway has been stymied by the observation of thromboembolic complications in pilot clinical trials as a result of the expression of CD154 on platelets (5). Nevertheless, understanding the altered differentiation programs initiated in alloreactive T cell populations under conditions of CD154 blockade remains an important goal in the ongoing pursuit to harness the therapeutic potential of this pathway.

In order to study the effects of CD40/CD154 pathway blockade on innate immune responses and donor-reactive T cell responses to a transplant, we employed an allogeneic skin graft (SG) model in which anti-CD154 monoclonal antibodies (mAb) were administered in combination with donor specific transfusion (DST) for the induction of immune tolerance following transplantation, as previously described (4, 6, 7). DST injection provides a large bolus of antigen presented by relatively inert APCs (8), stimulating antigen-specific T cell activation

by providing "signal one." Several other groups have also demonstrated the potent effects of combined DST and costimulation blockade in the prolongation of islet, cardiac, skin and kidney allograft survival in murine and nonhuman primate models (4, 6-11). Although it has been generally accepted that CD154 costimulation blockade leads to anergy (12) or deletion (13, 14) of recently activated T cells, the mechanism by which DST and anti-CD154 mAb synergize to induce these effects on the alloreactive T cell population remains incompletely understood.

The combination of CD154 blockade and the inhibition of polymorphonuclear cells (PMNs) have also been shown to have synergistic effects in prolonging allograft survival (15). Because chemokines that recruit innate immune cells are expressed early in the wound healing process of skin grafts (16), we aimed to determine whether blockade of CD154 could also impair the chemotactic signals attracting cells to the transplanted graft, thereby preventing further damage. During instances of tissue injury and inflammation, platelets are activated and recruited to the site of injury. Platelets have also been shown to express high levels of CD154 on their surface (17). CD154 expressed on platelets interacts with CD40 on the surface of endothelial cells during wound healing, which leads to increased expression of inflammatory adhesion receptors, such as E-selectin, ICAM-1 and VCAM-1. Furthermore, Henn and colleagues demonstrated that human umbilical vein endothelial cells cultured *in vitro* with activated platelets increased their production of leukocyte attracting chemokines, such as IL-8 (17). We hypothesized that if the interaction between activated platelets and endothelial cells involved in the revascularization of the skin allograft were leading to increased inflammation

and promoting increased cellular infiltration, then could treatment with CD154 blockade inhibit this interaction and prevent leukocyte recruitment? In this study, we observed that animals treated with CD154 blockade had significantly impaired expression of CXCL1/KC, CCL3/MIP-1α (macrophage inflammatory protein-1α), and CCL5/RANTES (regulated upon activation normal T cell expressed and secreted) in the allografts compared to untreated controls. Finally, we show that anti-CD154 treatment and DST, either as monotherapies or in combination, resulted in reduced macrophage and $CD8^+$ T cell infiltration, while anti-CD154 treatment alone was sufficient to also significantly diminish neutrophil infiltration into the donor allografts.

In order to assess the differential impact of DST and anti-CD154 mAb on programming donor-reactive $CD8^+$ T cell expansion, contraction, and differentiation over time, we performed longitudinal analyses on the donor-reactive $CD8^+$ T cell responses. We hypothesized that the previously observed deletion of graft-reactive $CD8^+$ T cells following anti-CD154/DST treatment was the result of differential programming of these cells following encounter with alloantigen (8, 12). Recently, studies of viral-specific $CD8^+$ T cell responses have revealed programmed differentiation of antigen-specific T cells into either long-lived memory precursors or short-lived effectors as early as day 4 post-infection (18). These differentially programmed cells can be segregated on the basis of their expression of KLRG-1 (killer cell lectin-like receptor G-1), in that KLRG-1^{high} cells represent short-lived effectors while KLRG-1^{low} antigen-specific $CD8^+$ T cells represent long-lived memory precursors (18, 19). Our data revealed that treatment with either anti-CD154 or

DST induced distinct differentiation programs in graft-reactive $CD8^+$ T cell responses. Specifically, anti-CD154 treatment functioned to reduce the magnitude of the alloreactive T cell response by delaying $CD8^+$ T cell expansion and increasing the proportion of KLRG-1^{high} short-lived effector cells. In contrast, DST treatment accelerated alloreactive $CD8^+$ T cell expansion but inhibited differentiation into multi-cytokine producing cells.

MATERIALS AND METHODS

Mice

B6-Ly5.2/Cr (H2-K^b, CD45.1) and BALB/c (H2-K^d, CD45.2) mice were obtained from the National Cancer Institute (Charles River, Frederick, MD).

Skin Transplantation, Donor Specific Transfusion and Antibody Treatment

Full thickness BALB/c tail and ear skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages for 6 days. DST was the adoptive transfer of whole splenocytes, given as a single dose of 10^7 splenocytes i.v. on the day of transplantation. When designated, mice were treated with costimulation blockade treatment with hamster monoclonal anti-mouse CD154 (MR1, BioExpress, West Lebanon, NJ). Treatment was administered i.p. at a 500μg dose on the day of transplantation as well as on days 2, 4, 6 post transplantation. Skin grafts were monitored over time and declared as rejected when <10% viable graft remained.

Activated T Cell Surface Staining and TruCount Analysis

At indicated time points, splenocytes were removed and disrupted with glass slides. Cells were stained with antibodies against CD4 PacBlue (Caltag), CD8 PacOrange (Caltag), CD62L FITC (BD Pharmingen), CD44 APC (eBiosciences), and KLRG-1 PE (Southern Biotech). Absolute counts were obtained by using TruCount tubes (BD Pharmingen). Samples were run on a LSR II Flow Cytometer (BD Pharmingen). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

T cell Intracellular Cytokine Staining

To measure IFN-γ (FITC, eBiosciences) and TNF (PE, BD Pharmingen) secretion in donor reactive cells, single cell suspensions of responder splenocytes from transplanted mice were placed into 96-well flat-bottom plates (10^6 per well). BALB/c splenocytes (10^6 per well) were used as stimulators and co-cultured with responder splenocytes. Cells were cultured in the presence of 10μg/mL Brefeldin A for 4 hours and processed with an intracellular staining kit (BD Biosciences), according to manufacturer's instructions. Samples were run on a LSR II Flow Cytometer (BD Pharmingen). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Histology and IHC Quantification

Skin grafts were removed and placed in cryomolds with OCT Embedding Compound (Tissue-Tek, Hatfield, PA) and frozen on dry ice on day 7 post transplantation. Longitudinal sections of skin grafts were cut 5 μm thick with a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost Plus microscope slides. Slides were fixed with 100% acetone and used for H&E as well as immunohistochemical staining. Neutrophil Marker (Santa Cruz Biotechnology, Santa Cruz, CA) and F4/80 (Abcam) were used for $CD8^+$ T cell, neutrophil and macrophage immunohistochemical detection, respectively, by 3,3 diaminobenzidine (DAB) peroxidation and counterstained with haematoxylin. An Olympus BX43 microscope was used for visualization.

The whole slide digital images were captured using the Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA) system with a 20× objective. Images were viewed and analyzed with ScanScope software using positive pixel count algorithm (Aperio). Several fields (8-10) of epidermis and dermis areas were measured in each section. The ratio of total strong positive vs. area measured was used as the quantifying parameter.

RNA Isolation and Chemokine RT-PCR

Mice were sacrificed and skin grafts were extracted and placed at 4^{o} C in RNA*later* (Qiagen) until ready for use. RNA was isolated from skins using RNeasy Fibrous Tissue kit (Qiagen). Reverse transcription of RNA into cDNA was performed using TaqMan reverse transcription kit (Roche). CXCL1, CCL3, CCL5 real-time PCR assays (Applied Biosystems) were run on a 7900HT Real-Time PCR System (ABI).

Statistical Analysis

Prism software was used to perform log-rank Kaplan-Meier statistical analyses on skin graft survival curves. For longitudinal analysis of T cell expansion and differentiation, two-way ANOVA tests were performed, followed by Tukey post-test on significant results. For analysis of innate cell infiltration, RT-PCR, cytokine producing cells and KLRG-1 expression, one-way ANOVA tests were performed, followed by Tukey post-test on significant results.

RESULTS

Anti-CD154 and DST synergize to protect allogeneic grafts from rejection

To assess the effects of CD40/CD154 pathway blockade on skin graft survival, we used a well-established tolerizing regimen consisting of DST in conjunction with anti-CD154 monoclonal antibody treatment (6). B6-Ly5.2/Cr mice (H2-K^b, CD45.1) were transplanted with allogeneic BALB/c (H2-K^d, CD45.2) skin grafts (Figure 2.1A). DST consisted of a single intravenous infusion of 10^7 allogeneic BALB/c splenocytes administered at the time of transplantation. Similar to previous reports using this model (6), untreated animals rapidly rejected their skin grafts (MST 13d). Anti-CD154 mAb monotherapy resulted a modest, but significant, delay in graft rejection as compared to untreated mice (MST 17d, p=0.039), while DST monotherapy resulted in rapid allograft rejection similar to untreated animals (MST 13d). In contrast, combined treatment with anti-CD154 mAb and DST resulted in significant prolongation of skin graft survival, as treated animals exhibited a MST of 50 days (p=0.0002, compared to untreated mice) (Figure 2.1B).

Anti-CD154 treatment alters recruitment of innate immune cells following transplantation

In vitro culture of CD154-expressing platelets with CD40-expressing endothelial cells has been shown to significantly increase their expression of vascular adhesion molecules and production of leukocyte attracting chemokines (17). We hypothesized that if the interaction between activated platelets and the endothelial cells involved in revascularization of the graft were leading to increased

inflammation, then treatment with CD40/CD154 pathway blockade could inhibit this interaction and prevent leukocyte recruitment. In order to assess the impact of DST and/or CD154 blockade on cellular infiltration, we measured the amount of infiltrating cells in allografts on day 7 post-transplantation via immunohistochemical staining for $CD8^+$ T cells (Figure 2.2A), macrophages (Figure 2.2C), and neutrophils (Figure 2.2E) cells. Quantification of the number of strongly positive pixels per μm^2 revealed that either DST or CD154 blockade individually or in combination diminished $CD8^+$ T cell infiltration into allografts (Figure 2.2B). Staining for F4/80 revealed that all treatment groups exhibited reduced macrophage infiltration into allografts (Figure 2.2D). In contrast, only anti-CD154, either in the presence or absence of DST, resulted in profound diminution of neutrophil-specific anti-Gr-1 staining in the transplanted allograft (Figure 2.2F).

CD154 blockade induces differential chemokine gradient and neutrophil recruitment to transplanted grafts

Given the differential migration of cells into the transplanted skin grafts in the presence of CD154 blockade vs. DST, we aimed to determine if CD40/CD154 pathway blockade and DST differentially influenced the chemokine gradients recruiting these cells into grafts. In order to assess this potential difference between the two treatments, we directly analyzed the transplanted skin graft for CXCL1 (KC), CCL3 (MIP-1α), and CCL5 (RANTES) expression. On day 7 post-transplantation, skin grafts were extracted and real-time PCR of the cDNA from the skin graft samples was performed.

First, we measured the RNA expression level of one of the primary neutrophil chemoattractants, KC/CXCL1 (20). The relative expression of this gene was significantly attenuated in skin grafts of all animals treated with anti-CD154 mAb either as a monotherapy (0.21±0.09, $p<0.0001$) or in combination with DST (0.26±0.11, $p<0.0001$) when compared to untreated animals. In contrast, DST alone did not significantly reduce CXCL1 expression compared to untreated controls (Figure 2.3A).

MIP-1α and RANTES have also been associated with the recruitment of both innate and adaptive immune cells including monocytes and T cells (20). Therefore, we interrogated the expression of these chemokines in the transplanted grafts in the presence of CD40/CD154 pathway blockade, hypothesizing that alterations in the level of these chemokines might underlie the reduced infiltration of these cells into the allograft (Figure 2.2A-D). Real-time PCR analysis also revealed that both MIP-1α and RANTES were significantly attenuated in animals treated with CD154-blockade (Figure 2.3B, C). Furthermore, similar to KC/CXCL1 gene expression, DST did not significantly diminish the expression of MIP-1α and RANTES in the explanted grafts.

Anti-CD154 and DST independently alter the kinetics of expansion of activated $CD44^{high}$ $CD62L^{low}$ $CD8^{+}$ T cells over time

The orchestration of alloreactive immune responses are primarily mediated by activated donor-specific T cells. Therefore, we investigated the magnitude and kinetics of expanding $CD8^{+}$ T cell populations following transplantation in the presence of anti-CD154 and/or DST by tracking the frequency and absolute number

of antigen-experienced $CD8^+$ T cells over time. Antigen-experienced $CD8^+$ T cells were defined as those that had lost expression of CD62L and acquired expression of CD44. In naïve B6 animals, approximately $4.5\pm0.72x10^5$ cells of the peripheral $CD8^+$ T cell compartment were $CD62L^{low}$ and $CD44^{high}$ cells. Untreated recipients of allogeneic skin grafts developed large numbers of antigen-experienced cells with a peak of expansion at day 10 post-transplantation ($2.36\pm0.55x10^6$). Following resolution of this effector cell population into memory, untreated recipients possessed $1.0\pm0.32x10^6$ effector/ memory phenotype T cells at day 50. Anti-CD154 mAb monotherapy resulted in a delayed expansion of activated $CD8^+$ T cells, with a peak at day 14 ($9.45\pm1.56x10^5$). Furthermore, the magnitude of this peak was significantly reduced compared to the peak response of untreated mice ($p=0.0133$). In contrast, DST monotherapy accelerated the expansion of activated $CD8^+$ T cells starting as early as day 7 ($8.29\pm1.32x10^5$, $p=0.059$ as compared to untreated controls) and peaking on day 10 ($9.78\pm0.65x10^5$). Accumulation of activated $CD8^+$ T cells in the presence of DST treatment was moderately (but not statistically significantly) reduced compared to untreated controls at day 10 ($p=0.069$). The combination of CD154 blockade and DST led to a significantly greater diminution of both the magnitude and kinetics of expansion of graft-reactive $CD8^+$ T cells. In particular, the peak of expansion of these cells was delayed (day 14) compared to untreated controls. Furthermore, the magnitude of the peak of the alloreactive $CD8^+$ T cell response following combined anti-CD154 and DST (day 14) was significantly diminished compared with the peak expansion of untreated mice (day 10) ($6.18\pm0.78x10^5$ vs. $2.36\pm0.55x10^6$, respectively; $p=0.0026$) (Figure 2.4B).

Anti-CD154 and DST have distinct effects on the programmed differentiation of alloreactive CD8$^+$ T cells into cytokine-producing effectors

To assess the effects of anti-CD154 and DST on the programmed differentiation of alloreactive CD8$^+$ T cells into cytokine-producing effectors, splenocytes from skin grafted animals treated with anti-CD154, DST, or the combination were isolated at the indicated timepoints, stimulated *ex vivo* with BALB/c splenocytes, and subjected to intracellular cytokine staining (Figure 2.5A, B). Data showed that as early as day 7 post-transplantation, splenic CD8$^+$ T cells from untreated animals began to differentiate into multi-functional TNF and IFN-γ producing T cells. At day 10, untreated mice developed a peak of IFN-γ producing donor-reactive CD8$^+$ T cells ($1.96\pm0.70x10^5$). Similar to its effects on the expansion of activated CD44^{high} CD62L^{low} CD8$^+$ T cells, DST mediated an early expansion of IFN-γ producing graft-specific CD8$^+$ T cells with a peak response at day 7 ($9.02\pm0.47x10^4$) (Figure 2.5B). In contrast, anti-CD154 mAb monotherapy delayed CD8$^+$ T cell differentiation into IFN-γ producing cells, such that a modest peak was observed 14 days post-transplantation ($0.22\pm0.21x10^5$) (Figure 2.5B). Finally, combined treatment with anti-CD154 and DST significantly impaired the activation of cytokine-producing donor-reactive CD8$^+$ T cells compared to untreated controls, such that IFN-γ$^+$ TNF$^+$ alloreactive CD8$^+$ T cells were virtually undetectable (Figure 2.5A).

We observed similar numbers of alloreactive IFN-γ-producing effectors in the untreated and DST treated groups on day 7 post-transplantation (Figure 2.5B). However, by day 10, this number expanded in the untreated animals, while it

contracted in the DST treated animals. In order to understand the nature of the T cell program that led to these disparate outcomes, we sought to determine the fraction of $CD44^{high}$ $CD62L^{low}$ activated T cells in these animals that produced cytokines following *ex vivo* restimulation. On day 7, 17.10±3.49% of antigen-experienced $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells isolated from untreated animals produced IFN-γ (Figure 2.5C, left panel). In contrast, although DST resulted in early accumulation of $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells, a significantly lower fraction of these cells had differentiated into IFN-γ producers on day 7 compared with untreated animals (8.08±0.64%, $p<0.05$).

These results indicated that DST decreased the frequency of differentiated IFN-γ producers as a percentage of the total activated T cell population. Therefore, we next interrogated whether this treatment also affected T cell differentiation into multi-cytokine producing effectors. To test this, IFN-γ producing $CD8^+$ T cells were analyzed for their co-production of TNF. In untreated animals, the large majority of IFN-γ producing $CD8^+$ T cells co-produced TNF (77.89±3.04%) upon *ex vivo* restimulation. However, DST treatment significantly impaired the ability of graft-reactive $CD8^+$ T cells to differentiate into IFN-γ^+ TNF^+ multi-cytokine producers compared with untreated controls (53.27±2.17%, $p=0.0028$) (Figure 2.5D).

Similar to its effect on total burst size and differentiation of T cells, CD40/CD154 pathway blockade also impaired the development of alloreactive $CD8^+$ memory T cells. Specifically, treatment with anti-CD154 resulted in a reduced accumulation of IFN-γ producing alloreactive $CD8^+$ T cells at day 50 post-transplantation compared with untreated controls ($0.45±0.17x10^4$ vs.

7.35±0.84x10^4, respectively; p<0.0001). DST monotherapy also diminished the persistence of IFN-γ producing alloreactive $CD8^+$ T cells (1.07±0.03x10^4, p<0.0001) at day 50 compared with untreated animals. Finally, the combination of CD154 blockade and DST even more profoundly reduced the alloreactive $CD8^+$ memory T cell population compared with untreated controls (0.02±0.02x10^4 vs. 7.35±0.84x10^4, respectively; p<0.0001) (Figure 2.5A, B, far right panels).

Anti-CD154 treatment alters the ratio of alloreactive $CD8^+$ KLRG-1^{low} long-lived memory precursors to KLRG-1^{high} short-lived effectors

The results of this study indicated that CD154 blockade delayed and diminished the accumulation of alloreactive $CD8^+$ T cells during the immune response to a transplant. In order to interrogate the molecular mechanisms underlying this effect, we examined the expression of a cell surface protein known to be associated with the differentiation of short-lived effector T cells that exhibit rapid contraction *in vivo*. The increased expression of KLRG-1 early during T cell responses has recently been shown to be upregulated on short-lived effector cells following antigen stimulation while the lower expression of KLRG-1 is associated with a long-lived memory T cell program (18). We analyzed the expression of KLRG-1 on alloreactive $CD8^+$ T cells on day 7 post-transplantation. In untreated animals, 44.12±2.30% of antigen-experienced $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells expressed increased levels of KLRG-1 by day 7. In contrast, CD40/CD154 pathway blockade led to a marked reduction in the long-lived memory precursor KLRG-1^{low} $CD8^+$ T cell population, and a commensurate increase in the frequency of KLRG-1^{high}

short-lived effectors compared with untreated controls (58.68±4.62% vs. 44.12±2.30%, respectively; $p<0.05$) (Figure 2.6A, B). DST monotherapy did not significantly alter KLRG-1 expression on alloreactive $CD8^+$ T cells (35.15±1.2%). Finally, similar to anti-CD154 monotherapy, combined anti-CD154 and DST significantly increased the frequency of KLRG-1^{high} short-lived alloreactive $CD8^+$ T cells compared with untreated controls (57.87±3.97% vs. 44.12±2.30%, respectively; $p<0.05$).

DISCUSSION

In this manuscript, we assessed the impact of CD154 blockade and DST on innate immune responses and the programmed differentiation of alloreactive $CD8^+$ T cell responses longitudinally following transplantation. We interrogated the effects of CD40/154 pathway blockade and DST treatment on innate immune cell involvement in graft rejection. El-Sawy *et al.* had previously demonstrated that depletion of PMNs alleviated cellular infiltration and prevented cardiac allograft rejection (15). Therefore, we aimed to investigate the effects of CD40/CD154 pathway blockade on innate immune responses directly in the graft. We observed that CD154 costimulation blockade resulted reduced infiltration of macrophages and neutrophils into the grafted skin. The ability of CD154 blockade to impair innate immunity has also been observed in other systems, in that in a murine model of arterial vessel injury, treatment with anti-CD154 monoclonal antibodies significantly impaired innate immune cell infiltration into the carotid arteries (21). In an antigen non-specific model of ischemia and reperfusion injury in liver transplants, Shen *et al.* demonstrated that animals treated with anti-CD154 had reduced injury to livers compared to untreated or anti-IFN-γ treated animals (22). Taken together, our data demonstrate that not only are $CD8^+$ T cell responses inhibited by CD154 blockade, but also innate immune cell trafficking into allografts may be dependent on CD40-CD154 interactions.

There are two potential hypotheses for the decreased innate immune cell migration into allografts in anti-CD154 treated recipients. Due to the expression of CD154 on platelets, its interaction with inflamed activated endothelial cells during

the revascularization process of the skin graft induces the increased expression of inflammatory adhesion molecules E-selectin, ICAM-1 and VCAM-1 (17). In one-way mixed lymphocyte reactions, the inhibition of adhesion molecules prevented the secretion of inflammatory chemokines MIP-1α, MCP-1 and IL-8 (23), of which MIP-1α was predominantly secreted by monocyte in an autocrine manner (24). We hypothesize that by preventing the upregulation of adhesion molecules on endothelial cells with CD154 blockade, the migration of inflammatory cells into the transplanted tissue could be inhibited. Therefore, we posited that the blockade of the CD40/CD154 pathway could indirectly reduce chemokine gradients released by infiltrating macrophages at the site of inflammation and preventing further damage to the transplanted graft. Secondly, the reduced cellular infiltration could be secondary effect of altered CD8+ T cell programming and reduced $CD8^+$ T cell activation and infiltration into the local environment, which could also lead to a dampened chemokine gradient to recruit innate immune cells. In this setting, we hypothesize that the inhibition of the CD40/CD154 pathway signals could lead to reduced $CD4^+$ T cell licensing of antigen presenting cells, necessary for $CD8^+$ T cell activation (25-27). The altered programming and activation of alloreactive $CD8^+$ T cells, as described in this manuscript, could lead to their reduced migration potential into allografts. In both fully- and minor-mismatched skin transplantation models, it has been described that CCL5 (RANTES) gene expression is delayed during graft rejection (16, 28). Furthermore, the increased expression of RANTES in allografts was associated with increased $CD8^+$ T cell infiltration during acute rejection (29).

Consistent with these two potential mechanisms, we showed that CD40/CD154 pathway blockade reduced the expression of leukocyte chemoattractants CXCL1, CCL3, and CCL5, which was reflected in the decreased infiltration of $CD8^+$ T cells, macrophages and neutrophils in the allograft. Taken together, these studies support the hypothesis that the local environment of the graft recruits innate immune cells into transplanted organs due to injury at the site of the transplant. In this study, we demonstrate that CD154-blockade also impacts the local microenvironment either directly or indirectly to diminish leukocyte recruitment and infiltration into the graft and prevent it from further injury.

We further demonstrated that treatment with anti-CD154 and DST induced distinct differentiation programs in alloreactive T cell populations, in that treatment with anti-CD154 delayed the expansion and accumulation of activated $CD62L^{low}$ $CD44^{high}$ $CD8^+$ T cells, while DST accelerated this process. Furthermore, anti-CD154 also resulted in delayed and reduced $CD8^+$ T cell differentiation into IFN-γ producing cells. Although the eventual emergence of alloreactive $CD8^+$ T cells in anti-CD154 treated animals could be attributed to the waning effects of the antibody following cessation of treatment, this is not likely since *in vivo* administration of MR-1 has been shown to persist in animals for up to three weeks (30). Therefore, the delayed $CD8^+$ T cell response in animals treated with CD40/CD154 blockade monotherapy is likely due to a CD154-independent breakthrough response, and not due to incomplete blockade of the pathway in this system. Importantly, this study revealed a novel effect of CD40/CD154 pathway blockade on T cell differentiation, specifically the ability of CD154 blockade to increase the frequency of $CD8^+$ KLRG-

1^{high} short-lived effector cells, and correspondingly decrease the frequency of KLRG-1^{low} long-lived memory precursors. While KLRG-1 has been shown to be present on exhausted $CD8^+$ T cells at later timepoints during the course of an immune response, its expression early during the response is associated with a short-lived effector cell that is destined to die during the contraction phase of the response (18). These data indicate that one mechanism by which CD154 blockade mediates antigen-specific $CD8^+$ T cell deletion is through the induction of KLRG-1 expression. Previous studies have shown that increased expression of KLRG-1 can be attributed to increased antigen duration and increased inflammation (19, 31). Further investigation into the mechanisms by which CD154 blockade also increases KLRG-1 expression in the context of transplantation is warranted.

In contrast, DST treatment resulted in the accelerated activation of $CD8^+$ T cells. Despite this accelerated activation, differentiation into competent IFN-γ secreting effectors was significantly inhibited, and of these IFN-γ-producing cells, a lower percentage of IFN-γ^+ TNF^+ double producers was observed compared with untreated controls. This pattern of cytokine expression is reminiscent of $CD8^+$ T cell exhaustion, wherein T cells first lose the ability to make TNF, followed by the loss of IFN-γ, before being deleted altogether (32). Because $CD8^+$ T cell exhaustion is facilitated by exposure to high dose antigen, we posit that DST could be initiating a process similar to T cell exhaustion by exposing the cells to high dose antigen presented on relatively inert APCs (8). Importantly, however, the distinct effects of anti-CD154 and DST on T cell programming, when combined, synergized in profoundly reducing antigen-specific T cell populations. This was true both in terms

of numbers of activated alloreactive $CD8^+$ T cells and IFN-γ producers, at all time points over the course of the immune response to the allograft. Thus, the accelerated T cell activation observed in DST treated recipients, when combined with the inhibitory signals associated with CD40/CD154 pathway blockade, produces a catastrophic exhaustive event for the cell, resulting in failure to mount an effective $CD8^+$ T cell response followed by prolonged protection of the graft.

From these data, we conclude that anti-CD154 and DST work through distinct mechanisms to inhibit cellular infiltration and the expansion and differentiation of donor-reactive $CD8^+$ T cells and result in alloreactive T cell deletion and loss of effector function following transplantation. As renewed interest in therapeutic blockade of the CD154/CD40 pathway gains momentum due to promising results using anti-CD40 monoclonal antibodies in translational models (33), understanding the cellular and molecular mechanisms by which blockade of the CD154/CD40 pathway functions to inhibit alloreactive T cell responses is critical to guide rational development of immunosuppressive regimens incorporating these therapeutics for use in transplantation.

Figure 2.1.

Anti-CD154 and DST synergize to protect allogeneic grafts from rejection.

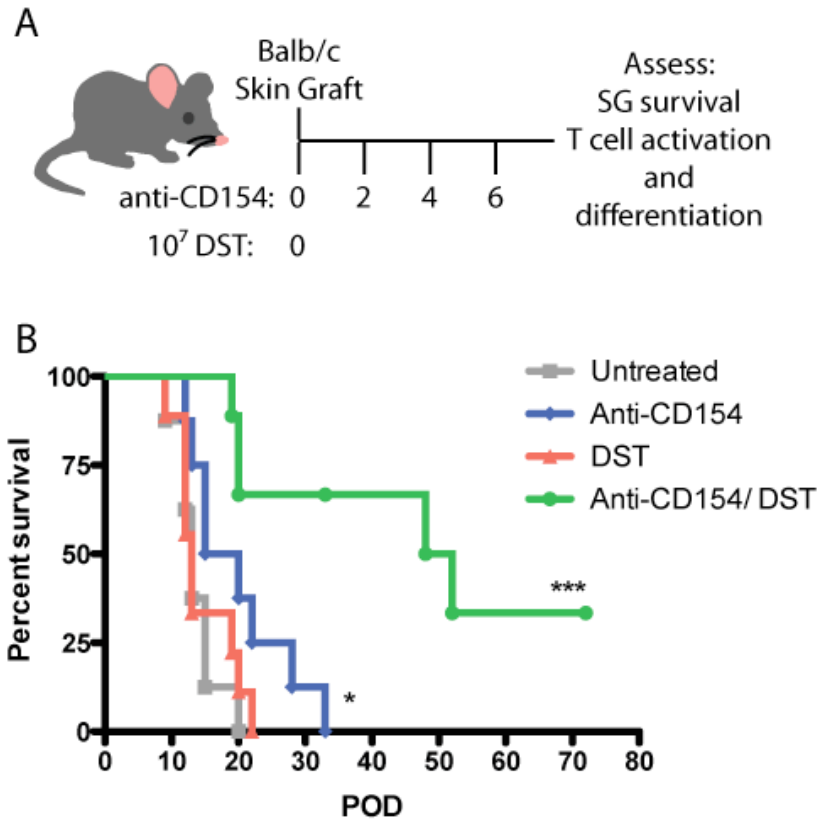


Figure 2.1. Anti-CD154 and DST synergize to protect allogeneic grafts from rejection. A. B6-Ly5.2/Cr mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c splenocytes (DST) and/ or anti-CD154 monoclonal antibody (500 μg on D0, 2, 4, 6), where indicated. B. Allo-skin grafts in untreated mice had an MST of 13 days. Monotherapy with either CD40/CD154 pathway blockade or DST led to rapid rejection of the allograft with MSTs of 17.5d ($p=0.039$) and 13d (p=n.s.), respectively. Anti-CD154 and DST combined treated significantly prolonged allograft survival to 50 days ($p=0.0002$). Data are summary of two experiments of 4-5 mice per group. *$p<0.05$, ***$p<0.001$.

Figure 2.2.

Anti-CD154 treatment alters recruitment of immune cells following transplantation.

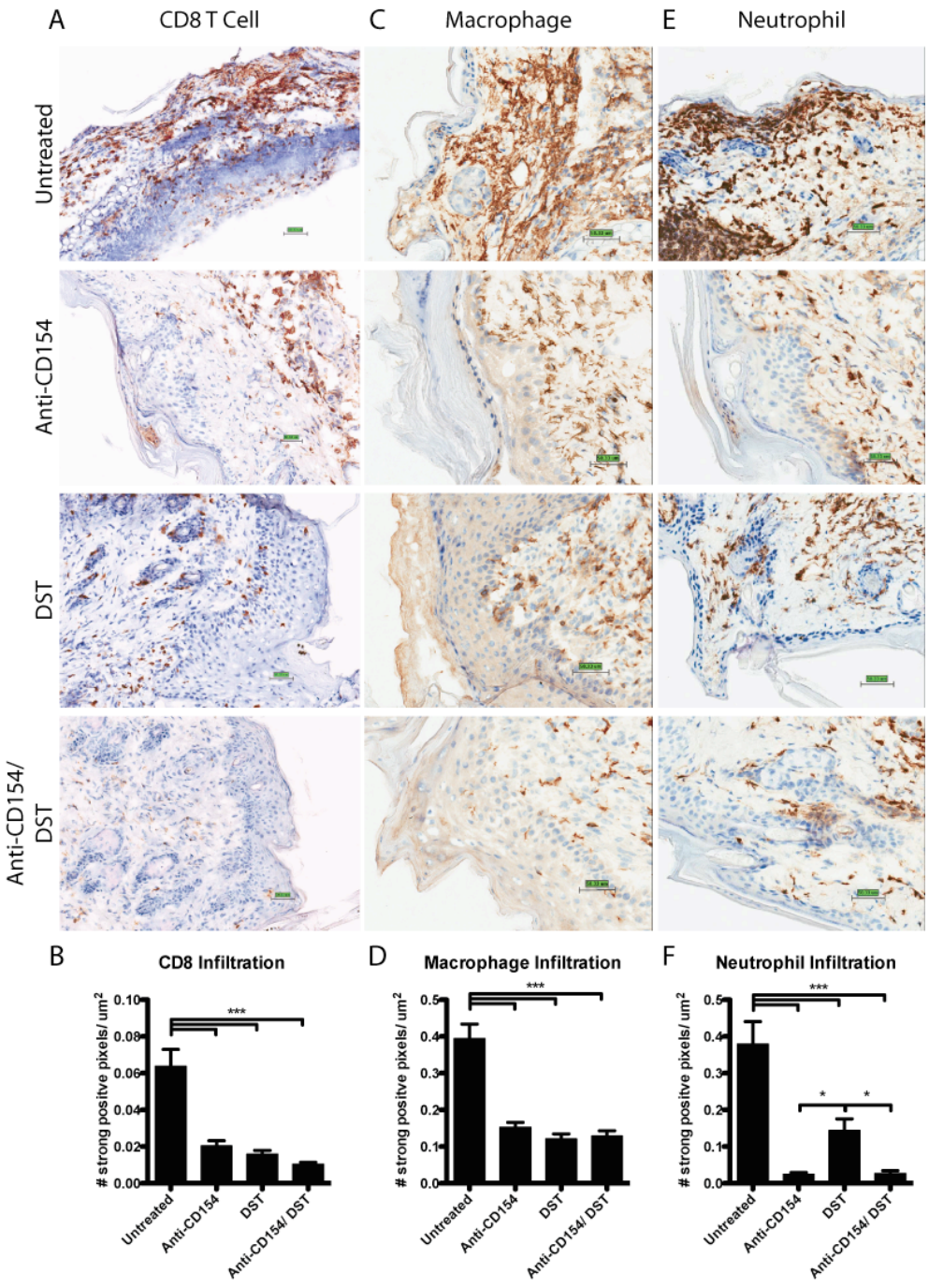


Figure 2.2. Anti-CD154 treatment alters recruitment of immune cells following transplantation. B6.SJL mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/ or anti-CD154 mAb, where indicated. Day 7 explanted allo-skin grafts were stained for CD8, A, F4/80, C, and Gr-1, E, to determine the level of $CD8^+$ T cell, macrophage, and neutrophil infiltration, respectively. Histological analysis of $CD8^+$ T cell, B, macrophage, D, and neutrophil, F, infiltration was digitally measured in 8-17 fields of epidermis and dermis. The ratio of total strongly positive pixels to total area was determined. Data are a summary of two experiments with three mice per group. $^{**}p<0.01$, $^{***}p<0.001$.

Figure 2.3.

CD154 blockade induces differential chemokine gradient and neutrophil recruitment to transplanted grafts.

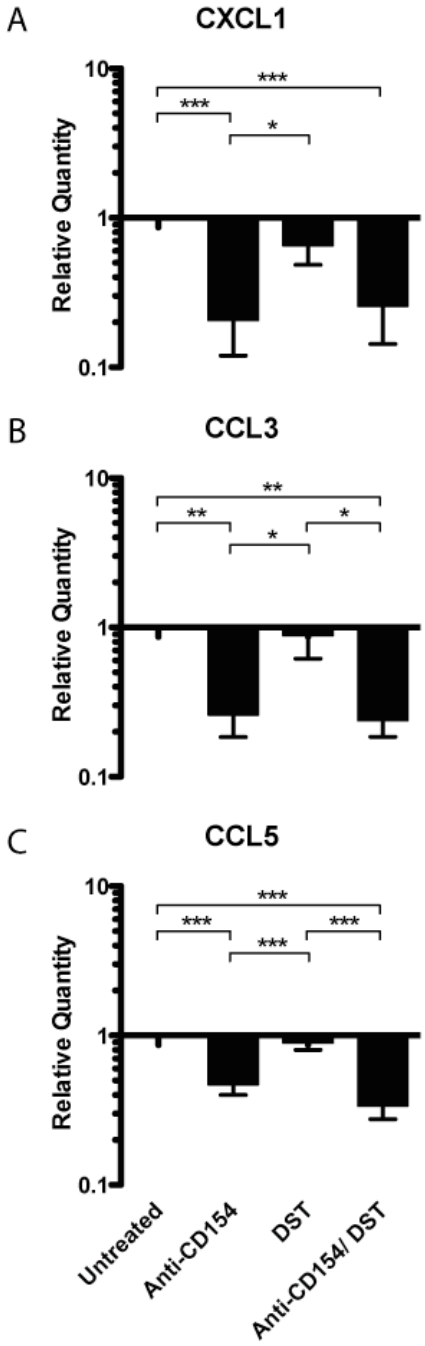


Figure 2.3. CD154 blockade induces differential chemokine gradient and neutrophil recruitment to transplanted grafts. B6.SJL mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/ or anti-CD154 mAb, where indicated. On day 7, skin grafts were explanted and processed for RNA extraction. Real time PCRs for chemokines CXCL1/ KC, CCL3/ MIP-1α, and CCL5/ RANTES were performed from cDNA. Data are summary of two experiments with three mice per group. *p<0.05, **p<0.01, ***p<0.0001.

Figure 2.4.

Anti-CD154 and DST independently alter the kinetics of expansion of activated CD44^{high} CD62L^{low} CD8^{+} T cells over time.

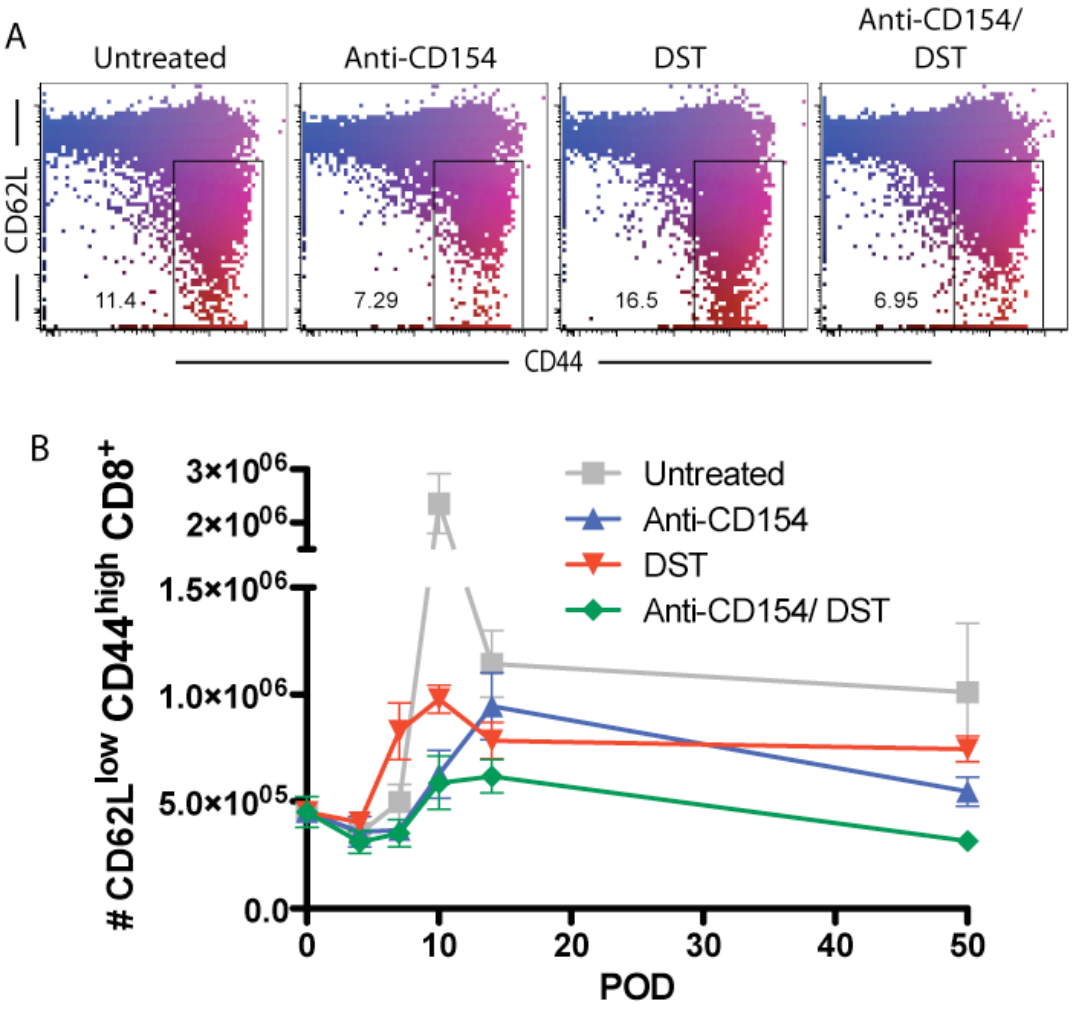


Figure 2.4. Anti-CD154 and DST independently alter the kinetics of expansion of activated CD44^{high} CD62L^{low} CD8^{+} T cells over time. B6-Ly5.2/Cr mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/or anti-CD154 mAb, where indicated. A. Representative flow plots of CD44^{high} and CD62L^{low} CD8^{+} T cells isolated from spleens of mice on day 7 post-transplantation. B. Expansion kinetics of activated CD8^{+} T cells after allo-transplantation. Data are summary of two experiments with three mice per group.

Figure 2.5.

Anti-CD154 and DST have distinct effects on the programmed differentiation of alloreactive CD8+ T cells into cytokine-producing effectors.

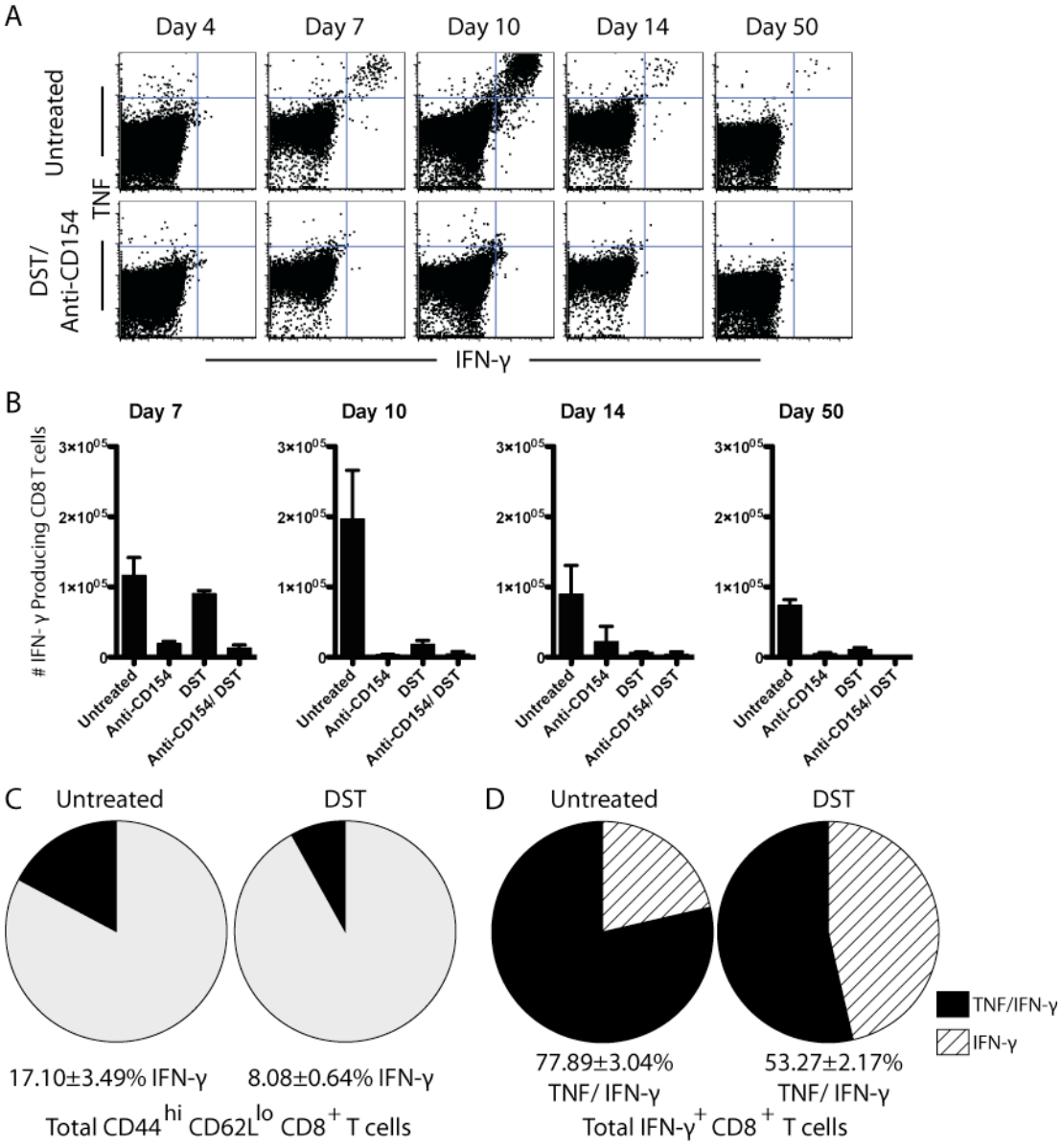


Figure 2.5. Anti-CD154 and DST have distinct effects on the programmed differentiation of alloreactive $CD8^+$ T cells into cytokine-producing effectors. B6-Ly5.2/Cr mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/ or anti-CD154 mAb, where indicated. A. Representative flow plots of TNF and IFN-γ producing $CD8^+$ T cells after *ex vivo* restimulation with BALB/c splenocytes, isolated from spleens of mice at day 7 post-transplantation. B. Absolute count of total IFN-γ producing $CD8^+$ T cells in the spleen over time following *ex vivo* restimulation. C. Pie charts represent total activated $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells. The black wedges represent the frequency of activated $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells that produce IFN-γ on day 7 post-transplantation ($p<0.05$). D. Pie charts represent all IFN-γ producing $CD8^+$ T cells. The striped wedges represent the IFN-γ-only producing population and black segments represent the TNF/IFN-γ double producing population in untreated vs. DST treated mice on day 7 ($p=0.0028$). Data are summary of two experiments with three mice per group.

Figure 2.6.

Anti-CD154 treatment alters the ratio of alloreactive CD8^{+} KLRG-1^{low} long-lived memory precursors to KLRG-1^{high} short-lived effectors.

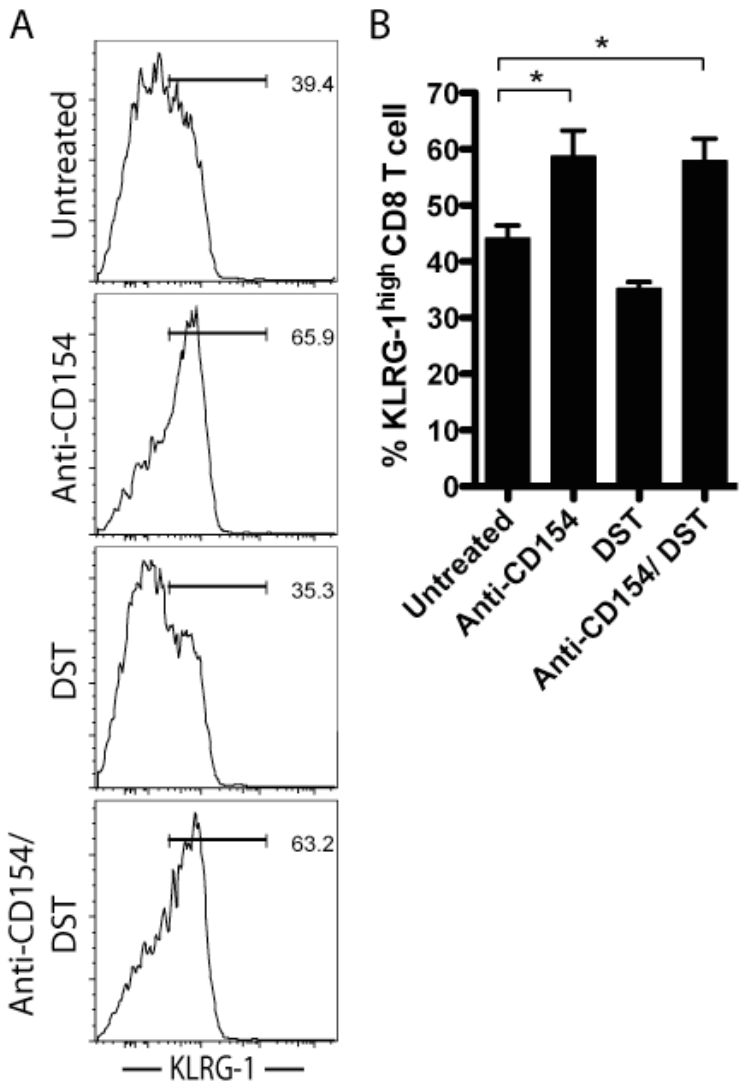


Figure 2.6. Anti-CD154 treatment alters the ratio of alloreactive CD8$^+$ KLRG-1^{low} long-lived memory precursors to KLRG-1^{high} short-lived effectors. B6-Ly5.2/Cr mice were transplanted with BALB/c skin grafts and were treated with 10^7 BALB/c DST and/ or anti-CD154 mAb, where indicated. A. Representative flow plots of KLRG-1 expression on antigen experienced CD44^{high} CD62L^{low} CD8$^+$ T cells at day 7 post-transplantation. B. Frequency of KLRG-1^{high} antigen experienced CD44^{high} CD62L^{low} CD8$^+$ T cells on day 7. Data are summary of two experiments with three mice per group.

REFERENCES

1. Halloran PF. 2004. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 351: 2715-29

2. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Jr., Knechtle SJ. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* 94: 8789-94

3. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381: 434-8

4. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, Mordes JP, Rossini AA. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* 92: 9560-4

5. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114

6. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA. 1998. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA4. *J Clin Invest* 101: 2446-55

7. Zheng XX, Markees TG, Hancock WW, Li Y, Greiner DL, Li XC, Mordes JP, Sayegh MH, Rossini AA, Strom TB. 1999. CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J Immunol* 162: 4983-90

8. Phillips NE, Markees TG, Mordes JP, Greiner DL, Rossini AA. 2003. Blockade of CD40-mediated signaling is sufficient for inducing islet but not skin transplantation tolerance. *J Immunol* 170: 3015-23

9. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc Natl Acad Sci U S A* 93: 13967-72

10. Pearl JP, Xu H, Leopardi F, Preston E, Kirk AD. 2007. CD154 blockade, sirolimus, and donor-specific transfusion prevents renal allograft rejection in cynomolgus monkeys despite homeostatic T-cell activation. *Transplantation* 83: 1219-25

11. Preston EH, Xu H, Dhanireddy KK, Pearl JP, Leopardi FV, Starost MF, Hale DA, Kirk AD. 2005. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. *Am J Transplant* 5: 1032-41

12. Quezada SA, Fuller B, Jarvinen LZ, Gonzalez M, Blazar BR, Rudensky AY, Strom TB, Noelle RJ. 2003. Mechanisms of donor-specific transfusion

tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation. *Blood* 102: 1920-6

13. Margenthaler JA, Kataoka M, Flye MW. 2003. Donor-specific antigen transfusion-mediated skin-graft tolerance results from the peripheral deletion of donor-reactive CD8+ T cells. *Transplantation* 75: 2119-27

14. van Maurik A, Fazekas de St Groth B, Wood KJ, Jones ND. 2004. Dependency of direct pathway CD4+ T cells on CD40-CD154 costimulation is determined by nature and microenvironment of primary contact with alloantigen. *J Immunol* 172: 2163-70

15. El-Sawy T, Belperio JA, Strieter RM, Remick DG, Fairchild RL. 2005. Inhibition of polymorphonuclear leukocyte-mediated graft damage synergizes with short-term costimulatory blockade to prevent cardiac allograft rejection. *Circulation* 112: 320-31

16. Kondo T, Novick AC, Toma H, Fairchild RL. 1996. Induction of chemokine gene expression during allogeneic skin graft rejection. *Transplantation* 61: 1750-7

17. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-4

18. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. 2008. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205: 625-40

19. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27: 281-95

20. Rollins BJ. 1997. Chemokines. *Blood* 90: 909-28

21. Li G, Sanders JM, Bevard MH, Sun Z, Chumley JW, Galkina EV, Ley K, Sarembock IJ. 2008. CD40 ligand promotes Mac-1 expression, leukocyte recruitment, and neointima formation after vascular injury. *Am J Pathol* 172: 1141-52

22. Shen X, Reng F, Gao F, Uchida Y, Busuttil RW, Kupiec-Weglinski JW, Zhai Y. 2010. Alloimmune activation enhances innate tissue inflammation/injury in a mouse model of liver ischemia/reperfusion injury. *Am J Transplant* 10: 1729-37

23. Lukacs NW, Kunkel SL, Burdick MD, Strieter RM. 1993. The production of chemotactic cytokines in an allogeneic response. The role of intercellular adhesion molecule-1 and lymphocyte function-associated antigen-3. *Am J Pathol* 143: 1179-88

24. Fahey TJ, 3rd, Tracey KJ, Tekamp-Olson P, Cousens LS, Jones WG, Shires GT, Cerami A, Sherry B. 1992. Macrophage inflammatory protein 1 modulates macrophage function. *J Immunol* 148: 2764-9

25. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478-80

26. Ridge JP, Di Rosa F, Matzinger P. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-8

27. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-3

28. Watarai Y, Koga S, Paolone DR, Engeman TM, Tannenbaum C, Hamilton TA, Fairchild RL. 2000. Intraallograft chemokine RNA and protein during rejection of MHC-matched/multiple minor histocompatibility-disparate skin grafts. *J Immunol* 164: 6027-33

29. Koga S, Novick AC, Toma H, Fairchild RL. 1999. CD8+ T cells produce RANTES during acute rejection of murine allogeneic skin grafts. *Transplantation* 67: 854-64

30. Janeway CA, Rosen FS. 1966. The gamma globulins. IV. Therapeutic uses of gamma globulin. *N Engl J Med* 275: 826-31

31. Floyd TL, Koehn BH, Kitchens WH, Robertson JM, Cheeseman JA, Stempora L, Larsen CP, Ford ML. 2011. Limiting the amount and duration of antigen exposure during priming increases memory T cell requirement for costimulation during recall. *J Immunol* 186: 2033-41

32. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77: 4911-27

33. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA, Robertson JM, Leopardi FV, Strobert EA, Iwakoshi NN, Reimann KA, Ford ML, Kirk AD, Larsen CP. 2011. Nondepleting Anti-CD40-Based Therapy Prolongs Allograft Survival in Nonhuman Primates. *Am J Transplant*

Chapter 3

Antigen-Specific Induced Foxp3$^+$ Regulatory T Cells Are Generated Following CD40/CD154 Blockade

In Press in Proceedings of National Academy of Sciences, 2011

ABSTRACT

Blockade of the CD40/CD154 pathway potently attenuates T cell responses in models of autoimmunity, inflammation, and transplantation. Indeed, CD40 pathway blockade remains one of the most powerful methods of prolonging graft survival in models of transplantation. Despite this effectiveness, the cellular and molecular mechanisms underlying the protective effects of CD40 pathway blockade are incompletely understood. Furthermore, the relative contributions of deletion, anergy and regulation have not been measured in a model in which donor-reactive $CD4^+$ and $CD8^+$ T cell responses can be assessed simultaneously.

To investigate the impact of CD40/CD154 pathway blockade on graft-specific T cell responses, a transgenic mouse model was used wherein recipients containing OVA-specific $CD4^+$ and $CD8^+$ TCR transgenic T cells were grafted with skin expressing OVA in the presence or absence of anti-CD154 and donor-specific transfusion. Results indicated that CD154 blockade altered the kinetics of donor-reactive $CD8^+$ T cell expansion, delaying differentiation into IFN-γ^+ TNF^+ multi-functional cytokine producers. The eventual differentiation of cytokine-producing effectors in tolerant animals coincided with the emergence of an antigen-specific $CD4^+$ $CD25^{hi}$ $Foxp3^+$ T cell population, which arose not from endogenous natural T_{reg}, but were peripherally generated from naïve $Foxp3^-$ precursors.

INTRODUCTION

Blockade of the CD40/CD154 pathway has long been appreciated as a potent means of inhibiting alloreactive T cell responses and prolonging graft survival. Despite this pronounced effect on allograft survival in both murine and non-human primate models (1-3), clinical trials using anti-CD154 monoclonal antibodies (mAb) in transplantation were halted due to thromboembolic complications, likely as a result of the expression of CD154 on platelets (4, 5). Because recent studies using anti-CD40 mAbs in both murine and non-human primate models demonstrated comparable efficacy to CD154 blockade, however, significant interest in the pathway remains (6-9).

Early studies by Parker *et al.* described the treatment combination of anti-CD154 and donor-specific transfusion (DST) for the induction of immune tolerance in transplantation (3). Several groups have since shown the potent effects of this combined therapy in the prolongation of islet (3, 10, 11), cardiac (12), skin (10, 13), and kidney (14, 15) allograft survival in murine and nonhuman primate models. Although costimulation blockade has been associated with T cell anergy (16) or deletion (17, 18), elucidation of the precise independent and synergistic effects of DST and anti-CD154 on the kinetics of donor-reactive $CD4^+$ and $CD8^+$ effector T cell expansion has been limited due to our inability to identify and track graft-specific T cells in fully allogeneic models.

Importantly, studies by Taylor *et al.* demonstrated that $CD4^+$ $CD25^+$ T_{reg} are required for tolerance induced via CD40/CD154 pathway blockade in a graft-versus-host model (19). Although this report revealed a requirement for T_{reg} during

tolerance induction, it is not known whether the T_{reg} required are pre-existing, thymically-derived T_{reg}, or if CD40/CD154 blockade induces the generation of peripherally-elicited T_{reg}. Furthermore, the temporal relationship between the *in vivo* accumulation of T_{reg} and deletion of graft-specific effectors following CD40/CD154 blockade has not been investigated.

To address these questions, we used an OVA-expressing transgenic mouse model (20), which allowed us to directly track both donor-reactive $CD4^+$ and $CD8^+$ T cell responses simultaneously over time. While previous studies have used transgenic models to assess the degree of T cell deletion following CD154/CD40 blockade (16, 21), none have systematically interrogated its impact on the kinetics of both $CD4^+$ and $CD8^+$ T cell expansion and acquisition of effector function. Our results indicated that while CD40/CD154 blockade alone delayed donor-reactive $CD4^+$ and $CD8^+$ T cell expansion and differentiation, the addition of DST was required for antigen-specific T cell deletion. Importantly, anti-CD154 combined with DST also led to peripheral conversion of $Foxp3^-$ donor-reactive $CD4^+$ T cells into $Foxp3^+CD25^+$ donor-specific T_{reg}.

MATERIALS AND METHODS

Mice

B6-Ly5.2/Cr (H2-K^b, CD45.1) and C57BL/6 (H2-K^b, CD45.2) mice were obtained from the National Cancer Institute (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 (20) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained in accordance with Emory University Institutional Animal Case and Use Committee guidelines (Atlanta, GA). OT-II x RAG$^{-/-}$ transgenic mice were purchased from Taconic Farms (Germantown, NY). All animals were housed in pathogen-free animal facilities at Emory University.

Donor Specific Transfusion and Adoptive Transfers

For DST administration, mOVA spleens were processed into single cell suspensions, and 10^7 splenocytes were given prior to transplantation. For adoptive transfers of donor-reactive T cells, spleen and mesenteric LNs of OT-I and OT-II mice were processed and stained with monoclonal antibodies for CD4 and CD8 (both from Invitrogen), Thy1.1 and Vα2 (BD Pharmingen) for flow cytometry analysis. Cells were resuspended in PBS and $1.5x10^6$ of each Thy1.1$^+$ OT-I and OT-II were injected i.v. In some experiments CD45.2$^+$ OT-II x RAG$^{-/-}$ cells were adoptively transferred into CD45.1$^+$ recipients.

Skin Transplantation and Antibody Treatment

Full thickness tail and ear skins were transplanted onto dorsal thorax of recipient mice and secured with adhesive bandages. Where indicated, mice were treated with 500μg hamster monoclonal anti-mouse CD154 (MR-1, BioExpress, West Lebanon, NJ) on days 0, 2, 4, and 6 post transplantation.

Surface Stains and Flow Cytometry

Spleens or draining axillary and brachial LNs were stained for CD4 and CD8 (both from Invitrogen); Thy1.1, CD44 and CD62L (all from BD Pharmingen); and KLRG-1 (Southern Biotech). Samples were analyzed using a multicolor LSRII FACS machine (BD Biosciences). Data was analyzed using FlowJo software (Treestar, San Carlos, CA).

Intracellular Cytokine Staining

Responder splenocytes were stimulated with 10nM $OVA_{257-264}$ (SIINFEKL, Emory University Microchemical Core Facility) and 10μM $OVA_{323-339}$ peptides in the presence of 10μg/mL Brefeldin A for 4 hours. Intracellular staining kit was used to detect TNF and IFN-γ (all from BD Pharmingen), and IL-2 (eBiosciences), according to manufacturer's instructions.

In vivo *Cytotoxicity Assay*

C57BL/6 (CD45.2) splenocytes were split into two populations and stained with either 2.5μM or 0.25μM of CFSE. Cells either remained unpulsed or were pulsed

with 1μM SIINFEKL peptide, respectively. Once mixed at equal ratios ($5x10^5$ cells of each), cells were injected i.v. into experimental mice (CD45.1). At 18hr post injection, lysis of target cells was assessed, as previously described (22).

Histology

Skin grafts were removed and frozen in cryomolds with OCT Embedding Compound (Tissue-Tek, Hatfield, PA) on day 7 post transplantation. Longitudinal sections of grafts were cut 5 μm thick with a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost Plus microscope slides and fixed with 100% acetone. Anti-mouse Foxp3 (eBiosciences) was used for Foxp3 immunohistochemical detection by 3,3 diaminobenzidine (DAB) peroxidation and counterstained with haematoxylin. A Zeiss LSM 510 META point scanning laser confocal microscope was used for immunofluorescent staining visualization. $Foxp3^+$ cells were quantified by counting positive cells in ten high-power fields (HPF, x400 magnification).

T_{reg} Staining

Splenocytes and draining lymph nodes were processed and stained with antibodies to CD4 and CD8 (both from Invitrogen); Thy1.1 or CD45.2, Vα2 and CD25 (all from BD Pharmingen). Intracellular staining with anti-Foxp3 was performed using intranuclear staining kit (eBiosciences), according to manufacturer's instructions.

Statistical Analysis

Survival data were plotted on Kaplan-Meier curves and log-rank tests were performed. For longitudinal analysis of T cells, two-way ANOVA tests were performed, followed by Bonferroni post-test on significant results. For single timepoint analysis of T cell accumulation and phenotypes (MFI), 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).

RESULTS

Combined DST/CD154 blockade prolongs skin graft survival and prevents cellular infiltration

Although many groups have studied the impact of CD40 pathway blockade on graft survival and T cell responses (16, 19, 21, 23), the precise mechanism by which inhibition of CD40 mediated signals attenuates donor-reactive T cell responses remains incompletely understood. In fully allogeneic models, anti-CD154 mAbs combined with DST has been shown to significantly delay rejection, but the ability to track alloreactive T cells is dependent on cytokine production. This limits the ability to study donor-specific cells that may have become non-responsive due to the absence of CD40/CD154-mediated signals. Therefore, to assess the contribution of T cell non-responsiveness to the protective effect of CD154 blockade on skin graft (SG) survival, naïve donor-reactive OT-I and OT-II T cells were adoptively transferred into naïve recipients that were then grafted with OVA-expressing skin (Fig. 1A). Resulting SG survival in this transgenic model reflected those previously observed in allogeneic systems (13). Untreated mice rapidly rejected SGs with a median survival time (MST) of 13.5 days, while treatment with anti-CD154/DST significantly prolonged survival to over 100 days (Fig. 1B).

CD154 blockade/DST inhibits antigen-specific $CD8^+$ T cell expansion

To identify the relative contributions of T cell deletion vs. anergy/exhaustion, the kinetics of donor-reactive $CD8^+$ T cell expansion and contraction were measured over time following transplantation and treatment with anti-CD154/DST on days 0,

2, 4, and 6 post-transplant. Spleens of untreated mice developed a donor-reactive T cell response that peaked on days 7 to 10. CD154 blockade led to a significant delay (day 10) in the donor-reactive T cell response, while this response was significantly accelerated (day 4) in DST treated animals. Finally, combined anti-CD154/DST treatment led to minimal expansion of donor-reactive T cells over background on day 4 (Fig. 2A, B). These effects of CD154 blockade on donor-reactive T cell expansion kinetics were further confirmed in the axillary and brachial draining lymph nodes (LNs) (Sup. Fig. 1A, B).

To determine whether CD154/CD40 pathway blockade limits antigen-specific T cell differentiation, we analyzed markers of activation (CD44 and CD62L) and a marker of short-lived effector lineage (KLRG-1) (24) in each treatment group on day 7 post-transplantation. We observed that CD154 blockade, both in the presence and absence of DST, decreased the frequencies of $CD44^{high}$ and $CD62L^{low}$ antigen-specific $CD8^+$ T cells and increased the frequency of $KLRG\text{-}1^{high}$ antigen-specific $CD8^+$ T cells compared with untreated controls (Fig. 2C).

CD154 blockade/DST prevents antigen-specific $CD8^+$ T cell cytokine production and cytolytic potential

Although increased donor-reactive T cell expansion is an important indicator of potential allograft rejection, it does not speak to the functionality of these cells following transplantation. We therefore interrogated the role of the CD40/CD154 pathway in inducing the differentiation of competent TNF and IFN-γ cytokine-producing effectors following transplantation (Fig. 3A). We observed a minimum

threshold of 10^5 graft-specific cytokine secreting cells following transplantation in all the groups that underwent rejection. This threshold number of cells was observed on days 7 and 10 for untreated animals (Fig. 3B). DST accelerated the emergence of dual cytokine producing cells to day 4, while anti-CD154 delayed the differentiation of these cells until day 10 post-transplantation (Fig. 3B). Only the DST/anti-CD154 group (which did not undergo rejection) failed to achieve this threshold number of cytokine producing cells at any time-point (Fig. 3B).

To further investigate the impact of CD154 blockade on T cell differentiation, *in vivo* cytotoxicity assays were performed. Anti-CD154 treatment did not attenuate the antigen-specific cytolysis of target cells relative to untreated controls. However, DST monotherapy significantly reduced cytolysis of target cells on day 7, perhaps due to the rapid contraction of antigen-specific T cells at this timepoint. Consistent with the reduced expansion and cytokine production by donor-reactive T cells, anti-CD154/DST treatment significantly impaired cytolysis of target cells (Fig. 3C). Together, these data show that while CD154/CD40 pathway blockade delayed the accumulation of donor-reactive T cells, it did not prevent killing of donor-derived targets.

CD154 blockade reduces antigen-specific $CD4^+$ T cell accumulation and promotes migration of $Foxp3^+$ T_{reg} to the graft

To assess the availability of $CD4^+$ T cell help in this model, we analyzed the effect of CD154 blockade on donor-specific helper T cell activation. Donor-reactive $CD4^+$ T cells expanded with similar kinetics as $CD8^+$ T cells over time. Treatment

with either anti-CD154 or DST led to a substantially reduced accumulation of donor-reactive $CD4^+$ T cells on day 7 compared to untreated controls, but anti-CD154/DST treatment significantly impaired donor-reactive helper T cell responses (Fig. 4A, B).

Because T_{reg} have been shown to promote graft survival in several models (25), we assessed whether CD154 blockade resulted in the expansion of T_{reg} *in vivo*. Results revealed no gross change in the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in mice treated with anti-CD154 (Fig. 4C). We next asked whether anti-CD154 altered the ability of T_{reg} to accumulate in the graft (Fig. 4D). Immunohistochemical analysis of $Foxp3^+$ cells in SGs at day 7 revealed that anti-CD154/DST treatment led to a dramatic increase in T_{reg} infiltration compared to untreated controls.

Anti-CD154/DST promotes conversion of donor-reactive $CD4^+$ T cells into $CD25^+$ $Foxp3^+$ iT_{reg}

While no increase in total T_{reg} was observed in the draining LNs following CD40/CD154 blockade, the increased T_{reg} infiltration in SGs of these animals led us to hypothesize that a graft-specific T_{reg} population may be emerging in the context of CD40/CD154 blockade, but may be undetectable within the larger pool of non-graft-reactive T_{reg}. To test this, we analyzed donor-reactive $Thy1.1^+$ $CD4^+$ T cells in the LNs for Foxp3 expression. Adoptively transferred naïve OT-II T cells, prior to transplantation, did not express Foxp3 (Fig. 5A, top panel), while donor-reactive $CD4^+$ T cells in LNs of untreated mice developed a small population of Foxp3-expressing cells compared to naïve controls. At early time points, neither anti-CD154 nor DST alone significantly increased the frequency of $Foxp3^+$ cells among

donor-reactive $CD4^+$ T cells, compared to untreated controls. However, CD154 blockade resulted in a modest increase in the frequency of donor-specific $Foxp3^+$ $CD25^+$ $CD4^+$ T_{reg} at day 14. Interestingly, the combination of anti-CD154/DST treatment resulted in a significant increase in the frequency of graft-specific $Foxp3^+$ $CD25^+$ $CD4^+$ T_{reg} *in vivo* by day 7 (Fig. 5A, B). Because low frequencies of endogenous T_{reg} have been reported in RAG-sufficient OT-II T cells (26), we next asked whether the observed increase in accumulated graft-specific $Foxp3^+$ $CD25^+$ $CD4^+$ T cells was due to expansion of a small population of $Foxp3^+$ OT-II nT_{reg} that existed in the transferred cell preparation, or due to peripheral conversion of antigen-specific $CD4^+$ T cells into iT_{reg} following CD40/CD154 blockade. Therefore, we adoptively transferred $1.5x10^6$ $Thy1.1^+$ OT-I and $1.5x10^6$ RAG-deficient $CD45.2^+$ OT-II T cells, which lack Foxp3-expressing cells (26), into $CD45.1^+$ recipients. Mice received a mOVA skin graft and either remained untreated or were treated with combined anti-CD154/DST. On day 7 post-transplant, draining LNs were analyzed for the presence of antigen-specific $Foxp3^+$ $CD4^+$ T cells. Results indicated that anti-CD154/DST significantly induced the conversion of donor-reactive $CD4^+$ T cells into graft-specific $Foxp3^+$ $CD25^+$ iT_{reg} compared to untreated controls (Fig. 5C, D). Taken together, these data indicated that blockade of the CD40 pathway in the presence of DST resulted in the peripheral conversion of antigen-specific $CD4^+$ T cells into induced T_{reg}.

The ratio of T_{reg} to antigen-specific effector T cells has been previously identified as a predictor of the potential protective effects of T_{reg} (27). We therefore compared the relative level of accumulated donor-reactive $CD8^+$ T cells to that of donor-

specific Foxp3$^+$ CD25$^+$ CD4$^+$ T cells. As described above, untreated recipients substantially expanded donor-reactive CD8$^+$ T cells, while generating negligible levels of antigen-specific Foxp3$^+$ CD4$^+$ T cells (Fig. 5E, left panel). Conversely, anti-CD154/DST treatment dramatically increased the ratio of graft-specific Foxp3$^+$ CD4$^+$ T cells to donor-reactive CD8$^+$ effectors in the draining LNs over time (Fig. 5E, right panel).

DISCUSSION

In this study, we have elucidated the effects of CD40/CD154 pathway blockade on donor-reactive $CD4^+$ and $CD8^+$ T cell responses. From these data, we concluded that treatment with either DST or anti-CD154 resulted in mechanistically distinct modes of graft protection. Anti-CD154 treatment delayed the expansion and differentiation of donor-reactive $CD8^+$ T cells into multi-functional cytokine producing cells. Furthermore, CD154 blockade led to late conversion of donor-reactive $Foxp3^-$ $CD4^+$ T cells into $Foxp3^+$ iT_{reg}. This effect was observed in both RAG-sufficient and RAG-deficient antigen-specific T cells, in which it is well-established that no $FoxP3^+$ nT_{reg} exist (26). While prior studies have shown a role for regulation in the tolerance induced via DST/anti-CD154 (19, 28, 29), here we show that the mechanism underlying the observed increase in $Foxp3^+$ T_{reg} following exposure to DST/anti-CD154 is conversion of antigen-specific naïve T cell precursors into $Foxp3^+$ cells. We speculate that the conversion of naïve/effector $CD4^+$ T cells into iT_{reg} requires the presence of antigen, which is provided much earlier in the setting of DST than in mice treated with anti-CD154 monotherapy. Conversely, DST led to early expansion but abortive activation of donor-reactive $CD8^+$ T cells, with rapid contraction that likely contributed to decreased ability to lyse target cells by day 10. However, antigen-specific $Foxp3^+$ $CD25^+$ iT_{reg} were not induced following DST treatment in the absence of CD154 blockade. Thus, we conclude that this degree of abortive activation alone was insufficient to protect grafts from rejection. Only the combination of abortive activation and the early emergence of peripherally-induced iT_{reg} was able to sufficiently attenuate donor-reactive effector T cell responses and

prolong graft survival. These data suggest that an early increase in the ratio of T_{reg} to effector T cells may underlie the potent protective effects of anti-CD154/DST combined therapy.

What is the mechanism by which interruption of CD40/CD154 mediated signals induces the expression of Foxp3? Our favored hypothesis is that inhibition of CD40 signaling conditions APC or subsets of APC such that synaptic contact with antigen-specific T cells instructs them to become regulatory cells rather than activated effectors. This hypothesis is based on work demonstrating peripheral generation of $Foxp3^+$ T_{reg} following exposure to tolerogenic plasmacytoid DC (28). The specific cell surface or soluble mediators which function to regulate iT_{reg} conversion is an important area of future research, however, we predict that DC in which CD40 signaling is inhibited fail to present costimulatory molecules or secrete inflammatory cytokines in order to instruct $CD4^+$ T cells to differentiate into T_{reg}. For example, IL-6 has been shown to potently inhibit TGF-β mediated T_{reg} differentiation (30), and a recent study demonstrated that loss of CD40 signaling rendered DC deficient in IL-6 production (31). Alternatively, it is possible that anti-CD154 functions to inhibit CD40 signaling on another cell type. In particular, $CD8^+$ T cells have been shown to express CD40 and are capable of secreting inflammatory cytokines that might also inhibit iT_{reg} conversion (32, 33). Furthermore, peripheral conversion of naïve $CD4^+$ T cells into $Foxp3^+$ T_{reg} has also been observed following interruption or attenuation of TCR mediated signals. In particular, von Boehmer's group observed the conversion of $Foxp3^-$ $CD4^+$ T cells into $Foxp3^+$ T cells following exposure to low-dose antigen (34), and conversely iT_{reg} generation was inhibited by

high TCR stimulation (35). Peripheral generation of Foxp3$^+$ T cells has also been demonstrated following resolution of an acute systemic autoimmune disease (36). In addition, upon *in vivo* treatment of anti-CD4 mAb during transplantation, extra-thymic development of CD25^{hi} T_{reg} from CD25$^-$ precursors was observed (37). Thus, there are likely many mechanisms by which naïve CD4$^+$ T cells may be instructed to become Foxp3$^+$ regulatory cells in the periphery. Here, we demonstrate that *in vivo* blockade of the CD40/CD154 pathway is one of those mechanisms, and show that early emergence of these iT_{reg} correlates with graft survival.

Our data also indicate that a primary effect of CD154/CD40 blockade is to delay CD8$^+$ T cell expansion and differentiation. Previous reports have examined donor-reactive T cells at a single time point post-transplantation and have concluded that anti-CD154 mAb leads to deletion of CD4$^+$ and CD8$^+$ T cell responses (16, 21). However, analysis of the kinetics of the T cell response in the current study revealed that CD154/CD40 pathway blockade alone delayed, rather than deleted, antigen-specific CD4$^+$ and CD8$^+$ T cell responses, as both responses in anti-CD154 treated animals eventually reached the same magnitude as untreated controls. Since the half-life of anti-CD154 mAb (MR-1) is approximately 3 weeks *in vivo* and the last dose was given on day 6 post-transplant, we would not expect a significant drop in circulating blocking antibody during the time course studied (days 0-14). This indicates that these findings are not the result of an emerging response as the CD154 blockade wanes, but rather a "breakthrough" response, where graft-reactive CD8$^+$ T cells are delayed in their differentiation, but eventually develop into fully competent effectors even in the presence of blocking antibody. Conversely, our

analysis revealed that DST accelerated kinetics of donor-reactive $CD4^+$ and $CD8^+$ T cell expansion, a finding that is corroborated by previous studies showing that, at a single time point, donor-reactive T cells isolated from mice treated with DST exhibited increased CD44 expression, indicative of a more activated phenotype (21). Taken together, our analysis of the kinetics and magnitude of donor-reactive T cell responses suggest a mechanism in which T cells are strongly driven into division in the presence of DST, but are induced to delay differentiation in the presence of anti-CD154. In the presence of both reagents, the resulting "push and pull" results in donor-reactive T cell deletion or conversion to regulatory T cells. A similar finding is also observed in the setting of low donor-reactive T cell precursor frequency, in which minimal competition for antigen may provide increased access to APCs, cells are thus driven to divide earlier and more robustly (38-41). Through the provision of an increased source of antigen, DST may similarly decrease the T cell:APC ratio, thereby stimulating increased division. In both systems, robust division in the absence of costimulation leads to eventual deletion (40).

The use of congenically marked TCR transgenic cells of a defined specificity in this study was advantageous in that we were able to both identify populations of graft-specific T cells without relying on effector function for detection, and definitively address the issue of iT_{reg} conversion vs. expansion of T_{reg} from a pre-existing small population of $FoxP3^+$ T_{reg}. However, a potential caveat of this study is that it focused on the behavior of a monoclonal T cell population responding to a surrogate minor antigen, and thus it is possible that this system may not be fully comparable to a polyclonal T cell response to allogeneic tissues. Work to identify

polyclonal graft-specific T cells during transplantation in order to confirm these findings in a non-TCR transgenic system is underway.

Thus, in this manuscript, we conclude that the mechanism by which DST and anti-CD154 blockade synergize in order to protect the graft is through both the abortive response of donor-reactive $CD8^+$ T cells that have been driven into unsupported division and conversion of antigen-specific $CD4^+$ T cells into iT_{reg} following CD154 blockade. While the use of intact FcR-binding anti-CD154 is not a clinically viable strategy due to the potential for thromboembolic complications, blockade of this pathway remains the single most effective method of inducing transplantation tolerance in experimental models. Thus, studying the mechanisms by which it induces robust tolerance remains an important question for the field, with the hopes of developing alternate methods to inhibit CD154. While recent studies have used an RNAi approach to inhibit CD40 expression (42, 43), a similar approach could be envisaged for CD154 inhibition. Alternatively, non-cross-linking mAbs could be developed to antagonize CD154, much like nonactivating single chain F_V-based reagents have been developed in lieu of cross-linking anti-CD28 mAbs (44), which had resulted in a much more severe side-effect profile than anti-CD154 mAbs in pilot studies in humans (45). Thus, further elucidation of the mechanisms underlying the potency of CD154 blockade in inducing transplantation tolerance may allow for the identification of critical cellular and molecular pathways required for the establishment and maintenance of transplantation tolerance.

Figure 3.1.

CD154 blockade and DST prolongs skin graft survival.

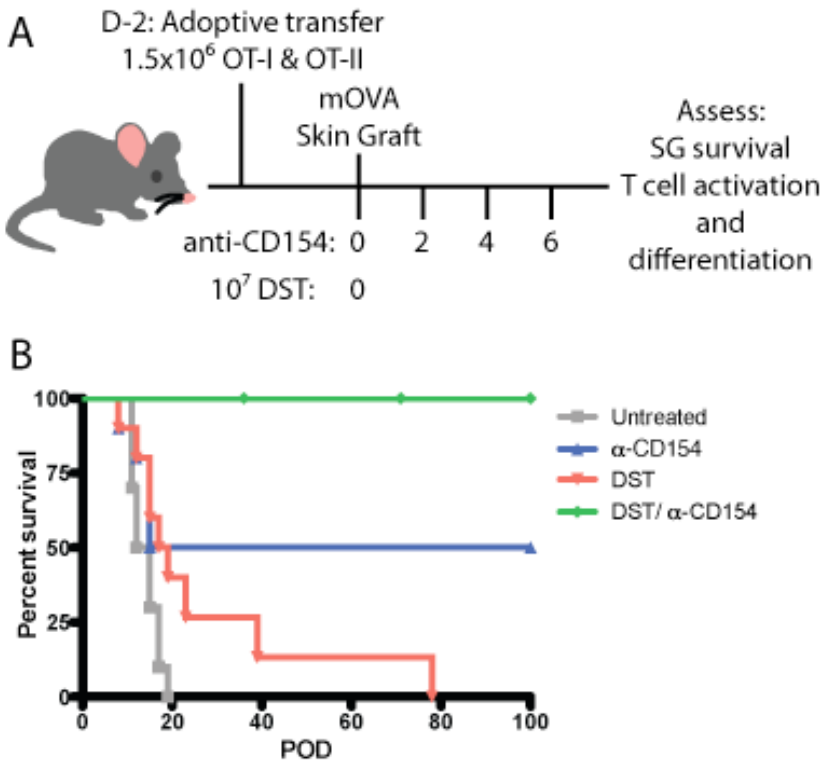


Figure 3.1. CD154 blockade and DST prolongs skin graft survival. A. Mice were adoptively transferred with $1.5x10^6$ of each OT-I and OT-II T cells two days prior to transplant. On day 0, mice were transplanted with mOVA skin graft and were treated with 10^7 mOVA DST and/or 500μg MR-1 where indicated. B. Untreated mice rejected SGs with an MST of 13.5 days. Anti-CD154 monotherapy led to bimodal survival of SGs with 50% of mice rejecting grafts with an MST of 15 days and 50% demonstrating indefinite survival ($p=0.0027$). DST monotherapy resulted in to an MST of 18 days ($p=0.031$), while combined anti-CD154/DST led to an indefinite survival of SGs ($p<0.0001$). Data are cumulative of two independent experiments with five mice per group.

Figure 3.2.

CD154 blockade delays expansion of antigen-specific CD8⁺ T cells but does not alter activation status at peak of response.

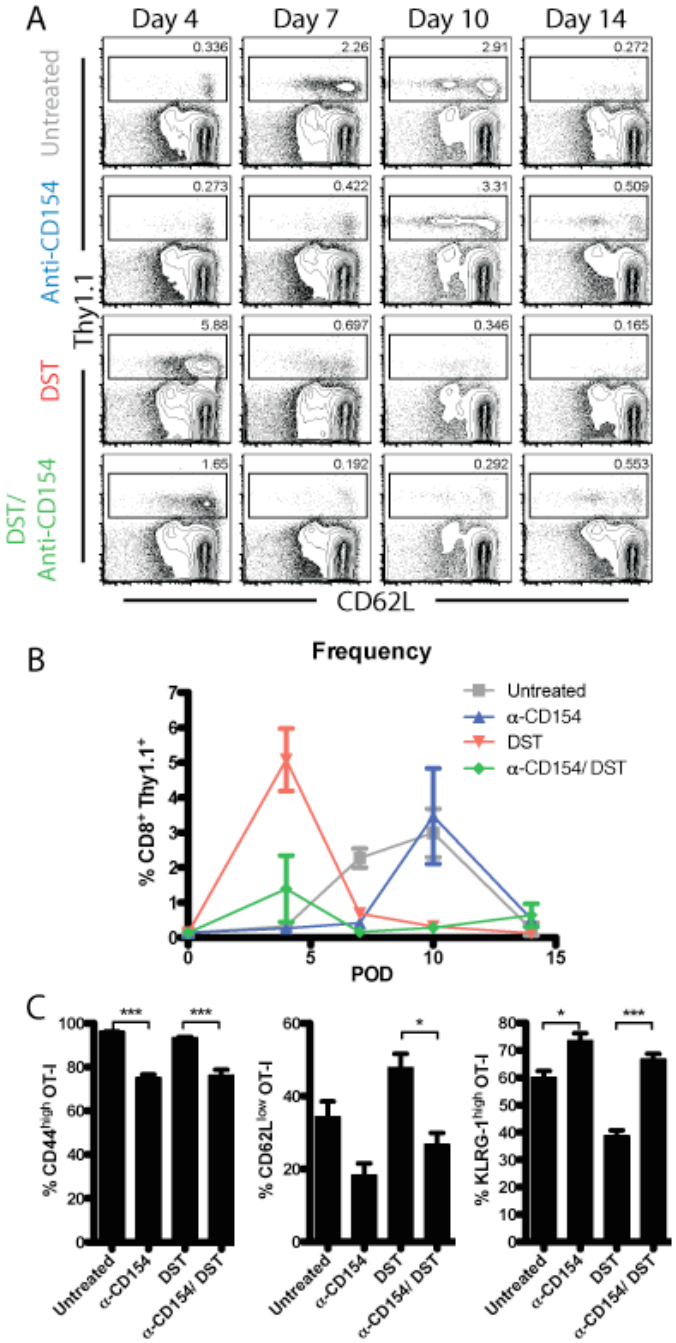


Figure 3.2. CD154 blockade delays expansion of antigen-specific CD8$^+$ T cells but does not alter activation status at peak of response. Mice were treated as described in Fig. 1 and sacrificed at the indicated timepoints. A. Concatenated flow plots of CD8$^+$ splenocytes; gates shown are on donor-reactive CD8$^+$ (Thy1.1$^+$) T cells. B. Frequencies of donor-reactive CD8$^+$ splenocytes are shown. OT-I T cell populations in untreated mice peaked at days 7-10 with 2.27%±0.27% and 2.98%±0.69%, respectively. Compared to untreated controls, anti-CD154 treatment delayed expansion of T cells (D10: 3.47%±1.37%), while DST accelerated expansion of OT-I T cells (D4: 0.32%±0.04% vs. 5.08%±0.98%, respectively; p=0.006). Combined treatment minimally expanded T cells (D4: 1.39%±0.95%). C. Activation markers on OT-I T cells on day 7 in the spleen. CD40/CD154 blockade reduced CD44 and CD62L upregulation, while promoting an increase of KLRG-1 expression. Data are summarized from three experiments with three mice per group. Statistics shown are mean±SEM. *p<0.05 and ***p<0.001

Figure 3.3.

CD154 blockade delays donor-reactive CD8⁺ T cells differentiation into multi-functional cytokine producing cells.

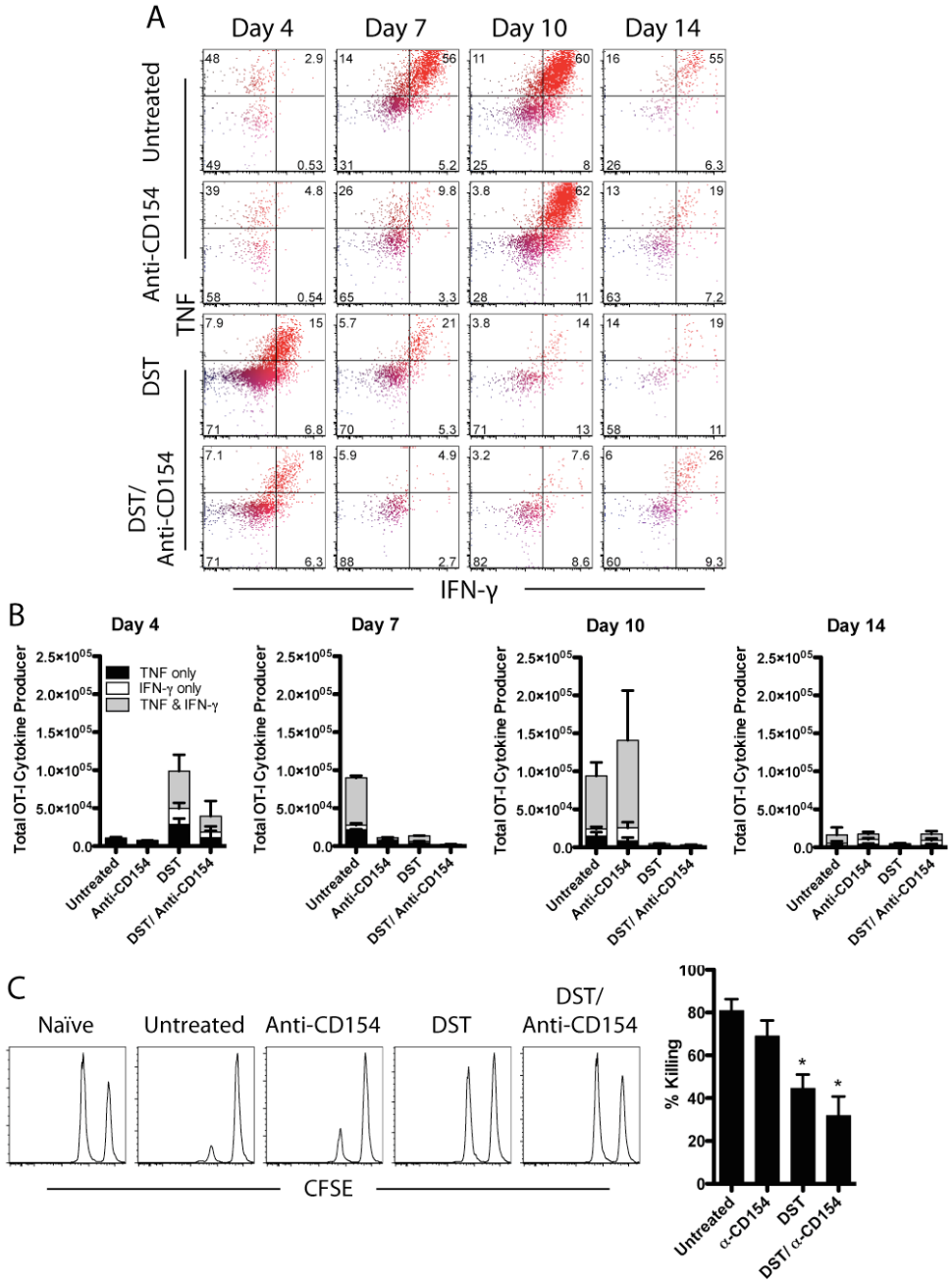


Figure 3.3. CD154 blockade delays donor-reactive CD8$^+$ T cells differentiation into multi-functional cytokine producing cells. Mice were treated as described in Fig. 1 and sacrificed at the indicated timepoints. A. Concatenated flow plots of intracellular cytokine staining in splenic OT-I T cells, following stimulation for 4hrs *in vitro* with SIINFEKL peptide. B. Cytokine production by splenic OT-I T cells, summarized from three experiments with three mice per group. Untreated mice developed into dual cytokine producers at day 7 ($6.22x10^4 \pm 0.29x10^4$). DST accelerated T cell production of TNF and IFN-γ (D4: $4.90x10^4 \pm 2.14x10^4$). Anti-CD154 delayed T cell differentiation (D10: $1.15x10^5 \pm 0.65x10^5$). Anti-CD154/DST inhibited differentiation (D4: $2.08x10^4 \pm 1.98x10^4$). C. Day 10 *in vivo* cytotoxicity assay. Peptide coated targets and unpulsed control targets were labeled with different concentrations of CFSE and adoptively transferred into recipients. After 18hrs, the ratio of unpulsed vs peptide pulsed targets remaining was assessed by flow cytometry. Relative to untreated controls, anti-CD154 treatment did not alter killing of target cells (91.01%±0.82% vs 66.59%±11.03%, respectively; p=0.379). DST treatment significantly impaired killing (55.52%±8.28%; p=0.003). Anti-CD154/DST significantly impaired cytolysis (28.26%±12.78%; p=0.001). Data are summarized from two experiments with five mice per group. Statistics shown are mean±SEM.

Figure 3.4.

Anti-CD154 and DST treatment reduces antigen-specific CD4⁺ T cell accumulation and promotes Foxp3⁺ T_{reg} cell migration to graft.

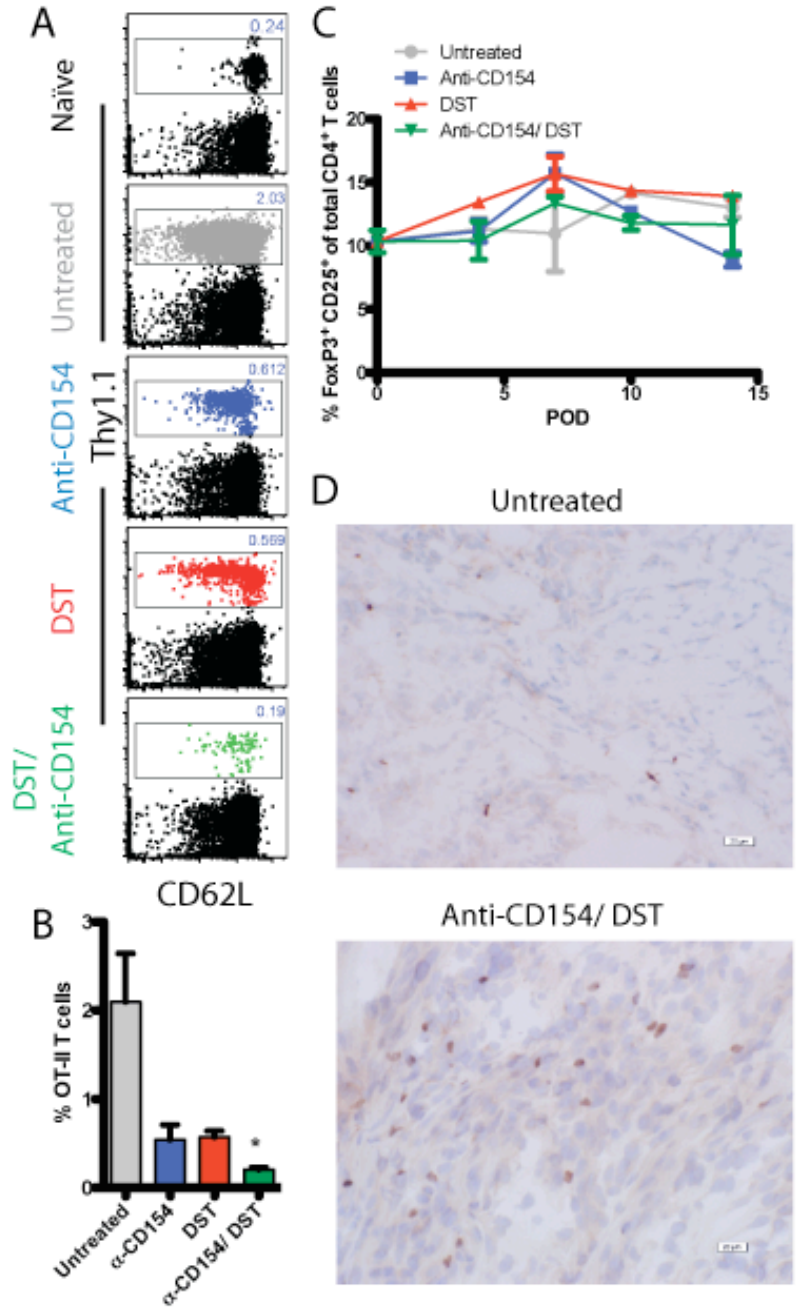


Figure 3.4. Anti-CD154 and DST treatment reduces antigen-specific $CD4^+$ T cell accumulation and promotes $Foxp3^+$ T_{reg} cell migration to graft. Mice were treated as described in Fig. 1 and sacrificed at the indicated timepoints. A. Day 7 concatenated flow plots of $CD4^+$ T cells in draining LNs, with gates identifying OT-II ($Thy1.1^+$) T cells. B. Donor-reactive $CD4^+$ T cells in the draining LNs. Anti-CD154 monotherapy reduced OT-II T cell levels compared to untreated controls (0.54%±0.17% vs 2.10%±0.55%, respectively; p=0.054). DST monotherapy reduced OT-II T cell levels (0.57±0.07%; p=0.051). Anti-CD154/DST significantly reduced OT-II levels (0.20%±0.02%; p=0.026). C. Frequency of total T_{reg} (both transgenic and endogenous) cells in the draining LNs over time. D. Day 7 $Foxp3^+$ cells in SGs. $Foxp3^+$ cells were counted in ten HPF (x400). Anti-CD154/DST treatment significantly increased $Foxp3^+$ cell infiltration compared to untreated controls (14.2±4.78/HPF vs. 4.95±0.22/HPF; respectively; p<0.0005). Data are summarized from three experiments with three mice per group. Statistics shown are mean±SEM.

Figure 3.5.

Anti-CD154 and DST treatment promote conversion of donor-reactive CD4⁺ T cells into CD25⁺ Foxp3⁺ iT_{reg}.

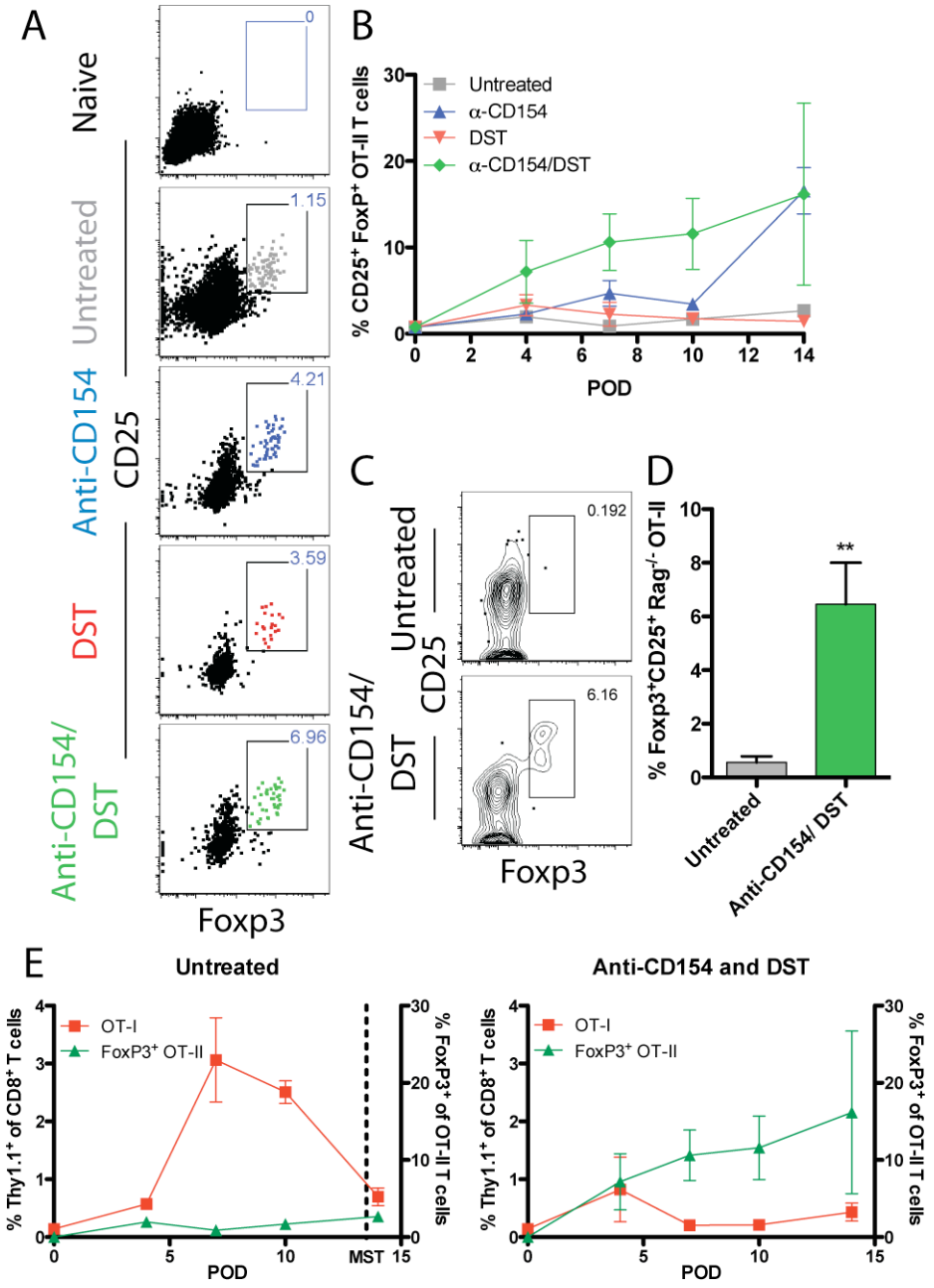
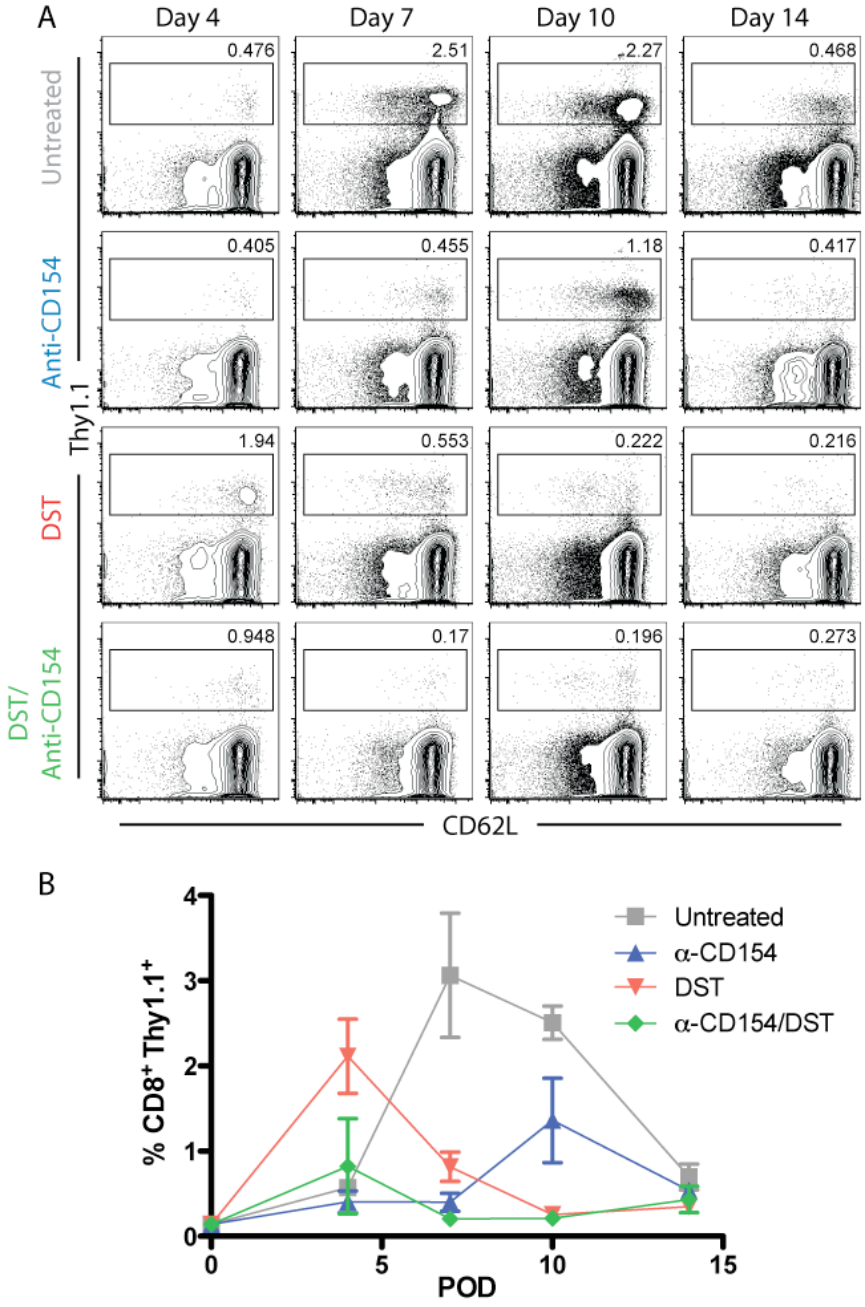


Figure 3.5. Anti-CD154 and DST treatment promote conversion of donor-reactive CD4$^+$ T cells into CD25$^+$ Foxp3$^+$ iT$_{reg}$. A. Day 7, concatenated flow plots of OT-II (Thy1.1$^+$) T cells in LNs, with gates on CD25$^+$ Foxp3$^+$ OT-II cells. B. Longitudinal analysis of Foxp3$^+$ OT-II T cells. On day 7, the frequency of T$_{reg}$ in untreated mice was 0.88%±0.44% of OT-II T cells. Anti-CD154 (4.68%±1.49%; p=0.071) and DST (2.27±1.37%; p=0.389) monotherapies slightly increased the frequencies of Foxp3-expressing T cells within the OT-II T cell compartment. Anti-CD154/DST significantly increased Foxp3 expression in OT-II T cells compared to untreated controls (10.61%±3.28%; p=0.042). On day 14, both anti-CD154 and anti-CD154/DST reached similarly high levels of iT$_{reg}$ conversion (16.57%±2.68% and 16.17%±10.53%, respectively; p=0.973). Data are summarized from three experiments with three mice per group. C, D. B6.SJL (CD45.1$^+$) were adoptively transferred with OT-I and RAG$^{-/-}$ OT-II T cells and treated with anti-CD154/DST. On day 7, draining LNs were analyzed for Foxp3-expressing OT-II T cells. C. Representative flow plots of CD45.2$^+$ RAG$^{-/-}$ OT-II T cells; gate represents CD25$^+$ Foxp3$^+$ iT$_{reg}$ cells. D. Combined CD154 blockade and DST significantly increased the conversion of OT-II T cells into Foxp3$^+$ iT$_{reg}$ compared to untreated controls (6.452±1.552% vs. 0.559%±0.225, p=0.0035). Data are summarized from two experiments with 3-5 mice per group. E. Relative frequencies of Thy1.1$^+$ OT-I T cells compared to peripherally converted Thy1.1$^+$ OT-II iT$_{reg}$ from untreated (left panel) and anti-CD154/DST treated (right panel) LNs. OT-I T cells are measured on the left y-axis, while the OT-II T$_{reg}$ are measured on the right y-axis. Data are summarized from three experiments with three mice per group. Statistics shown are mean±SEM.

Supplemental Figure 3.1.

CD154 blockade delays antigen-specific CD8⁺ T cell expansion in draining lymph nodes.



Supplemental Figure 3.1. CD154 blockade delays antigen-specific CD8$^+$ T cell expansion in draining lymph nodes. Mice were treated as described in Figure 1 and sacrificed at the indicated timepoints. A. We observed expansion and contraction of the donor-reactive CD8$^+$ T cells in the draining node with peaks spanning days 7 through 10 (3.06% ± 0.73% and 2.51% ± 0.19%, respectively). Treatment with DST led to an early expansion of the donor reactive CD8$^+$ T cells, peaking at day 4, compared to untreated controls (2.11% ± 0.44% vs. 0.57% ± 0.09%, respectively; p=0.026). Treatment with anti-CD154 monotherapy delayed expansion of OT-I T cells with a peak at day 10, at which time the magnitude of the response was similar to untreated controls (1.36% ± 0.49% vs. 2.51% ± 0.19%, respectively; p=0.098). Combined treatment with DST and anti-CD154 led to minimal expansion of donor-reactive CD8$^+$ T cells at day 4 over background (0.82% ± 0.56% vs. 0.14% ± 0.02%, respectively; p=0.205). B. Concatenated flow cytometry plots of CD8$^+$ T cells are shown, and gates shown represent the antigen specific OT-I T cells in the draining LNs. Data shown are summarized from three experiments with three mice per group. Statistics shown are mean±SEM.

REFERENCES

1. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, Fechner JH, Jr., Germond RL, Kampen RL, Patterson NB, Swanson SJ, Tadaki DK, TenHoor CN, White L, Knechtle SJ, Harlan DM. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 5: 686-93

2. Larsen CP, Alexander DZ, Hollenbaugh D, Elwood ET, Ritchie SC, Aruffo A, Hendrix R, Pearson TC. 1996. CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation* 61: 4-9

3. Parker DC, Greiner DL, Phillips NE, Appel MC, Steele AW, Durie FH, Noelle RJ, Mordes JP, Rossini AA. 1995. Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* 92: 9560-4

4. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114

5. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-4

6. Haanstra KG, Ringers J, Sick EA, Ramdien-Murli S, Kuhn EM, Boon L, Jonker M. 2003. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* 75: 637-43

7. Haanstra KG, Sick EA, Ringers J, Wubben JA, Kuhn EM, Boon L, Jonker M. 2005. Costimulation blockade followed by a 12-week period of cyclosporine A facilitates prolonged drug-free survival of rhesus monkey kidney allografts. *Transplantation* 79: 1623-6

8. Masunaga T, Yamashita K, Sakihama H, Hashimoto T, Hua N, Imai A, Inobe M, Miyazaki T, Todo S, Uede T. 2005. Dimeric but not monomeric soluble CD40 prolongs allograft survival and generates regulatory T cells that inhibit CTL function. *Transplantation* 80: 1614-22

9. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S, Rees P, Hendrix R, Price K, Kenyon NS, Hagerty D, Townsend R, Hollenbaugh D, Pearson TC, Larsen CP. 2005. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* 174: 542-50

10. Zheng XX, Markees TG, Hancock WW, Li Y, Greiner DL, Li XC, Mordes JP, Sayegh MH, Rossini AA, Strom TB. 1999. CTLA4 signals are required to optimally induce allograft tolerance with combined donor-specific transfusion and anti-CD154 monoclonal antibody treatment. *J Immunol* 162: 4983-90

11. Gordon EJ, Markees TG, Phillips NE, Noelle RJ, Shultz LD, Mordes JP, Rossini AA, Greiner DL. 1998. Prolonged survival of rat islet and skin xenografts in mice treated with donor splenocytes and anti-CD154 monoclonal antibody. *Diabetes* 47: 1199-206

12. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc Natl Acad Sci U S A* 93: 13967-72

13. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA. 1998. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA4. *J Clin Invest* 101: 2446-55

14. Preston EH, Xu H, Dhanireddy KK, Pearl JP, Leopardi FV, Starost MF, Hale DA, Kirk AD. 2005. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. *Am J Transplant* 5: 1032-41

15. Pearl JP, Xu H, Leopardi F, Preston E, Kirk AD. 2007. CD154 blockade, sirolimus, and donor-specific transfusion prevents renal allograft rejection in cynomolgus monkeys despite homeostatic T-cell activation. *Transplantation* 83: 1219-25

16. Quezada SA, Fuller B, Jarvinen LZ, Gonzalez M, Blazar BR, Rudensky AY, Strom TB, Noelle RJ. 2003. Mechanisms of donor-specific transfusion tolerance: preemptive induction of clonal T-cell exhaustion via indirect presentation. *Blood* 102: 1920-6

17. Margenthaler JA, Kataoka M, Flye MW. 2003. Donor-specific antigen transfusion-mediated skin-graft tolerance results from the peripheral deletion of donor-reactive CD8+ T cells. *Transplantation* 75: 2119-27

18. van Maurik A, Fazekas de St Groth B, Wood KJ, Jones ND. 2004. Dependency of direct pathway CD4+ T cells on CD40-CD154 costimulation is determined by nature and microenvironment of primary contact with alloantigen. *J Immunol* 172: 2163-70

19. Taylor PA, Noelle RJ, Blazar BR. 2001. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med* 193: 1311-8

20. Ehst BD, Ingulli E, Jenkins MK. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3: 1355-62

21. Iwakoshi NN, Mordes JP, Markees TG, Phillips NE, Rossini AA, Greiner DL. 2000. Treatment of allograft recipients with donor-specific transfusion and anti-CD154 antibody leads to deletion of alloreactive CD8+ T cells and prolonged graft survival in a CTLA4-dependent manner. *J Immunol* 164: 512-21

22. Barber DL, Wherry EJ, Ahmed R. 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. *J Immunol* 171: 27-31

23. Quezada SA, Bennett K, Blazar BR, Rudensky AY, Sakaguchi S, Noelle RJ. 2005. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol* 175: 771-9

24. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. 2008. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205: 625-40

25. Wood KJ, Sakaguchi S. 2003. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* 3: 199-210

26. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204: 1775-85

27. Warnecke G, Bushell A, Nadig SN, Wood KJ. 2007. Regulation of transplant arteriosclerosis by CD25+CD4+ T cells generated to alloantigen in vivo. *Transplantation* 83: 1459-65

28. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, Angeli V, Li Y, Boros P, Ding Y, Jessberger R, Trinchieri G, Lira SA, Randolph GJ, Bromberg JS. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 7: 652-62

29. Wang T, Ahmed EB, Chen L, Xu J, Tao J, Wang CR, Alegre ML, Chong AS. 2010. Infection with the intracellular bacterium, Listeria monocytogenes, overrides established tolerance in a mouse cardiac allograft model. *Am J Transplant* 10: 1524-33

30. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-8

31. Perona-Wright G, Jenkins SJ, O'Connor RA, Zienkiewicz D, McSorley HJ, Maizels RM, Anderton SM, MacDonald AS. 2009. A pivotal role for CD40-mediated IL-6 production by dendritic cells during IL-17 induction in vivo. *J Immunol* 182: 2808-15

32. Sun JC, Bevan MJ. 2004. Cutting edge: long-lived CD8 memory and protective immunity in the absence of CD40 expression on CD8 T cells. *J Immunol* 172: 3385-9

33. Bourgeois C, Rocha B, Tanchot C. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* 297: 2060-3

34. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-27

35. Molinero LL, Miller ML, Evaristo C, Alegre ML. 2011. High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-{kappa}B-Dependent Manner. *J Immunol* 186: 4609-17

36. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. 2006. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med* 203: 2785-91

37. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. 2004. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. *J Immunol* 172: 923-8

38. Marzo AL, Klonowski KD, Le Bon A, Borrow P, Tough DF, Lefrancois L. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol* 6: 793-9

39. Blair DA, Lefrancois L. 2007. Increased competition for antigen during priming negatively impacts the generation of memory CD4 T cells. *Proc Natl Acad Sci U S A* 104: 15045-50

40. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, Larsen CP. 2007. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med* 204: 299-309

41. Ford ML, Wagener ME, Hanna SS, Pearson TC, Kirk AD, Larsen CP. 2008. A critical precursor frequency of donor-reactive CD4+ T cell help is required for CD8+ T cell-mediated CD28/CD154-independent rejection. *J Immunol* 180: 7203-11

42. Pluvinet R, Petriz J, Torras J, Herrero-Fresneda I, Cruzado JM, Grinyo JM, Aran JM. 2004. RNAi-mediated silencing of CD40 prevents leukocyte adhesion on CD154-activated endothelial cells. *Blood* 104: 3642-6

43. Ripoll E, Pluvinet R, Torras J, Olivar R, Vidal A, Franquesa M, Cassis L, Cruzado JM, Bestard O, Grinyo JM, Aran JM, Herrero-Fresneda I. 2011. In vivo therapeutic efficacy of intra-renal CD40 silencing in a model of humoral acute rejection. *Gene Ther* 18: 945-52

44. Zhang T, Fresnay S, Welty E, Sangrampurkar N, Rybak E, Zhou H, Cheng XF, Feng Q, Avon C, Laaris A, Whitters M, Nagelin AM, O'Hara RM, Jr., Azimzadeh AM. 2011. Selective CD28 blockade attenuates acute and chronic rejection of

murine cardiac allografts in a CTLA-4-dependent manner. *Am J Transplant* 11: 1599-609

45. Suntharalingam G, Perry MR, Ward S, Brett SJ, Castello-Cortes A, Brunner MD, Panoskaltsis N. 2006. Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. *N Engl J Med* 355: 1018-28

Chapter 4

CD40/CD154 Blockade Inhibits Dendritic Cell Production of IL-6 and Alters Balance of Graft-Specific IL-17-Secreting T Cells and Foxp3$^+$ iT$_{reg}$

ABSTRACT

Blockade of the CD40/CD154 pathway remains one of the most effective means of promoting transplantation tolerance. However, the effects of CD40/CD154 blockade on dendritic cells (DCs) during the course of bone marrow transplant rejection are not well understood. To dissect the effects of CD154/CD40 blockade on DC activation *in vivo*, we generated hematopoietic chimeras in mice that expressed a surrogate minor antigen (OVA). Adoptive transfer of OVA-specific OT-I and OT-II T cells led to chimerism rejection, which was attenuated by treatment with CD154 blockade. Although anti-CD154 did not alter the expression of MHC and costimulatory molecules compared to untreated controls, DCs exhibited a significant reduction in inflammatory IL-6 and TNF cytokine secretion. Importantly, this decrease in inflammatory cytokine secretion by DCs coincided with reduced IL-17 production by antigen-specific T cells and an increase in antigen-specific $Foxp3^+$ regulatory T cells. Therefore, these results suggest that blockade of CD40 ligation on DCs during graft rejection impairs the provision of signal 3 to developing donor-reactive T cell populations, with a reduced impact on the provision of costimulation (signal two). These data suggest that therapies designed to target inflammatory cytokines during donor-reactive T cell activation may be beneficial in attenuating these responses and prolonging graft survival.

INTRODUCTION

In both bone marrow and solid organ transplantation models, specifically targeting graft-reactive T cell responses to prevent transplant rejection remains an important goal. Early studies by Larsen *et al.* demonstrated that combined blockade of CD40/CD154 and CD28/B7 costimulation signals significantly prolonged the survival of cardiac allografts in a murine model (1). Since then, several studies in murine and non-human primate models have shown that blockade of costimulatory signals promotes survival of bone marrow, skin, kidney, heart, and islet transplants (2-5). Blockade of the CD40/CD154 costimulatory pathway remains one of the most effective means of inducing long-term graft survival following transplantation. However, monoclonal antibodies designed to targeting CD154 resulted in thromboembolic events in early pilot studies in humans (6). Renewed interest in blockade of this pathway for the prevention of graft rejection has been sparked by promising results from several recent studies in non-human primate transplant models (7-9), and clinical trials using CD40 blockers in renal transplant recipients are now underway (10). Thus, the therapeutic potential of targeting this pathway is high, and understanding the effects of CD154/CD40 blockade in transplant models may uncover other novel downstream targets for therapeutic intervention.

Despite the clear efficacy of blockade of this pathway, the mechanisms underlying its effect are still under investigation. While one report indicated that anti-CD154 monoclonal antibodies may impact the outcome of graft rejection by specifically binding to and depleting antigen-specific $CD4^+$ T cells which express CD154 following activation (11), subsequent studies using anti-CD40 monoclonal

antibodies showed similar efficacy in both bone marrow and solid organ transplant models in mouse and non-human primates (7-9, 12-14). Thus, it is likely that blockade of the CD40/CD154 pathway, rather than antibody-mediated depletion of antigen-specific cells, plays a major role in the observed attenuation of graft rejection. In dendritic cells (DCs), ligation of CD40 by CD154 expressed on activated $CD4^+$ T cells leads to the activation of downstream signaling pathways, resulting in several key events that promote the generation of effective T cell responses. These include 1) increasing MHC expression which would enhance the strength the TCR signals (15, 16), 2) induction of costimulatory molecule expression (e.g., CD80, CD86, OX40L) which would increase the strength of "second signals" (17, 18), 3) increased production of pro-inflammatory cytokines (IL-12, IL-6, IL-1) sometimes referred to as "signal three" (19), and 4) increasing DC longevity (20, 21), thereby enhancing T cell priming via of all of the above mechanisms. However, very little is known about the effects of CD40 blockade on DC *in vivo* during tolerance induction.

The type and amount of cytokines secreted by DC during T cell activation profoundly impact the nature of the ensuing T cell response (22). Recently, IL-6 has been defined as an important switch factor for the differentiation of IL-17 secreting cells (23-26). IL-17 secretion has been observed in both $CD4^+$ and $CD8^+$ T cell populations in response to infections, and these cells have also been shown to play an important role in the pathogenesis of several different autoimmune diseases, including murine models of inflammatory bowel disease (27), experimental autoimmune encephalomyelitis (24), and autoimmune aplastic anemia (28). Seminal studies reported a requirement for TGF-β and IL-6 for the differentiation of

$CD4^+$ Th17 cells, while the absence of IL-6 induces $CD4^+$ to become $FoxP3^+$ T_{reg} in the presence of TGF-β (24). However, the signals involved in the differentiation of IL-17-secreting $CD8^+$ T cells are less well-described.

Here, we hypothesized that blockade of the CD40/CD154 pathway *in vivo* would result in altered DC phenotype and/or function, which could in turn result in sub-optimal T cell priming and in this way lead to protection of hematopoietic chimerism following bone marrow transplantation. In order to test this, we generated hematopoietic chimeras that expressed membrane-bound chicken ovalbumin (OVA) on all hematopoietically-derived cells. Subsequent adoptive transfer of OVA-specific $CD8^+$ OT-I and $CD4^+$ OT-II T cells led to rejection of the chimerism, a process that was attenuated by treatment with anti-CD154 mAb (MR-1). In order to dissect the effects of CD154/CD40 blockade on DC activation *in vivo*, antigen-bearing splenic DCs were isolated from recipients that had received OT-I and OT-II T cells in the presence or absence of anti-CD154 mAb. Results demonstrated that DCs derived from anti-CD154-treated recipients did not differ with regard to their expression levels of MHC or costimulatory molecules, as compared to untreated controls. However, DCs activated in the presence of CD154/CD40 blockade exhibited significantly reduced secretion of inflammatory cytokines, including IL-6. Because IL-6 has been shown to be critical for the differentiation of IL-17-secreting T cells (24, 29), we probed the impact of CD154/CD40 blockade on the reciprocal induction of bone marrow-specific IL-17 secreting cells vs iT_{reg}. Our data revealed that while inhibition of CD154/CD40 signaling enhanced the peripheral conversion of graft-specific iT_{reg}, it concomitantly

inhibited the induction of graft-specific IL-17 secreting T cells.

MATERIALS AND METHODS

Mice

C57BL/6 (CD45.2) and B6-Ly5.2/Cr (CD45.1) mice were obtained from the National Cancer Institute (Charles River, Frederick, MD). OT-I and OT-II TCR transgenic mice were bred to Thy1.1 congenic at Emory University. OT-II x $RAG^{-/-}$ TCR transgenic mice were purchased from Taconic and bred to Thy1.1 congenic animals at Emory University. mOVA mice (30) were a gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN) and were maintained at Emory University. All animals were housed in pathogen-free animal facilities at Emory University. All studies were approved by the Institutional Animal Care and Use Committee of Emory University.

Bone marrow isolation and establishment and screening of mOVA BM chimera

Recipient B6-Ly5.2/CR (CD45.1) (NCI) mice were treated one day prior to bone marrow adoptive transfer with 500μg of Busulfan (Busulfex, Otsuka America Pharmaceutical, Inc.) intraperitoneally. Bone marrow was flushed from femurs and tibias of mOVA ($CD45.2^{+}$) mice with saline using a 27g needle and was disrupted through the needle. BM cells were subsequently resuspended in saline and adoptively transferred i.v. at a dose of 20×10^6 cells per mouse. On the day of bone marrow infusion, recipients were treated with 500μg of both CTLA-4 Ig (Bristol-Myers Squibb) and anti-CD154 (MR1, BioExpress), followed by the same dose on days 2, 4, 6, to prevent an immune response against the OVA antigen. mOVA bone marrow chimeras were used in experiments at approximately 8-12 weeks post-

transplant. Hematopoietic chimerism was determined by staining peripheral blood with B220-PerCP, CD45.1-PE, and CD45.2-FITC (all from BD Pharmingen). Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo Software (Treestar, San Carlos, CA).

Adoptive transfers and antibody treatment

Spleens of OT-I and OT-II mice were disrupted with frosted glass slides and processed into a single cell suspension. Splenocytes were stained with CD4-APC, CD8-FITC, Vα2-PE and Thy1.1-PerCP monoclonal antibodies (BD Pharmingen) for flow cytometric analysis. Using TruCount beads (BD Pharmingen), absolute numbers of each cell population were obtained and $5x10^6$ OT-I and 10^6 OT-II T cells were adoptively transferred i.v. into recipient mice. Following adoptive transfer, animals were treated with 250μg of anti-CD154 (MR1, BioExpress) on days 0, 2, 4, and 6, in indicated groups, or left untreated in control groups.

Dendritic cells isolation and flow cytometric analysis

Spleens were removed from mice. One millilitre of 2mg/ml collagenase type 3 (Worthington Bio. Corp, NJ) in HBSS (with Ca^{+2}/Mg^{+2}) was injected into the spleen, which was then incubated with 2ml collagenase solution at 37°C /5%CO_2 for 30 min. Single cell suspensions were prepared after incubation by mashing the spleen using 3ml syringe plunger on a cell strainer (70μM) and washing cells with PBS. Single cell suspensions were stained for flow cytometric analysis with antibodies against CD11c-APC, CD11b-PerCP, CD8α-APC-Cy7, H-$2K^b$-FITC, CD80-PE, CD86-PE, and

CD40-FITC (all from BD Pharmingen). Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo Software (Treestar, San Carlos, CA).

Assessment of ex vivo *cytokine production by DCs*

DCs were isolated as described above and single cell suspensions were enriched by negative selection using magnetic beads coated with anti-CD19, CD90.1, and CD90.2 monoclonal antibodies (Miltenyi Biotec Inc.) according to the manufacturer's instructions. Purified DCs (purity >70%) were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 50μM 2-mercaptoethanol, streptomycin (100μg/ml), and penicillin (100 units/ml). DCs were then distributed in 200-μl aliquots ($6x10^5$ cells/well) to a 96-well plate and cultured for 24 hours at 37°C in 5% CO_2 in duplicate. The cultured cell supernatants were measured for the levels of inflammatory cytokines by a cytometric bead array (CBA) (BD Pharmingen) according to the manufacturer's instructions.

T cell intracellular cytokine staining

To measure cytokine production by antigen specific T cells, surface and intracellular stains were performed with monoclonal antibodies to CD8-Pacific Orange (Invitrogen), CD4-Pacific Blue (Invitrogen), Thy1.1-PerCP (BD Pharmingen), TNF-PE-Cy (BD Pharmingen), IFN-γ-FITC (BD Pharmingen), IL-17-PE (BD Pharmingen). Spleens of chimeric mice were processed into single cells suspensions and plated onto 96-well flat-bottom plates at 10^6 cells per well. Cells were stimulated with

10nM $OVA_{257-264}$ (SIINFEKL, Emory University Microchemical Core Facility) and 10μM $OVA_{323-339}$ in the presence of 10μg/mL of Brefeldin A for 5 hours. Cells were processed with an intracellular staining kit (BD Biosciences) according to manufacturer's instructions. As a positive control, cells were stimulated with 10ng/mL phorbol 12-myristate 13-acetate (PMA) and 1μg/mL ionomycin (Sigma). Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo Software (Treestar, San Carlos, CA).

Intracellular FoxP3 staining

Splenocytes were isolated and disrupted using frosted glass slides. Cells were surface stained with CD8-Pacific Orange (Invitrogen), CD4-Pacific Blue (Invitrogen), Thy1.1-PerCP (BD Pharmingen), and CD25-FITC (BD Pharmingen). Cells were processed with an intranuclear staining kit and stained with FoxP3-PE antibody (eBiosciences), according to manufacturer's instructions. Data were acquired on a LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo Software (Treestar, San Carlos, CA).

DC purification, RNA isolation and immune array

For DC isolation and purification, single cell suspensions were first enriched by negative selection using magnetic beads coated with anti-CD19, CD90.1, and CD90.2 monoclonal antibodies as described above (yielding ~70% purity of $CD11c^+$ cells). Following enrichment, $CD11c^+$ Thy 1.1^- cells were further purified by FACS sorting on a BD FACS Aria. Following FACS-sorted DC populations were >90% $CD11c^+$ cells.

RNAs from the sorted DCs were isolated using RNeasy Isolation kit (Qiagen). Reverse transcription of the RNA into cDNA was performed using Taqman reverse transcription kit (Roche). Mouse immune array cards (Applied Biosystems) were used to obtain a real-time analysis of the selected immune related genes. Arrays were run on a 7900HT Real-Time PCR System from ABI.

RESULTS

CD154/CD40 pathway blockade prevents antigen-specific T cell mediated destruction of OVA-expressing peripheral leukocytes

In an effort to better understand fundamental biology involved in the induction and/or abortion of an immune response following CD154/CD40 blockade, we developed a model in which antigen-specific $CD4^+$ and $CD8^+$ T cells recognized and rejected cognate antigen-bearing hematopoietic cells following bone marrow transplantation. Using an ovalbumin expressing transgenic mouse (mOVA, B6 background), we generated hematopoietic chimeras in which busulfan-treated B6 host mice ($CD45.1^+$) received a $CD45.2^+$ bone marrow transplant expressing a single defined alloantigen (OVA). Adoptive transfer of $5x10^6$ graft-specific $CD8^+$ (OT-I) and 10^6 graft-specific $CD4^+$ (OT-II) T cells were sufficient to induce rejection of the $CD45.2^+$ mOVA-expressing bone marrow cells such that by day 6, the number of donor-derived ($CD45.2^+$) peripheral B cells was significantly reduced (Figure 4.1A). Both $CD4^+$ and $CD8^+$ T cells were required to mediate this effect, as adoptive transfer of either OT-I or OT-II alone failed to result in rejection (Supplemental Figure 4.1). Importantly, treatment of animals with a short course of CD154 blockade (MR-1) on days 0, 2, 4, and 6 post-transplant resulted in protection of the bone marrow from rejection (Figure 4.1A, B). These results indicated that blockade of the CD154/CD40 pathway alone was sufficient to prevent T cell-mediated destruction of OVA-expressing peripheral leukocytes and preserve hematopoietic chimerism.

CD154/CD40 blockade attenuates antigen-specific T cell expansion and effector function

To address the potential mechanisms by which this protection occurred, we analyzed the antigen-specific $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T cell responses in MR-1 treated animals. Briefly, $5x10^6$ $Thy1.1^+$ $CD8^+$ OT-I and 10^6 $Thy1.1^+$ $CD4^+$ OT-II T cells were adoptively transferred into mOVA bone marrow chimeras and the activation and expansion of $Thy1.1^+$ $CD4^+$ and $CD8^+$ T cell populations were assessed. We observed that while graft-specific OT-I T cells expanded dramatically in untreated animals by day 4 post-transfer (Figure 4.2A, B), treatment with MR-1 resulted in a significant diminution in the antigen-specific $CD8^+$ T cell response ($p<0.0001$). A similar result was observed for antigen-specific $CD4^+$ T cell responses, which exhibited a more than two-fold reduction in the presence of MR-1 (Supplemental Figure 4.2). The functionality of the remaining graft-specific $CD8^+$ T cells was assessed by intracellular cytokine staining following *ex vivo* restimulation with cognate antigen. Results revealed that the frequency of TNF and IFN-γ-secreting $Thy1.1^+$ $CD8^+$ T cells was significantly diminished in animals treated with MR-1 compared to untreated controls (0.21±0.06% vs. 3.74±0.94%, respectively; $p<0.01$) (Figure 4.2C, D). Taken together, these results demonstrate that blockade of the CD154 pathway attenuates both expansion and differentiation of antigen-specific T cells in bone marrow chimeras.

CD154 blockade inhibits provision of signal three from DC, but not provision of signal one or signal two

The above results indicated that antigen-specific $CD8^+$ T cell responses were sharply attenuated in the presence of CD154 blockade. The DC licensing model of T cell activation suggests that CD154 expressed on activated $CD4^+$ T cells binds to CD40 expressed on DCs and functions to initiate the upregulation of class I and class II MHC and several costimulatory molecules, thus enhancing the priming of antigen-specific $CD8^+$ T cell responses. In order to assess the impact of CD154 blockade on DC licensing in this model, we first characterized the frequency and phenotypes of splenic DCs in mOVA bone marrow chimeras following the adoptive transfer of antigen-specific T cells. mOVA bone marrow chimeras contained $1.75\pm0.77x10^6$ total DC per spleen (Figure 4.3A), and > 90% of those were $CD45.2^+$, expressing the OVA antigen (data not shown). Adoptive transfer of OT-I and OT-II T cells did not result in a change in the number of splenic DCs at day 3 post-transfer, either in the presence or absence of MR-1 ($1.59\pm0.44x10^6$ and $2.04\pm1.04x10^6$, respectively, Figure 4.3B). In addition, the relative proportion of myeloid ($CD11c^+$ $CD11b^+$ $CD8\alpha^-$) vs. lymphoid ($CD11c^+$ $CD11b^-$ $CD8\alpha^+$) DC was not altered in the presence of MR-1 (Figure 4.3C-E). Thus, these data demonstrate that blockade of the CD154/CD40 pathway did not impact the overall quantity or myeloid/lymphoid phenotype of DCs in bone marrow chimera recipients.

We next examined the impact of CD154 blockade on the expression of MHC (signal one) and costimulatory (signal two) molecules on DCs. The DC licensing model predicts that CD40 ligation results in upregulation of MHC and costimulatory

molecules. This was confirmed in our model with experiments in which agonistic anti-CD40 monoclonal antibody (FGK4.5) was injected into mice and splenic DCs were assessed to express high levels of MHC and costimulatory molecules (data not shown). Next, we asked whether T cells could provide the CD40 stimulus necessary for DC licensing. In *in vitro* activation studies, as predicted, CD154 was upregulated on antigen-specific $CD4^+$ T cells (Supplemental Figure 4.3). Following adoptive transfer of OT-I and OT-II T cells into mOVA bone marrow chimeras, consistent with the DC licensing model, provision of T cell-derived CD154 signals resulted in a statistically significant increase in class I ($H\text{-}2K^b$) expression on the surface of splenic DCs at day 3 post-transfer (Figure 4.4). Likewise, expression of CD86 and CD40 were also increased (Figure 4.4). Surprisingly, however, treatment with MR-1 failed to attenuate the expression of class I MHC, or the expression of costimulatory molecules on splenic DCs during graft-specific T cell priming (Figure 4.4). These results suggest that diminution in the provision of either signal one or signal two is not the mechanism by which CD154 blockade attenuates graft-specific T cell responses and promotes long term graft survival.

Next, in order to assess the impact of CD154 blockade on the provision of signal three in this model, we conducted similar experiments in which antigen-specific $Thy1.1^+$ $CD4^+$ and $CD8^+$ T cells were adoptively transferred into mOVA hematopoietic chimeras. On days 1 and 3 post-transfer, $CD11c^+$ splenic DCs were FACS-sorted and either immediately processed for real-time PCR analysis of mRNA or cultured *in vitro* for 24h for supernatant assessement of cytokine production. Results demonstrated that the adoptive transfer of antigen-specific T cells resulted

in a profound increase in the ability of DC to produce pro-inflammatory cytokines. Specifically, DC isolated from T cell adoptive transfer recipients exhibited an increase in IL-6 and TNF at the protein level, and in IL-12 at the mRNA level (Figure 4.5 and Supplemental Figure 4.4A). Importantly, DCs isolated from mice that had been treated with CD154 blockade exhibited statistically significantly reduced IL-6 production, on both day 1 and day 3 post-T cell transfer (Figure 4.5). In addition, CD154 blockade also resulted in a decrease in TNF secretion (Supplemental Figure 4.4A). Furthermore, differences in the expression of IL-12p35 and IL-1β mRNA were detected, although neither were detected at the protein level in any of the groups, suggesting that the expression of these cytokines was below the limit of detection (Supplemental Figure 4.4B, C). These data suggest that, overall, a major effect of CD154 blockade (MR-1) in this system may be to inhibit the provision of inflammatory cytokines (signal three) to antigen-specific T cells during priming.

CD154/CD40 pathway blockade inhibits IL-17 production and induces Foxp3$^+$ iT$_{reg}$ conversion

The above results demonstrating that a prominent feature of *in vivo* CD154/CD40 pathway blockade was to inhibit IL-6 production by DCs led us to explore the potential impact of reduced IL-6 levels on the resultant antigen-specific T cell response. Splenocytes from mOVA bone marrow chimera recipients of OVA-specific OT-I and OT-II x RAG$^{-/-}$ T cells were isolated on day 7 post-transfer and restimulated *ex vivo* with OVA cognate antigens. We observed that donor antigen-stimulated splenocytes from untreated animals contained a small but discrete and

consistent population of OVA-specific $CD8^+$ OT-I T cells that secreted IL-17 in response to OVA antigen (1.37±0.32%). In contrast, donor antigen-stimulated splenocytes isolated from mice treated with MR-1 possessed a significantly reduced frequency of IL-17-secreting graft specific $CD8^+$ T cells (0.40±0.21%, p=0.045) (Figure 4.6A, B). Interestingly, no IL-17-secreting $CD4^+$ T cells were observed in either group. These data suggest that $CD8^+$ T cells responding to systemic hematopoietically-expressed antigen can differentiate into inflammatory IL-17 secreting $CD8^+$ T cells (T_c17), and that this process is attenuated by blockade of the CD154/CD40 pathway.

In addition to its emerging role as a potent inducer of type 17 responses, IL-6 also plays a critical negative regulatory role in the peripheral differentiation of induced regulatory T cells (iT_{reg}). As such, we hypothesized that CD154/CD40 blockade and the resulting decrease in IL-6 secretion might enhance the generation of iT_{reg} in this model. In order to test this we examined splenocytes from mOVA bone marrow chimeric animals, which had received antigen-specific RAG-deficient $CD4^+$ and $CD8^+$ T cells in the presence or absence of MR-1. Analysis of $CD4^+$ $Thy1.1^+$ splenocytes on days 10 and 24 post-transfer revealed that while untreated animals contained a minimal frequency (0.12±0.02%) of peripherally-induced iT_{reg}, animals treated with MR-1 contained a detectable population of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells at day 10 post transfer (0.41±0.13%), which continued to increase in frequency out to day 24 post-transfer (1.63±0.27%, p=0.0017) (Figure 4.6C-E). These data suggested that blockade of the CD154/CD40 pathway resulted in the enhancement of graft-specific regulatory T cell populations. Because absolutely no $Foxp3^+$ OT-II T cells

are present in OT-II x RAG$^{-/-}$ pre-transfer cell preparations (31) we conclude that blockade of this pathway promotes the peripheral conversion of graft-specific iT_{reg} from Foxp3^{-} precursors.

DISCUSSION

In this manuscript, we have suggested a mechanism by which blockade of CD40/CD154 provides protection of hematopoietic chimerism following bone marrow transplantation. We observed that although blockade of CD40/CD154 did not alter the level of expression of class I or class II MHC or costimulatory molecules on the surface of dendritic cells, it did significantly alter the differentiation of these cells, specifically with regard to their ability to secrete the inflammatory cytokines IL-6 and TNF. These results suggest that blockade of CD40 ligation on DC during the course of graft rejection critically impacts the provision of signal three to developing donor-reactive T cell populations, with less of an impact on the provision of costimulation (signal two). These data provide a mechanistic basis for the observed synergy between blockade of the CD40 and CD28 pathways, and suggest that therapies designed to target the provision of inflammatory cytokines during the generation of donor-reactive T cell responses may be beneficial in attenuating these responses and prolonging graft survival.

Our results demonstrating that MR-1 does not inhibit $CD4^+$ T cell-induced upregulation of class I or class II MHC on DCs are surprising, given the known role for CD154 in DC licensing. Indeed, our results confirmed reports by many other groups demonstrating that activated $CD154^+$ $CD4^+$ T cells lead to the upregulation of MHC and costimulatory molecules on DCs (32-34). There are two potential reasons that MR-1 fails to inhibit this activity. It is possible that MR-1 does not completely inhibit the ability of cell-associated CD154 to bind to CD40 expressed on the surface of DCs. CD154 binding in the presence of MR-1 might result in a partial signal

delivered to the APC, resulting in the upregulation of MHC and costimulatory molecules but not the elaboration of inflammatory cytokines. CD40 signaling in DCs is mediated by binding of individual TRAFs to the intracellular domain, and previous studies have revealed that the CD40 TRAF 2/3 binding site is critical for costimulatory molecule expression, while the TRAF 6 binding site is required for production of inflammatory cytokines (35). Thus, it is possible that binding of MR-1 inhibits the ability of TRAF 6 but not TRAF 2/3 to be recruited to the CD40 intracellular domain. Experiments to test this hypothesis are ongoing. An alternate possibility is that there are CD154-dependent mechanisms by which cognate T cells can upregulate costimulatory molecule expression (but not cytokine production) on DC.

Recently, IL-6 has been identified as a predominant inflammatory cytokine involved in the induction of T_h17 T cells (24, 29). While TGF-β is required for both T_h17 and T_{reg} differentiation, IL-6 has been implicated as the switch factor between these two T cell subtypes, providing the signals necessary for T cell differentiation into an inflammatory (T_h17) rather than a regulatory (T_{reg}) phenotype (24, 36). In this manuscript, we demonstrated that CD40/CD154 pathway blockade *in vivo* inhibited both IL-6 and TNF secretion by dendritic cells. These results are consistent with findings from other studies demonstrating that strong CD40-mediated signals are associated with increased IL-6 secretion by DCs (37). In addition, the inhibition of IL-6 and IL-17 was shown to protect cardiac allografts from rapid rejection (38), and the combined reduction of TNF and IL-6 has been shown in an *in vitro* system to reduce allograft specific T cell proliferation and

differentiation (39). Therefore, due to the observation that IL-6 production was dramatically decreased when CD40/CD154 pathway was blocked, we investigated the impact of anti-CD154 on T_h17 and T_{reg} differentiation. Interestingly, we did not observe differentiation of graft-specific $CD4^+$ T cells into IL-17 secreting $CD4^+$ T_h17 cells in untreated animals in this model (Figure 4.6), thus we cannot assess the impact of CD40/CD154 pathway blockade on $CD4^+$ T_h17 cell differentiation.

However, our results did reveal an inhibition in IL-17 production by antigen-specific $CD8^+$ T cells following CD154/CD40 blockade. IL-17 secreting $CD8^+$ T cells have recently been identified as playing important roles in several animal models of infection and transplantation. For example, in T-bet deficient animals, IL-17 secreting $CD8^+$ T cells have been reported in response to both allografts and viral infections (40-42). In cardiac allograft models, mice lacking T-bet developed costimulation blockade resistant rejection mediated by IL-17 secreting $CD8^+$ T cells (41, 42). Genetic deletion of both T-bet and Eomes transcription factors also resulted in the differentiation of LCMV-reactive $CD8^+$ T cells into IL-17-secreting cells, which were associated with a wasting syndrome in the infected animals (40). Although the differentiation requirements for $CD8^+$ T_c17 cells have not been well described, our results would suggest that IL-6 and/or TNF may be important for their development.

Consistent with known effects of IL-6 on $CD4^+$ T cell differentiation, we observed increased differentiation of naïve $CD4^+$ T cells into $FoxP3^+$ iT_{reg} in anti-CD154-treated animals in which IL-6 elaboration by DC was reduced. Peripheral conversion of iT_{reg} may provide a source of antigen-specific regulatory T cells that

can promote survival of a bone marrow transplant. The peripheral conversion of naïve T cells into iT_{reg} has been observed in several other models, including following *in vitro* stimulation in the presence of TGF-β (43, 44) and following *in vivo* activation in the presence of low dose antigen (45). Conversely, increased level of antigen-stimulation prevented the conversion of T cells into a regulatory phenotype (46). In addition, CD4 blockade has been shown to result in the generation if $CD25^{hi}$ iT_{reg} and to protect skin allografts from rejection (47). In this study, we show that blockade of the costimulatory molecule CD154 may also lead to the conversion of graft-specific $CD4^+$ T cells into graft-protective iT_{reg}.

Taken together, we have shown that although CD40/CD154 pathway blockade with MR-1 does not significantly alter the phenotype of dendritic cells, it does alter their cytokine secretion profile. This reduction in DC-derived inflammatory cytokines coincided with decreased antigen-specific $CD8^+$ T cell differentiation into T_c17 and increased antigen-specific $CD4^+$ T cell differentiation into iT_{reg}. Thus, targeting DC-derived inflammatory cytokines in clinical transplantation could lead to enhanced engraftment and improved outcomes.

Figure 4.1.

CD40/CD154 pathway blockade protects mOVA bone marrow chimerism against rejection.

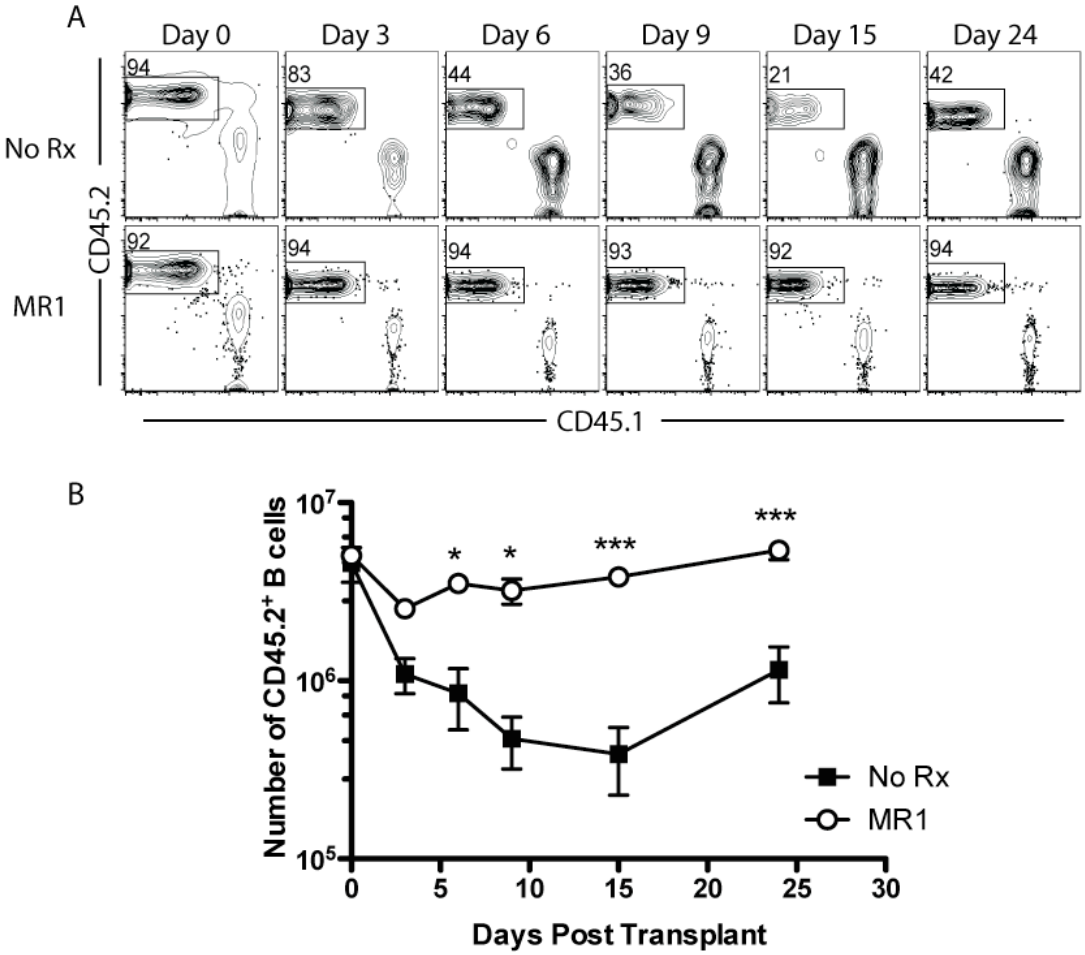


Figure 4.1. CD40/CD154 pathway blockade protects against T cell-mediated rejection of hematopoietic chimerism. On Day 0, $5x10^5$ OT-I and 10^6 OT-II T cells were adoptively transferred into mOVA bone marrow chimeric mice. At the time of transfer, mice were treated with 500 μg anti-CD154 mAb (MR-1), with continued treatments on days 2, 4 and 6, in indicated groups. A. Blood B cell chimerism was measured. Representative flow plots of remaining $B220^+$ cells, gating on the $mOVA^+$ ($CD45.2^+$) cells, over time. B. Total number of accumulated $CD45.2^+$ $mOVA^+$ B cells in the blood over time. Data are representative of three experiments with 4-5 mice per group. Statistics shown are mean±s.e.m. *$p \leq 0.05$, ***$p \leq 0.0001$.

Figure 4.2.

CD40/CD154 pathway blockade impairs bone marrow specific CD8⁺ T cell accumulation and differentiation.

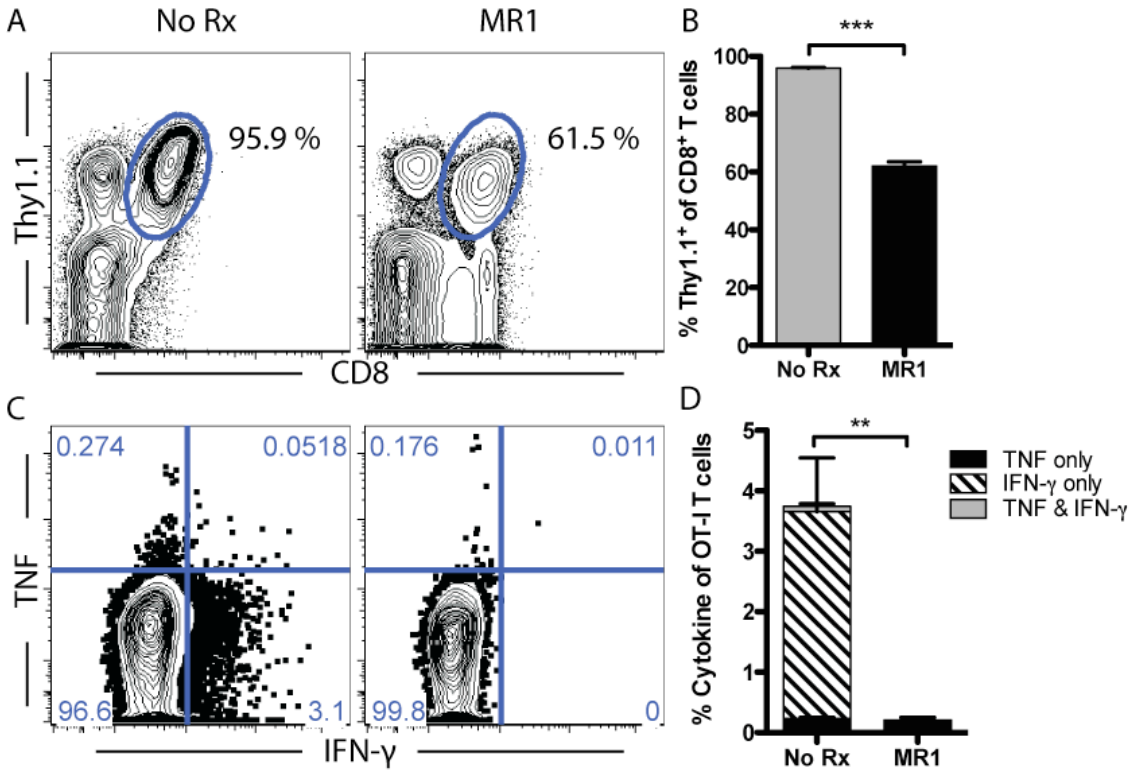


Figure 4.2. CD40/CD154 pathway blockade impairs bone marrow specific $CD8^+$ T cell accumulation and differentiation. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II, and treated with four doses of MR-1 on days 0, 2, 4, and 6, where indicated. A. Representative flow plots of cells in the spleens of treated mice. Data displayed are gated on $Thy1.1^+$ $CD8^+$ OT-I T cells on day 4 post-transfer. B. Frequency of accumulated OT-I T cells at day 4 post-transfer. C. Representative flow plots of intracellular TNF and IFN-γ cytokine staining of OT-I T cells after 4-hour *ex vivo* peptide stimulation. D. TNF and IFN-γ producing OT-I T cells at day 4 post-T cell transfer. Data are representative of two experiments with 4 mice per group. Statistics shown are mean±s.e.m. $^{**}p\leq0.01$, $^{***}p\leq0.0001$.

Figure 4.3.

CD40/CD154 pathway blockade does not impact the quantity of myeloid and lymphoid dendritic cells.

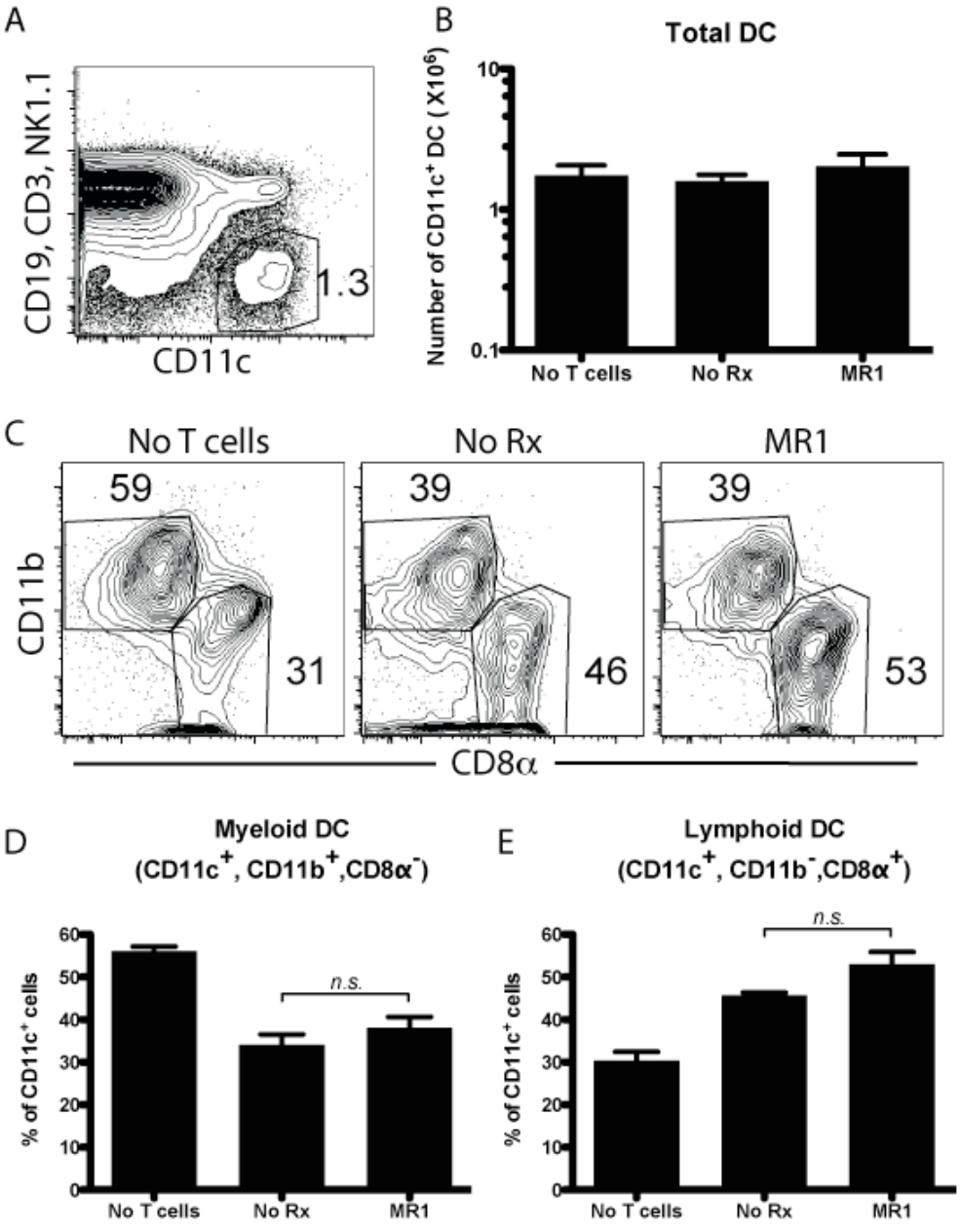


Figure 4.3. CD40/CD154 pathway blockade does not impact the quantity of myeloid and lymphoid dendritic cells. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II, and treated with four doses of MR-1, where indicated. A. Representative flow plot of cells gated on $CD11c^+$ dendritic cells, excluding B cells (CD19), T cells (CD3) and NK cells (NK1.1). B. Total DCs in spleen of mice prior to and post-T cell transfer in the presence or absence of MR-1, where indicated. C. Representative flow plots of dendritic cell populations, gated on $CD11c^+$. D. Frequency of myeloid ($CD11c^+$ $CD11b^+$ $CD8\alpha^-$) dendritic cells in spleens. E. Frequency of lymphoid ($CD11c^+$ $CD11b^-$ $CD8\alpha^+$) dendritic cells in spleens. Data are representative of 5 experiments with 3 mice per group. Statistics shown are mean±s.e.m.

Figure 4.4. CD40/CD154 pathway blockade does not impact dendritic cell differentiation and expression of costimulatory molecules.

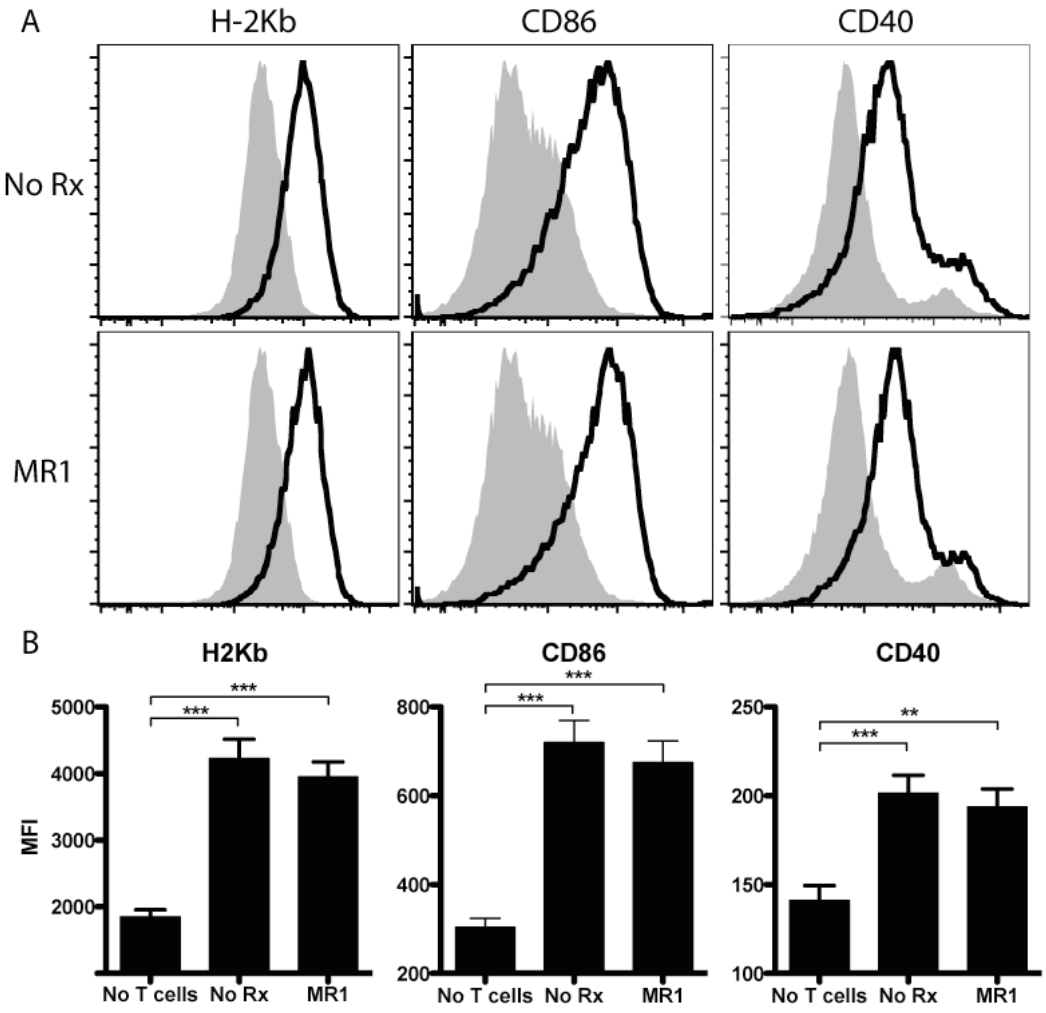


Figure 4.4. CD40/CD154 pathway blockade does not impact expression of MHC and costimulatory molecules on dendritic cells. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II, and treated with four doses of MR-1 on days 0, 2, 4, and 6, where indicated. Mice were sacrificed on day 3 and spleens were analyzed. A. Representative flow plots of $CD11c^+$ DCs expression of H-$2K^b$, CD86, and CD40 (black lines). Surface molecules on inactive $CD11c^+$ DCs prior to T cell transfer (shaded grey histograms). B. Relative expression of surface molecules on dendritic cells. Data are representative of 5 experiments with 3 mice per group. Statistics shown are mean±s.e.m. $^{**}p\leq0.01$, $^{***}p\leq0.0001$.

Figure 4.5.

CD40/CD154 pathway blockade impairs dendritic cell production of IL-6.

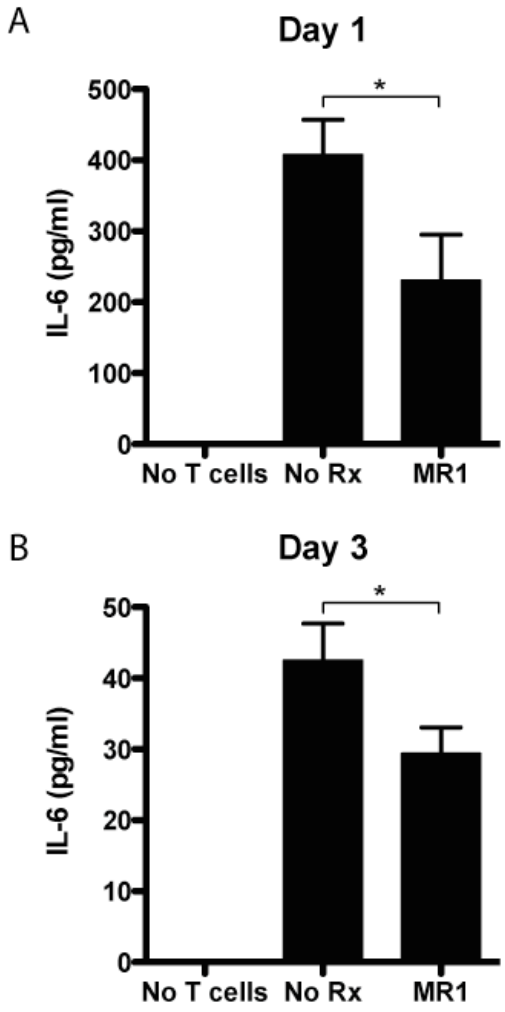


Figure 4.5. CD40/CD154 pathway blockade impairs dendritic cell production of IL-6. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II T cells and were treated with anti-CD154 on days 0, 2, 4, and 6, where indicated. Mice were sacrificed on days 1 (A) and 3 (B) post transfer. Splenic dendritic cells were isolated, FACS-sorted and cultured *in vitro* for 24 hours. Secretion of IL-6 cytokine by isolated DCs was measured in supernatants through a CBA analysis. Data are representative of two experiments with 3 mice per group. Statistics shown are mean±s.e.m. *$p \leq 0.05$.

Figure 4.6.

CD40/CD154 pathway blockade impairs T cell IL-17 production and promotes T_{reg} conversion.

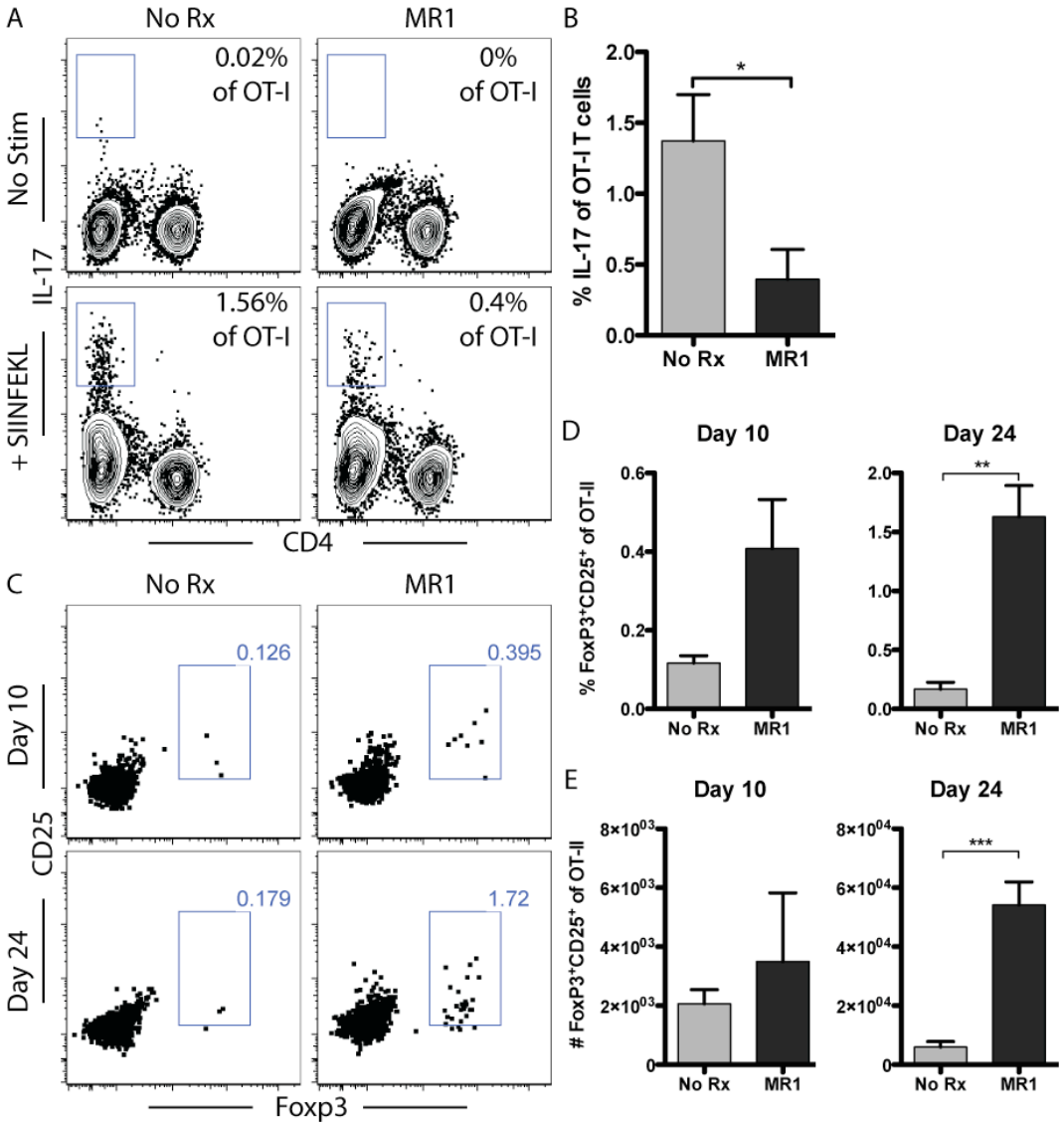
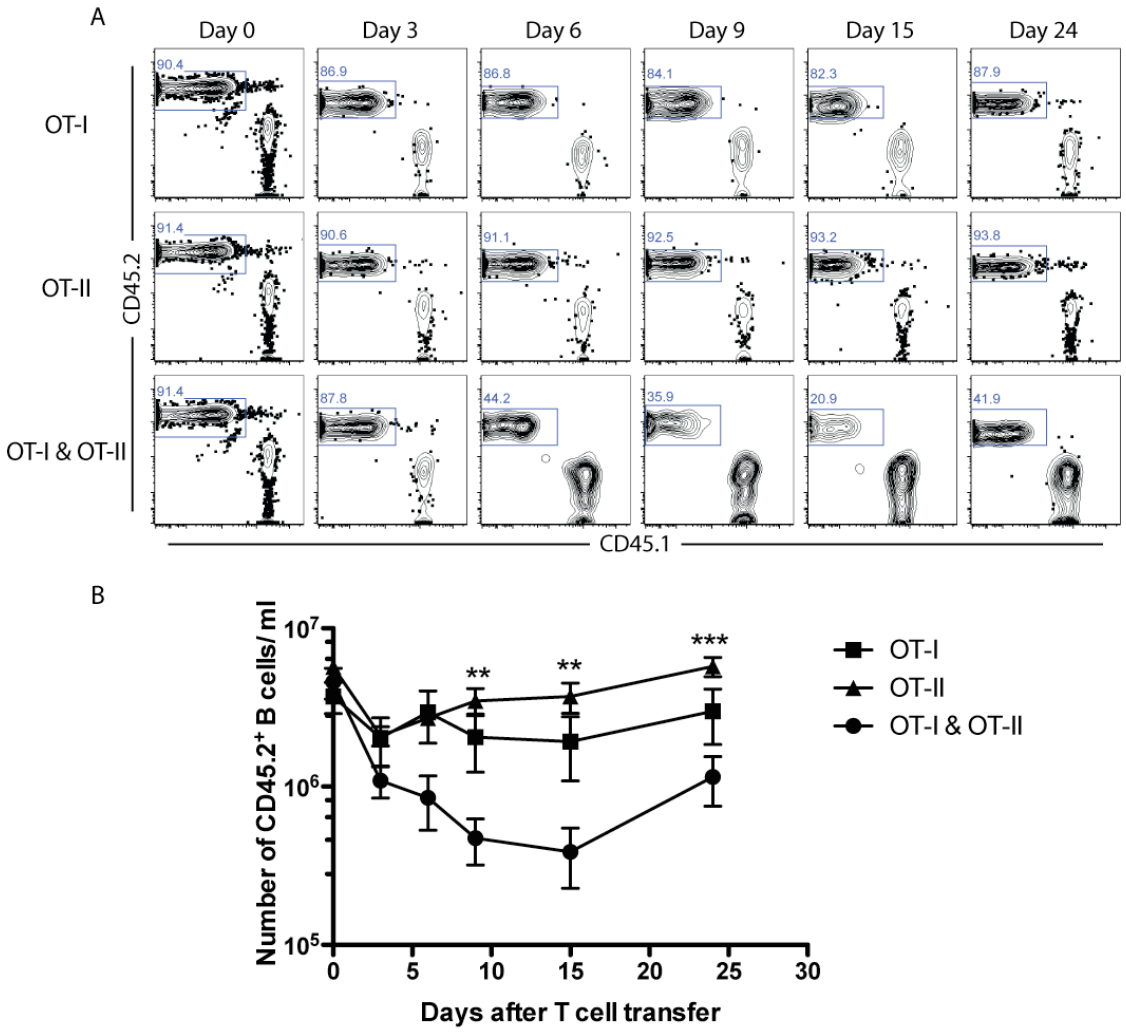


Figure 4.6. CD40/CD154 pathway blockade impairs T cell IL-17 production and promotes T_{reg} conversion. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-IIxRAG$^{-/-}$ T cells and were treated with four doses of anti-CD154 on days 0, 2, 4, and 6, where indicated. A. Representative flow cytometry plots of IL-17 cytokine production by OT-I and OT-II T cells. Splenocytes were stimulated *ex vivo* with cognate peptides for 4 hours. Data displayed are gated on Thy1.1$^+$ CD8$^+$ T cells. B. Frequency IL-17 producing OT-I T cells on day 7 post-transfer. C. Representative flow plots of OT-II T cells on day 10 and 24 post transfer. Data displayed are gated on Thy1.1$^+$ CD4$^+$ T cells. D. Frequency of isolated OT-II iT_{reg} in spleens on days 10 and 24. E. Total numbers of isolated OT-II iT_{reg} in spleens on days 10 and 24. Data are summary of two experiments with 4 mice per group. Statistics shown are mean±s.e.m. *p≤0.05, **p≤0.01, ***p≤0.0001.

Supplemental Figure 4.1.

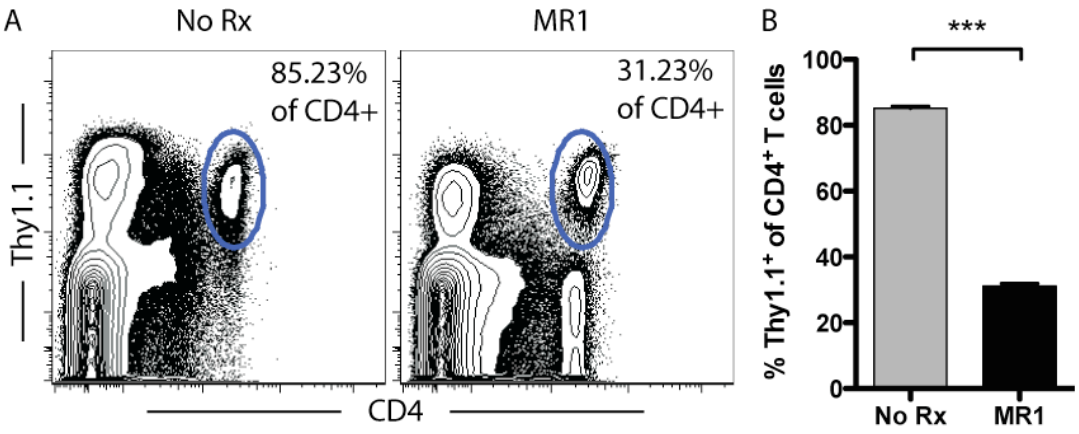
Antigen-specific CD4⁺ and CD8⁺ are both necessary to reject hematopoietic chimerism.



Supplemental Figure 4.1. Antigen-specific $CD4^+$ and $CD8^+$ are both necessary to reject hematopoietic chimerism. On Day 0, $5x10^5$ OT-I and/ or 10^6 OT-II T cells were adoptively transferred into mOVA bone marrow chimeric mice. At the time of transfer, mice were treated with 500 μg anti-CD154 mAb (MR-1), with continued treatments on days 2, 4 and 6, in indicated groups. A. Blood B cell chimerism was measured. Representative flow plots of remaining $B220^+$ cells, gating on the donor-derived $mOVA^+$ ($CD45.2^+$) cells, over time. B. Total number of accumulated $CD45.2^+$ $mOVA^+$ B cells in the blood over time. Data are representative of two experiments with 5 mice per group. Statistics shown are mean±s.e.m. $^{**}p\leq0.01$, $^{***}p\leq0.0001$.

Supplemental Figure 4.2.

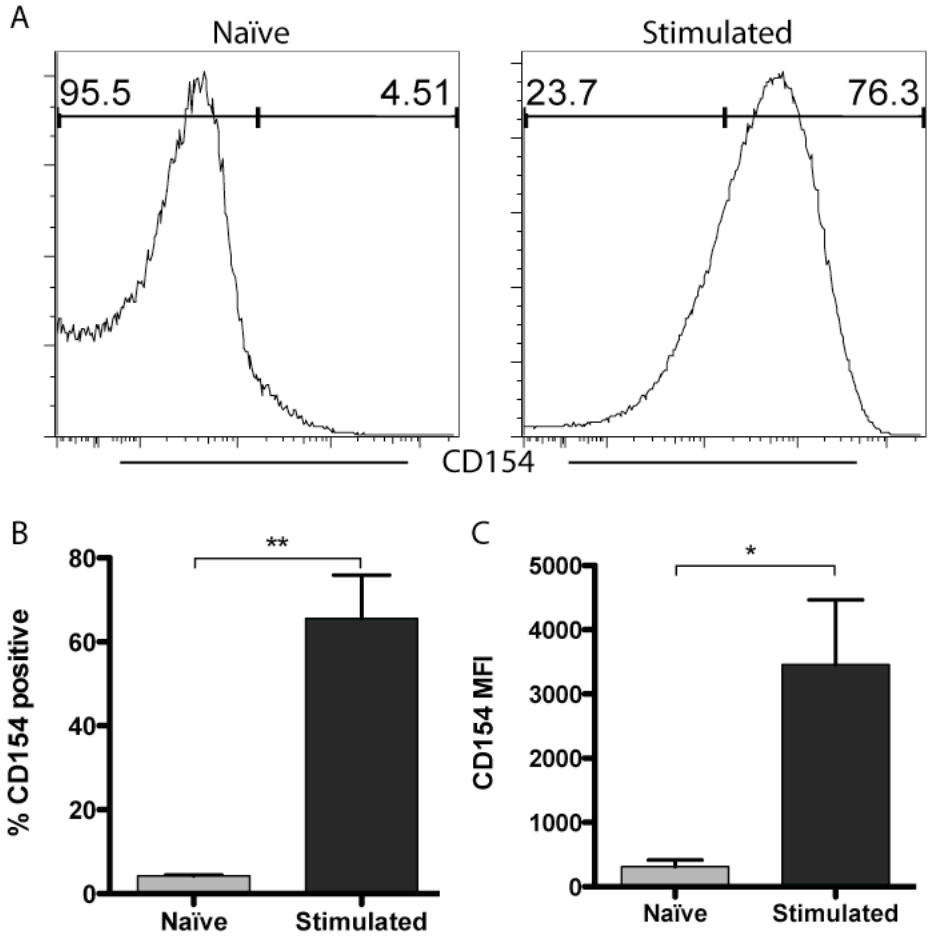
CD40/CD154 pathway blockade impairs graft-specific CD4⁺ T cell accumulation.



Supplemental Figure 4.2. CD40/CD154 pathway blockade impairs graft-specific $CD4^+$ T cell accumulation. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II, and treated with four doses of MR-1 on days 0, 2, 4, and 6, where indicated. A. Representative flow plots of $CD4^+$ T cells in the spleens of treated mice. Data displayed are gated on $Thy1.1^+$ $CD4^+$ T cells. B. Frequency of accumulated OT-II T cells at day 4 post-transfer. Data are representative of two experiments with 4 mice per group. Statistics shown are mean±s.e.m. ***$p \leq 0.0001$.

Supplemental Figure 4.3.

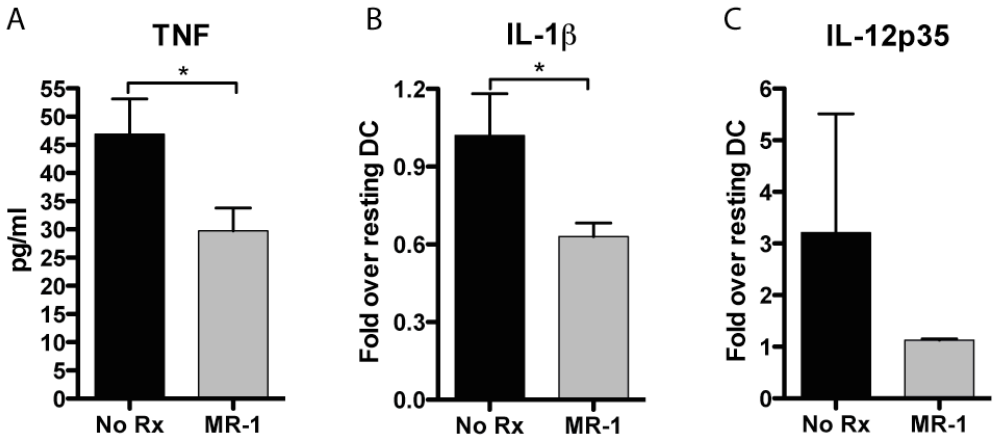
Antigen-specific OT-II T cells significantly upregulate CD154 expression upon stimulation.



Supplemental Figure 4.3. Antigen-specific OT-II T cells significantly upregulate CD154 expression upon stimulation. OT-II T cells were isolated from spleens of naïve animals and were stimulated for 4 days at $5x10^6$ per well in 24-well plates in the presence of 1 μM $OVA_{323-339}$. Cells were then counted and replated at 10^6 per well in 96-well plates with 10 μM concentration of $OVA_{323-339}$ in the presence of 1 μL/mL Monensin for 6 hour. A. Representative flow plots of CD154 expression in unstimulated (left) and pulsed (right) OT-II T cells. B. Frequency of $CD154^+$ OT-II T cells after *in vitro* stimulation. C. MFI of total CD154 expression on OT-II T cells after *in vitro* stimulation. Results are summary of three experiments with 3 replicates per group. Statistics shown are mean±s.e.m. *p≤0.05, **p≤0.001.

Supplemental Figure 4.4.

CD154 blockade diminishes proinflammatory cytokine production by DCs.



Supplemental Figure 4.4. CD154 blockade diminishes proinflammatory cytokine production by DCs. mOVA chimeric mice were adoptively transferred with $5x10^6$ OT-I and 10^6 OT-II T cells and were treated with anti-CD154 every other day, where indicated. Mice were sacrificed on day 3. A. Secretion of TNF by isolated splenic DCs was measured *ex vivo* by CBA analysis. Results are a summary of two experiments with a total of 8-9 mice per group. B. Splenic dendritic cells were isolated, FACS-sorted and cultured *in vitro* for 24 hours. Expressions of inflammatory cytokines were analyzed by real-time PCR, as described in *Materials and Methods*. There was no difference detected in G-CSF, GM-CSF, CXCL-10, CXCL-11, IL-10, IL-12p40, IL-13, IL-15, IL-18, IL-1α, or IL-4. Results are summary of three experiments with three mice per group. Statistics shown are mean±s.e.m. *$p \leq 0.05$.

REFERENCES

1. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. 1996. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381: 434-8

2. Hancock WW, Sayegh MH, Zheng XG, Peach R, Linsley PS, Turka LA. 1996. Costimulatory function and expression of CD40 ligand, CD80, and CD86 in vascularized murine cardiac allograft rejection. *Proc Natl Acad Sci U S A* 93: 13967-72

3. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, Fechner JH, Jr., Germond RL, Kampen RL, Patterson NB, Swanson SJ, Tadaki DK, TenHoor CN, White L, Knechtle SJ, Harlan DM. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 5: 686-93

4. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Jr., Knechtle SJ. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* 94: 8789-94

5. Markees TG, Phillips NE, Gordon EJ, Noelle RJ, Shultz LD, Mordes JP, Greiner DL, Rossini AA. 1998. Long-term survival of skin allografts induced by donor splenocytes and anti-CD154 antibody in thymectomized mice requires CD4(+) T cells, interferon-gamma, and CTLA4. *J Clin Invest* 101: 2446-55

6. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114

7. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA, Robertson JM, Leopardi FV, Jonhson BE, Strobert EA, Iwakoshi NN, Reimann KA, Ford ML, Kirk AD, Larsen CP. Non-Depleting CD40-Specific Therapy Prolongs Allograft Survival in Nonhuman Primates. *Am J Transplant* In press

8. Page A, Srinivasan S, Singh K, Russell M, Hamby K, Deane T, Sen S, Stempora L, Price AA, Strobert E, Reimann KA, Kirk AD, Larsen CP, Kean LS. CD40 Blockade Combines with CTLA4Ig and Sirolimus to Produce Mixed Chimerism in a MHC-Defined Rhesus Macaque Transplant Model. *Am J Transplant* In press

9. Thompson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbutt G, Rayat GR, Cano J, Song M, Jiang W, Strobert E, Rajotte R, Pearson T, Kirk AD, Larsen CP. 2011. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. *Am J Transplant* 11: 947-57

10. Watanabe M, Yamashita K, Suzuki T, Kamachi H, Kuraya D, Koshizuka Y, Ogura M, Yoshida T, Sakai F, Miura T, Todo S. 2011. Long-Term Acceptance of Islet Allografts by ASKP1240 (4D11), a Fully Human Anti-CD40 Monoclonal Antibody in Cynomolgus Monkeys. *American Journal of Transplantation* 11: 80-

11. Monk NJ, Hargreaves RE, Marsh JE, Farrar CA, Sacks SH, Millrain M, Simpson E, Dyson J, Jurcevic S. 2003. Fc-dependent depletion of activated T cells

occurs through CD40L-specific antibody rather than costimulation blockade. *Nat Med* 9: 1275-80

12. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S, Rees P, Hendrix R, Price K, Kenyon NS, Hagerty D, Townsend R, Hollenbaugh D, Pearson TC, Larsen CP. 2005. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* 174: 542-50

13. Haanstra KG, Ringers J, Sick EA, Ramdien-Murli S, Kuhn EM, Boon L, Jonker M. 2003. Prevention of kidney allograft rejection using anti-CD40 and anti-CD86 in primates. *Transplantation* 75: 637-43

14. Gilson CR, Milas Z, Gangappa S, Hollenbaugh D, Pearson TC, Ford ML, Larsen CP. 2009. Anti-CD40 monoclonal antibody synergizes with CTLA4-Ig in promoting long-term graft survival in murine models of transplantation. *J Immunol* 183: 1625-35

15. Saemann MD, Diakos C, Kelemen P, Kriehuber E, Zeyda M, Bohmig GA, Horl WH, Baumruker T, Zlabinger GJ. 2003. Prevention of CD40-triggered dendritic cell maturation and induction of T-cell hyporeactivity by targeting of Janus kinase 3. *Am J Transplant* 3: 1341-9

16. Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. *Nature* 392: 245-52

17. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811

18. Hochweller K, Anderton SM. 2005. Kinetics of costimulatory molecule expression by T cells and dendritic cells during the induction of tolerance versus immunity in vivo. *Eur J Immunol* 35: 1086-96

19. van Kooten C, Banchereau J. 2000. CD40-CD40 ligand. *J Leukoc Biol* 67: 2-17

20. Miga AJ, Masters SR, Durell BG, Gonzalez M, Jenkins MK, Maliszewski C, Kikutani H, Wade WF, Noelle RJ. 2001. Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur J Immunol* 31: 959-65

21. De Smedt T, Pajak B, Klaus GG, Noelle RJ, Urbain J, Leo O, Moser M. 1998. Antigen-specific T lymphocytes regulate lipopolysaccharide-induced apoptosis of dendritic cells in vivo. *J Immunol* 161: 4476-9

22. O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, Kanno Y. 2011. Genomic views of STAT function in CD4+ T helper cell differentiation. *Nat Rev Immunol* 11: 239-50

23. Korn T, Bettelli E, Oukka M, Kuchroo VK. 2009. IL-17 and Th17 Cells. *Annu Rev Immunol* 27: 485-517

24. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-8

25. Perona-Wright G, Jenkins SJ, O'Connor RA, Zienkiewicz D, McSorley HJ, Maizels RM, Anderton SM, MacDonald AS. 2009. A pivotal role for CD40-mediated IL-6 production by dendritic cells during IL-17 induction in vivo. *J Immunol* 182: 2808-15

26. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8: 942-9

27. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6: 1123-32

28. de Latour RP, Visconte V, Takaku T, Wu C, Erie AJ, Sarcon AK, Desierto MJ, Scheinberg P, Keyvanfar K, Nunez O, Chen J, Young NS. 2010. Th17 immune responses contribute to the pathophysiology of aplastic anemia. *Blood* 116: 4175-84

29. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, Vollmar P, Stritesky GL, Kaplan MH, Waisman A, Kuchroo VK, Oukka M. 2008. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 105: 18460-5

30. Ehst BD, Ingulli E, Jenkins MK. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3: 1355-62

31. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med* 204: 1775-85

32. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478-80

33. Ridge JP, Di Rosa F, Matzinger P. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-8

34. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-3

35. Kobayashi T, Walsh PT, Walsh MC, Speirs KM, Chiffoleau E, King CG, Hancock WW, Caamano JH, Hunter CA, Scott P, Turka LA, Choi Y. 2003. TRAF6 is a critical factor for dendritic cell maturation and development. *Immunity* 19: 353-63

36. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* 441: 231-4

37. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. 2009. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells. *Proc Natl Acad Sci U S A* 106: 876-81

38. Chen L, Ahmed E, Wang T, Wang Y, Ochando J, Chong AS, Alegre ML. 2009. TLR signals promote IL-6/IL-17-dependent transplant rejection. *J Immunol* 182: 6217-25

39. Shen H, Goldstein DR. 2009. IL-6 and TNF-alpha synergistically inhibit allograft acceptance. *J Am Soc Nephrol* 20: 1032-40

40. Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, Hunter CA, Wherry EJ, Lindsten T, Reiner SL. 2008. Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science* 321: 408-11

41. Yuan X, Ansari MJ, D'Addio F, Paez-Cortez J, Schmitt I, Donnarumma M, Boenisch O, Zhao X, Popoola J, Clarkson MR, Yagita H, Akiba H, Freeman GJ, Iacomini J, Turka LA, Glimcher LH, Sayegh MH. 2009. Targeting Tim-1 to overcome resistance to transplantation tolerance mediated by CD8 T17 cells. *Proc Natl Acad Sci U S A* 106: 10734-9

42. Burrell BE, Csencsits K, Lu G, Grabauskiene S, Bishop DK. 2008. CD8+ Th17 mediate costimulation blockade-resistant allograft rejection in T-bet-deficient mice. *J Immunol* 181: 3906-14

43. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-86

44. Taylor PA, Lees CJ, Blazar BR. 2002. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood* 99: 3493-9

45. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-27

46. Molinero LL, Miller ML, Evaristo C, Alegre ML. 2011. High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-{kappa}B-Dependent Manner. *J Immunol* 186: 4609-17

47. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. 2004. Alloantigen-induced CD25(+)CD4(+) regulatory T cells can develop in vivo from CD25(-)CD4(+) precursors in a thymus-independent process. *Journal of Immunology* 172: 923-8

Chapter 5

Cutting Edge: Rapamycin Augments Pathogen-Specific but Not Graft-Reactive CD8$^+$ T Cell Responses

Originally published in *The Journal of Immunology*.

Ferrer, I.R., Wagener, M.E., Robertson, J.M., Turner, A.P., Araki, K., Ahmed, R., Kirk, A.D., Larsen, C.P., Ford, M.L. 2010. Cutting Edge: Rapamycin Augments Pathogen-Specific but not Graft-Reactive CD8$^+$ T Cell Responses. *J. Immunol.* 185: 2004-8

ABSTRACT

Recent evidence demonstrating that exposure to rapamycin during viral infection *increased* the quantity and quality of antigen-specific T cells poses an intriguing paradox, since rapamycin is used in transplantation to dampen, rather than enhance, donor-reactive T cell responses. In this report, we compared the effects of rapamycin on the antigen-specific T cell response to a bacterial infection versus a transplant. Using a transgenic system in which the antigen and the responding T cell population were identical in both cases, we observed that treatment with rapamycin augmented the antigen-specific T cell response to a pathogen, while it failed to do so when the antigen was presented in the context of a transplant. These results suggest that the environment in which an antigen is presented alters the influence of rapamycin on antigen-specific T cell expansion, and highlights a fundamental difference between antigen presented by an infectious agent as compared to an allograft.

INTRODUCTION

Transplantation is a life-saving treatment option for many forms of end-stage organ disease. The advent of new immunosuppressive agents over the last thirty years has dramatically increased the graft survival of almost all types of organ and tissue transplantation. One such agent, rapamycin, was isolated in the early 1970's from *Streptomyces hygroscopicus* and was found to potently inhibit cell proliferation and therefore possess immunosuppressive effects (1). Despite its current widespread use for the prevention of kidney allograft rejection (2), the precise effects of rapamycin on different cell types involved in rejection, including effector T cells, dendritic cells (DC) and regulatory T cells is an area of intense investigation (1). Rapamycin exerts its effect by targeting the mammalian target of rapamycin (mTOR) (3), a serine/threonine protein kinase which has a pervasive role in many aspects of both the innate and adaptive immune response (1). Several studies exist to suggest that blockade of mTOR by rapamycin retards dendritic cell maturation and inhibits antigen uptake and presentation by DC (4) and also attenuates T cell proliferation by inhibiting the G1➔S transition. Furthermore, rapamycin has been shown by many groups to enhance the generation and function of regulatory T cells (5, 6), potentially further promoting its immunosuppressive effects during transplantation.

Recent studies in virally infected mice that had been treated with rapamycin revealed surprising and as-yet unappreciated effects on the expansion and retention of viral-specific $CD8^+$ T cells. Specifically, Araki *et al.* measured the antigen-specific $CD8^+$ T cell response following infection of mice infected with lymphocytic

choriomeningitis virus (LCMV) in the presence or absence of rapamycin (7). In contrast to the expected result based on rapamycin's known function as an immunosuppressant, this study revealed that treatment with rapamycin instead increased the quantity and quality of virus-specific memory T cells. Treatment of both mice and rhesus macaques with rapamycin resulted in an increased response to live virus or vaccination, respectively. Using RNAi knockdown of mTOR, the regulatory-associated protein of mTOR (RAPTOR), or the rapamycin-binding protein FKB12, these studies also demonstrated that inhibition of mTOR functioned in a T cell intrinsic manner to enhance the quantity and quality of antigen-specific T cells (7).

Because this drug is used in many clinical transplantation immunosuppressive regimens (2), we sought to address whether treatment with rapamycin resulted in an increase in the T cell response to additional types of pathogens, such as bacterial infection, and also to study the effect of rapamycin on the expansion and retention of donor-reactive $CD8^+$ T cells following transplantation. In short, we sought to compare the effect of rapamycin on the $CD8^+$ T cell response to a pathogen to its effect on the $CD8^+$ T cell response to a transplant. In order to accomplish this, we made use of a transgenic system in which the same monoclonal TCR transgenic T cells responded to a bacterial pathogen infection or a skin graft. OT-I T cells recognized an epitope (SIINFEKL/K^b) that was expressed by both *Listeria monocytogenes* (LM-OVA)(8) and by donor skin under the control of the β-actin promoter (9). In comparing the $CD8^+$ T cell responses by identical monoclonal cell populations to the same epitope in the

setting of either pathogen infection or transplantation, we found that treatment with rapamycin resulted in very disparate effects on the antigen-specific cell populations. While rapamycin augmented the antigen-specific $CD8^+$ T cell response to a bacterium, it failed to do so in response to a transplant.

MATERIALS AND METHODS

Mice

Adult male 6- to 8-week old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TCR transgenic OT-I and OT-II mice were purchased from Taconic, Inc. and bred onto the Thy1.1$^+$ background. Act-mOVA mice were produced by Dr. Marc Jenkins, Univ. of Minnesota (9). Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

T Cell Adoptive Transfers

OT-I and OT-II Thy1.1$^+$ TCR transgenic T cells were harvested from spleen. The frequency of OT-I or OT-II T cells was determined prior to adoptive transfer by staining with anti-Vα2 (used by both TCRs) and anti-CD8 or anti-CD4, respectively (Pharmingen, San Diego, CA).

Listeria infection and rapamycin treatment

Forty-eight hours prior to *Listeria* infection, naïve B6 mice received an i.v. injection of 10^4 OT-I T cells. Similar results were observed if mice received both OT-I and OT-II T cells (data not shown). Mice were then infected with 10^4 colony-forming units (CFU) of *Listeria monocytogenes* expressing ovalbumin (8) gene i.p. on day 0. Where indicated, mice were treated with 1.5 ug/day rapamycin (Rapamune, Wyeth Pharmaceuticals) on days 0-10 post-infection or post-transplant as previously described (7).

Skin Grafting

Forty-eight hours prior to mOVA skin grafting, mice received an i.v. injection of 10^6 OT-I and OT-II T cells, to mimic the higher precursor frequencies observed in allospecific immune responses (10). Skin grafts (~1 cm^2) were transplanted onto the dorsal thorax of recipient mice and secured with an adhesive bandage for 5 days. Rejection was defined as 90% loss of viable epidermal tissue.

Flow Cytometric Analyses for Frequency and Absolute Number

Splenocytes were stained with Thy1.1-PerCP, CD8-PacOrange, and CD4-PacBlue (BD Pharmingen) for flow cytometric analysis on a BD LSRII flow cytometer. Absolute numbers of antigen-specific T cells were determined by TruCount Bead Analysis (Pharmingen). Data were analyzed using FlowJo Software (Treestar, San Carlos, CA).

Intracellular Cytokine Staining

Cells were incubated for 4 hours with 10 nM $OVA_{257-264}$ (SIINFEKL), and 10 μg/ml Brefeldin A (Pharmingen), and processed using an intracellular staining kit (Pharmingen).

Statistical Analyses

Groups were compared by Mann-Whitney non-parametric test (GraphPad Prism Software, La Jolla, CA).

RESULTS

Rapamycin treatment resulted in increased antigen-specific CD8⁺ effectors following infection with OVA-expressing Listeria monocytogenes

In order to assess the effects of treatment with rapamycin on the antigen-specific $CD8^+$ T cell response following a bacterial infection, we adoptively transferred $Thy1.1^+$ OT-I T cells specific for $OVA_{257-264}/K^b$ into naïve B6 recipients, which were then infected with an OVA-expressing *Listeria monocytogenes* (LM-OVA) in the presence or absence of rapamycin. In this model infectious bacteria are cleared from the spleen and liver by day 5 post-infection (data not shown). At the peak of the antigen-specific $CD8^+$ T cell response (day 10 post-infection), frequency of OVA-specific OT-I T cells in the peripheral blood was quantified by flow cytometry (Figure 5.1A). We observed an increase in the frequency of antigen-specific T cells at day 10 post-infection in the rapamycin-treated animals as compared to untreated controls (18.4% ± 1.9% vs 8.9% ± 1.8%, respectively, p=0.001) (Figure 5.1B). Longitudinal analysis revealed that the enhancing effects of rapamycin were observed at all timepoints, including memory, indicating that these observations were not simply due to altered kinetics of expansion of the antigen-specific $CD8^+$ T cell response (data not shown). Consistent with recently published results in a viral infection model (7), these data indicated that treatment with rapamycin enhanced the antigen-specific T cell response following bacterial infection.

Rapamycin treatment did not result in increased donor-reactive CD8$^+$ T cell responses following skin transplantation

In order to determine whether rapamycin resulted in an increased frequency of donor-reactive memory T cells following transplantation, we used the same transgenic mouse model to identify and track T cells responding to the graft in the presence or absence of rapamycin. Briefly, mice received 10^6 Thy1.1$^+$ OVA-specific CD8$^+$ OT-I T cells two days prior to receiving an OVA-expressing skin graft. Mice were then left untreated or were treated with rapamycin, and were sacrificed on day 10 post-transplant. Results indicated that the frequency of the donor-reactive CD8$^+$ populations was not increased in the rapamycin treated recipients at the peak of the response (Figures 5.1D, E). This result was also true for the draining LN (data not shown). The minimal effect of rapamycin was not due to failure of Thy1.1$^+$ T cells to be recruited into the response, since all Thy1.1$^+$ cells underwent division following engraftment as measured by CFSE dilution (data not shown). Furthermore, the differential effect of rapamycin shown in Figure 5.1B vs Figure 5.1D was not simply due to differences in precursor frequency, as increasing the number of adoptively transferred T cells to 10^6 in recipients of LM-OVA still resulted in an increase in the frequency of antigen-specific T cells following rapamycin treatment as compared to untreated controls (data not shown). Therefore, these results suggest an intrinsic difference in the effect of rapamycin on the T cell response to a graft vs. a pathogen.

Rapamycin altered CD62L expression on CD8$^+$ T cells responding to a pathogen but not a graft

In addition to enhancing the quantity of antigen-specific T cells following *Listeria* infection, we found that treatment with rapamycin also altered the quality of the antigen-specific CD8$^+$ T cell response. CD62L has been used as a marker to differentiate between effector and memory T cells at memory time points (11). Central memory T cells (CD62L^{hi}) possess increased proliferative capacity and increased ability to mount a secondary response as compared to CD62L^{lo} cells. Therefore, the presence of more CD62L^{hi} cells in the rapamycin-treated recipients may signify an increase in the quality of these memory T cells. Specifically, treatment with rapamycin during *Listeria* infection resulted in an increased frequency of CD62L^{hi} Thy1.1$^+$ cells (17.6 ±1.6% CD62L^{hi} vs. 10.8 ±1.1% in untreated controls, p=0.0046, Figure 5.1C). These results indicated that in addition to augmenting the frequency of pathogen-specific T cells, rapamycin also impacted the degree of differentiation of these cells. Therefore, we next sought to address whether treatment with rapamycin resulted in increased CD62L expression in donor-reactive CD8$^+$ T cells stimulated by a skin graft. In contrast to our observations in the bacterial infection model, we found that splenocytes analyzed on day 10 post-transplant in rapamycin-treated recipients did not demonstrate an increase in CD62L expression (Figure 5.1F).

Rapamycin differentially impacted the absolute number of IFN-γ-secreting cells generated in response to LM-OVA vs. an mOVA skin graft

As shown in Figure 1, we observed increased quantity and quality of antigen-specific $CD8^+$ T cell populations in response to a bacterial infection following treatment with rapamycin. To further assess the quality of antigen-specific T cell responses generated against a pathogen vs. a graft in the presence of rapamycin, we examined the ability of these antigen-specific T cells to produce cytokines following *ex vivo* restimulation (Figure 5.2). Following *ex vivo* restimulation with OVA peptide, $CD8^+$ T cells derived from splenocytes of rapamycin-treated LM-OVA-infected recipients exhibited an *increased* absolute number of IFN-γ-secreting T cells as compared to untreated LM-OVA infected controls (Figure 5.2A, B). In sharp contrast, we observed a modest *decrease* in the absolute number of IFN-γ-producing cells isolated from rapamycin-treated mOVA skin graft recipients in response to antigen restimulation (Figure 5.2C, D). In summary, the immunostimulatory effect of rapamycin on antigen-specific T cell populations observed in the context of a pathogen was not observed in the context of a transplant.

Simultaneous infection and transplantation did not result in increased donor-specific T cell immunity in rapamycin-treated recipients

From the experiments presented above we concluded that rapamycin exhibited disparate effects on identical antigen-specific T cells that were responding in the context of a bacterial infection versus a transplant. Because mTOR has also been shown to be involved in signaling downstream of Toll-like receptors (TLRs)

(12), we hypothesized that TLR signaling or other inflammatory signals associated with a bacterial pathogen might be required for the enhancing effects of rapamycin on antigen-specific T cell responses. As such, we examined the donor-reactive T cell response to a skin graft in the presence of a concomitant bacterial infection. Briefly, naïve B6 recipients were adoptively transferred with Thy1.1$^+$ OT-I and OT-II T cells and received a mOVA skin graft. On the day of graft placement, mice also were infected with wild-type (non-OVA-expressing) LM. Results demonstrated that concomitant infection with LM in the presence of rapamycin failed to result in the augmentation of OVA-specific CD8$^+$ T cell populations in response to the graft (Figure 5.3A, B). Furthermore, we observed no difference in the expression of CD62L as we had in OVA-specific T cell populations responding to LM-OVA (data not shown). Also in contrast to our observations of OT-I T cells stimulated by LM-OVA, we observed a modest decrease in the number of IFN-γ-producing donor-reactive T cells in rapamycin-treated mice receiving a concurrent LM infection and mOVA skin graft as compared to untreated controls (Figure 5.3C). These data therefore demonstrated that TLR-mediated stimulation or other pathogen-associated inflammation was not sufficient to explain the disparate effects of rapamycin on antigen-specific T cell responses following stimulation by a pathogen vs. a transplant.

DISCUSSION

Because rapamycin was widely appreciated to attenuate immune responses through mechanisms such as altered DC differentiation and increased T_{reg} populations (13, 14), recent evidence demonstrating that exposure to rapamycin during the course of an immune response to viral infection increased the quantity and quality of antigen-specific T cells (7) poses an intriguing paradox for the field of immunology. In this report, we have attempted to reconcile these seemingly disparate findings by directly comparing the effects of rapamycin on an antigen-specific T cell response to a bacterial infection versus a skin graft. By employing a transgenic system in which both the antigen of interest and the responding monoclonal T cell population were identical in both models, we observed that treatment with rapamycin augmented the antigen-specific T cell response to bacterial infection, while failing to do so when the same antigen was presented in the context of a transplant. These results suggest a fundamental difference in the effects of rapamycin on the expansion of T cell populations in response to an infectious agent as compared to an allograft. They also serve to mitigate concern that treatment with rapamycin might paradoxically augment donor-reactive T cell responses.

In addition to its known role as a signaling component downstream of the T cell receptor/CD3 complex, mTOR has also been shown to participate in the signaling cascade downstream of many TLRs (12). Therefore, we speculated that the observed difference in the effects of rapamycin on pathogen- and donor-reactive T cell responses might be due to engagement of TLR/innate immune pathways

during the bacterial infection, either on antigen-presenting cells (4, 13, 14) or on the T cells themselves (15). These pathways would presumably not be engaged following transplantation of a skin graft. However, results indicated that infection with non-OVA-expressing *Listeria* concurrently with transplantation of mOVA skin grafts did not result in augmentation of anti-OVA T cell responses. One potential caveat of this experiment might be the question of whether the *Listeria* was present in the same local environment as the graft-specific T cells during priming, in order to influence the effect of rapamycin on these cells. However, Chong and colleagues have demonstrated that *Listeria* infection concurrent with transplantation resulted in the prevention of tolerance induction (16), signifying that LM infection-derived inflammatory signals are likely to reach graft-specific T cells in this model, and that the presence of a concurrent bacterial infection and engagement of innate immune mechanisms does influence the quality of anti-donor T cell responses. Importantly, however, our results indicate that this effect was not impacted by the presence or absence of rapamycin. If activation of innate immune mechanisms is not responsible, what other factors could account for the observed discrepancy of the effect of rapamycin on pathogen-specific vs graft-specific T cell responses? One possibility is that duration of antigen presentation may modulate the impact of rapamycin on antigen-specific T cell responses. Further experiments are required to test this hypothesis.

In addition, these data may have relevance to the issue of heterologous alloreactivity. Our group and others have shown that prior or concurrent pathogen infection can augment the allospecific T cell response and alter the outcome of an

allograft (16-18). This has been attributed in part to molecular mimicry (19), bystander activation (16) and other reasonably simple mechanisms. It would appear from our results that in the presence of rapamycin, presentation of the same antigen delivered either by a graft or a pathogen to the same T cell could evoke markedly different responses. As such, the outcome of stimulation with a cross-reactive antigen cannot be assumed to adversely effect allograft survival. Moreover, the order of presentation (pathogen or allograft first), and presence of immunosuppression at the time of initial presentation may significantly influence the ultimate outcome.

In summation, these results highlight disparate effects of rapamycin on T cell responses to pathogens and donor tissue, and underscore the fact that there are many facets to the mTOR signaling pathway in immune cells that are still poorly understood. Still, the fact that previous studies and data presented here indicate that rapamycin might paradoxically enhance the antigen-specific $CD8^+$ T cell response to viral or bacterial pathogens suggests that treatment of transplant recipients with rapamycin monotherapy may simultaneously increase immunity to a virus or vaccine (7) while inhibiting the response to an allograft. The impact of rapamycin on pathogen-specific $CD8^+$ T cell responses in the context of other immunosuppressive agents is an important area of future research.

Figure 5.1.

Rapamycin enhanced the antigen-specific T cell response to OVA in the context of a bacterial infection but not a transplant.

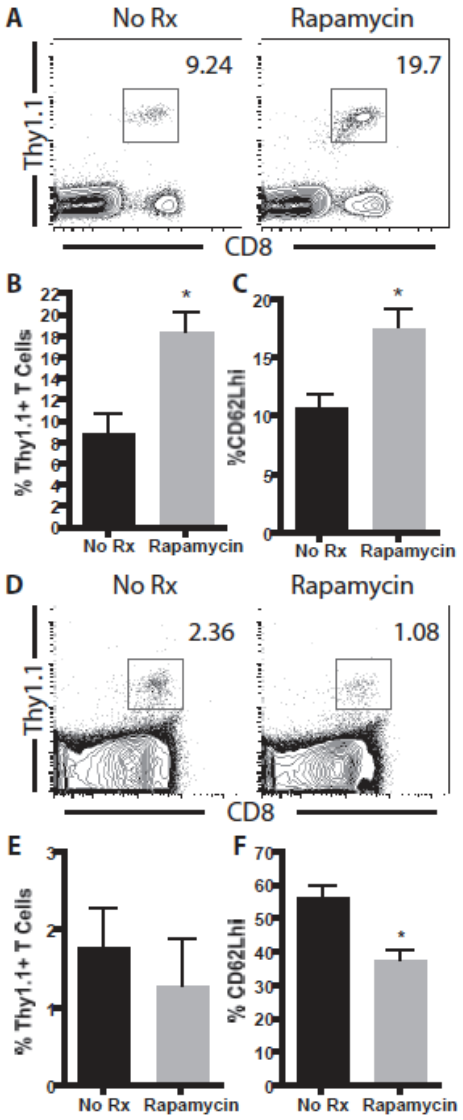


Figure 5.1. Rapamycin enhanced the antigen-specific T cell response to OVA in the context of a bacterial infection but not a transplant. A-C, 10^4 Thy1.1$^+$ OT-I T cells were adoptively transferred into naïve B6 recipients, which were then infected with 10^4 CFU of OVA-expressing LM-OVA. Rapamycin was administered intraperitoneally in 500 µl of sterile PBS from days 0-10 post-infection. Analysis of splenocytes indicated that the day 10 frequency (A, B) or % CD62L^{hi} (C) of the donor-reactive CD8$^+$ population was significantly increased in the rapamycin treated recipients ($p<0.005$). D-F, 10^6 Thy1.1$^+$ CD8$^+$ OT-I and CD4$^+$ OT-II T cells were adoptively transferred into B6 recipients two days prior to receiving an OVA-expressing skin graft in the presence or absence of rapamycin. Analysis of splenocytes indicated that the day 10 frequency (D, E) or % CD62L^{hi} (F) of the donor-reactive CD8$^+$ population was not increased in the rapamycin treated recipients. Results are cumulative analyses of three independent experiments with five mice per treatment group.

Figure 5.2.

Rapamycin altered IFN-γ production in antigen-specific T cell populations following pathogen infection.

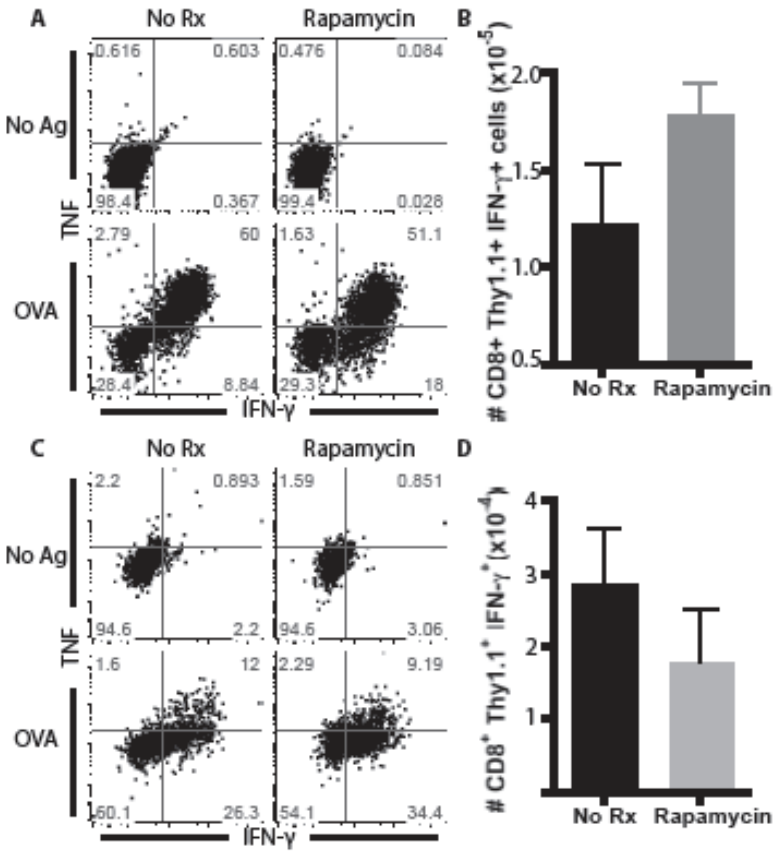


Figure 5.2. Rapamycin altered IFN-γ production in antigen-specific T cell populations following pathogen infection. A, Splenocytes from LM-OVA infected mice, which were either left untreated or treated with rapamycin were restimulated with OVA peptide and stained for the presence of IFN-γ or TNF. B, Treatment with rapamycin resulted in an increase in the number of total IFN-γ^+ T cells. C, Splenocytes from mOVA grafted mice, which were untreated or treated with rapamycin, were restimulated with OVA peptide and stained for the presence of IFN-γ or TNF. D, Treatment with rapamycin did not result in an increase in the number of total IFN-γ^+ T cells. Experiments were performed three times independently with 5 mice per group.

Figure 5.3

Simultaneous infection and transplantation did not result in increased donor-specific immunity in rapamycin treated recipients.

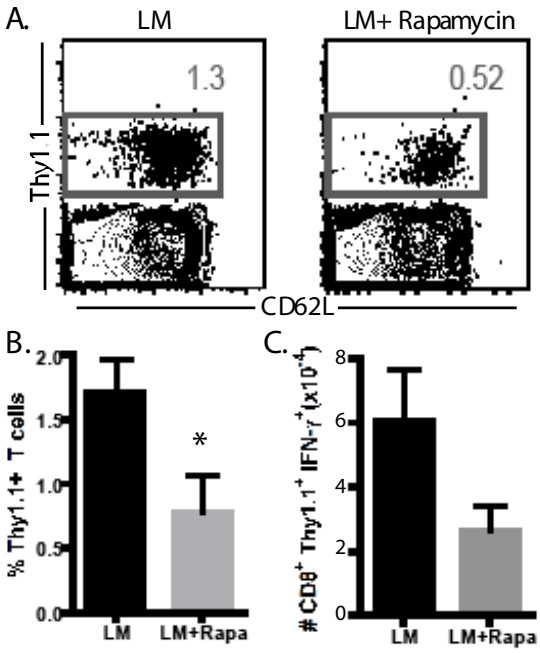


Figure 5.3. Simultaneous infection and transplantation did not result in increased donor-specific immunity in rapamycin treated recipients. 10^6 Thy1.1$^+$ OVA-specific CD8$^+$ OT-I and CD4$^+$OT-II T cells were adoptively transferred into B6 recipients. Recipients were infected with 10^4 CFU of wild-type *Listeria* in the presence or absence of rapamycin. Analysis of splenocytes indicated that the frequency of donor-reactive CD8$^+$ population was not increased in the rapamycin treated recipients (A, B ($p<0.01$)). Absolute numbers of IFN-γ producing Thy1.1$^+$ cells were calculated by TruCount analysis (C). Experiments were performed three times independently with five mice per group.

REFERENCES

1. Thomson AW, Turnquist HR, Raimondi G. 2009. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 9: 324-37

2. Morath C, Arns W, Schwenger V, Mehrabi A, Fonouni H, Schmidt J, Zeier M. 2007. Sirolimus in renal transplantation. *Nephrol Dial Transplant* 22 Suppl 8: viii61-viii5

3. Wullschleger S, Loewith R, Hall MN. 2006. TOR signaling in growth and metabolism. *Cell* 124: 471-84

4. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N, Pulendran B. 2008. Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 9: 1157-64

5. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, Shokat KM, Fisher AG, Merkenschlager M. 2008. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A* 105: 7797-802

6. Haxhinasto S, Mathis D, Benoist C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205: 565-74

7. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108-12

8. Shen H, Slifka MK, Matloubian M, Jensen ER, Ahmed R, Miller JF. 1995. Recombinant Listeria monocytogenes as a live vaccine vehicle for the

induction of protective anti-viral cell-mediated immunity. *Proc Natl Acad Sci U S A* 92: 3987-91

9. Ehst BD, Ingulli E, Jenkins MK. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3: 1355-62

10. Ford ML, Koehn BH, Wagener ME, Jiang W, Gangappa S, Pearson TC, Larsen CP. 2007. Antigen-specific precursor frequency impacts T cell proliferation, differentiation, and requirement for costimulation. *J Exp Med* 204: 299-309

11. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4: 225-34

12. Saemann MD, Haidinger M, Hecking M, Horl WH, Weichhart T. 2009. The multifunctional role of mTOR in innate immunity: implications for transplant immunity. *Am J Transplant* 9: 2655-61

13. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. 2007. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* 178: 7018-31

14. Raimondi G, Sumpter TL, Matta BM, Pillai M, Corbitt N, Vodovotz Y, Wang Z, Thomson AW. 2010. Mammalian target of rapamycin inhibition and alloantigen-specific regulatory T cells synergize to promote long-term graft survival in immunocompetent recipients. *J Immunol* 184: 624-36

15. Rahman AH, Cui W, Larosa DF, Taylor DK, Zhang J, Goldstein DR, Wherry EJ, Kaech SM, Turka LA. 2008. MyD88 plays a critical T cell-intrinsic role in supporting CD8 T cell expansion during acute lymphocytic choriomeningitis virus infection. *J Immunol* 181: 3804-10

16. Wang T, Chen L, Ahmed E, Ma L, Yin D, Zhou P, Shen J, Xu H, Wang CR, Alegre ML, Chong AS. 2008. Prevention of allograft tolerance by bacterial infection with Listeria monocytogenes. *J Immunol* 180: 5991-9

17. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, Wherry EJ, Onami T, Lanier JG, Kokko KE, Pearson TC, Ahmed R, Larsen CP. 2003. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 111: 1887-95

18. Pantenburg B, Heinzel F, Das L, Heeger PS, Valujskikh A. 2002. T cells primed by Leishmania major infection cross-react with alloantigens and alter the course of allograft rejection. *J Immunol* 169: 3686-93

19. Macdonald WA, Chen Z, Gras S, Archbold JK, Tynan FE, Clements CS, Bharadwaj M, Kjer-Nielsen L, Saunders PM, Wilce MC, Crawford F, Stadinsky B, Jackson D, Brooks AG, Purcell AW, Kappler JW, Burrows SR, Rossjohn J, McCluskey J. 2009. T cell allorecognition via molecular mimicry. *Immunity* 31: 897-908

Chapter 6—Unpublished Results

Rapamycin Augments CD8$^+$ T Cell Responses to Latent and Chronic Infections but Not Heterologous Immune Graft-Reactive Responses

ABSTRACT

Recent reports have demonstrated that rapamycin, an agent commonly used in the prevention of solid organ transplant rejection, augments T cell immune responses to infectious agents. In contrast, we have shown that while rapamycin augments $CD8^+$ T cell responses to a pathogen infection; it diminishes T cell responses towards a skin graft. These results begged the question: are pathogen- and graft-reactive T cells fundamentally different with regard to the way they respond in the presence of rapamycin?

In order to address this question, we used a transgenic mouse model system where both transplanted grafts and pathogen infections expressed the same OVA epitope (SIINFEKL) to which the adoptively transferred $CD8^+$ OT-I T cells could respond. In these studies, we determined that pathogen-elicited memory $CD8^+$ T cell responses were enhanced with rapamycin treatment only in response to a secondary pathogen infection. Conversely, the same pathogen-elicited memory $CD8^+$ T cell responses were inhibited following a response to a transplanted graft. We further determined that $CD8^+$ T cells responded similarly towards several types of transplants, including skin grafts, vascularized heart grafts and transfusions. Although the precise mechanisms behind the differential effects of rapamycin on T cell responses are still poorly understood, we determined that both the presence of both antigen presenting cells and secondary lymphoid compartments were important for T cell activation in the presence of rapamycin.

INTRODUCTION

In the 1970's, rapamycin was originally isolate from soil samples on Easter Island and was identified as possessing potent anti-fungal properties isolated from *Streptomyces hygroscopicus* (1, 2). Since its discovery, rapamycin has been found to possess several immunosuppressive effects, such as inducing apoptosis in dendritic cells, inhibiting the upregulation of costimulatory molecules and preventing antigen uptake and presentation (3-5). Treatment with rapamycin has been shown to attenuate T cell proliferation, to sequester T cells in lymphoid tissues, to promote T cell anergy, and to enhance the generation of regulatory T cells through the upregulation of Foxp3 in naïve T cells (6-10).

Considering these well-established immunosuppressive effects of rapamycin, recent studies on lymphocytic choriomeningitis virus (LCMV) viral-specific $CD8^+$ T cell responses revealed that treatment with rapamycin, surprisingly, increased the quantity and quality of virus-specific memory T cells (11). Treatment of both mice and rhesus macaques with rapamycin resulted in an increased response to both live virus and vaccination, respectively. Because this drug is commonly used as part of combined treatment regimens in the prevention of transplantation rejection (12), we interrogated whether rapamycin treatment also triggered an augmented T cell response against *Listeria monocytogenes* bacterial infection compared with that towards a transplanted graft. Upon closer analysis of $CD8^+$ T cell responses in the setting of either pathogen infection or transplantation, we determined that treatment with rapamycin resulted in very disparate effects on the antigen-specific cell populations. While rapamycin augmented the antigen-specific $CD8^+$ T cell

response to a bacterium, it failed to do so in response to a transplant (13). Because of these contrasting effects on $CD8^+$ T cell responses, we aimed to investigate the fundamental differences between T cell responses to pathogens versus transplanted grafts in the presence of rapamycin treatment.

In order to address this question, we used a transgenic mouse model system that allowed us to track $CD8^+$ T cell responses to the same epitope being presented in the context of either an infection or a graft. Using this model we are able to track antigen-specific $CD8^+$ T cells (OT-I) that respond to the SIINFEKL peptide presented on H2-K^b MHC. As the source for transplant tissues, we used mOVA that ubiquitously express OVA under the control of the β-actin promoter (14). Finally, we employed different pathogen constructs that also expressed the SIINFEKL epitope to which the OT-I T cells respond, including *Listeria monocytogenes*-OVA (15), γ-herpesvirus-68.OVA (16), and polyomavirus-SIINFEKL. In studying the pathogen-elicited memory $CD8^+$ T cell population, we determined that rapamycin did not enhance secondary T cell responses to a transplanted graft compared with those responding to a secondary pathogen infection. Immune responses to vascularized grafts and transfusions in the presence of rapamycin also developed similarly as antigen-specific T cell responses to a peripheral skin graft. Although we determined that rapamycin significantly augmented pathogen-specific $CD8^+$ T cell responses against transplantation-specific pathogens, such as herpesvirus and polyomavirus, these responses require antigen to be presented on hematopoietically-derived APCs in secondary lymphoid organs.

MATERIALS AND METHODS

Mice

Adult male 6- to 8-week old B6-Ly5.2/Cr (H2-K^b, CD45.1) mice were purchased from the National Cancer Institute (Frederick, MD). TCR transgenic OT-I mice were purchased from Taconic Farms (Germantown, NY) and bred onto the Thy1.1$^+$ background at Emory University. Act-mOVA mice were donated by Dr. Marc Jenkins, University of Minnesota (14). Alymphoplastic (aly/aly) mice obtained from CLEA Japan, Inc. (Tokyo, Japan) and bred at Emory University (17). Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

T Cell Adoptive Transfers

OT-I Thy1.1$^+$ TCR transgenic T cells were harvested from spleen and mesenteric lymph nodes. The frequency of OT-I T cells was determined prior to adoptive transfer by staining with anti-Vα2 (used by both TCRs) and anti-CD8 or anti-CD4, respectively (Pharmingen, San Diego, CA). Two days prior to infections and grafting, mice received an i.v. injection of 10^6 OT-I T cells.

Flow Cytometric Analyses for Frequency and Absolute Number

Splenocytes were stained with Thy1.1 PerCP, CD8 PacOrange, and CD4 PacBlue (BD Pharmingen) for flow cytometric analysis on a BD LSRII flow cytometer. Absolute numbers of antigen-specific T cells were determined by TruCount Bead Analysis

(Pharmingen). Data were analyzed using FlowJo Software (Treestar, San Carlos, CA).

Intracellular Cytokine Staining

Cells were incubated for 4 hours with 10 nM $OVA_{257-264}$ (SIINFEKL), and 10 μg/ml Brefeldin A (BD Pharmingen), and processed using an intracellular staining kit (BD Pharmingen). Cytokine production was detected with antibodies for TNF PE and IFN-γ Fitc (BD Pharmingen).

Listeria-OVA, γHV-68-SIINFEKL and Polyomavirus-SIINFEKL infections and rapamycin treatment

Listeria monocytogenes (expressing the ovalbumin gene) infection dose was 10^4 colony-forming units (CFU) (15) i.p. on day 0. Gamma-HV68.OVA was donated by Samuel Speck (Emory University, GA) (16). Animals were infected with 10^5 plaque-forming units (PFU) of γ-HV68.OVA injected i.p., where indicated. Polyomavirus-SIINFEKL was donated by Aron Lukacher (Emory University, GA). Animals were infected with 10^5 PFU of Polyomavirus-SIINFEKL through a footpad injection (sub-cutaneous), where indicated.

Mice were treated with 1.5 ug/day rapamycin (Rapamune, Wyeth Pharmaceuticals) daily from the day 0 through day 10 post-infection or post-transplant as previously described (11, 13).

Skin and Heart Grafting and Donor Specific Transfusion

Two days following OT-I T cell adoptive transfer, full-thickness skin grafts (~1 cm^2) were transplanted onto the dorsal thorax of recipient mice and secured with an adhesive bandage for 5 days. Rejection was defined as 90% loss of viable epidermal tissue. Two days after OT-I T cell adoptive transfers, B6-Ly5.2/Cr naïve mice were transplanted with a primarily vascularized mOVA heart grafts.

For donor specific transfusions, mOVA splenocyted were processed into single cell suspensions in PBS and 10^7 cells were injected either i.p. or i.v., where indicated.

Full Bone Marrow Chimerism and Splenectomy

Naïve B6-Ly5.2/Cr (H2-K^b, CD45.1^+) mice were lethally irradiated with 1000 rads one day prior to bone marrow transplantation. Bone marrow from mOVA (H2-K^b, CD45.2^+) animals was flushed from femurs and tibias with PBS using a 25g needle. Animals were injected with $2x10^7$ mOVA bone marrow cells i.v. and treated with 500 μg of each CTLA-4 Ig (Bristol-Myers Squibb) and anti-CD154 (MR1, BioExpress) on days 0, 2, 4, and 6 post transfer to ensure engraftment. The reverse chimerism was also performed: B6-Ly5.2/Cr bone marrow into mOVA mice, followed by treatment with CTLA-4 Ig and anti-CD154, as described above. Bone marrow chimerism levels were measured by flow cytometry, staining for CD45.1, CD45.2, and CD11c.

An incision was made in skin and peritoneum above the spleen. Vessels were tied off with 7.0 Vicryl absorbably sutures (McKesson) and spleen was excised. Peritoneum and skin were closed with absorbable sutures (5.0 Vicryl, McKesson).

Isolation of lymphocytes from livers for flow cytometry

Livers lymphocytes were islolated as previously described (18). Briefly, mice were sacrificed with CO_2 and perfused with 1% (w/v) collagenase IV (Gibco, Co.) in PBS. Livers were cut into small pieces and were collagenase digested for 20 min at 37^{o} C. After homogenizing through a 100 μm mesh cell strainer (Falcon, BD Pharmingen), hepatocytes were pelleted, followed by lymphocyte separation using a Percoll gradient (Sigma-Aldrich). Interface layer contained the liver lymphocytes that were analyzed using flow cytometry.

Statistical Analyses

When comparing two groups a student's t-test was performed. When comparing three or more groups, a one-way ANOVA analysis was performed followed by a Tukey comparison test on the significant results (GraphPad Prism Software, La Jolla, CA).

RESULTS

Rapamycin treatment does not enhance pathogen-elicited memory CD8$^+$ T cell responses to transplanted graft

Preformed memory T cells to pathogen and environmental agents pose a great risk to transplantation rejection through heterologous immunity (19-21). As it has been previously described, up to 45% of viral reactive T cell clones possess cross-reactivity to at least one HLA allele (22). Therefore, since pathogen-elicited memory T cells mediate increased graft rejection, and rapamycin increases pathogen-specific memory T cell responses, we aimed to test whether treatment with rapamycin could enhance heterologous immune T cell responses to transplanted grafts.

In order to address this question, 10^6 OT-I T cells were adoptively transferred into naïve B6 mice followed by a *listeria monocytogenes*-OVA infection. Upon resolution of memory T cell responses (>30 days), mice were either reinfected with LM-OVA or were transplanted with a mOVA skin graft in the presence or absence of daily rapamycin treatment (Figure 6.1A). On day 7 post-secondary challenge, results indicated that treatment with rapamycin significantly enhanced the pathogen-elicited CD8$^+$ T cell response to a secondary pathogen infection compared to untreated controls ($3.04\pm1.42x10^6$ vs. $0.21\pm0.13x10^5$, respectively; p=0.036). Conversely, treatment with rapamycin did not significantly affect pathogen-elicited memory T cell secondary immune responses to the transplanted skin graft compared with untreated animals ($4.74\pm1.17x10^4$ vs. $3.86\pm1.99x10^4$, respectively; p=n.s.) (Figure 6.1B, C).

We further assessed the impact of rapamycin on memory $CD8^+$ T cell differentiation into cytokine producing cells following either a pathogen or transplant rechallenge. On day 7 post-challenge, the pathogen-elicited memory T cells from the spleens of either LM-OVA infected or mOVA skin graft transplanted animals were *ex vivo* restimulation with SIINFEKL epitope. Although rapamycin treatment did not significantly alter the frequency of IFN-γ producing cells compared to untreated controls, following *ex vivo* restimulation, the total number of IFN-γ-secreting pathogen-rechallenged $CD8^+$ T cells was significantly higher than untreated controls ($2.18\pm0.96x10^6$ vs. $0.16\pm0.10x10^6$, respectively; p=0.0297). This difference in IFN-γ producing cells was due to the increased accumulation of total pathogen-specific $CD8^+$ T cells in the presence of rapamycin compared to untreated animals. In contrast, because the overall accumulation of pathogen-elicited memory $CD8^+$ T cells responding to the transplanted graft was not altered with rapamycin treatment, the number of IFN-γ producing graft-reactive $CD8^+$ T cells remained unchanged following treatment with rapamycin compared with untreated animals ($2.61\pm0.92x10^4$ vs. $2.13\pm1.25x10^4$, respectively; p=n.s.).

Rapamycin does not augment $CD8^+$ T cell responses to vascularized grafts or to donor specific transfusions

When comparing immune responses to a *Listeria monocytogenes* infection versus that to a transplanted skin graft, the site and mode of antigen recognition may be very different. Because antigens draining from a skin graft are presented peripherally in the draining lymph nodes and the antigens presented following i.p.

Listeria inoculation occurred centrally in the spleen, we asked whether different methods of antigen exposure during transplantation could alter the $CD8^+$ T cells responses following treatment with rapamycin. Therefore, following adoptive transfers of 10^6 OT-I T cells, recipient animals were transplanted with vascularized heterotopic mOVA heart grafts in the presence or absence of rapamycin (Figure 6.2A). On day 10 post-transplantation, spleens were analyzed for the accumulation of mOVA heart-specific OT-I T cells. Our results demonstrated that treatment with rapamycin did not augment the $CD8^+$ T cells responses to the transplanted heart graft compared with untreated controls (Figure 6.2B, C).

We also assessed the effect of rapamycin therapy on $CD8^+$ T cell responses to antigen in suspension through donor specific transfusion (infusion of 10^7 mOVA splenic cells) given either i.p. or i.v. (Figure 6.2A). Similar to $CD8^+$ T cell responses following the vascularized heart transplants, rapamycin did not augment the antigen-specific $CD8^+$ T cell responses to transfusions given by either i.p. or i.v. routes compared with untreated controls (Figure 6.2B, C).

Hematopoietically derived antigen-presenting cells are necessary for $CD8^+$ T cell priming

From the previous results, we determined that the location of the antigen presentation from the transplanted graft or infection did not contribute to the enhancing effects of rapamycin. We aimed to determine whether antigen presentation was occurring on the surface of hematopoietically derived antigen presenting cells or directly on the surface of the transplanted graft. When T cells are

activated by parenchymal cells, the $CD8^+$ T cell responses are significantly blunted with an increased level of apoptosis (23). Therefore, we utilized a mOVA transgenic reverse bone marrow chimera system where the OVA antigen is presented either by hematopoietically derived APCs or by the surrounding tissues and parenchymal cells. In this model, naïve B6-Ly5.2/Cr ($CD45.1^+$) animals were transplanted with mOVA ($CD45.2^+$) bone marrow, resulting in the expression of mOVA antigen only on the hematopoietically derived antigen presenting cells. Conversely, naïve mOVA mice were transplanted with B6-Ly5.2/Cr bone marrow, resulting in the depletion of mOVA antigen from the bone marrow leading to the expression of antigen only on the peripheral parenchymal cells (Figure 6.3A). Three weeks post-bone marrow transplantation, dendritic cell chimerism levels were assessed by measuring the frequency of $CD45.1^+$ vs. $CD45.2^+$ cells in the $CD11c^+$ population. Using the protocol described in the *materials and methods*, we were able to obtain nearly 100% dendritic cell chimerism in either of the two types of bone marrow transplant recipients (Figure 6.3B).

Over four weeks post-bone marrow transplantation and upon confirmation of chimerism levels, 10^6 OT-I T cells were adoptive transferred into the bone marrow chimeras in the presence or absence of rapamycin treatment. Data showed that, as expected, when antigen was presented by hematopoietically derived APCs, antigen-specific $CD8^+$ T cells expanded. In contrast, this response was significantly ablated following treatment with rapamycin (Figure 6.3C, D, left panels). Conversely, upon adoptive transfer of OT-I T cells into animals that only presented antigen on parenchymal cells, $CD8^+$ T cell responses were significantly blunted.

Minimal accumulation of adoptive transferred cells was detected in either untreated or rapamycin treated mice (Figure 6.3C, D, right panels). In these experiments we have confirmed that antigen presentation on hematopoietically derived APCs is necessary to significantly activate antigen-specific $CD8^+$ T cells, this interaction is not sufficient to augment T cell responses following treatment with rapamycin.

Rapamycin augments $CD8^+$ T cell responses to transplantation-specific pathogens

Although in previous experiments we utilized *Listeria monocytogenes* to assess the effect of rapamycin on antigen-specific $CD8^+$ T cell responses, this pathogen does not commonly lead to complications transplantation patients. Therefore, we aimed to test whether rapamycin also enhanced $CD8^+$ T cell responses to other pathogens that commonly afflict transplantation patients. We used two viral constructs that express the SIINFEKL epitope, to which OT-I T cells respond. The first construct is γ-HV68.OVA, a herpes virus from the same family as CMV that upon reactivation may induce detrimental immune responses against a transplanted graft. The second construct is murine polyomavirus (PyV)-SIINFEKL, a member of the polyomavirus family which include JC and BK viruses which can cause progressive multifocal leukoencephalopathy (PML) in transplantation patients due to excessive immunosuppression, upon viral reactivation. Using a similar adoptive transfer model of OT-I transgenic T cells, we assessed antigen-specific $CD8^+$ T cell responses to either γ-HV68.OVA or PyV-SIINFEKL.

On day 10 post-adoptive transfers, the antigen-specific $CD8^+$ T cell responses were assessed in the presence or absence of rapamycin treatment. Similar to responses following LM-OVA infection, infection with γ-HV68.OVA led to a significantly augmented $CD8^+$ T cell response with rapamycin treatment compared with untreated animals ($5.41\pm0.71x10^5$ vs. $1.56\pm0.61x10^5$, respectively; p=0.0035). Likewise, PyV-SIINFEKL infection led to a significantly increased accumulation of antigen-specific $CD8^+$ T cells in the spleens of rapamycin treated animals compared with untreated controls ($11.09\pm0.10x10^5$ vs. $5.22\pm1.92x10^5$, respectively; p=0.025) (Figure 6.4A, B).

We further wanted to assess the impact of rapamycin therapy on the differentiation of the pathogen-specific T cells into multi-cytokine producing cells. On day 10 post-infection, splenic $CD8^+$ T cells were restimulated *ex vivo* with SIINFEKL peptide and analyzed for TNF and IFN-γ production. Results indicated that treatment with rapamycin did not significantly impact the frequency of total IFN-γ producing antigen-specific $CD8^+$ T cells towards γ-HV68.OVA or PyV-SIINFEKL compared with their respective untreated controls (γ-HV68: 58.96±3.09% vs. 61.88±2.62%, respectively; and PyV: 51.52±1.52% vs. 40.80±1.09%, respectively). Conversely, due to the increased accumulation of antigen specific $CD8^+$ T cells, the total number of IFN-γ-producing antigen-specific $CD8^+$ T cells was significantly increased following treatment with rapamycin. Specifically, on day 10 post-γ-HV68.OVA infection, rapamycin significantly increased the number of total IFN-γ producing OT-I T cells compared with untreated controls ($3.32\pm0.50x10^5$ vs. $0.96\pm0.37x10^5$, respectively, p=0.0071). Similarly, following infection with PyV-

SIINFEKL, rapamycin significantly augmented the number of OT-I T cells producing IFN-γ compared with untreated controls ($5.75\pm0.68x10^5$ vs. 2.13±0.80x105, respectively; p=0.010).

Secondary lymphoid organs are necessary to induce potent pathogen-specific CD8⁺ T cell responses

Because rapamycin has been shown to increase the frequency of CD62L expressing cells (11, 13), and the expression of this marker promotes T cell migration into the lymph nodes, we interrogated whether the enhancing effects of rapamycin required antigen presentation in secondary lymphoid organs. In order to address this question, we used aly/aly mice, that lack peripheral lymph nodes (17), as recipients. A week prior to infection, we splenectomized the recipient animals in order to remove all secondary lymphoid organs. In order to avoid the adoptive transfer of extra antigen presenting cells, OT-I T cells were purified prior to transfer. Mice were then infected with PyV-SIINFEKL in the presence or absence of rapamycin treatment (Figure 6.5A).

On day 10 post-infection, antigen-specific $CD8^+$ T cell accumulation in the blood was assessed. Results demonstrated that untreated mice lacking secondary lymphoid organs were able to mount a response against a polyomavirus infection to similar levels as naïve wild type animals ($1.84\pm0.14x10^4$ vs. $1.64\pm0.84x10^4$ in 100 μL of blood, respectively; p=n.s.). Conversely, when mice were treated with rapamycin during the infection, the antigen-specific $CD8^+$ T cell responses diverged. Rapamycin treatment significantly decreased the OT-I T cell response in the blood of

mice lacking secondary lymphoid organs compared with wild type animals that developed a significantly augmented T cell response with rapamycin treatment ($0.35\pm0.12x10^4$ vs. $4.27\pm1.23x10^4$ in 100 μL of blood, respectively; $p<0.05$) (Figure 6.5B, C). Further analysis of the pathogen specific $CD8^+$ T cell response revealed that rapamycin augmented the OT-I T cell response in wild type mice by 260.2% compared with untreated animals. In stark contrast, rapamycin treatment in mice lacking secondary lymphoid organs led to an 80.5% reduction in the antigen-specific T cell response compared with untreated controls (Figure 6.5D).

Because similar levels of pathogen-reactive T cells in the blood of animals lacking peripheral lymphoid organs compared with wild type animals were observed, we interrogated whether the absence of peripheral lymphoid organs instilled pressure on other organs to prime the $CD8^+$ T cells against polyomavirus infection. Thus, we assessed the accumulation of antigen-specific $CD8^+$ T cells in the liver of infected mice. On day 10 post-infection, both splenectomized aly/aly and wild type mice developed a large population of pathogen-specific $CD8^+$ T cells in their livers. Although, surprisingly, untreated mice lacking secondary lymphoid organs displayed a significant increase in the pathogen-reactive $CD8^+$ T cell population in their livers compared with wild type mice ($1.75\pm0.15x10^6$ vs. $0.14\pm0.02x10^6$, respectively; $p<0.001$). These data demonstrate that even in the absence of the conventional secondary lymphoid compartment, the presence of a systemic infection, such as polyomavirus, will induce a pressure on other organs to prime the pathogen-specific T cells. However, following treatment with rapamycin, pathogen-reactive $CD8^+$ T cell responses were significantly impaired in mice lacking

secondary lymphoid organs compared with untreated controls ($0.50\pm0.31x10^6$ vs. $1.75\pm0.15x10^6$, respectively; $p<0.01$). This result suggests that although T cells are being primed in extra-lymphoid organs, the microenvironment is not sufficient to create an augmented $CD8^+$ T cell response in the presence of rapamycin treatment.

DISCUSSION

Because of the well-appreciated immunosuppressive effects of rapamycin on adaptive immune responses, the discovery that rapamycin indeed augmented pathogen-reactive T cell responses warranted investigation (11, 13). We first interrogated rapamycin's effect on heterologous immune responses. The process of heterologous immunity is described as the increased alloreactivity against a transplanted graft due to either a concurrent or prior infection. The mechanism of action behind heterologous immunity has been attributed to both molecular mimicry (24) and bystander activation (20). In order to test the effect of rapamycin on heterologous immunity, we used the *Listeria*-OVA bacterial construct to prime the pathogen-specific $CD8^+$ T cell response. The secondary challenge was either another infection with *Listeria*-OVA or a mOVA skin graft transplant. One minor caveat of this experimental system is that it does not completely reflect heterologous immune responses because the $CD8^+$ T cells respond to identical epitopes during either the pathogen infection or the transplant. However, this model allows us to standardize immune responses so that T cell receptors may have the same affinity to the cognate antigen, where the only variable is the microenvironment of stimulation and the treatment course. Our results indicated that treatment with rapamycin enhanced pathogen-elicited memory $CD8^+$ T cell responses towards a secondary pathogen infection, but not to the transplanted graft. Thus, we concluded that treatment with this drug may not enhance heterologous immune responses and promote graft rejection. Although rapamycin is not an effective monotherapy for transplantation survival, this finding could argue for the

transient increase in treatment during reactivation episodes of infectious agents. Although treatment with this drug would not significantly impair immune responses against the graft, it could accelerate adaptive immune responses against the invading pathogen.

In unpublished observations by Araki *et al*, rapamycin did not augment pathogen-specific $CD8^+$ T cell responses to infection with chronic strain of LCMV (clone-13) as compared to acute infections with LCMV Armstrong. One large difference between these two types of infection is the persistence of the infection. If the prolonged antigen-presentation were contributing to the reduced responses towards a graft, we would hypothesize that the same would be true for other persistent infections. However, infection with the latent virus, γ-HV68, and the persistent virus, polyoma, did not hinder the enhancing effects of rapamycin treatment. Although no significant differences were observed at the peak of $CD8^+$ T cell responses, there remains a possibility that rapamycin may diminish pathogen-specific $CD8^+$ T cell responses during later time points, as persistent infections further induce T cell non-responsiveness and exhaustion (25).

Another confounding factor behind the LCMV clone-13 studies is that the virus not only induces a chronic viral infection, but also persists in multiple organs and cell types, including parenchymal cells. Because the priming of antigen-specific cytotoxic T cells by parenchymal cells has been shown to significantly diminish the T cell response and induce apoptosis through the upregulation of mitochondrial pro-apoptotic molecules (23), we sought to investigate whether antigen-presentation of graft-related antigens could be occurring directly in the graft

parenchyma rather than on hematopoietically-derived APCs. Creating reverse bone marrow chimeras, where the OVA cognate antigen was only expressed by either the surrounding parenchymal cells or by the hematopoietically-derived cells, allowed us to change the site of antigen presentation and assess the impact of rapamycin on antigen-specific $CD8^+$ T cell responses. No augmentation in the antigen-specific $CD8^+$ T cell response was observed when cells were primed by hematopoietically-derived APCs in the presence of rapamycin. Furthermore, peripheral lymphoid organs were necessary for the enhancing effects of rapamycin on pathogen-specific $CD8^+$ T cell responses. Although the liver compensated for the lack of peripheral lymphoid tissues in infected animals in order to produce a protective immune response, rapamycin did not increase pathogen-reactive $CD8^+$ T cell responses when primed in non-lymphoid organs.

Taken together, these data suggest that pathogen-elicited and graft-reactive $CD8^+$ T cell responses have different activation programs in the presence of rapamycin. These results demonstrate that although antigen-presentation by professional APCs may be important to produce a significant $CD8^+$ T cell response, the inflammation and added stimulus from an infectious pathogen may be required for the enhancing effects of rapamycin treatment. Although the fundamental mechanisms underlying the differential effects of rapamycin on graft- vs. pathogen-specific immune responses remain unclear, this remains as an important area of future investigations.

Figure 6.1.

Rapamycin treatment does not enhance pathogen-elicited memory CD8⁺ T cell responses to transplanted graft.

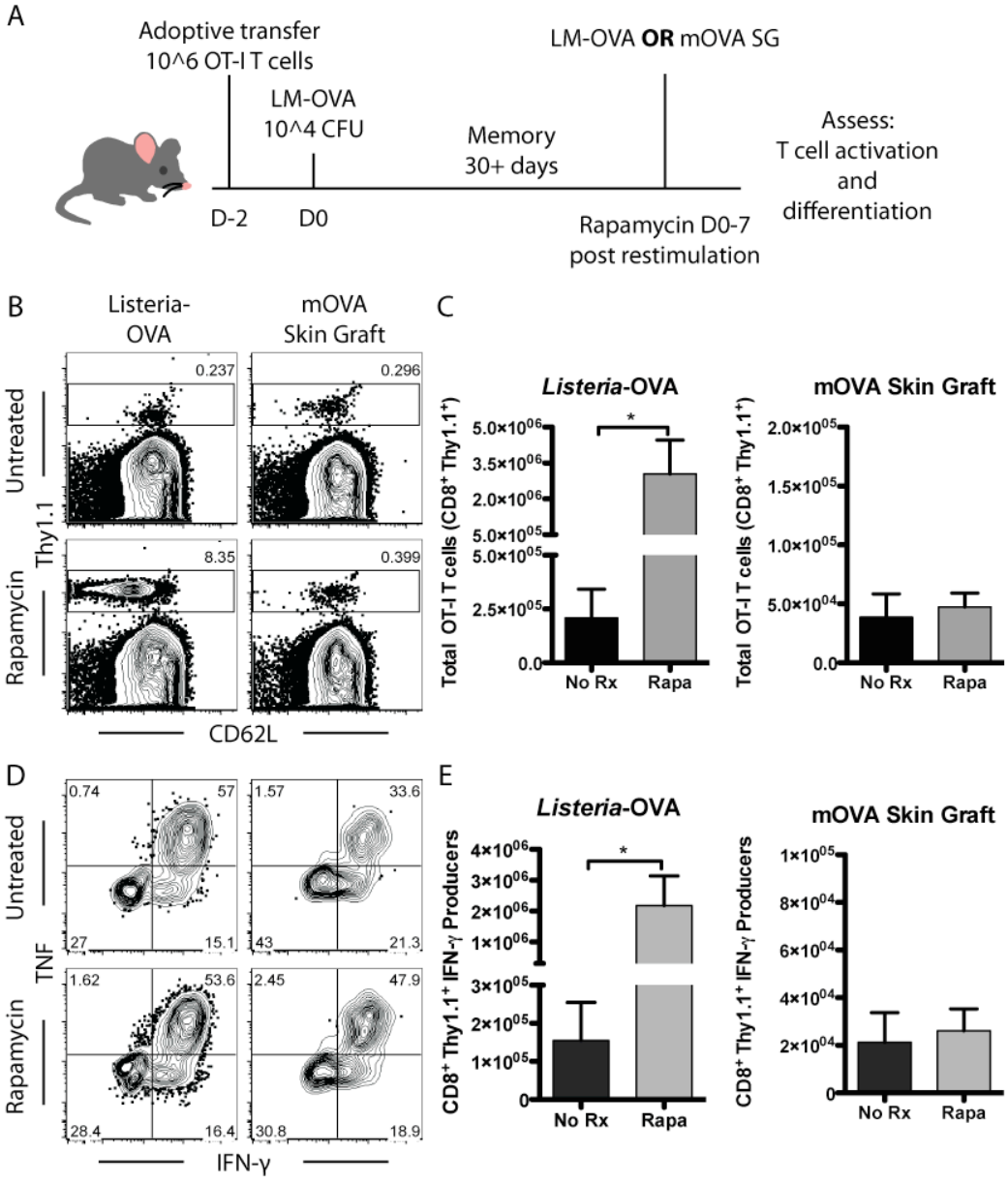


Figure 6.1. Rapamycin treatment does not enhance pathogen-elicited memory CD8$^+$ T cell responses to transplanted graft. A. Experimental design. Briefly, animals were adoptively transferred with 10^6 OT-I CD8$^+$ T cells two days prior to infection with 10^4 CFU of LM-OVA. After >30 days, animals were either reinfected with 10^4 CFU LM-OVA or transplanted with LM-OVA skin graft and treated with 1.5 μg rapamycin daily (d0-7), where indicated. B. Day 7 post-secondary challenge, representative flow plots of accumulated CD8$^+$ T cells in the spleen, gating on Thy1.1$^+$ OT-I T cells. C. Total counts of OT-I T cells in the spleens at day 7 post-rechallenge. D. Day 7 post-rechallenge, splenic OT-I T cells were restimulated *ex vivo* with SIINFEKL epitope for 4hr and TNF and IFN-γ production was measured. Representative flow plots of OT-I T cell production of cytokines. E. Total count of IFN-γ producing OT-I T cells on day 7 following rechallenge. *$p<0.05$.

Figure 6.2.

Rapamycin does not augment CD8⁺ T cell responses to vascularized grafts or to donor specific transfusions.

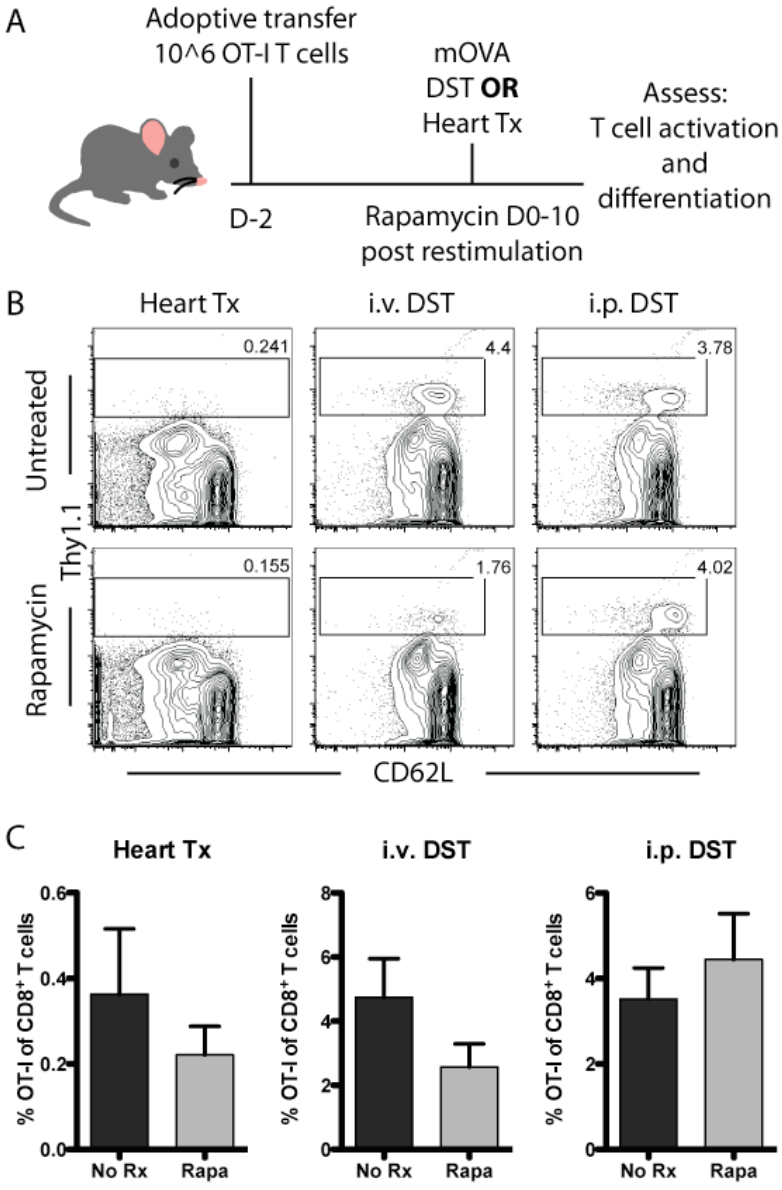


Figure 6.2. Rapamycin does not augment $CD8^+$ T cell responses to vascularized grafts or to donor specific transfusions. A. Experimental design. Briefly, animals were adoptively transferred with 10^6 OT-I $CD8^+$ T cells two days prior to transplantation or infusion. mOVA vascularized heart grafts were transplanted heterotopically. For DST, 10^7 splenocytes were injected either i.p. or i.v., where indicated. B. On day 10 post-transplantation or post-infusion, spleens were assessed for the accumulation of donor-specific $CD8^+$ T cells specific for SIINFEKL. Representative flow plots of $CD8^+$ T cells, gating on the $Thy1.1^+$ OT-I T cells. C. Summary of frequency of $CD8^+$ T cells in spleens of transplanted or transfused animals on day 10.

Figure 6.3.

Hematopoietically derived antigen-presenting cells are necessary for CD8⁺ T cell priming.

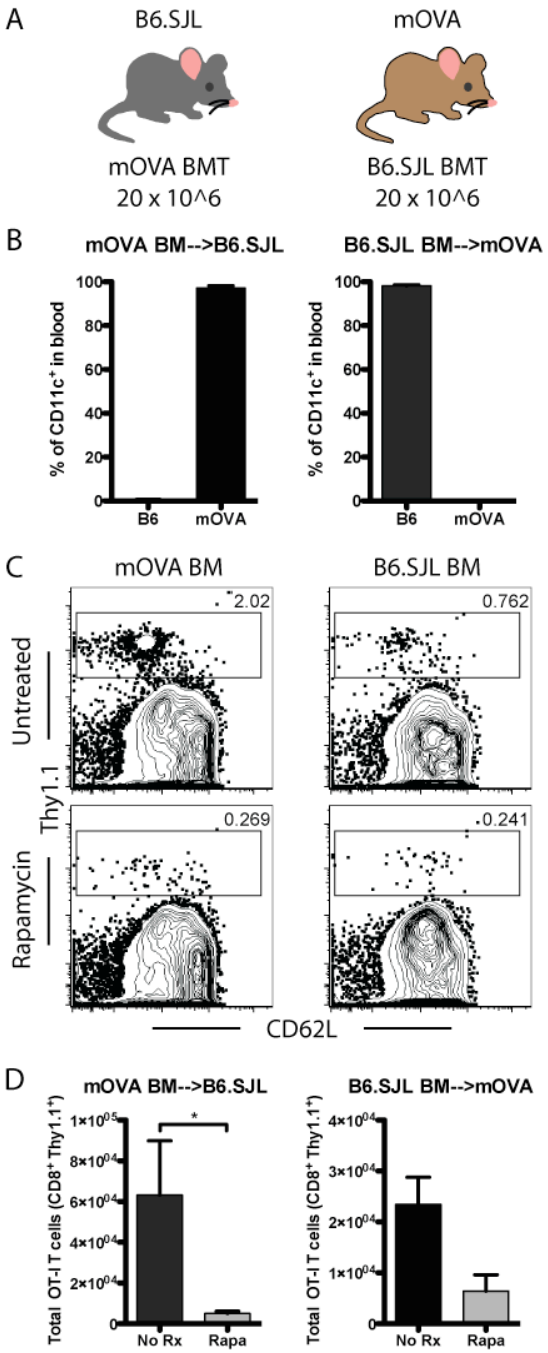


Figure 6.3. Hematopoietically derived antigen-presenting cells are necessary for CD8$^+$ T cell priming. A. Experimental design. Naïve B6-Ly5.2/Cr (H2-K^b, CD45.1$^+$) mice were injected with 2x10^7 mOVA (H2-K^b, CD45.2$^+$) bone marrow cells i.v. and treated with CTLA-4 Ig and anti-CD154 on days 0, 2, 4, and 6 post transfer to ensure engraftment (left column). The reverse chimerism protocol was also performed: B6-Ly5.2/Cr bone marrow into mOVA mice (right column). B. Three weeks following bone marrow transplantation, dendritic cell chimerism levels were measured by staining for CD45.1$^+$ and CD45.2$^+$ cells in the CD11c$^+$ population. C. Accumulation of OT-I T cells in the spleen of chimeric mice was measured on day 10 post-adoptive transfer, in the presence or absence of rapamycin treatment. Representative flow plots of total CD8$^+$ T cells, gating on antigen-specific Thy1.1$^+$ OT-I T cells. D. Summary of frequency of CD8$^+$ T cells in spleens of chimeric animals on day 10 post-transfer. *$p<0.05$.

Figure 6.4

Rapamycin augments CD8⁺ T cell responses to transplantation-specific pathogens.

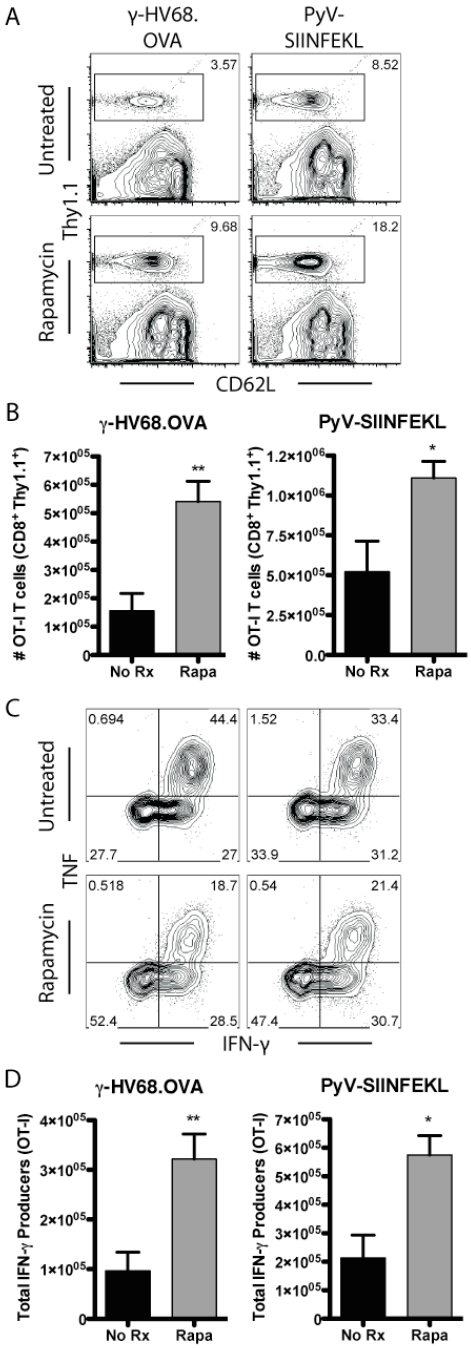


Figure 6.4. Rapamycin augments CD8$^+$ T cell responses to transplantation-specific pathogens. Naïve B6-Ly5.2/Cr mice were adoptively transferred with 10^6 OT-I T cells two days prior to infection. Animals were infected with 10^5 PFU of either γ-HV68.OVA (i.p.) or PyV-SIINFEKL (sub-cu.) in the presence or absence of 1.5 μg daily rapamycin. On day 10 post-infection, mice were sacrificed and splenic antigen-specific OT-I T cells were analyzed. A. Representative flow plots of total splenic CD8$^+$ T cells, gating on the Thy1.1$^+$ antigen-specific OT-I T cells. B. Summary of total number of accumulated OT-I T cells in spleens. C. Splenocytes were restimulated *ex vivo* with SIINFEKL peptide. Representative flow plots of antigen-specific OT-I T cells producing IFN-γ and TNF. D. Total number of IFN-γ producing OT-I T cells in spleens on day 10 post-infection

Figure 6.5.

Secondary lymphoid organs are necessary to induce potent pathogen-specific CD8⁺ T cell responses.

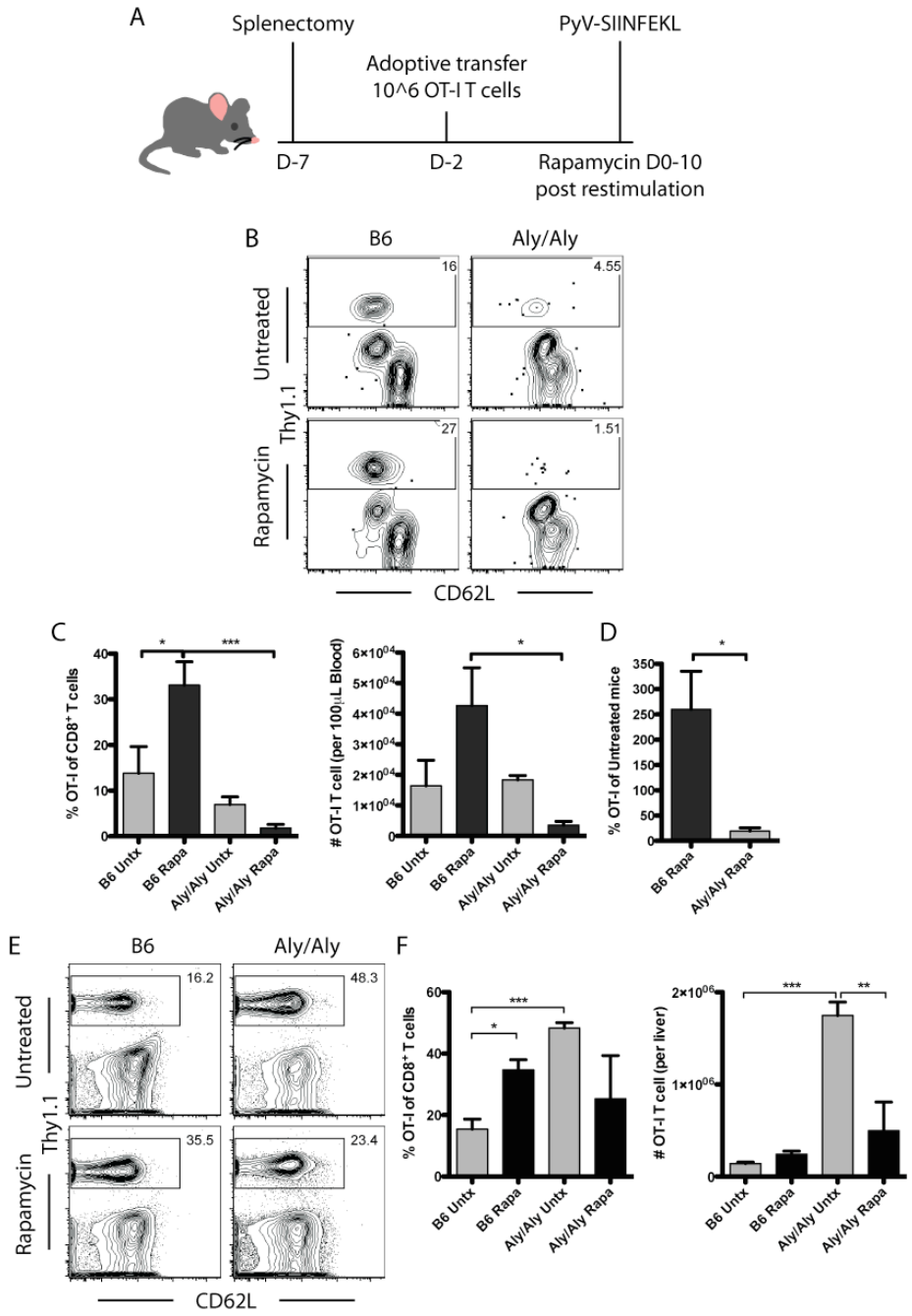


Figure 6.5. Secondary lymphoid organs are necessary to induce potent pathogen-specific CD8$^+$ T cell responses. A. Experimental design. Aly/aly mice were splenectomized 7 days prior to adoptive transfer of 10^6 OT-I T cells (MACs purified). Two days post-transfer, mice were infected with 10^5 PFU PyV-SIINFEKL, and treated with 1.5 μg daily rapamycin, where indicated. Control groups consisted of wild type B6-Ly5.2/Cr non-splenectomized mice that were adoptively transferred with the same purified OT-I T cell preparation prior to PyV-SIINFEKL infection, in the presence or absence of rapamycin treatment. B. On day 10 post-infection, mice were bled to assess the accumulation of antigen-specific CD8$^+$ T cells. Representative flow plots of total CD8$^+$ T cells in the blood of infected mice, with gating on Thy1.1$^+$ antigen-specific CD8$^+$ T cells. C. Summary of frequency (left panel) and total number (right panel) of OT-I T cells in the blood of infected mice on day 10. D. Change in frequency of accumulated OT-I T cells following treatment with rapamycin compared with untreated animals, in the blood. E. On day 10 post-infection, livers were processed to assess the accumulation of antigen-specific CD8$^+$ T cells. Representative flow plots of total CD8$^+$ T cells, gated on Thy1.1$^+$ antigen-specific CD8$^+$ T cells. D. Summary of frequency (left panel) and total number (right panel) of OT-I T cells in the liver of infected mice on day 10.

REFERENCES

1. Sehgal SN, Baker H, Vezina C. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J Antibiot (Tokyo)* 28: 727-32

2. Vezina C, Kudelski A, Sehgal SN. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot (Tokyo)* 28: 721-6

3. Woltman AM, de Fijter JW, Kamerling SW, van Der Kooij SW, Paul LC, Daha MR, van Kooten C. 2001. Rapamycin induces apoptosis in monocyte- and CD34-derived dendritic cells but not in monocytes and macrophages. *Blood* 98: 174-80

4. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, Thomson AW. 2003. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood* 101: 4457-63

5. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. 2007. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol* 178: 7018-31

6. Gilbert KM, Weigle WO. 1993. Th1 cell anergy and blockade in G1a phase of the cell cycle. *J Immunol* 151: 1245-54

7. Sinclair LV, Finlay D, Feijoo C, Cornish GH, Gray A, Ager A, Okkenhaug K, Hagenbeek TJ, Spits H, Cantrell DA. 2008. Phosphatidylinositol-3-OH kinase

and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat Immunol* 9: 513-21

8. Haxhinasto S, Mathis D, Benoist C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205: 565-74

9. Song K, Wang H, Krebs TL, Danielpour D. 2006. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. *EMBO J* 25: 58-69

10. Song J, Salek-Ardakani S, So T, Croft M. 2007. The kinases aurora B and mTOR regulate the G1-S cell cycle progression of T lymphocytes. *Nat Immunol* 8: 64-73

11. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108-12

12. Morath C, Arns W, Schwenger V, Mehrabi A, Fonouni H, Schmidt J, Zeier M. 2007. Sirolimus in renal transplantation. *Nephrol Dial Transplant* 22 Suppl 8: viii61-viii5

13. Ferrer IR, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP, Ford ML. 2010. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. *J Immunol* 185: 2004-8

14. Ehst BD, Ingulli E, Jenkins MK. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3: 1355-62

15. Shen H, Slifka MK, Matloubian M, Jensen ER, Ahmed R, Miller JF. 1995. Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. *Proc Natl Acad Sci U S A* 92: 3987-91

16. Braaten DC, Sparks-Thissen RL, Kreher S, Speck SH, Virgin HWt. 2005. An optimized CD8+ T-cell response controls productive and latent gammaherpesvirus infection. *J Virol* 79: 2573-83

17. Miyawaki S, Nakamura Y, Suzuka H, Koba M, Yasumizu R, Ikehara S, Shibata Y. 1994. A new mutation, aly, that induces a generalized lack of lymph nodes accompanied by immunodeficiency in mice. *Eur J Immunol* 24: 429-34

18. Pillarisetty VG, Shah AB, Miller G, Bleier JI, DeMatteo RP. 2004. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J Immunol* 172: 1009-17

19. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, Wherry EJ, Onami T, Lanier JG, Kokko KE, Pearson TC, Ahmed R, Larsen CP. 2003. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 111: 1887-95

20. Wang T, Chen L, Ahmed E, Ma L, Yin D, Zhou P, Shen J, Xu H, Wang CR, Alegre ML, Chong AS. 2008. Prevention of allograft tolerance by bacterial infection with Listeria monocytogenes. *J Immunol* 180: 5991-9

21. Pantenburg B, Heinzel F, Das L, Heeger PS, Valujskikh A. 2002. T cells primed by Leishmania major infection cross-react with alloantigens and alter the course of allograft rejection. *J Immunol* 169: 3686-93

22. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, van der Hoorn MA, Kester MG, Doxiadis, II, Falkenburg JH, Claas FH, Heemskerk MH. 2010. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* 115: 3146-57

23. Gruber A, Cannarile MA, Cheminay C, Ried C, Marconi P, Hacker G, Brocker T. 2010. Parenchymal cells critically curtail cytotoxic T-cell responses by inducing Bim-mediated apoptosis. *Eur J Immunol* 40: 966-75

24. Macdonald WA, Chen Z, Gras S, Archbold JK, Tynan FE, Clements CS, Bharadwaj M, Kjer-Nielsen L, Saunders PM, Wilce MC, Crawford F, Stadinsky B, Jackson D, Brooks AG, Purcell AW, Kappler JW, Burrows SR, Rossjohn J, McCluskey J. 2009. T cell allorecognition via molecular mimicry. *Immunity* 31: 897-908

25. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77: 4911-27

Chapter 7

Discussion

TARGETED IMMUNOSUPPRESION: CD40/CD154 PATHWAY BLOCKADE

Summary of three CD40/CD154 blockade experimental models

In the first part of this thesis, we discussed the effect of CD40/CD154 pathway blockade with an anti-CD154 blocking antibody, MR1. Through different experimental models, we were able to gleam different information relating to the mechanism by which anti-CD154 may be protecting the graft against rejection.

In chapter 2, we used an allogeneic skin graft model where animals were treated with both anti-CD154 and donor specific transfusion (DST) as a way to induce transplantation tolerance. In these studies, we determined that CD40/CD154 pathway blockade specifically inhibits the accumulation of chemoattractants including KC, MIP-1α, and RANTES within the skin graft to inhibit the infiltration of both innate and adaptive immune cells, such as neutrophils, macrophages and $CD8^+$ T cells. The use of this allogeneic skin graft model has also allowed us study the effect of anti-CD154 on the overall alloreactive $CD8^+$ T cell response. We also observed that DST accelerated the activation of $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells, although with reduced differentiation into multi-cytokine producing cells. On the other hand, treatment with anti-CD154 led to a delay in the accumulation of $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells, while their ability to produce IFN-γ was significantly hampered. Interestingly, anti-CD154 treatment was also associated with the increased expression of KLRG-1 on $CD8^+$ T cells early in the immune response, which correlated with the reduced accumulation of long-lived memory precursor cells (1, 2). From these data, we concluded that the "push-and-pull" from these two treatments had detrimental effects on the activation of $CD8^+$ T

cells leading to a significantly reduced expansion of $CD44^{high}$ $CD62L^{low}$ $CD8^+$ T cells, expressing high levels of KLRG-1 with minimal cytokine production.

In chapter 3, in order to be able to specifically track the antigen-specific T cell responses, we transitioned into a transgenic model of skin graft transplantation also using combined anti-CD154 and donor specific transfusion as a tolerance-inducing regimen. Once again, in this transgenic system, we observed that DST accelerated, while anti-CD154 delayed, the expansion and accumulation of donor-reactive $CD8^+$ T cells compared with untreated controls. Furthermore, the treatment with both of these therapies led to opposing effects on donor-reactive $CD8^+$ T cell differentiation into multi-cytokine producing cells. Although neither of the two treatments led to changes in the accumulation of total regulatory T cells, skin grafts analyzed from animals treated with anti-CD154 showed increased T_{reg} infiltration. Further analysis of donor-specific $CD4^+$ T led to a previously unobserved conversion of donor-reactive $CD4^+$ T cells into induced $Foxp3^+$ $CD25^+$ regulatory T cells following treatment with anti-CD154 (Figure 7.1).

In chapter 4, we used a transgenic bone marrow chimera experimental model to study the effects of CD40/CD154 pathway blockade on antigen presenting cells (APCs) and on bone-marrow reactive $CD4^+$ and $CD8^+$ T cells. Since treatment with anti-CD154 inhibits CD40-CD154 interactions, we presumed that blockade of this pathway would also inhibit the activation of APCs. Surprisingly, treatment with anti-CD154 did not inhibit the upregulation of class I MHC or costimulatory molecules, including CD40, CD86 and ICAM-1. In contrast, CD154 blockade led to the inhibition of inflammatory cytokines such as IL-6, TNF, IL-1β and IL-12p35

(Figure 7.2). Similar to studies in the skin graft models, we determined that, indeed, anti-CD154 reduced the accumulation of both $CD4^+$ and $CD8^+$ T cells, while preventing their differentiation into IFN-γ producing cells. Furthermore, treatment with CD40/CD154 pathway blockade not only reduced the accumulation of IL-17 producing bone-marrow reactive $CD8^+$ T cells, but also, led to the increased conversion of bone-marrow specific $CD4^+$ T cells into induced T_{reg}.

Different experimental models for analysis of CD40/CD154 pathway blockade

In studying the effects of CD40/CD154 costimulation blockade on prolonging transplantation survival, we utilized different model systems to both detect differences and confirm similarities in T cell responses following treatment with anti-CD154. In chapter 2, the use of a fully allogeneic skin graft model allowed us to study alloantigen reactive T cells in the midst of the full inflammatory milieu of responses towards an allogeneic transplant. Unlike the transgenic skin graft model described in chapters 3 and 4, we are unable to track antigen-specific $CD4^+$ and $CD8^+$ T cells through surface markers as no tetramers are available for the detection of alloantigen-specific T cells due to the unknown T cell epitopes reactive during allogeneic responses. Consequently, the only method of tracking the skin graft-specific $CD4^+$ and $CD8^+$ T cells in an allogeneic model is through the detection of cytokine production following *ex vivo* restimulation. Although analysis of cytokine production allows us to track graft-reactive T cells, we cannot determine whether either of the treatments rendered the graft-reactive T cells anergic or non-responsive. Interestingly, in the fully allogeneic model, we were able to detect

changes in KLRG-1 expression in the antigen experienced $CD44^{high}$ $CD62L^{low}$ population of $CD8^+$ T cells. Specifically, treatment with anti-CD154 led to an increased frequency of KLRG-1^{high} antigen-experienced $CD8^+$ T cells, and correspondingly a decrease in the frequency of KLRG-1^{low} long-lived memory precursors. While KLRG-1 has been shown to be expressed on exhausted $CD8^+$ T cells at later timepoints during the course of an immune response, its expression early during the response is associated with short-lived effector cells that are destined to die during the contraction phase of the response (2). These data indicate that one mechanism by which CD154 blockade mediates $CD8^+$ T cell deletion is through the induction of KLRG-1 expression. Previous studies have shown that increased expression of KLRG-1 can be attributed to increased antigen duration and increased inflammation (1, 3). Similarly, in our antigen-specific T cell transgenic model, we observed a similar induction in KLRG-1 expression on OT-I T cells in animals treated with anti-CD154 mAb. Although further investigation into the mechanisms by which CD154 blockade may increase KLRG-1 expression is warranted, we concluded that since these T cells will likely be programmed for exhaustion or even deletion, the use of transgenic models could provide us with the means by which to study the donor-reactive T cells prior to their eventual non-responsive state.

Therefore, to specifically study the effects of CD40/CD154 pathway blockade on the donor-specific T cells, we moved into an antigen-specific transgenic skin graft model. Similar to the fully allogeneic system, we demonstrated that DST and anti-CD154 synergize to prolong antigen-specific skin graft survival. Furthermore, DST

accelerated the activation of donor-specific T cell responses, while CD40/CD154 pathway blockade delayed the accumulation and differentiation of the donor-reactive T cells into multi-cytokine producing cells. In contrast, we were able to report a previously unobserved finding: the conversion of donor-reactive $CD4^+$ T cells into induced graft-specific $CD25^+$ $Foxp3^+$ regulatory T cells. When we analyzed the overall accumulation of regulatory T cells in the draining lymph nodes, no significant changes in T_{reg} levels between anti-CD154 and DST treated animals were observed. However, upon analysis of the donor-reactive $CD4^+$ T cell population, we were able to demonstrate that the conversion of $CD4^+$ T cells into iT_{reg} was present in the antigen-specific T cell population. This finding further highlights the strength of the transgenic model system, where we can specifically track donor-reactive T cell responses; donor-specific iT_{reg} would not be detectable in the allogeneic model.

Although the mechanisms behind the peripheral conversion of naïve $CD4^+$ T cells into $Foxp3^+$ regulatory T cells is not fully established, this conversion is likely the result of multiple factors. The conversion of $Foxp3^-$ $CD4^+$ T cells into $Foxp3^+$ T cells has been observed following exposure to low-dose antigen (4), and conversely high TCR stimulation has been shown to inhibit iT_{reg} generation (5). In addition, upon *in vivo* treatment with blocking anti-CD4 mAb during transplantation, extra-thymic development of $CD25^{high}$ T_{reg} from $CD25^-$ precursors was reported (6). Here, we demonstrate CD154 engagement preferentially guides $CD4^+$ T cell differentiation away from iT_{reg}.

Although this model allows us to specifically track donor-reactive T cell responses, our ability to assess the effects of CD40/CD154 pathway blockade on

antigen-presenting cells is limited. In this system, DST is likely the primary method of antigen recognition by T cells early in the response. Upon encounter with donor-reactive T cells, the APCs are quickly targeted for deletion. Consequently, in order to study the effects of CD40/CD154 pathway blockade on dendritic cell activation, we utilized a bone marrow chimera model using the transgenic OVA system as the target antigen. In this model, antigen-specific T cells synchronously activate upon encounter with antigen presented on hematopoietically-derived APCs. In this model, we found that blockade of the CD40/CD154 pathway also led to the conversion of donor-reactive $CD4^+$ T cells into induced bone marrow specific $Foxp3^+$ $CD25^+$ regulatory T cells, similar to the skin graft model system. Unlike the skin graft models, the process of creating the bone marrow chimeras led to a substantially increased frequency (>90%) of $CD11c^+$ dendritic cells in the spleen that also expressed our cognate antigen, OVA. Therefore, activated dendritic cells that expressed OVA could be more easily detected after the adoptive transfer of antigen-specific T cells prior to their deletion. Through these studies, we discovered that treatment with anti-CD154 did not fully block the activation signals in dendritic cells, as we still observed an increase in both class I MHCs and costimulatory molecules. However, blockade of the CD40/CD154 pathway did lead to a reduced ability of activated dendritic cells to produce inflammatory cytokines following antigen activation, including TNF, IL-12p35, IL-1β, and IL-6 (Figure 7.2). Because of the reduced IL-6 production by DCs, we analyzed the bone marrow reactive T cells for IL-17 secretion. This reactivity has previously been undetected in solid organ transplantation models, including our transgenic skin graft system. Surprisingly,

although no IL-17 production was detected in antigen-specific $CD4^+$ T cell population, we observed that a significant frequency of antigen-specific $CD8^+$ T cells produced IL-17 following *ex vivo* restimulation in untreated animals, which was inhibited with anti-CD154 treatment. Although the role these cells play during immune responses has yet to be fully dissected, IL-17-producing $CD8^+$ T cells have recently been identified as playing important roles in several animal models of infection and transplantation. For example, in T-bet deficient animals, IL-17 secreting $CD8^+$ T cells have been reported in response to both allografts and viral infections (7-9). In cardiac allograft models, mice lacking T-bet also developed costimulation blockade resistant rejection mediated by IL-17-secreting $CD8^+$ T cells (8, 9). Although the differentiation requirements for $CD8^+$ T_c17 cells have not been well described, our results would suggest that IL-6 and/or TNF may be important for their development.

Small molecule inhibitors for targeted CD40/CD154 pathway blockade

From these data, we confirmed that CD40/CD154 pathway blockade has potent inhibitory effects on innate and adaptive immune responses. When studying immune responses to transplanted grafts, the objective of future research will be to focus on ways to provide the same inhibitory effects of anti-CD154 without utilizing antibodies against the surface molecule. One way to potentially provide the same inhibitory effects of anti-CD154 is through the development of small molecule inhibitors against stimulatory signals downstream of CD40 in antigen presenting cells. Upon ligation of CD40 by CD154, TNF receptor associated factors (TRAFs)

bind to the intracellular cytoplasmic domain of CD40, which triggers the activation of NF-κB and JNK signaling (10-15). The binding of the different TRAF molecules provides distinct non-redundant signals within the antigen presenting cells, leading to either the activation or inhibition of donor-reactive T cell responses. The interactions of some TRAF molecules to the cytoplasmic tail of CD40 are mutually exclusive to others. As previously described, there is considerable overlap in the binding sites of TRAF1, TRAF2, and TRAF3 (16), where the interaction of one molecule with the CD40 cytoplasmic domain prevents the binding of the other molecules. Therefore, it is possible that signaling through a particular TRAF molecule could be responsible for the detrimental stimulatory signals against a transplanted organ. For example, CD40 signaling through TRAF2 has been shown to increase JNK and NF-κB mediated signals, and to promote TRAF3 degradation (17), which could lead to enhanced T cell activation. In contrast, because signaling through TRAF3 has been associated with certain negative regulatory effects, such as decreased JNK and NF-κB activation and reduced immunoglobulin production by B cells, it seems unlikely that this molecule could contribute to activation signals against a transplanted organ (18, 19). Conversely, the binding of TRAF6 to the cytoplasmic tail of CD40 triggers the activation of JNK, inducing the secretion of proinflammatory cytokines such as IL-1, IL-6, and IL-12 (15, 20-22). Thus, it is possible that blockade of the CD40/CD154 pathway inhibits the ability of either TRAF2 or TRAF6, but not TRAF3 to be recruited to the CD40 intracellular domain to induce DC activation. Targeted deletion of individual TRAF binding domains could allow us to test this hypothesis. If one of these pathways is playing a key role in the

activation of alloantigen-reactive T cells, the development of intracellular small molecule inhibitors could provide the targeted therapeutic in preventing transplantation rejection, with similar efficacy as with treatment with anti-CD154, but without the thromboembolic complications.

TO TARGET CD40 OR CD154? THAT IS THE QUESTION

Blocking CD40 in lieu of CD154 for skin allograft survival in mice

Due to the beneficial effects of CD40/CD154 pathway blockade in transplantation, increased interest in the pathway has arisen. Recently, the effects of different isotypes of an anti-CD40 blocking antibody have been analyzed for use in the prevention of skin allograft rejection in mice (23). In this study, the use of anti-CD40 IgG_{2b} isotype to block CD40/CD154 signaling prolonged skin graft survival, while treatment with the IgG_1 isotype switch variant of the same antibody construct accelerated immune responses against the graft. Furthermore, treatment with anti-CD40 IgG_{2b} (7E1-G2b) led to similar survival times as compared with treatment with anti-CD154 (MR1). In these experiments, animals treated with either anti-CD40 IgG_{2b} or anti-CD154, in combination with CTLA4-Ig, displayed skin graft survival rates over 100 days in a highly permissive (BALB/c→C3H) allograft model. Importantly, however, it was not published that the same animals treated with anti-CD40 IgG_{2b} rapidly rejected their skin grafts starting at day 100; while anti-CD154 treated mice displayed indefinite survival of skin grafts (unpublished observations). Interestingly, in the same transgenic bone marrow chimerism survival studies we use in chapter 4, preliminary experiments suggested that similar

to untreated animals, treatment with anti-CD40 IgG_{2b} did not protect against minor mismatched (mOVA) bone marrow rejection upon adoptive transfer of the antigen-specific $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T cells. Futhermore, upon analyzing the bone marrow-reactive $CD4^+$ and $CD8^+$ T cell responses in our bone marrow chimerism model, we observed no difference in the suppressive effects of anti-CD40 IgG_{2b} compared with untreated animals (unpublished observations). In contrast with animals treated with anti-CD154 (MR1), animals treated with anti-CD40 IgG_{2b} displayed no suppression of $CD8^+$ T cell activation and expansion and did not develop of donor-specific iT_{reg}. These studies reveal that the readout of a study is very important when comparing the effects of two treatments. In one case, the preliminary analysis of skin graft survival in a permissive model seemed sufficient to demonstrate that CD40/CD154 blockade with 7E1 IgG_{2b} could protect against rejection. However, closer analysis of T cell responses *in vivo* may reveal that treatment with anti-CD154 vs. anti-CD40 antibodies does not result in the same graft-specific immune responses.

New therapies targeting the CD40/CD154 pathway in allotransplantation

Over the last twenty years, targeting CD40/CD154 pathway has been widely used to prevent rejection in several experimental models of transplantation including skin, kidney, islet, and bone marrow. During the development of a CD154 blocker, one of the first human homologue anti-CD154 monoclonals (5C8) was tested in the prevention of renal allograft rejection in non-human primates (24). Either alone or in combination with the CD80/CD28 blocker, CTLA4-Ig, 5C8

significantly prolonged kidney survival rates in rhesus macaques up to 100- or 150-days. Although treatment with this therapeutic significantly prolonged kidney allograft survival, it did not prevent lymphocytic infiltration into the transplanted kidney. This increased infiltration was a sign that the kidneys were not fully tolerized, and was a potential predictor for future transplant rejection in the surviving macaques.

H106 was another human anti-CD154 antibody tested in pre-clinical models. This antibody has been used in the prevention of renal allograft rejection (25) and in the prolongation of bone marrow transplant chimerism levels (26) in nonhuman primates. In renal allograft models, treatment with H106 in combination with CTLA4-Ig and sirolimus, significantly prolonged kidney survival rates. However, treatment with H106 only transiently prolonged (25). Treatment with H106 promoted chimerism induction due to the prevention of allo-specific T cell proliferation. Although combined therapy of H106 with belatacept and sirolimus aided in prolonging chimerism survival, the weaning from the either the CD154 blocker or that of sirolimus triggered rapid rejection of chimerism, associated with increased allo-T cell proliferation (26).

Because targeting CD154 has been associated with thromboembolic complications due to its expression on platelets (27, 28), new therapeutics have been developed to specifically target CD40 to inhibit allo-immune responses and promote transplant survival. Chi220, a chimeric mouse anti-human monoclonal antibody against CD40, has been described as a partially agonistic antibody that not only fully blocks CD40 molecules, but also weakly facilitates proliferation of

stimulated B cells (29). Despite this minor level of proliferation, Chi220 has been used in the prevention of transplantation rejection in non-human primates. In a kidney transplantation study, the use of Chi220 in combination with CTLA4-Ig significantly prolonged renal allograft survival compared to CTLA4-Ig monotherapy (25). Similarly, the use of Chi220 in combination with belatacept promoted euglycemia and prolonged alloislet survival in nonhuman primates (29). In these studies, the use of Chi220 resulted in the depletion of serum B cells, which were repopulated once treatment ceased (25, 29). Furthermore, continued treatment with the depleting anti-CD40 antibody led to significant detrimental effects following CMV primary infections in macaques, which were not controlled due to the absence of naïve antibody-producing B cells specific to the virus (25). On the other hand, since antibody-producing memory B cells typically downregulate surface molecules, animals with prior CMV exposure remained protected against CMV reactivation (29).

Recently, a new mouse anti-CD40 monoclonal antibody (3A8) has been identified for use in the prevention of transplant rejection. Similar to Chi220, 3A8 has been described as a partial agonist antibody that impairs alloantigen-specific T cell proliferation, but does not inhibit CD154 binding to CD40, which induced the upregulation of costimulatory molecules CD80/CD86 on the surface of B cells (30). Unlike treatment with Chi220, 3A8 did not induce depletion of B cells. In rhesus macaques, combined treatment of 3A8 with sirolimus and basiliximab induction therapy led to significantly prolonged alloislet survival, leading to euglycemia, compared with either 3A8 alone or sirolimus/ basiliximab treatment groups.

Therapies targeting the CD40/CD154 pathway in xenotransplantation

The anti-CD154 humanized antibody, H106, has also been used in the prevention of xenoislet rejection (31, 32). In studies by Cardona *et al.*, rhesus macaques were transplanted with either neonatal or adult porcine islets followed by treatment with H106 in combination with the CD80/CD28 blocker, belatacept, and sirolimus (31, 32). Survival rates of the neonatal porcine islets were significantly prolonged with the anti-CD154 H106 mAb compared with untreated controls (31). On the other hand, the treatment with H106 only mildly prolonged survival rates of adult porcine islets in rhesus macaques (2/5 recipients) (32).

Once again, due to fears of thromboembolic complications with CD154-targeting antibodies, the anti-CD40 mAb Chi220 has also been studied as a therapeutic for CD40/CD154 blockade in the prevention of neonatal porcine islet transplantation rejection in rhesus macaques (33). In these studies, all recipients received basiliximab induction therapy, belatacept and sirolimus in the presence or absence of Chi220 treatment. Results indicated that Chi220 significantly prolonged xenoislet survival (MST 59 days) compared to animals not receiving Chi220 treatment (2/3 failure to engraft). However, treatment with H106 using the same base immunosuppression protocol led to xenograft survival of >140 days (31). Thus, the discrepancy in survival times between these two treatments suggests there may be a different mechanism of action in the protection of the transplant following treatment with CD154-blockade compared with the temporary delay in rejection with anti-CD40 treatment.

In conclusion for these studies, the use of either anti-CD154 or anti-CD40 monoclonal antibodies in the prevention of transplantation rejection has drastically different effects on immune responses. Some antibodies have signal blocking properties; some stimulate antigen-presenting cell activation; while others deplete immune cells that may play an important role in protective immunity. Although we have observed that targeting CD40 may improve allograft survival, the development of this therapeutic comes with several caveats and questions. Will *in vivo* administration of the antibody stimulate antigen-presenting cells? Will the antibody deplete B cells necessary for protective immunity? Is the treatment actually effective at protecting against transplantation rejection?

All of these factors may be playing a role in the reduced efficacy of Chi220 and 3A8 compared to the anti-CD154 monoclonal antibodies. As described in studies with Chi220 treatment, the temporary depletion of B cells could aid in the prolongation of the transplanted graft through the reduced activation of T cell responses, but this poses a significantly increased risk in hindering protective immunity against novel pathogen infections. Although a CMV infection may not be life-threatening in non-immunocompromised individuals, the increased inflammation and damage due to the infection increases the risk for transplantation rejection. Furthermore, decreased control of JC virus reactivation in patients could increase the potential of progressive multifocal leukoencephalopathy (PML), a lethal demyelinating disease caused by JC virus lysis of oligodendrocytes in the brain of immunosuppressed individuals. Additionally, treatment with either of these CD40-blocking antibodies (Chi220 and 3A8) led to the significantly increased activation of

responding B cells *in vitro*. As described with treatment with 7E1-IgG_1, once the APCs are activated and upregulate costimulatory molecules, the responding T cells become more reactive (23). Consequently, upon diminution or cessation of other immunosuppressants, the more reactive antigen-presenting cells could effectively induce a detrimental T cell response against the transplanted graft. Also, due to the increased activation of antigen presenting cells, the question of therapeutic efficacy arises. In the above neonatal porcine xenoislet transplant studies, we provided direct comparisons for CD40 vs. CD154 blockade using the same base immunosuppressant protocols. Studies revealed that treatment with H106 led to islet survival rates of over 140 days, while in animals treated with Chi220, islets survived less than 60 days. Furthermore, in the mouse skin allograft experiments comparing 7E1-IgG_{2b} (anti-CD40) and MR1 (anti-CD154), animals treated with 7E1-IgG_{2b} rapidly rejected skin grafts after 100 days, while those treated with MR1 appreciated indefinite skin graft survival.

Taken together, we speculate that the use of antibodies targeting the CD40 molecule could provide a false sense of security during the course of the treatment and could prove to be detrimental to both graft survival and protective immunity against pathogens. Although this remains untested in the non-human primates, but based on our studies in the chimerism model, we suspect that the suboptimal protection of anti-CD40 mAb may be due to the lack of T_{reg} induction by these therapies and the subsequent sustained protection of the graft. We propose that future development of therapeutics targeting the CD40/CD154 pathway should focus on a CD154-based therapeutic as it could not only be more effective against

transplantation rejection, but also less detrimental against infectious agents. However, considering the thromboembolic complications behind targeting CD154, novel therapies should focus on non-crosslinking therapeutics such as the development of inhibitory F_{ab} fragments against CD154 or other targeted small molecule inhibitors against intracellular signaling molecules, as mentioned above.

ASSESSING IMPORTANT INTERACTIONS INHIBITED BY CD154-BLOCKADE

Several mechanisms of antigen-specific $CD4^+$ and $CD8^+$ T cell activation have been described following CD40-CD154 interactions. Even though blockade of the CD40/CD154 pathway has been described as one of the most effective therapies inducing transplantation tolerance, it remains unclear which CD4-CD8-APC interactions need to be inhibited to induce the protective effects of anti-CD154.

Blockade of this pathway could lead to the inhibition of three potential downstream activation signals. The first interaction involves the classically accepted pathway of $CD8^+$ T cell activation through APC "licensing" by antigen-specific $CD4^+$ T cells (34-36). In this model, the antigen-specific $CD4^+$ T cell recognizes its cognate antigen on an APC and provides the activation signals by engaging CD40. Through this licensing process, the CD40-CD154 interaction primes the APC to upregulate other costimulatory molecules (CD80, CD86, 4-1BB, OX40, etc.), to increase the expression of class I and class II MHC, and to induce the production of proinflammatory cytokines (IL-1, IL-6 IL-12), all of which can subsequently drive antigen-specific $CD8^+$ T cell activation (37-39). Although it is not clear which of these inflammatory responses are inhibited by CD40/CD154 pathway

blockade and are responsible for graft-specific immune responses, our results would suggest that perhaps the inhibition of "signal three" may be critical to protecting grafts against rejection.

In recent studies, CD40 expression on $CD8^+$ T cells has been identified as an important factor in the activation and development of long-lived memory $CD8^+$ T cells responding to soluble antigens (40). However, the need for direct interaction between the $CD4^+$ and the $CD8^+$ T cell for activation has been controversial, as the presence of CD40 has been deemed unnecessary in the activation of $CD8^+$ T cells against acute *Listeria monocytogenes*, LCMV or influenza virus infections (41, 42). We speculate that the differences in priming of T cells in a non-infectious model, such as towards a soluble antigen or a transplant, could promote direct T-to-T interactions leading to graft-specific responses. Conversely, in the setting of acute infections, the increased inflammation and engagement of toll-like receptors on APCs could overwhelm the system and circumvent the need for direct interactions between the $CD4^+$ and $CD8^+$ T cells.

The final potential activation signal inhibited by anti-CD154 treatment is retrograde, or reverse, signaling into the $CD4^+$ T cell (43-45). The significance of this activation signal remains controversial due to the very short cytoplasmic domain of CD154, which could prevent the binding of potential downstream activation molecules. However, in future studies, we need to confirm whether this reverse signal is playing any role in activating antigen-specific $CD4^+$ T cells, and subsequently promoting $CD8^+$ T cell activation.

Although we hypothesize that the primary mechanism behind CD40/CD154 pathway blockade is through the prevention of APC licensing, the role the other interactions play during transplantation rejection have yet to be determined. In order to assess the significance of these interactions in activating graft-reactive T cell responses, we have bred both OT-I and OT-II T cells onto $CD40^{-/-}$ and $CD154^{-/-}$ backgrounds, respectively. In preliminary experiments, we used the conversion of donor-reactive $CD4^+$ T cells into graft-specific $CD25^+$ $Foxp3^+$ regulatory T cells as the primary readout of CD40/CD154 inhibition. In initial experiments, we adoptively transferred OT-I and $CD154^{-/-}$ OT-II T cells into naïve mice that received a mOVA donor-specific transfusion followed by a mOVA skin graft. In this system, where all three cellular interactions were inhibited, the absence of CD154 led to conversion of the donor-reactive $CD4^+$ T cells into iT_{reg}. However, when wild type OT-II T cells were co-transferred with the $CD154^{-/-}$ OT-II T cells prior to transplantation, the conversion of iT_{reg} was completely ablated. This result suggests that either APC licensing or $CD8^+$ T cell help was occurring through the wild type $CD4^+$ T cell, resulting in activating, rather than inhibitory, signals to the donor-reactive $CD4^+$ T cells.

We then tested the importance of direct interactions between $CD4^+$ and $CD8^+$ T cells by adoptively transferring wild type OT-II and $CD40^{-/-}$ OT-I T cells into naïve animals, followed by mOVA skin graft and DST treatment. If direct T-to-T cell interactions were contributing to T cell activation, the prevention of these signals would lead to increased T_{reg} conversion. However, following inhibition of T-to-T interactions, no T_{reg} conversion was observed. These preliminary results suggest

that APCs are being licensed and are activating the $CD4^+$ and $CD8^+$ T cells through either direct binding or soluble secreted factors, while circumventing any suppressive effects behind the lack of activating CD40 signals in the antigen-specific $CD8^+$ T cells.

In order to assess the importance of CD40-expression on antigen-presenting cells and APC licensing, we used $CD40^{-/-}$ recipient animals, which were adoptively transferred with purified wild type OT-I and OT-II T cells, and transplanted them with $CD40^{-/-}$ mOVA graft with $CD40^{-/-}$ DST treatment. If blockade of APC licensing were an important factor behind the protective effects of CD40/CD154 pathway blockade, then we would expect a high level of $CD4^+$ T cell conversion into iT_{reg}. However, no conversion of iT_{reg} was observed in this fully knockout model. Although these results contradict the expected importance of blocking APC licensing in the conversion of iT_{reg}, we speculate that the complete absence of CD40 signals transduced into the APC may lead to detrimental effects on APC survival (46). As observed in our chimerism studies, treatment with anti-CD154 (MR1) only partially inhibits APC activation signals, preventing the production of proinflammatory cytokines while still allowing for the upregulation of certain costimulatory molecules. Taken together, these data suggest that partial signals through CD40 are important for APC survival while the inhibition of other activation signals may be necessary for the induction of T_{reg} conversion, however further studies are warranted to test the importance of the different activation signals.

PARADOXICAL ASPECTS OF RAPAMYCIN IN TRANSPLANTATION (47)

Impact of rapamycin on CD8⁺ T cell differentiation in response to pathogens

Given this history as an immunosuppressive and anti-proliferative agent, it came as a great surprise when investigators began to interrogate the impact of rapamycin monotherapy on antigen-specific T cell responses during the course of viral or bacterial infection. Clinical observations published over the last decade hinted at the idea that patients treated with rapamycin demonstrated better outcomes with regard to cytomegalovirus (CMV) disease and were better able to control CMV viremia than patients treated with standard calcineurin inhibitor-based immunosuppression following transplantation (48). The authors of the study speculated that the underlying mechanism involved rapamycin-mediated attenuation of viral replication, as these viruses co-opt host machinery in order to replicate (48). Recently, however, in two studies published in *Nature* in 2009, Araki *et al.* and Pearce *et al*. demonstrated a paradoxical immunostimulatory effect of rapamycin on the $CD8^+$ memory T cell response following pathogen infection (49, 50). Administration of rapamycin during the priming phase was found to increase the number of virus-specific memory T cells in LCMV-infected animals. Furthermore, when rapamycin was administered only during the contraction phase of the response, the antigen-specific T cells did not increase in quantity, but rather increased in quality, acquiring a more central memory-like phenotype ($CD62L^{hi}$ KLRG-1^{lo} $CD27^{hi}$ Bcl-2^{hi}), and increased proliferative capacity upon rechallenge (Figure 7.3). In a series of elegant RNAi knock-down experiments, the authors showed that this enhancement in the antigen-specific response was a T cell-intrinsic

effect. These results were further corroborated in a rhesus macaque model in which animals receiving rapamycin exhibited increased recall responses and enhanced differentiation of memory T cells following vaccination with modified vaccinia Ankara (49, 51). Since both the macaque and murine studies involved non-replicative viruses (or virus-like particles), these data provide further evidence that the direct impact of mTOR inhibition on viral replication is likely not the mechanism by which viral load might be attenuated following treatment with rapamycin. Instead, unlike the previously described effects of mTOR inhibition on dendritic cells, T_{reg}, or other immune compartments (52), both studies point to a direct effect of rapamycin on $CD8^+$ T cells to enhance both the quantity and quality of memory T cell differentiation in response to pathogen exposure *in vivo*.

What is the mechanism underlying this effect? The explanation could be linked to the ability of rapamycin to enhance fatty acid oxidation (FAO) in responding T cells. Studies in other cell types, including smooth muscle cells and hepatocytes, have demonstrated that inhibition of mTOR with rapamycin results in increased FAO along with reduced glucose utilization (53, 54). Importantly, the transition from glycolysis to FAO was recently shown to be critical for effector to memory transition in $CD8^+$ T cells (50). In particular, T cells lacking TRAF6 were shown to be deficient in their ability to undergo FAO following growth factor withdrawal, and also exhibited a reduced ability to differentiate into memory T cells. However, treatment with the diabetes drug metformin restored FAO and enhanced differentiation of memory T cells, both in TRAF6-deficient as well as wild-type T cells (50). Similar results were observed in $CD8^+$ T cells following treatment with

rapamycin; thus, rapamycin may act on effector T cells to facilitate the metabolic switch from glycolysis to FAO, thereby enhancing memory T cell differentiation. Another possible mechanism by which rapamycin may augment the generation of T cell memory is by impacting the relative balance of expression of the transcription factors T-bet and Eomesodermin in $CD8^+$ T cells (55). Specifically, blocking mTOR through rapamycin decreases the expression of T-bet, which is highly expressed in effector T cells, and promotes expression of Eomesodermin, which is highly expressed in memory T cells. Taken together, these data suggest possible mechanism(s) underlying the immunostimulatory effects of rapamycin on pathogen-specific T cell responses, and imply that exposure to rapamycin may produce different outcomes depending on the cell cycle and metabolic state of a given cell or population.

Fundamental differences in the effect of rapamycin on graft versus pathogen specific T cells

Given these findings on the effects of rapamycin on pathogen-specific T cell responses, the question of whether rapamycin also augmented antigen-specific $CD8^+$ T cell response during transplantation arose. Was this enhancing effect of rapamycin being masked by the presence of other immunosuppressants following transplantation? Or was there something fundamentally different about the way rapamycin affects T cells responding to cognate antigen in the context of a graft versus a pathogen?

To address these questions, a recent study from our group directly compared the impact of rapamycin on $CD8^+$ T cells responding to a graft versus a pathogen, using a system in which the same epitope was presented to a monoclonal T cell population in both circumstances (56). We observed that while treatment with rapamycin dramatically increased the antigen-specific $CD8^+$ T cell response to the pathogen, the identical T cell population responding to antigen in the context of a graft failed to be enhanced in the presence of rapamycin (Figure 7.3). As observed by Araki *et al.*, antigen-specific T cells responding to the pathogen also exhibited enhanced quality, by increased expression of CD62L, which is associated with enhanced development into a central memory phenotype (49, 57), while antigen-specific T cells responding to a graft failed to exhibit this change. These results suggested that the environment in which an antigen is presented alters the influence of rapamycin on antigen-specific T cell expansion, and highlight a fundamental difference between antigen presented by an infectious agent as compared to an allograft. What is not known is why or how mTOR inhibition through rapamycin treatment exerts such disparate effects on T cells responses. Interestingly, concomitant infection of skin graft recipients with a bacterial pathogen did not restore the enhancing effects of rapamycin on the graft-specific T cells, demonstrating that TLR- or other pathogen-associated inflammatory signals provided *in trans* were not sufficient to precipitate the rapamycin-induced augmentation (56). These data suggested a principal difference in the way rapamycin impacts T cells responding to antigen presented in the context of a pathogen versus that of a graft. Based on studies observing antigen-specific T cell

responses in the absence of antigen presenting cells or peripheral lymphoid organs, we concluded that antigen presentation in secondary lymphoid organs are necessary, but not sufficient to induce the enhancing effects of rapamycin. Furthermore, we speculate that the effects of rapamycin may not be T cell-intrinsic in the setting of a transplanted graft, as we did not observe enhanced T cell responses to polyomavirus infection in the absence of peripheral lymphoid organs. However, the mechanism underlying this phenomenon remains to be determined, and is a critically important issue for the field of transplantation.

Taken together, the implications of these results are far-reaching. First, donor-reactive memory T cells have been shown to pose a potent barrier to graft survival following transplantation, as evidenced by basic studies in mouse models and the fact that clinical studies revealed a correlation between pre-transplant frequency of donor-reactive memory T cells and incidence of acute rejection within the first year (58, 59). These memory T cells can arise in unsensitized individuals due to molecular mimicry between pathogen-derived and alloantigens via prior infections or exposure to environmental antigens (60). Molecular mimicry between pathogen- and allo-antigens is now known to be more common than previously appreciated: recent evidence indicated that 45% of virus-specific memory T cell clones obtained from normal healthy humans possessed cross-reactivity with an alloantigen (61). From our pathogen-elicited memory T cell induction experiments, we concluded that in the presence of rapamycin, presentation of cognate or cross-reactive antigen delivered either by a graft or a pathogen to the same T cell could

evoke markedly different responses and prevent heterologous immune T cell responses.

In addition, the finding that rapamycin enhances the antigen-specific $CD8^+$ T cell response to pathogens suggests that treatment of transplant recipients with rapamycin monotherapy may increase immunity to viruses or vaccines (49). However, the ability of rapamycin to augment pathogen-specific $CD8^+$ T cell responses in the context of other immunosuppressive agents is completely unknown. Currently, the standard of care in most instances is to reduce the intensity of immunosuppression if patients experience infectious complications. As such, further studies are required to inform and direct clinical use of rapamycin as an adjunct therapy for boosting protective immunity to both live infections as well as vaccines in transplant recipients.

Another fork in the road: mTOR inhibition inhibits T_h cell differentiation

The studies discussed above demonstrated that inhibition of mTOR through rapamycin results in an increase in the magnitude and functionality of $CD8^+$ pathogen-specific memory T cell responses. Conversely, studies on $CD4^+$ pathogen-specific T cells failed to demonstrate an enhancement in the function and magnitude of the response. Specifically, Delgoffe and colleagues used a cre-lox system to ablate the mTOR gene in T cells only (62). Their work demonstrated that in response to an acute viral infection, $CD4^+$ T cells deficient in mTOR failed to express the T_h transcription factors T-bet, GATA3, and RORγt, and therefore failed to polarize into Th1, Th2, and Th17 subsets following activation, despite overall normal activation (i.e. expression of activation markers and IL-2 production) (62) (Figure 7.4). The

authors attributed this to an attenuation of STAT-mediated signaling downstream of the respective cytokine receptors required for T_h polarization. Thus, inhibition of mTOR may have disparate effects on $CD4^+$ versus $CD8^+$ T cell populations, enhancing antigen-specific $CD8^+$ populations while inhibiting differentiation of $CD4^+$ T cells. Again, the precise mechanisms underlying this dichotomy remain unknown.

Rapamycin and regulation: An important facet of its immunosuppressive effects

Another intriguing aspect of rapamycin is that it has consistently been found to augment rather than suppress T_{reg} generation, functionality, and survival (52) (Figure 7.4). For example, studies have shown that T_{regs} cultured *in vitro* with rapamycin exhibited increased levels of expansion as compared to untreated controls (63).

Although prolonged rapamycin treatment has been associated with reduced peripheral T cell numbers due to impaired thymic output, this effect seems to be reversed in the T_{reg} compartment (64). Specifically, rapamycin has been shown to increase the proportion of Foxp3-expressing $CD4^+$ T cells among recent thymic emigrants. This effect is compounded by the fact that rapamycin promotes survival of natural T_{regs} while increasing the upregulation of Foxp3 in conventional T cells and enhancing T_{reg} function (65). One purported mechanism underlying this effect is the fact that mTOR inhibition can result in reduced Akt phosphorylation and hence increased Smad3 activity, which has been shown to lead to an increase in Foxp3 expression (66).

Rapamycin effects on antigen presenting cells: immunosuppressive and immunostimulatory

Consistent with its effects on T cell differentiation, rapamycin may also impart paradoxical effects on dendritic cell differentiation and maturation. For a number of years, mTOR inhibition in DCs has been associated with inhibited activation and upregulation of costimulatory molecules on the DC cell surface, and reduced IL-12 and TNF secretion after exposure to IL-4 in *in vivo* and *in vitro* models (67). Furthermore, the effects of mTOR inhibition in plasmacytoid DCs, which are typically associated with the host response to viral infections, resulted in reduced secretion of type I IFNs by these antigen presenting cells (68).

In contrast to these well-appreciated inhibitory effects of rapamycin on DC differentiation and maturation, recent studies have demonstrated paradoxical effects of rapamycin on macrophages and myeloid DC (69). Specifically, mTOR inhibition with rapamycin led to both enhanced production of IL-12 and reduced production of IL-10 in macrophages and myeloid DCs following TLR or bacterial stimulation. Furthermore, rapamycin was shown to increase autophagy by antigen presenting cells, leading to enhanced antigen presentation of *M. tuberculosis*. This increase in autophagy in the presence of rapamycin promoted increased T_h1 type responses and protected mice from a lethal bacterial challenge (70). Thus, rapamycin appears have the capacity to exert immunosuppressive or immunostimulatory effects on DC populations; again, the conditions and parameters modifying this impact remain unknown.

Rapamycin as a fountain of youth: impact on longevity and survival

In perhaps the most surprising development in rapamycin biology is the unanticipated role it plays in increasing longevity and decreasing incidence of death from all causes. While experience in clinical transplantation has demonstrated an association of rapamycin with hyperlipidemia, thrombocytopenia, and impaired wound healing (71), inactivation of mTOR had previously been shown to increase lifespan in invertebrates including yeast, nematodes, and fruit flies. Recently, a study assessed the impact of administration of oral rapamycin on lifespan in both male and female mice of different strains and at different test sites across the country. Although the study targeted a much higher level of rapamycin in the blood than currently used in transplantation (60-70 ng/mL vs. 8-12 ng/mL, respectively), results demonstrated that whether initiated at either middle or old age (270 and 600 days, respectively), rapamycin increased the median and maximum lifespan in both genders (72). Distribution of disease phenotypes was not different between the groups. The authors speculated that mTOR inhibition may increase survival by postponing deaths from cancer or by mimicking the anti-aging effects of caloric restriction. Furthermore, the immunostimulatory effects of rapamycin on pathogen-specific $CD8^+$ T cell responses, discussed above, would certainly suggest that treatment with rapamycin might decrease or delay death from infectious etiologies (49, 50).

CONCLUDING REMARKS

Although much is known about CD40/CD154 pathway blockade and rapamycin treatment, this dissertation brings new insight on the underlying effects of these therapeutics on graft- and pathogen-specific T cell responses, respectively. We have demonstrated that anti-CD154 treatment enhances graft-specific iT_{reg} induction, while treatment with rapamycin promotes viral-specific T cell responses. These two therapies fulfill different, but equally important, requirements of immunosuppressant regimens for transplantation. Although several questions remain behind (A) which specific signals are necessary to be blocked to induce graft protection with anti-CD154 and (B) what the mechanisms are behind the enhancing effects of rapamycin on protective immunity, these studies provide a promising potential for future transplantation therapies without rendering patients completely immunosuppressed.

Figure 7.1.

Effects of DST and CD40/CD154 pathway blockade treatments on antigen-specific $CD4^+$ and $CD8^+$ T cells.

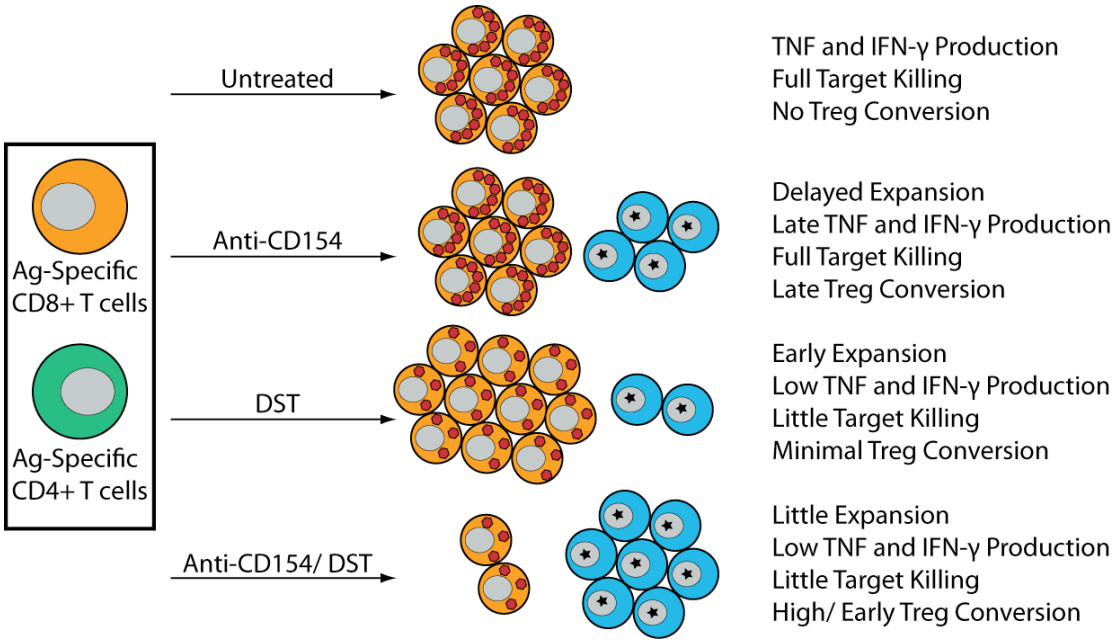


Figure 7.1. Effects of DST and CD40/CD154 pathway blockade treatments on antigen-specific $CD4^+$ and $CD8^+$ T cells. Treatment with DST and anti-CD154 have completely divergent effects on antigen-specific T cells. DST accelerates $CD8^+$ T cell activation while preventing their differentiation into multi-cytokine producing cells. In contrast, anti-CD154 treatment delays antigen-specific $CD8^+$ T cell expansion, but still permits their differentiation. Finally, while neither treatment significantly produces regulatory T cells, the combination of the two therapeutics results in the early conversion of antigen-reactive $CD4^+$ T cells into graft-specific iT_{reg}. Orange cells are the antigen-specific $CD8^+$ T cells. Green cells are the antigen-specific $CD4^+$ T cells. Red hexagons represent amount of cytokine production and target killing. Blue cells represent the converted $CD4^+$ T cells into iT_{reg}, with Foxp3 (black stars) in the nucleus.

Figure 7.2.

Inhibition of CD40-mediated signals results in partial antigen presenting cell activation.

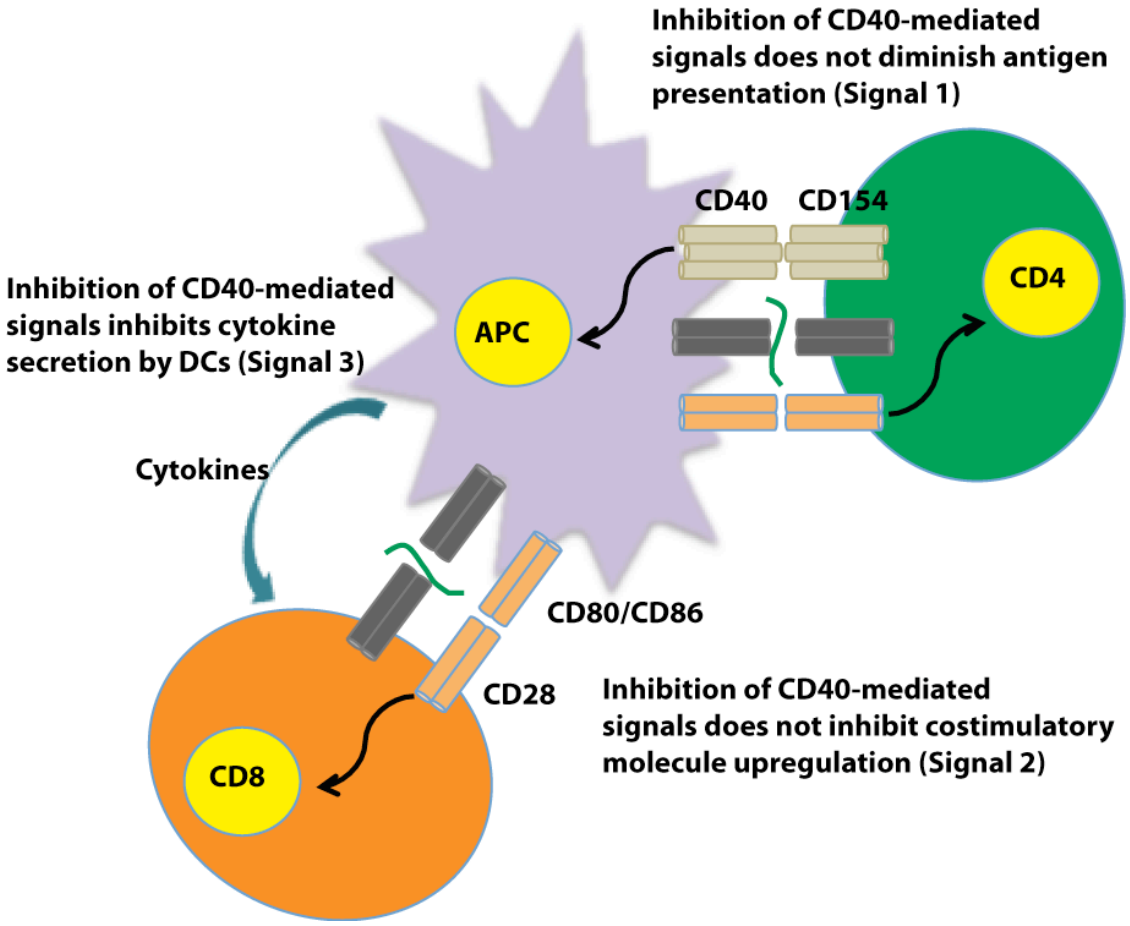


Figure 7.2. Inhibition of CD40-mediated signals results in partial antigen presenting cell activation. Following both CD40- and CD154-targeting antibodies, antigen presenting cells (dendritic cells and B cells) upregulate both class I MHC and costimulatory molecules. Conversely, as described in chapter 4, the inhibition of CD40/CD154 pathway inhibited dendritic cell specific secretion of inflammatory cytokines, such as TNF, IL-1β, IL-12p35, and IL-6.

Figure 7.3.

Differential effects of rapamycin therapy on antigen-specific CD8⁺ T cell responses following infection or transplantation.

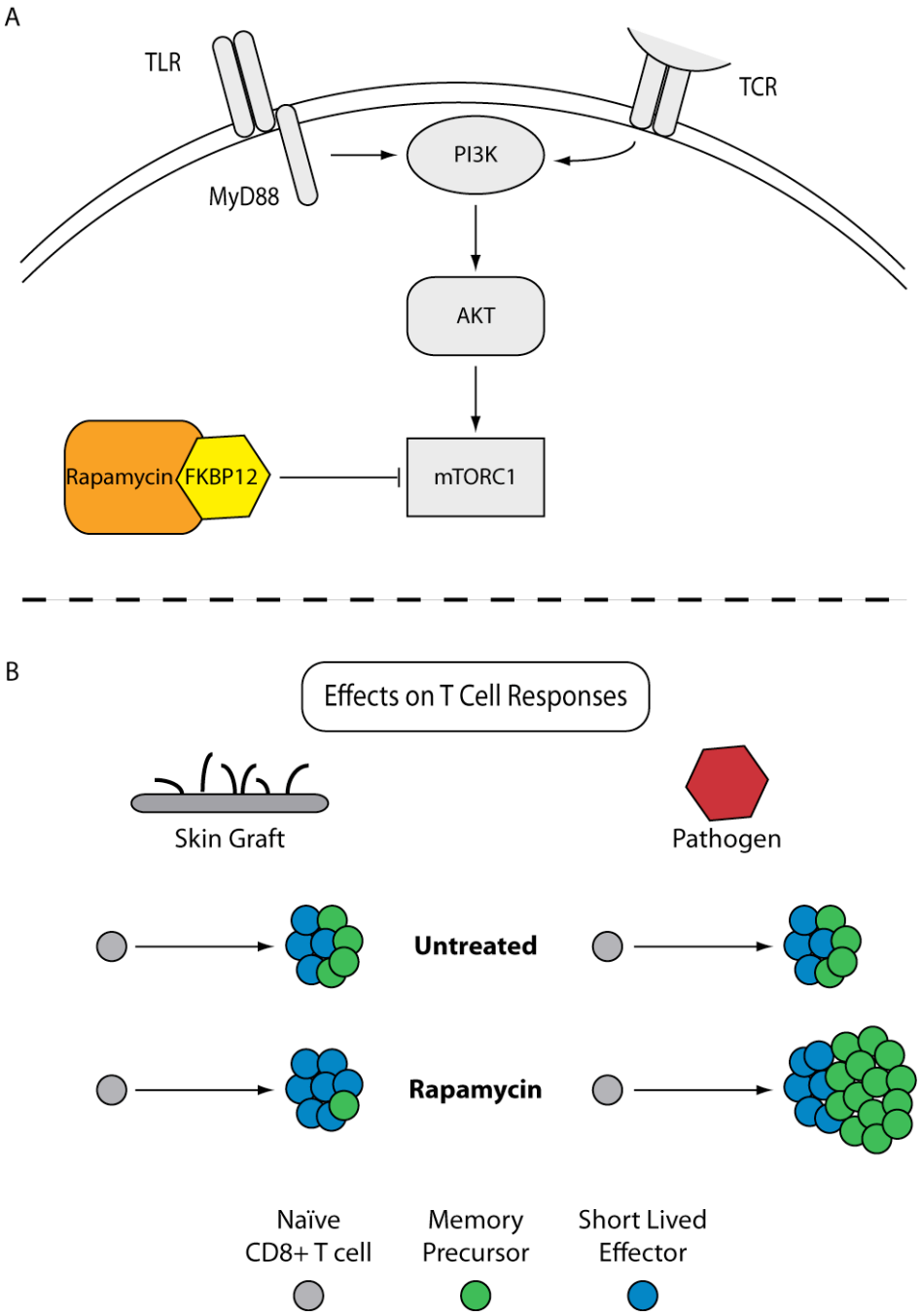


Figure 7.3. Differential effects of rapamycin therapy on antigen-specific CD8$^+$ T cell responses following infection or transplantation. A. PI3K activity results in Akt phosphorylation and subsequently mTOR complex 1 activation, which is blocked by the complex of rapamycin bound to FKBP12. B. In blocking the mTOR pathway in a transplantation model, rapamycin minimally impacts expansion of donor-reactive CD8$^+$ T cells, while attenuating differentiation of these cells into CD62L-expressing central memory-like cells. In contrast, mTOR blockade with rapamycin leads to enhanced activation and expansion of antigen-specific CD8$^+$ T cells during the course of an infection, while promoting the development of CD62L^{hi} CD8$^+$ central memory-like cells.

Figure 7.4.

Reduced signaling through the mTOR complex alters differentiation of CD4⁺ T cell responses.

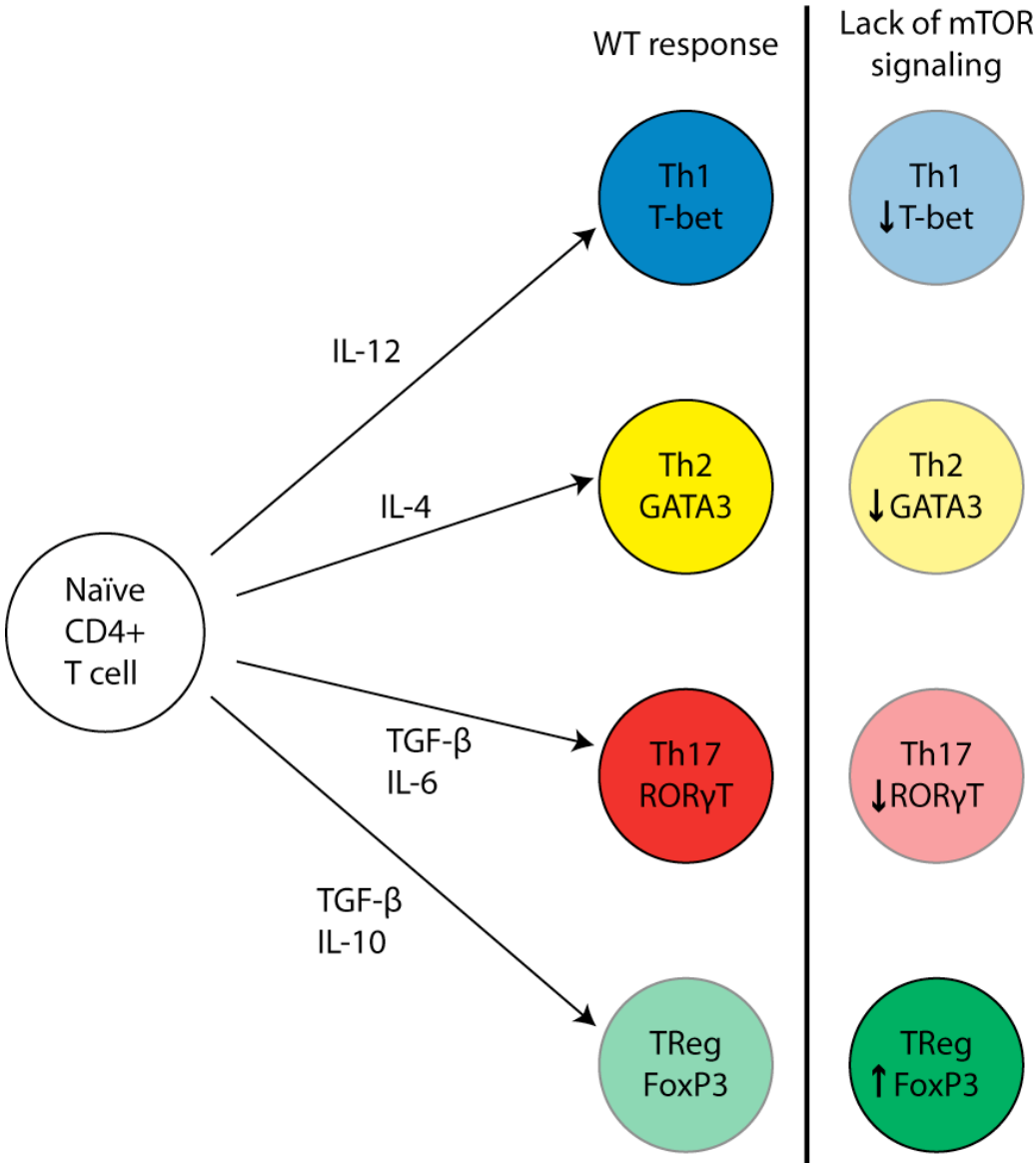


Figure 7.4. Reduced signaling through the mTOR complex alters differentiation of CD4$^+$ T cell responses. The lack of signaling through mTOR in antigen-specific CD4$^+$ T cells leads to reduced polarization into either T_h1, T_h2, or T_h17 T cells. In contrast, mTOR inhibition by rapamycin leads to enhanced development of regulatory T cells.

Table I. Dichotomous effects of rapamycin in immunobiology

Immunosuppressive	Immunostimulatory
↓$CD4^+$ T cell differentiation	↑$CD8^+$ T cell memory differentiation
↑Treg development	↑$CD8^+$ T cell activation
↓Response to skin graft	↑Response to pathogen
↓DC maturation	↑IL-12 production by DCs
Additional Contradictory Effects	
↑Survival	↑Hyperlipidemia ↑Thrombocytopenia ↓Wound healing

REFERENCES

1. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, Gapin L, Kaech SM. 2007. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27: 281-95

2. Sarkar S, Kalia V, Haining WN, Konieczny BT, Subramaniam S, Ahmed R. 2008. Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205: 625-40

3. Floyd TL, Koehn BH, Kitchens WH, Robertson JM, Cheeseman JA, Stempora L, Larsen CP, Ford ML. 2011. Limiting the amount and duration of antigen exposure during priming increases memory T cell requirement for costimulation during recall. *J Immunol* 186: 2033-41

4. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6: 1219-27

5. Molinero LL, Miller ML, Evaristo C, Alegre ML. 2011. High TCR Stimuli Prevent Induced Regulatory T Cell Differentiation in a NF-{kappa}B-Dependent Manner. *J Immunol* 186: 4609-17

6. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. 2004. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. *J Immunol* 172: 923-8

7. Intlekofer AM, Banerjee A, Takemoto N, Gordon SM, Dejong CS, Shin H, Hunter CA, Wherry EJ, Lindsten T, Reiner SL. 2008. Anomalous type 17

response to viral infection by CD8+ T cells lacking T-bet and eomesodermin. *Science* 321: 408-11

8. Yuan X, Ansari MJ, D'Addio F, Paez-Cortez J, Schmitt I, Donnarumma M, Boenisch O, Zhao X, Popoola J, Clarkson MR, Yagita H, Akiba H, Freeman GJ, Iacomini J, Turka LA, Glimcher LH, Sayegh MH. 2009. Targeting Tim-1 to overcome resistance to transplantation tolerance mediated by CD8 T17 cells. *Proc Natl Acad Sci U S A* 106: 10734-9
9. Burrell BE, Csencsits K, Lu G, Grabauskiene S, Bishop DK. 2008. CD8+ Th17 mediate costimulation blockade-resistant allograft rejection in T-bet-deficient mice. *J Immunol* 181: 3906-14
10. Cheng G, Cleary AM, Ye ZS, Hong DI, Lederman S, Baltimore D. 1995. Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* 267: 1494-8
11. Ishida TK, Tojo T, Aoki T, Kobayashi N, Ohishi T, Watanabe T, Yamamoto T, Inoue J. 1996. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc Natl Acad Sci U S A* 93: 9437-42
12. Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K, Aizawa S, Watanabe T, Mosialos G, Kieff E, Yamamoto T, Inoue J. 1996. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *J Biol Chem* 271: 28745-8

13. Nakano H, Oshima H, Chung W, Williams-Abbott L, Ware CF, Yagita H, Okumura K. 1996. TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. *J Biol Chem* 271: 14661-4

14. Rothe M, Sarma V, Dixit VM, Goeddel DV. 1995. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 269: 1424-7

15. Baccam M, Woo SY, Vinson C, Bishop GA. 2003. CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-kappa B, AP-1, and C/EBP. *J Immunol* 170: 3099-108

16. Pullen SS, Miller HG, Everdeen DS, Dang TT, Crute JJ, Kehry MR. 1998. CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* 37: 11836-45

17. Hostager BS, Haxhinasto SA, Rowland SL, Bishop GA. 2003. Tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient B lymphocytes reveal novel roles for TRAF2 in CD40 signaling. *J Biol Chem* 278: 45382-90

18. Xie P, Hostager BS, Bishop GA. 2004. Requirement for TRAF3 in signaling by LMP1 but not CD40 in B lymphocytes. *J Exp Med* 199: 661-71

19. Hostager BS, Bishop GA. 1999. Cutting edge: contrasting roles of TNF receptor-associated factor 2 (TRAF2) and TRAF3 in CD40-activated B lymphocyte differentiation. *J Immunol* 162: 6307-11

20. Baccam M, Bishop GA. 1999. Membrane-bound CD154, but not CD40-specific antibody, mediates NF-kappaB-independent IL-6 production in B cells. *Eur J Immunol* 29: 3855-66

21. Kobayashi T, Walsh PT, Walsh MC, Speirs KM, Chiffoleau E, King CG, Hancock WW, Caamano JH, Hunter CA, Scott P, Turka LA, Choi Y. 2003. TRAF6 is a critical factor for dendritic cell maturation and development. *Immunity* 19: 353-63

22. Mackey MF, Wang Z, Eichelberg K, Germain RN. 2003. Distinct contributions of different CD40 TRAF binding sites to CD154-induced dendritic cell maturation and IL-12 secretion. *Eur J Immunol* 33: 779-89

23. Gilson CR, Milas Z, Gangappa S, Hollenbaugh D, Pearson TC, Ford ML, Larsen CP. 2009. Anti-CD40 monoclonal antibody synergizes with CTLA4-Ig in promoting long-term graft survival in murine models of transplantation. *J Immunol* 183: 1625-35

24. Kirk AD, Harlan DM, Armstrong NN, Davis TA, Dong Y, Gray GS, Hong X, Thomas D, Fechner JH, Jr., Knechtle SJ. 1997. CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc Natl Acad Sci U S A* 94: 8789-94

25. Pearson TC, Trambley J, Odom K, Anderson DC, Cowan S, Bray R, Lin A, Hollenbaugh D, Aruffo A, Siadak AW, Strobert E, Hennigar R, Larsen CP. 2002. Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* 74: 933-40

26. Kean LS, Adams AB, Strobert E, Hendrix R, Gangappa S, Jones TR, Shirasugi N, Rigby MR, Hamby K, Jiang J, Bello H, Anderson D, Cardona K, Durham MM, Pearson TC, Larsen CP. 2007. Induction of chimerism in rhesus macaques

through stem cell transplant and costimulation blockade-based immunosuppression. *Am J Transplant* 7: 320-35

27. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391: 591-4

28. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. 2000. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114

29. Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S, Rees P, Hendrix R, Price K, Kenyon NS, Hagerty D, Townsend R, Hollenbaugh D, Pearson TC, Larsen CP. 2005. Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* 174: 542-50

30. Badell IR, Thompson PW, Turner AP, Russell MC, Avila JG, Cano JA, Robertson JM, Leopardi FV, Strobert EA, Iwakoshi NN, Reimann KA, Ford ML, Kirk AD, Larsen CP. 2011. Nondepleting Anti-CD40-Based Therapy Prolongs Allograft Survival in Nonhuman Primates. *Am J Transplant*

31. Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, Bello-Laborn H, Hacquoil B, Strobert E, Gangappa S, Weber CJ, Pearson TC, Rajotte RV, Larsen CP. 2006. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med* 12: 304-6

32. Cardona K, Milas Z, Strobert E, Cano J, Jiang W, Safley SA, Gangappa S, Hering BJ, Weber CJ, Pearson TC, Larsen CP. 2007. Engraftment of adult porcine islet

xenografts in diabetic nonhuman primates through targeting of costimulation pathways. *Am J Transplant* 7: 2260-8

33. Thompson P, Cardona K, Russell M, Badell IR, Shaffer V, Korbutt G, Rayat GR, Cano J, Song M, Jiang W, Strobert E, Rajotte R, Pearson T, Kirk AD, Larsen CP. 2011. CD40-specific costimulation blockade enhances neonatal porcine islet survival in nonhuman primates. *Am J Transplant* 11: 947-57
34. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature* 393: 478-80
35. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393: 480-3
36. Ridge JP, Di Rosa F, Matzinger P. 1998. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393: 474-8
37. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* 18: 767-811
38. Curtsinger JM, Lins DC, Mescher MF. 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med* 197: 1141-51

39. Hochweller K, Anderton SM. 2005. Kinetics of costimulatory molecule expression by T cells and dendritic cells during the induction of tolerance versus immunity in vivo. *Eur J Immunol* 35: 1086-96

40. Bourgeois C, Rocha B, Tanchot C. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* 297: 2060-3

41. Sun JC, Bevan MJ. 2004. Cutting edge: long-lived CD8 memory and protective immunity in the absence of CD40 expression on CD8 T cells. *J Immunol* 172: 3385-9

42. Lee BO, Hartson L, Randall TD. 2003. CD40-deficient, influenza-specific CD8 memory T cells develop and function normally in a CD40-sufficient environment. *J Exp Med* 198: 1759-64

43. van Essen D, Kikutani H, Gray D. 1995. CD40 ligand-transduced co-stimulation of T cells in the development of helper function. *Nature* 378: 620-3

44. Poudrier J, van Essen D, Morales-Alcelay S, Leanderson T, Bergthorsdottir S, Gray D. 1998. CD40 ligand signals optimize T helper cell cytokine production: role in Th2 development and induction of germinal centers. *Eur J Immunol* 28: 3371-83

45. Blair PJ, Riley JL, Harlan DM, Abe R, Tadaki DK, Hoffmann SC, White L, Francomano T, Perfetto SJ, Kirk AD, June CH. 2000. CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. *J Exp Med* 191: 651-60

46. Miga AJ, Masters SR, Durell BG, Gonzalez M, Jenkins MK, Maliszewski C, Kikutani H, Wade WF, Noelle RJ. 2001. Dendritic cell longevity and T cell persistence is controlled by CD154-CD40 interactions. *Eur J Immunol* 31: 959-65

47. Ferrer IR, Araki K, Ford ML. 2011. Paradoxical aspects of rapamycin immunobiology in transplantation. *Am J Transplant* 11: 654-9

48. Ozaki KS, Camara NO, Galante NZ, Camargo LF, Pacheco-Silva A. 2005. Decreased Cytomegalovirus infection after antilymphocyte therapy in sirolimus-treated renal transplant patients. *Int Immunopharmacol* 5: 103-6

49. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. 2009. mTOR regulates memory CD8 T-cell differentiation. *Nature* 460: 108-12

50. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y. 2009. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460: 103-7

51. Turner AP, Shaffer, V. O., Araki, K., Martens, C., Turner, P. L., Gangappa, S., Ford, M. L., Ahmed, R., Kirk, A. D., Larsen, C. P. 2011. Sirolimus Enhances the Magnitude and Quality of Viral-Specific CD8+ T cell Responses to Vaccinia Virus Vaccination in Rhesus Macaques. *Am J Transplant* In press

52. Thomson AW, Turnquist HR, Raimondi G. 2009. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 9: 324-37

53. Brown NF, Stefanovic-Racic M, Sipula IJ, Perdomo G. 2007. The mammalian target of rapamycin regulates lipid metabolism in primary cultures of rat hepatocytes. *Metabolism* 56: 1500-7

54. Sipula IJ, Brown NF, Perdomo G. 2006. Rapamycin-mediated inhibition of mammalian target of rapamycin in skeletal muscle cells reduces glucose utilization and increases fatty acid oxidation. *Metabolism* 55: 1637-44

55. Rao RR, Li Q, Odunsi K, Shrikant PA. 2010. The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity* 32: 67-78

56. Ferrer IR, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP, Ford ML. 2010. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. *J Immunol* 185: 2004-8

57. Araki K, Youngblood B, Ahmed R. 2010. The role of mTOR in memory CD8 T-cell differentiation. *Immunol Rev* 235: 234-43

58. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, Wherry EJ, Onami T, Lanier JG, Kokko KE, Pearson TC, Ahmed R, Larsen CP. 2003. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest* 111: 1887-95

59. Heeger PS, Greenspan NS, Kuhlenschmidt S, Dejelo C, Hricik DE, Schulak JA, Tary-Lehmann M. 1999. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 163: 2267-75

60. Macdonald WA, Chen Z, Gras S, Archbold JK, Tynan FE, Clements CS, Bharadwaj M, Kjer-Nielsen L, Saunders PM, Wilce MC, Crawford F, Stadinsky B, Jackson D, Brooks AG, Purcell AW, Kappler JW, Burrows SR, Rossjohn J, McCluskey J. 2009. T cell allorecognition via molecular mimicry. *Immunity* 31: 897-908

61. Amir AL, D'Orsogna LJ, Roelen DL, van Loenen MM, Hagedoorn RS, de Boer R, van der Hoorn MA, Kester MG, Doxiadis, II, Falkenburg JH, Claas FH, Heemskerk MH. 2010. Allo-HLA reactivity of virus-specific memory T cells is common. *Blood* 115: 3146-57

62. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832-44

63. Battaglia M, Stabilini A, Roncarolo MG. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105: 4743-8

64. Coenen JJ, Koenen HJ, van Rijssen E, Kasran A, Boon L, Hilbrands LB, Joosten I. 2007. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant* 39: 537-45

65. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. 2007. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant* 7: 1722-32

66. Haxhinasto S, Mathis D, Benoist C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205: 565-74

67. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, Thomson AW. 2003. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood* 101: 4457-63

68. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N, Pulendran B. 2008. Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 9: 1157-64

69. Saemann MD, Haidinger M, Hecking M, Horl WH, Weichhart T. 2009. The multifunctional role of mTOR in innate immunity: implications for transplant immunity. *Am J Transplant* 9: 2655-61

70. Jagannath C, Lindsey DR, Dhandayuthapani S, Xu Y, Hunter RL, Jr., Eissa NT. 2009. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med* 15: 267-76

71. Halloran PF. 2004. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 351: 2715-29

72. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* 460: 392-5