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Coinhibitory Receptor Control of T Cell Responses in Transplantation

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Immunology and Molecular Pathogenesis

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Coinhibitory Receptor Control of T Cell Responses in Transplantation

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B.S., Beloit College, 2010

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Abstract

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T lymphocytes are known to be key players in the cellular rejection of allografted tissue following solid organ transplantation. Immunotherapeutic strategies to prevent rejection following transplantation frequently involve targeted blockade of T cell costimulatory pathways. We propose that in addition to costimulation blockade, harnessing the power of coinhibitory molecules that are selectively upregulated on memory-like cells will be critical to controlling donor-specific immune responses.

Here we have performed a series of studies to assess the contribution of coinhibitory receptors to the control of alloreactive T cell responses following transplantation in humans and animal models. We demonstrate that the T cell coinhibitory molecules 2B4 and TIGIT are expressed on CD28null effector memory CD4+ and CD8+ T cells that are associated with freedom from rejection following renal transplantation in humans. Further exploration of these pathways indicates that while 2B4 functions to control alloreactive T cells by limiting their glycolytic metabolism, and subsequently proliferation and recruitment into the alloreactive anti-donor response, antibody-mediated agonism of TIGIT signaling prolongs graft survival in a mouse model of skin transplantation.

Finally, we show programming of antigen specific $CD8^+$ T cells responding to graft and pathogen are dissimilar, and that antigen-specific $CD8^+$ T cells primed by a skin graft contract faster than those primed by infection, yet are able to expand more rapidly upon rechallenge. Additionally, the expression of CD127 at a memory time point suggests graft-elicited $CD8^+$ antigen specific T cells are maintained in a less terminally-differentiated state compared to gHV-elicited $CD8^+$ antigen specific T cells, despite fewer cells being present at that time point.

Taken together, the data presented here suggest that the surface marker expression, metabolic prolife, and functional capacity of T cells depends on the priming conditions and may be used to predict immunologic risk following transplantation.

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Chapter 1

Introduction

Introduction

Part I: Transplantation is a life-saving therapy for end-stage organ disease

Transplantation is a life-saving therapy for end-stage organ disease, and while close to 34,770 transplants from approximately 16,467 donors were performed in the United States between January and December of 2017, there are still 114,997 candidates on the waiting list for solid organ transplants as of March 2018[1]. Upwards of 50% of individuals who undergo transplantation in the United States receive kidney allografts as treatment for end-stage renal disease, or ESRD, which occurs as the kidneys shut down after a long-term struggle to filter waste from the blood. Following the kidney, the liver and pancreas are the most commonly transplanted organs in the US (1). While hematopoietic stem cell (HSC) transplantation is also common in the US, with approximately 8500 operations performed in 2016, currently therapies for solid organ and HSC transplantation are very different, and the latter will not be considered here [2].

Though the idea of transferring tissue from one human to another has existed for thousands of years, as is document historically in ancient China and Greece, the concept as we know it began in the late 19th century, when Alexis Carrel described vascular anastomoses, ushering a new era of surgical techniques that would go on to enable the earliest predecessors of transplantation [3, 4]. Interestingly, Dr. Carrel knew the allografts were failing but didn't know why, and the biology behind this rejection remained a mystery for many years [5]. A breakthrough came in the 1940s when Dr. Ray Owens, studying freemartin cattle at the University of Wisconsin, observed dizygotic twins that shared a circulatory system during fetal development were tolerant of skin grafts from one another [6]. Peter Medawar and his colleagues at Mill Hill in London went on to perform a series of illuminating experiments describing how mice that were exposed to spleen cells from a genetically disparate strain during fetal development were later able to accept skin grafts from the same donor strain as adults [7]. These remarkable findings set the stage for the beginning of clinical transplantation, and in December of 1954, Joseph Murray lead a team of surgeons at the Brigham Hospital in Boston in successfully transplanting a kidney from one identical twin to another, a graft which remained functional for close to a decade post-surgery [8] .

With the stage set by these major medical advances, physicians and laboratory scientists alike began to more closely exam the benefits & challenges of clinical immunosuppression. In the earliest of days, the

observation was made that azathioprine could inhibit cellular proliferation, and it began to be used routinely following transplantation [9]. Dr. Thomas Starzl began using the steroid prednisone to control immune responses following transplantation in the early 1960s [10]. At roughly that same time, J. L. Gowens and colleagues made a series of seminal observations that uncovered lymphocytes as the primary mediators of allograft rejection [11, 12], after which Okt3 began to be used to deplete T cells prior to transplantation [13] . Another key finding that changed the clinical care of transplant recipients and their long-term outcomes was Roy Calne's discovery that the use of cyclosporine A alone was able to inhibit rejection [14].

While these immunosuppressive reagents have enabled thousands of individuals to receive transplants, and has prolonged the life of many, notable challenges remain. Current regimens are generally non-specific, meaning that they do not only target those cells of the immune system that are responsible for graft rejection, and have significant off-target toxicities. Additionally, we lack a reliable mechanism to 'tune' immunosuppressive medications, and frequently when transplant recipients experience viral reactivation of cytomegalovirus or Epstein-Barr virus, for example, which are usually innocuous in immunocompetent individuals, the only clinical solution is to reduce the amount of immunosuppression on board, and hope that this doesn't result in a rejection episode. Therefore, going forward, ideal immunotherapeutic strategies will reduce rejection but allow for the generation and maintenance of protective anti-pathogen immune responses. Working within an organ allocation system where the biggest impediment to transplantation is a limited donor pool, designing successful strategies to promote long-term graft survival is of the utmost importance. The work presented in this dissertation aims to address mechanisms by which we may selectively target donor-reactive cells, leaving immunity to foreign infections in tact, and improving the quality of life for transplant recipients.

Transplant recipients must take intensive immunosuppressive drug regimens following surgery in order to maintain the stability and function of their allografts. Calcenurin inhibitors (CNIs), which disrupt all T cell activation, have classically been used to suppress the immune system following transplantation. These drugs have highly toxic and undesirable side-effect profiles that compromise both the graft and recipient. Additionally, this approach leaves patients severely immunocompromised and many experience devastating infections, the only treatment for which is to lessen the degree of immunosuppression used, thereby enhancing the likelihood of graft

rejection. As our understanding of the involvement of the adaptive immune system in organ rejection has advanced, new strategies have been designed to specifically target and inhibit the *de novo* activation of T cells via the blockade of costimulatory molecules. While these protocols have improved graft function, they have been accompanied by increased rates of acute rejection. Research in mice and non-human primates has demonstrated that costimulation-blockade resistant organ rejection following transplantation is primarily mediated by donor-reactive $CD8^+$ T cells [2] [3].

Controlling alloreactive T cell responses in an antigen-specific manner is imperative for improving long-term outcomes in pediatric and adult transplant recipients. The work described herein summarizes what is currently known about the role of costimulatory and coinhibitory signaling molecules in the context of transplantation and addresses the gaps in our understanding of cosignaling in transplant immunobiology. Ultimately, we aim to describe novel T cell signaling molecules that may be manipulated to attenuate graft rejection mediated by alloreactive T cells following transplantation while simultaneously allowing for the development of functional anti-pathogen immune responses.

Part II: Characterizing Innate Alloimmune Responses After Solid Organ Transplantation

Innate immune cells are involved in initiating responses that result in graft rejection

Allorecognition is a term used to describe the process by which the immune system recognizes foreign tissue via non-autologous major histocompatibility (MHC) gene products. While there are a number of processes involved in allorecognition that are unique to the perception of and response to a foreign graft, common principles of immunological antigen recognition dictate the development of non-self responses. All arms of the immune response are involved in alloreactivity and graft rejection and therefore to fully understand alloreactivity we must begin with a discussion of the mechanisms by which immune responses are initiated and develop.

While the rejection of an allograft is primarily executed by T and B lymphocytes, as will be discussed in detail later, the intiation of these adaptive responses is mediated by the innate immune system. In considering the intiation of an immunological response following transplantation, it is first important to remember that solid organ and bone marrow transplantation requires invasive and lengthy surgical procedures, that inherently cause

injury to the recipient. Secondly, while all efforts are made to make the procurement process as expedient as possible, there is always a period of time between the extraction of the donor organ and its transplantation into the recipient. It is well known that during this time the organ is highly susceptible to ischemia reperfusion injury, resulting in necrosis and extracellular matrix disruption [4].

Trauma experienced by tissues during surgery and subsequent ischemic damage causes release of molecules that activate host pathogen recognition receptors (PRRs), including members of the Toll-like receptor (TLR) family, some nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and certain RIG-like helicases (RLHs) [4]. The activation of immune cells following the release of these molecules from pathogens or physical injury have been the focus of active study since their description and discussion in seminal works by Charles Janeway (the self-/non-self discrimination hypothesis) [5] and Polly Matzinger (the danger hypothesis) [6] in the early 1990s. The observation that molecules released following surgical trauma or ischemia reperfusion injury can trigger PRR signaling and subsequent immune activation was made by Land et al., in 1994 [7]. More recent work has demonstrated that some inflammatory responses following tissue injury in transplantation are mediated by TLR4, and that, more broadly, these PRR responses are initiated by danger signals derived from necrotic cells and extracellular disruption [8] [9] [10, 11]. These include proteoglycans like biglycan [12], glycosaminoglyans (including hyaluronan) , heat shock proteins, and fibronectin variants such as fibronectin extra domain A [13] [14] [15] [16, 17] [18]. The triggering of inflammatory signaling cascades downstream of PRRs in the context of transplantation has been shown to be dependent on the same mediators as in responses to infection with a pathogen, as studies have shown that the propagation of these signals can be MyD88-dependent [19]. Interestingly, even in the presence of costimulation blockade, to be discussed in depth later, administration of TLR ligands such as LPS inhibits tolerance [20-22]. Additionally, other innate sensing mechanisms, such as signaling through the receptor for advanced glycation endproducts (RAGE) are involved in rejection, as blockade of RAGE promotes allograft survival [23].

The majority of work done in the last 20-30 years to understand sensing and recognition of alloantigen in the context of transplantation has focused on the role of dendritic cells. More recently however, a role for monocytes in detecting and initiating responses to non-self antigen has become better appreciated. In a mouse

model of transplantation, it has been shown that monocytes produce more inflammation in response to exposure to allogeneic cells compared to autologous cells [24]. This finding suggests that monocytes are capable of, in some capacity, distinguishing between self- and non-self. Additionally, the Lakkis group recently showed that encounter with alloantigen drives the differentiation of monocytes into inflammatory dendritic cells that produce inflammatory cytokines and promote T cell proliferation [25]. The same study yielded the observation that differentiation and activation of monocytes in response to allografts enhances T cell-mediated graft rejection and concluded that recognition of alloantigen by monocytes and their subsequent differentiation into T cell activating DCs bridges innate and adaptive immune responses following transplantation.

All arms of the immune response are involved in rejection

Following the sensing and subsequent activation of antigen presenting cells via the PRR pathways described above; other innate cells play a critical role in the first stages of alloimmune responses.

Macrophages, while not as potent activators of alloreactive T cells as DCs, infiltrate allografts and secrete cytokines, phagocytose necrotic debris, and produce ROS and iNOS [26]. This promotes T cell cytotoxicity, and ultimately, graft rejection. It has been shown that at the time of acute cellular rejection (ACR), graft infiltrate may be composed of up to 40-60% macrophages [27, 28]. In support of this role for macrophages in helping to mediate rejection, the Chadban group showed that depletion of macrophages within 24 hours of transplantation with chlodronate liposomes causes a reduction in allograft damage [29].

Neutrophils, also known as polymorphonuclear leukocytes (PMN or PML), are short-lived granulocytes that are poised to rapidly traffic to inflammatory sites and contribute to tissue damage via the release of proinflammatory cytokines as well as direct cytotoxicity [30]. The surgical trauma involved in transplantation induces a strong inflammatory response that quickly results in induction of adhesion molecules on endothelial surfaces and promotes production of chemokines, including Gro-alpha and MIP2, which recruit neutrophils to the site of injury [31-34]. While accumulation of neutrophils in the allograft following transplantation leads to amplification of the early immune response, this initial wave of PMN activity is short lived as these cells die rapidly [35-37]. The contribution of PMN to graft infiltration and inflammation has been confirmed by a number

of reports demonstrating that pathology is attenuated in animals lacking key PMN-attracting cytokines, or PMN themselves [35, 38-40].

Eosinophils are hematopoietically-derived granulocytes that play an important role in allergic inflammation and disease via their secretion of cationic granule proteins and by production of proinflammatory cytokines [4]. Recently, reports have described important roles for eosinophils in remodeling of airway tissues and in inflammatory gut diseases [41, 42]. While eosinophils have been long been recognized as contributing the development of allergic inflammation, they are not readily considered when discussing transplant rejection, although recent work has indicated that other granulocytes, such as mast cells, are involved in generation of tolerance through regulatory T cell-dependent mechanisms [43]. As illustrated in the preceding sections, interest has grown in investigating the role of innate cells in transplant tolerance and rejection in the last two decades. In the early and middle part of the 1990s, a number of studies were published regarding the involvement of eosinophils and eosinophilia in acute cellular rejection [44-46]. Interestingly, in a study published by the Dhillon group, biopsies revealed that infiltration of liver allografts by eosinophils is a statistically significant diagnostic feature of acute cellular rejection [47]. The presence of graft-infiltrating eosinophils has also been observed in renal allografts [48]. Additionally, an early report concluded that eosinophilia within allografts was actively associated with rejection, and not a bystander effect [49]. Monitoring eosinophilia as a diagnostic tool is of clinical importance in renal transplantation as well as liver transplantation as the presence of eosinophils in the urine precedes increases in serum creatinine and histological rejection [50]. Eosinophils may also play a key role in contributing to the physical rejection of allografts by promoting local inflammation and secretion of cytotoxic effector molecules [51].

Natural killer (NK) cells are the third largest subset of lymphocytes after B and T cells, whose survival and functional capacity relies on IL-2 and IL-15 signaling [52-54]. These cells, derived from the fetal liver, are profoundly heterogeneous and express a wide array of both stimulatory and inhibitory receptors on their cell surface. NK cells express a family of killer-cell immunoglobulin-like receptors (KIRs), and a number of common inhibitory receptors, such as CD94 and NKG2A, all of which are specific for self-MHC class I molecules [55]. The process known as NK cell “licensing” rests on the principal of the missing-self hypothesis

and describes how binding of self class I MHC molecules by inhibitory receptors on the cell surface can induce tolerance to self antigens, but can also permit the destruction of other target cells [56, 57]. Importantly, NK receptors are polymorphic between individuals, allowing for diverse recognition of MHC molecules across a species [58].

While the current consensus in the transplant immunobiology community is that NK cells alone are not necessary or sufficient for the graft rejection, it is known that NK cells traffic into and are found in large numbers in the allografts of patients experiencing rejection [58, 59]. Interestingly, NK cells play a dual role in allograft rejection and the establishment of transplant tolerance. While, as mentioned previously, NK cells alone are not sufficient to promote rejection [60], they contribute to rejection by promoting the maturation of antigen presenting cells via the production of proinflammatory cytokines such as IFN-γ and TNF-α, as well as by promoting the activities of alloreactive T cells [58, 61-63]. Induction of regulatory T cells (T_{regs}), which are thought to be of major importance for the establishment of transplant tolerance, can be abrogated by NK cell activation [64]. On the other hand, a recent study also showed that high CD56 expression correlates with a phenotypic subset of NK cells without cytolytic function that appear to have important regulatory properties via their production of cytokines that modulate the function of other immune cells [65]. NK cells have also been observed to be involved in the regulation of alloreactive T cells, and are required for the induction of tolerance to fully mismatched islet allografts [66, 67].

Much work has been done to study the dual role of NK cells in graft acceptance and long-term survival following MHC mismatched bone marrow transplantation (BMT). While it is appreciated that NK cells are critical effectors in rejection and graft-versus-host disease (GVHD) following BMT, they also play a primary role in the desirable graft-versus-leukemia phenomenon seen in cancer patients [68, 69]. NK cells may also promote other effects that are desirable in the context of transplantation, as recent experimental models utilizing CD28 costimulation blockade have shown that NK cell depletion is required for long-term graft survival, as they are critical for the induction of tolerance [62, 63, 66, 67]. NK cells mediate this important role in tolerance induction following transplantation by destroying graft-derived antigen presenting cells. In this way, they are able to reduce presentation of alloantigen to host alloreactive T cells, which has been demonstrated to be a key

component of long-term allograft survival in the context of costimulatory blockade [66]. The dual role of NK cells in transplant tolerance is likely to be fundamentally related to the differentiation and activation state of these cells during various phases of the immune response. While in a resting state, NK cells are unable to facilitate the rejection of skin allografts, these same cells, when stimulated by IL-15, are able to cause rapid graft rejection even in the absence of T and B lymphocytes [58].

Each of the aforementioned components of the immune system play a critical role in the recognition of transplanted allografts and the subsequent immune response mounted against foreign tissue antigens. However, it is the cells of the adaptive immune system, primarily T and B lymphocytes that are ultimately responsible for coordinating and executing allograft destruction and rejection. The following section discusses these processes in more detail.

Part III: Characterizing Adaptive Alloimmune Responses Following Solid Organ Transplantation

MHC compatability & direct and indirect recognition of alloantigens

The ability of adaptive immune cells to recognize and respond to foreign antigens depends largely on the processing and presentation of antigens on major histocompatibility complex (MHC) molecules. Note that the term MHC is used to describe these molecules in mice, the human equivalent is known as the human leukocyte antigen, or HLA, molecules. MHC molecules are encoded by highly polymorphic genes and expressed on both antigen-presenting and non-immune cells [70]. There are two similar but distinct classes of protein products encoded by these genes, MHC class I and class II molecules. $CD4^+$ T cells recognize peptides presented by class II molecules, while those peptide fragments associated with class I MHC molecules are recognized by $CD8^+$ T cells [70]. MHC I molecules are expressed ubiquitously on a wide variety of cell types throughout the body, while class II MHC molecules are found only on professional antigen-presenting cells, such as dendritic cells (DCs), macrophages, and B cells. The specific ability of $CD8^+$ and $CD4^+$ to recognize antigens presented on class I and class II molecules, respectively, was established in a series of experiments by Zinkernagel and Doherty published in *Nature* in 1974. It was in this seminal study that it was demonstrated that in order for a cytotoxic $CD8^+$ T cell to kill an infected target cell, the $CD8^+$ cell and target cell must be share a class I MHC

allele [71] and follow up studies confirmed that MHC restriction also determines the ability of $CD4^+$ T cells to recognize their cognate antigens.

The molecular mechanisms that govern MHC restriction and regulate tissue compatability are of critical importance to the study of transplant immunobiology, as they play a major role in dictating the outcome of graft acceptance and rejection. "Priming," describes the initial recognition of donor antigens by graft recipient's immune system. The process of priming follows these principals, and can occur via two primary methods that are not mutually exclusive, known as the direct and indirect pathways presentation of alloantigens. In the direct alloantigen recognition, a donor-derived antigen-presenting cell (APC) presents peptide fragments on allogeneic MHC, resulting in recognition of antigen in the context of foreign MHC by alloreactive T cells in the recipient [30]. In the indirect pathway, recipient APCs take up and process allogeneic MHC molecules present in the graft. These allogeneically derived peptides are then presented to recipient alloreactive T cells in the context of self-MHC [30].

While the direct and indirect pathways account for the majority of antigen presentation leading to the generation of allospecific immune responses, there are also MHC-independent mechanisms by which non-self antigens can be recognized in the context of transplantation. Minor histocompatibility antigens (mH) are the products of polymorphic genes outside of MHC loci that vary between genetically distinct individuals of the same species. Following processing, these antigens are predominantly presented indirectly on host MHC. A number of minor histocompatibility antigens have been identified in humans, and it is thought that there maybe up to 40 in inbred strains of mice [72]. These minor histocompatibility antigens are of clinical importance as they are implicated in rejection and graft-versus-host disease (GVHD) in human patients who receive HLA-identical transplants [72]. These principles of MHC restriction underlie the ability of lymphocytes to recognize and respond to both self and foreign antigens. The ability of both T and B cells to reject allografts depends on recognition of alloantigen processed and presented on MHC molecules, and transplant recipients are carefully MHC matched to their donors to reduce discordance and enhance the likelihood of graft survival.

B cells secrete alloantibody that underlies AMR

Seminal studies have shown that allografts are not rejected in mice lacking T cells, and researchers have noted that the passive transfer of antibody at the time of transplantation does not accelerate graft rejection [73]. Thus, historically, T cells have been thought to be the major lymphocyte subset involved in rejection, although more recently, a broader role for B cells in the rejection of allografts has been appreciated [74]. The development of chronic rejection, which is mediated by both T and B cells, is delayed, meaning that it can occur months to years after transplantation. Hyperacute rejection, however, occurs within hours following transplantation and is the result of pre-existing anti-donor alloantibodies, known as recipient pre-sensitization, leading to damage from immediate complement fixation and alloantibody deposition [73].

The contribution of B cells and antibody to graft failure and loss began to receive more attention as a correlation was observed between graft pathology (associated with impaired function and survival), the presence of donor HLA-specific antibody, and complement deposition on graft endothelial cells [73]. Then, in 1970, the Russell group made a groundbreaking discovery: the emergence of lesions on kidney allografts was associated with the presence of anti-donor HLA antibodies [75]. 20 years later, in the 1990s, two major findings added to the growing body of evidence supporting a role for antibody in graft rejection. In 1991, complement deposition, specifically C4d deposition, in the microvasculature became a recognized clinical marker for antibody-mediated complement fixation within the graft, and was associated with the presence of donor-specific antibody [76, 77]. A year later, Philip Halloran and colleagues made the striking observation that severe acute renal allograft rejection occurred in individuals with elevated levels of donor-specific antibody [78]. Since these early observations were made, much work has been done to define hyperacute, acute, and chronic antibody-mediated rejection, and the detection of donor-specific antibody is now an invaluable tool in clinical diagnosis of transplant rejection.

Damage inflicted by antibodies produced by allospecific plasma B cells is the hallmark of acute antibody-mediated rejection (AAMR). AAMR is clinically defined by 4 major features: I) notable clinical manifestation of graft dysfunction, II) histologically detectable tissue injury (including but not limited to tubular injury and the infiltration of capillaries by innate immune cells or thrombi), III) the presence of C4d deposition, and IV)

identification of circulating donor-specific antibodies in the sera [79, 80]. This type of acute rejected has been observed in roughly 6-7% of renal transplant cases, and has been noted in upwards of 30% of for causes biopsies taken for assessment of acute rejection [79-83].

Donor HLA-specific antibody has also been shown to be involved in chronic graft rejection. A recent study with renal transplant recipients at multiple centers showed that frequency of graft failure at one year was higher in patients who developed alloantibodies compared to those who did not [84]. These antibodies may be the result of pre-sensitization (i.e., due to blood transfusions or pregnancy), however the development of *de novo* donor-specific antibodies has also been identified as a risk factor for early allograft loss [85-87]. Chronic antibody mediated rejection is associated with glomerulopathy, arteriopathy, and deposition of C4d in capillaries and/or glomeruli [88-91]. Taken together, C4d deposition can occur prior to clinically recognizable graft rejection and its detection is a key diagnostic tool in identification of individuals at risk of multiple types antibody-mediate rejection.

The Biology of Allograft Rejection

Having discussed the concert of cells and signals necessary to sense non-self and initate an immune response, I will now briefly discuss the biological processes by which allografts are rejected. The involvement of T cells in allograft rejection has been appreciated for many decades, with specific discussion of a role for both cytotoxic and helper cells begin in the 1980s [92]. Both $CD8^+$ cytotoxic lymphocytes (CTLs) and $CD4^+$ Th1 cells secrete IFN-γ and IL-2 upon engagement with alloantigen, promoting the further activation of graft-infiltrating CTLs, NK cells, and monocytes [93]. Allograft rejection can occur rapidly in a process known as acute rejection, or can be delayed from the time of transplantation, resulting in long-term chronic rejection.

Acute Cellular And Antibody-Mediated Rejection

Acute cellular rejection is primarily mediated by CTLs that directly kill target cells in the allograft, specifically donor endothelial cells [30]. mRNAs associated with T cell cytotoxic killing abilities are upregulated, including CXCR3, CXCL9, CXCL10, and CXCL11, Tbet, FasL, CTLA-4, perforin, Granzyme and

FasL, which are sometimes used to clinically diagnose ACR [93]. Together, increased cytotoxicity and inflammatory infiltration result in microvascular endotheliatis, intimal arteritis, and tubulitis, cumulating in acute rejection, tissue destruction, and subsequent graft loss [94]. As discussed previously, acute antibody-mediated rejection occurs when alloantibodies bind to graft endothelial cells. $CD4^+$ Th2 cells provide help to B cells, enabling the differentiation of plasma cells and subsequent production of alloantibody [93]. In addition to the fixation of complement (particularly C4d), which induces lysis, direct binding of alloantibody to endothelial cells likely initiate graft cell signaling cascades that promote inflammation [30]. Despite advances in surgical techniques and the improvement of post-transplant immunosuppressive regimens, about 10% of adult transplant recipients experiencing at least one acute rejection episode within the first 12 months following transplantation [1].

Chronic Rejection

Although rates of acute rejection have been declined over the last ten years, chronic rejection remains a problem. Chronic rejection occurs as alloantibody deposition and direct cellular cytotoxicity induce the proliferation of smooth muscle cells in vascularized grafts [30]. Over time, this ischemic damage results arterial changes that are further exaggerated by both immunologic and non-immunologic factors. Eventually, these lesions become occlusive and blood flow to graft parenchyma is inhibited, resulting in fibrotic necrosis and, ultimately, graft loss.

T Cells in Allograft Rejection: Precursor frequency of alloreactive T cells

Following encounter with alloantigens, APCs process and display these peptides to cognate T cells. Upon recognition of their antigen in the form of a peptide:MHC (pMHC) complex, alloreactive T cells divide and proliferate rapidly before migrating into the graft to perform their effector functions. Examining the factors that control the kinetics of these T cell responses following transplantation in order to effectively contain these donor-reactive cells has become of great interest. In particular, investigation of how precursor frequency impacts the fate of T cell responses has been an active area of research in the last decade. Work has shown that T cell

commitment to a given lineage depends on the frequency of antigen-specific cells and that the overall capacity of these cells to proliferate depends on the amount of competition [95]. A seminal study by Leo Lefrançois' group demonstrated that when the initial precursor frequency of antigen specific cells was low, cells develop into a pool of effector memory T cells (T_{EM}) that are stable and do not differentiate further into central memory-like (T_{CM}) cells, suggesting that many cells must be present to undergo the transition from T_{EM} to T_{CM} [95]. Interestingly, regardless of the precursor frequency of antigen-specific responder cells, the size of the T cell compartment is flexible, as reports have shown that it can expand to adapt to new cells generated in response to new challenges, but that this can affect the stability of pre-existing cells and result in the loss of protective immunity [96, 97]. The initial studies defining the importance of precursor frequencies in determining the outcome of T cell responses were performed using animal models bacterial and viral disease. These findings have been supported by experiments performed in the context of transplantation as well, as the initial frequency of antigen-specific cells dictates differentiation programming and plays a key role in determining the length of graft survival [98]. Interestingly, precursor frequency may also control costimulation requirements (to be discussed in more detail later) by regulating rounds of division and differentiation status, thereby influencing the expression patterns of certain costimulatory molecules.

Naïve vs. memory lymphocytes in rejection

Following a primary immune response, the majority of effector cells undergo massive apoptosis during the contraction phase. A small fraction of these cells, however, persist and are retained in a quiescent state, forming a pool of memory cells. This immunologic memory is the foundation for long-term protection of the host. Memory cells are poised to respond more quickly and effectively upon subsequent exposure to the same antigen, and multiple factors contribute to this rapid recall response. Unlike naïve T cells (T_N), memory cells are maintained in an antigen and MHC independent manner, allowing them to be preserved for extended periods of time [99]. In contrast to naïve cells, which must be activated in secondary lymphoid organs, some memory cells constantly circulate within lymphoid and non-lymphoid tissues and can recognize antigen and mount recall responses outside of traditional lymphoid structures [100, 101]. Concordantly, memory T cells have a lower

"activation threshold" compared to naïve cells, which is to say that their requirements for activation are less stringent than those of naïve cells, allowing them to proliferate, secrete cytokine, and perform their effector functions within hours of restimulation [102].

The memory T cell pool is composed of different subsets, and while their development relies on $CD4^+$ T cell-help, each has unique characteristics, costimulation requirements, and regulatory mechanisms [103, 104]. Effector memory T cells, from hereon referred to as T_{EM} cells, are defined by their expression of homing molecules; T_{EM} cells lack the CC-chemokine receptor 7 (CCR7) and have low levels of CD62L (L-selectin) expression [105]. T_{EM} are found throughout the body, not just in lymphoid compartments, and are able to immediately perform their effector functions upon antigenic re-challenge, including production of IFN-γ, perforin, and IL-4 [105, 106]. Central memory cells (T_{CM}), which are $CCR7^+$ and $CD62L^{hi}$, are primarily found in secondary lymphoid organs. While unable to rapidly perform effector functions, T_{CM} cells proliferate and differentiate quickly upon antigen encounter, and secrete IL-2 [105, 107]. Of note, work has shown that the conditions under which naïve T cells are primed have a lasting impact on the cells ultimate phenotype and function following contraction and the progression to memory status [108].

Both T_{EM} and T_{CM} are retained over time via homeostatic proliferation that is independent of antigen exposure and their inherent ability to survive in a resting state via homeostatic turnover [109]. Seminal work by Pippa Marrack's group showed that preservation of resting $CD8^+$ memory cells is promoted by IL-15 and opposed by IL-2 [110]. A number of factors underlie the ability of these cells to mount and execute rapid recall responses. As memory cells are generally thought to be derived from effector cells (though there is some debate), they represent a heterogeneous population of cells that is enriched for antigen specific cells [111]. This increased frequency of antigen-specific cells allows these cells to mount responses exponentially larger and faster than those of naïve T cells. What's more, many memory T cells are thought to be hyperresponsive to antigen due to enhanced TCR signaling as a result of enriched antigen receptor signaling-associated molecules compared to naïve cells [112, 113]. Additionally, T_{EM} cells are able to mount secondary responses more rapidly than naïve T cell because of their ability recognize antigen in the periphery, which is due in large part to their programmed loss of CCR7 and CD62L, which is accompanied by upregulation of tissue-specific adhesion molecules and

chemokine receptors [114]. For these reasons, T_{EM} cells do not need to spend time or expend energy traveling to secondary lymphoid organs in order to come into contact with antigen-loaded APCs, thus making them more rapid and effective mediators of secondary immune response.

Lymphocyte subsets have distinct requirements for costimulatory signaling

In addition to having unique phenotypic and functional characteristics, naïve and memory T cells also differ in their requirements for costimulation. Importantly, studies have shown that activation of naïve T cells requires more costimulatory signaling than memory T cells [102, 115, 116]. Many factors are thought to impact the costimulatory requirement of T cells. Generally, naïve $CD4^+$ T cells can only be activated when antigen is presented by professional APCs, while memory $CD4^+$ cells can respond to antigen on dendritic cells, macrophages, and resting or activated B cells [115]. This suggests that memory T cells are less dependent on accessory costimulatory molecules than naïve cells are. The requirement for costimulation in the memory T cell compartment is in part dictated by the amount and duration of exposure to antigen during the primary response [117]. Memory cells can mount secondary responses in response to less antigen than is necessary to initiate a primary response [116]. Additionally, memory cells need less B7 stimulation and can be activated in the complete absence of CD40 signaling [116]. The ability of effector cells to mount responses in the absence of costimulation has been well documented in the transplant literature, where it is clear that some T cell responses are less dependent on costimulation as not all allograft rejection can be prevented by dual blockade of CD28 and CD40 signaling [98].

While the loss of CD28 on $CD8^+$ T cells does not inherently prevent their proliferation or inhibit their effector functions, peripheral tolerance cannot be achieved in its absence [118]. This finding may be in part explained by the fact that dependence on CD28 costimulation is controlled by a balance of proteins that regulate NFκB activity downstream of the receptor, and that NFκB signaling may play a different role in expansion and effector functions and the programs needed to achieve tolerance induction [119]. CD28 can also be downregulated on cells with the $CD4^+$ T cell compartment. CD28 loss renders these cells resistant to CTLA4-Ig-mediated inhibition, but these same cells have decreased proliferation capacity and increased sensitivity to apoptosis [120].

Viral exposure can induce downregulation of CD28 on $CD8^+$ T cells and lead to increased alloreactivity as assessed by the mixed lymphocyte reaction (MLR) [121]. Taken together, these data demonstrate that the lower activation threshold of memory T cells is in part due to a reduced requirement for costimulatory signaling and suggest that CD28 downregulation is an indicator of terminal differentiation, and may underlie costimulation blockade resistant rejection following transplantation. It is with this background that I will proceed to discuss the biology and function of a selected few key costimulatory molecules involved in regulating T cell responses following transplantation.

Part IV: T Cell Function is Tightly Regulated by a Balance of Costimulatory and Coinhibitory Signals

The development, differentiation, activation, and function of T cells are all regulated by a fine-tuned balance of cell intrinsic and extrinsic cues. T cell activation requires three distinct signals: Signal One, Signal Two, and Signal Three. Signal One is the binding of the T cell receptor to a peptide in the binding cleft of an MHC receptor on an antigen-presenting cell. Signal Two, which will be the main focus of this section, is binding of costimulatory receptors on the surface of the T cell to their ligands on the APC. Signal Three is the presence of cytokines that promote T cell activation and differentiation. The consideration of the activity and regulation of Signal Two is of critical importance for the development of immunosuppressive regimens to effectively inhibit donor-reactive T cells following transplantation. Strategies to block certain costimulatory pathways following transplantation can abrogate alloreactive T cell responses and assist in the induction of transplant tolerance has been an area of active research since the 1990s [122]. Indeed, early works showed blockade of classic costimulatory molecules like CD28, CD40, and CD40L effectively promoted long-term allograft survival and even operational tolerance [123-125]. However, subsequent examination of cosignaling pathways have highlighted many intricacies in cosignaling paradigms that have required a revision of our approach to costimulation blockade following transplantation. First, T cell fate is dictated by a constellation of signals during development, initial activation, and proliferation – it's a balance of costimulatory and coinhibitory signals that must constantly be held in tension for the proper functional outcome of a T cell response. Next, a single costimulatory molecule may play a different role in the development and function of Th1 vs. Th2 vs. Th17 cells

and the same is true for naïve vs. memory cells. The overall goal in considering the biology and mechanism of costimulatory signaling in the context of transplantation as will be discussed below is to give appropriate attention to the fact that distinct costimulatory molecules may be expressed on alloreactive cells compared to those that are harmless or even those that promote tolerance. I will therefore discuss each of these molecules in light of the potential to target them specifically to inhibit alloreactive T cells and promote tolerance.

CD28:B7 family

The CD28 molecule (as well as the structurally related cosignaling molecules ICOS and CTLA-4) is formed by covalently bonded heterodimers of paired variable domain-like Ig domains that are attached to a transmembrane domain with a cytoplasmic region containing tyrosine-based signaling motifs [126]. While the importance of CD28 as a critical T cell costimulatory molecule has been appreciated in the T cell activation paradigm for quite some time, it's full crystal structure was not reported until 2005 [126]. CD28 binds to the glycoproteins B7-1 (CD80) and B7-2 (CD86). B7-1 was first identified as the B lymphoblast antigen (BB-1) on activated B cells by the Clark group in 1982 [127]. Subsequent work by Gordon Freeman and colleagues showed that B7-1 is expressed on murine B cells after BCR crosslinking, and is induced on monocytes following exposure to IFN-γ [128]. Ten years after the discovery of B7-1, prompted by the observation that T cell responses in B7-1 deficient mice were still able to be antagonized by CTLA-4 signaling and stimulated via CD28, Lee Nadler's group identified a second molecule in humans that delivers costimulatory signals via CD28 and coinhibitory signals through CTLA-4, and termed it B7-2 [129]. This finding was also reported simultaneously in *Nature* by Azuma and colleagues [130]. Just months later, the Nadler group identified and cloned the murine homolog of B7 [131].

CD28 is constituatively expressed on over 90% of naïve $CD4^+$ T cells and on approximately 50% of naïve $CD8^+$ T cells [128] [132]. Early reports suggested that CD3 ligation without stimulation of CD28 induces anergy, suggesting CD28 is a critical regulatory of peripheral tolerance [133]. While this is true in some cases, more recent studies have shown that some T cell subsets are less dependent on signaling through CD28 to mount productive responses [118-120]. Genomic expression programs downstream of CD28 began to be elucidated

with cDNA microarrays in the early 2000s, suggesting that CD28 signaling promotes cell proliferation and survival by enhancing IL-2 production, elevating levels of cell cycle proteins, and increasing the expression of pro-survival factors and anti-apoptotic genes [134, 135]. The molecular basis of CD28s costimulatory properties lies in protein tyrosine kinases in its cytoplasmic domain, resulting in phosphorylation of critical scaffold proteins, including LAT, Vav, and SLP-76. These molecules then recruit PLCγ1 and Grb2:SOS complexes, leading to a downstream cascade which culminates in nuclear activation of NFAT, AP-1, and NFκB [135]. These signals are amplified by PI3K, which binds a Tyr(P)-Met-Xaa-Met motif in CD28's cytoplasmic tail, which promotes TCR microclustering and permits entrance into lipid rafts, thereby bringing other critical signaling molecules into close physical proximity, and enabling synaptic localization [133].

The transplant immunobiology field has long been interested in the therapeutic blockade of CD28, with the understanding that inhibition of CD28 and abrogation of downstream signaling may prevent activation of *de novo* donor-specific T cell responses and potentially dampen memory T cell recall responses. Inhibition of CD28 signaling has long been pursued prevent alloreactive T cell responses following transplantation, and thereby promote graft maintenance and long-term tolerance. Unfortunately, early attempts to inhibit CD28 signaling with an anti-CD28 monoclonal antibody had disastrous outcomes. As reported in the New England Medical Journal in 2006 by Suntharalingam and colleagues, the first clinical trial with anti-CD28 was performed in six healthy men. The anti-CD28 treatment was given intravenously, and a systemic inflammatory cytokine storm was observed in 6/6 patients within 90 minutes [136]. Each subject then became critically ill, with pulmonary injury, widespread intravascular coagulation, and renal failure [136]. While all 6 patients survived after receiving extensive critical care, the trial was extremely disappointing. Because of the failure of this, blockade of CD28 signaling has been performed with CTLA-4Ig, which has been one of the most classic methods of experimental and clinical costimulation blockade. CTLA-4Ig binds to B7-1 and B7-2 and inhibits proliferation of TCR transgenic (Tg) cells *in vivo* [137]. Research in a number of animal models has demonstrated that CTLA-4Ig can be used to induce tolerance. CTLA-4Ig has been used to tolerize $CD4^+$ T cells in a model of intestinal transplantation [138] and activation-induced cell death (AICD)-promoting reagents (like rapamycin) are used in conjunction with CD28-based costimulation blockade to promote tolerance in mice [139] and to prevent islet allograft rejection in

NHP [140]. These data demonstrate that CD28 is an important therapeutic target for costimulation blockade to prevent rejection. The development of CTLA-4Ig into belatacept, and the generation of CD28-specific domain antibodies will be discussed in detail later.

ICOS: B7h

A CD28 homolog, the inducible costimulator of T cells (ICOS) is another critical costimulator of T cell responses. As its name implies, ICOS, an approximately 60 kDa member of the Ig superfamily, is not expressed on naïve T cells but is induced on T cells upon activation [141]. Once bound by its ligand, ICOS-L (B7-h1), ICOS increases T cell proliferation and cytokine production [142]. Work has shown that ICOS signaling is not required for early events following T cell activation, and it is thought that ICOS is more important than CD28 for the induction of cytokine production in recently activated T cells [143]. This is supported by evidence that while ICOS is dispensable for IL-2 production, it is critical for the production of IL-10, and loss of ICOS results in decreased production of IL-4, IL-5, and IFN-γ [144]. Interestingly, ICOS is readily expressed, on effector and regulatory T cells as well as B cells, where it plays an important role in the synthesis and secretion of IgG [142] [143]. Ligation of ICOS by ICOS-L, which is expressed on DCs and B cells as well as some endothelia, induces recruitment of PI3K to a phosphotyrosine motif in its cytoplasmic tail, although, unlike CD28, ICOS is unable to recruit Grb-2 [144]. ICOS expression is enhanced by binding of the Fos family member Fra2 to AP-1 and is also regulated by Fyn, NFAT, and ERK [145, 146].

The role of ICOS in alloreactive T cell responses has been of interest to the transplant immunobiology community for over a decade. In a model of murine cardiac transplantation administration of an anti-ICOS monoclonal antibody or ICOS-Fc inhibited acute rejection, resulting in prolonged allograft survival [147]. Furthermore, when ICOS blockade was given in conjunction with anti-CD40L and DST therapy, no evidence of vasculopathy or chronic rejection was detected [147]. Interestingly, the efficacy of ICOS blockade is dependent on the time at which the therapy is given. Work by the Sayegh group demonstrated that while the administration of anti-ICOS treatment at the time of transplant can reduce the production of alloantibody and the expansion of $CD4^+$ T cells, it must be given post-transplantation in order to suppress $CD8^+$ T cell responses [148]. These data

indicate that ICOS plays an important role in regulating the differentiation and effector capacity of T cells and is thus a relevant target for further investigation in the context of therapeutic costimulation blockade to improve graft survival following transplantation.

CTLA-4:B7 family

CTLA-4, discovered by the Ledbetter group in 1991 [149], is a heterodimer composed of Ig domains [126]. CTLA-4, like CD28, binds to B7-1 and B7-2, however the net result of these interactions are inihibitory with respect to T cell activation. Importantly, CTLA-4 binds B7-1 and B7-2 with 10-20 times greater affinity than CD28 does [135]. CTLA-4 is not expressed on naïve T cells; rather it is induced only after activation [150, 151] as its surface expression is largely dependent on calcium flux. Upon activation, CTLA-4 travels to the immune synapse and is endosomally recycled to and from the cell surface [150]. CTLA-4 is kept at the cell surface when its cytoplasmic tail is phosphorylated, preventing it from interacting with the clathrin adaptor AP-2, and preventing its endocytosis [135]. CTLA-4 exerts its inhibitory effects in a variety of ways. First, it may outcompete CD28 for ligands [152]. Second, CTLA-4 inhibits lipid raft formation and decreases accumulation of LAT [153]. Third, CTLA-4 has been shown to regulate TCR zeta chain stability in microclusters [154]. Finally, the cytoplasmic tail of CTLA-4 binds known inhibitory phosphatases. When bound to CTLA-4, SHP-2 dephosphorylates TCR zeta chains, limiting signaling downstream of the TCR [155]. CTLA-4 also binds PP2A via a 3 amino acid residue motif in its cytoplasmic tail, thereby suppressing IL-2 production [156]. Taken together, CTLA-4 inhibits the formation of the IS and reduces TCR signaling.

While the inhibitory effects of CTLA-4 on T cell proliferation can occur either after direct ligation by B7-1/B7-2 or following TCR stimulation and CTLA-4 crosslinking [157], it is clear that negative regulatory signaling downstream of CTLA-4 is critical for the control of T cell response. Studies have shown that CTLA-4 deficient animals develop profound lymphoproliferative diseases and autoimmunity [158, 159] and that blockade of CTLA-4 results in increased anti-tumor T cell responses [160]. These observations have provoked discussion of how CTLA-4 signaling is involved in the establishment of tolerance. Considering the role of CTLA-4 in tolerance is critical for a proper discussion of therapeutics aimed at B7-1 and B7-2 to prevent T cell costimulation

via CD28 as this strategy also inhibits important negative regulatory signaling via CTLA-4. Work in a murine model of minor- and major-mismatched cardiac allotranplantation shows that antibody-mediate blockade of CTLA-4 accelerated graft rejection [161]. Similar results were reported regarding the establishment of tolerance in a model of staphylococcal enterotoxin B challenge [162]. Zheng and colleagues demonstrated that administration of CTLA-4Ig could induce long-term cardiac allograft survival in B7-1 deficient recipients [163]. Indeed, long-term cardiac allograft survival was achieved with CTLA-4Ig treatment, however this tolerance was broken when anti-CTLA-4 was given perioperatively. These findings highlight the importance of balancing costimulatory and coinhibitory signaling in T cells.

PD-1:PD-L1/PD-L2

Programmed death 1 (PD-1), an Ig superfamily member, is a critical negative regulator of T cell responses. PD-1 is expressed on $CD4^+$ and $CD8^+$ T cells, while it's ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) are expressed on activated APCs [141]. PD-L1 is also found on some parenchymal and endothelial cells, where its expression is critical for tolerance induction [164]. PD-1 contains ITSM and ITIM domains in its cytoplasmic tail that are phosphorylated upon ligation [165]. These sites are then bound by the phosphatases SHP-1 and SHP-2, whose recruitment may counteract positive signaling in the immunological synapse, thus inhibiting early events following TCR activation [165]. Thus, PD-1 plays an important role in inhibiting early events following TCR activation and is also responsible for downregulating the expression of anti-apoptotic molecules and transcription factors associated with T cell effector function [165]. Ligation of PD-1 on T cells by either of its ligands also results in decreased proliferation and cytokine production [166]. The blockade of PD-1 in mice results in increased T cell activation and the development of autoimmune disease with age [167].

Interestingly, PD-1 appears to have a dual effect in the outcome of transplantation. Blocking PD-L1 can accelerate graft rejection by expanding alloreactive T cells [168]. However, another report demonstrated that administration of PD-L1-Ig promotes inhibitory PD-1 signaling and prevents allorejection [169]. This same study showed that when given in conjunction with anti-CD40L or low-dose rapamycin, PD-L1-Ig can induce tolerance. These studies indicate that PD-1 stimulation can result in different outcomes depending on the ligand

and context. Most importantly, these publications emphasize the importance of the PD-1 pathway in regulating tissue-specific immune responses following transplantation.

CD40:CD154

CD40 was discovered in 1990 by the Goodwin group as a receptor for tumor necrosis factor (TNF) [170]. Its ligand, CD40L (or CD154) is a trimeric member of the TNF family and is similar to 41BB and OX40 [171], which will be discussed briefly later. While CD40 is expressed on antigen presenting and endothelial cells, CD40L is expressed on the surface activated T cells and its cleavage products also exist in a soluble form [172] [173]. While my discussion of CD40/CD40L signaling will primarily focus on its roles in T cell function and allograft rejection, it is important to note that this pathways is also critical for the development and activation of some other immune cells [172]. CD40 ligation increases the expression of costimulatory molecules on APCs and promotes production of cytokines that are important for lymphocyte proliferation, such as IL-12 [174]. Ligation of CD40 also induces secretion of proinflammatory cytokines by monocytes [175]. Signaling via the CD40/CD40L pathway is essential for promoting B cell expansion and function, and is required for T-dependent antibody responses as it supports the formation of germinal centers and promotes antibody isotype class switching [176]. Furthermore, cells stimulated via the BCR and CD40L are resistant to killing by cytolytic T cells, demonstrating CD40L has an important role in maintaining antigen-specific B cell responses [177].

Evidence exists that CD40L itself can act as a receptor of costimulatory signals. Indeed, crosslinking of CD40L on $CD4^+$ T cells augmented proliferation and enhanced IL-4 production [178]. Signaling via the CD40/CD40L pathway is critical for mounting CD4 T cell responses, as these cells fail to expand after antigen encounter *in vitro* in mice deficient in CD40 or CD40L [179]. Restoration of this critical signaling pathway via *in vivo* infusion of soluble CD40 can reverse B cell impairment and initiate formation of germinal centers [180]. The costimulatory nature of the CD40/CD40L pathway is due to a cascade of signaling events that results in NFκB activation and the upregulation of anti-apoptotic molecules [172].

In the context of transplantation, treatment regiments utilizing anti-CD40L monoclonal antibodies (mAbs) prolong heart and islet allograft survival in mice [181, 182]. Mechanistically, CD40L blockade works to promote

long-term graft survival by inhibiting activation of effector T cells, preventing the formation of alloantibody, and dampening innate inflammatory cascades [181]. Blockade of this pathway in conjunction with CD28 blockade has been shown to induce operational tolerance in large animal models, as Kirk et al demonstrated that combined treatment with CTLA-4Ig and anti-CD40L promotes long-term allograft survival in a nonhuman primate model of renal transplantation [183]. Larsen and colleagues showed that dual CD28/B7 and CD40/CD40L blockade synergize to prolong graft survival by blocking clonal expansion of alloreactive T cells and preventing acute and chronic rejection [125]. It is important to note that CD40 is also expressed on tissue endothelial cells. When ligated by CD40L, endothelia increase expression of adhesion molecules critical for leukocyte recruitment, including E-selectin, ICAM-1, and VCAM-1, and therefore blocking CD40L serves to inhibit inflammation and lymphocyte recruitment within the allograft [184]. While CD40L blockade has been successfully used to promote long-term allograft acceptance and tolerance induction in animal models of transplantation for many years, problems have arisen when these strategies were applied to non-murine models. When CD40L blockade was utilized in a non-human primate model of renal transplantation, severe thromboembolic complications occurred [185]. This was attributed by CD40L expression on activated platelets and endothelia, since signaling through CD40L on these cells can induce endothelial inflammation that compromises the integrity of the vasculature [186]. It has since been shown that binding of anti-CD40L on platelets is mediated by FcgRIIA (CD32) [187]. Clinical trials exploring the therapeutic benefits of CD40L blockade were halted, and though some published studies utilized ketorolac to prevent thromboembolisms [188], clinical trials have not resumed. In light of these studies, it is clear that more research is required before CD40/CD40L therapies can be re-introduced in the clinic.

CD27:CD70 and CD30:CD30L

CD27 is another important costimulatory TNF superfamily receptor. CD27 is constituatively expressed on naïve T and B cells, as well as on NK cells [189]. CD27 binds CD70, which is primarily expressed on APCs and induced on T and B cells following activation and is functionally important for the development and activation of T cells, and delivers costimulatory signals to T cells independently of CD28 [190] [191]. CD27, in

conjunction with the CD40/CD40L signaling pathway, plays a role in the induction of Th-dependent B cell responses [192, 193]. CD27/CD70 signaling is required for the development of primary and memory responses to viral infections [194]. Antibody-mediated blockade of CD70 allows for normal $CD4^+$ T cells but impairs the proliferation and effector functions of $CD8^+$ cells as well as the generation of $CD8^+$ memory [195]. In a model of murine cardiac transplantation, antagonism of CD70 resulted in prolonged allograft survival, and when used in conjunction with CTLA4Ig, long-term survival [195].

CD30 is a costimulatory molecule differentially expressed on T cells with noted enrichment in the effector memory compartment [141, 196]. Interestingly, CD30 is constituatively expressed on T_{regs} and binds CD30L expressed on APCs and parenchymal cells [141, 197]. CD30 can be actively shed from memory T cells, and measurement of circulating levels of CD30 in the serum has been used a biomarker for rejection following transplantation [198, 199]. Manipulation of CD30 signaling is also relevant to transplant as it has been shown that this pathway is involved in initiating apoptosis of memory cells as CD30-expressing T_{regs} can trigger the death of $CD8^+$ memory T cells upon CD30L binding [200]. While these findings have raised interest in the therapeutic potential of CD27- and CD30-targeted strategies following transplantation, more research is necessary to determine how best to harness this pathway to reduce rejection and promote tolerance.

OX40:OX40L

OX40 is a costimulatory member of the TNFR superfamily. While OX40 is transiently expressed on $CD4^+$ and $CD8^+$ T cells after activation, its ligand, OX40L, is induced on pAPCs following CD40 ligation or TLR stimulation [201]. Three OX40 monomers come together to engage trimeric OX40L, and moves into lipid rafts upon binding [202]. In addition to binding PI3K, the cytoplasmic domain of OX40 can recruit and bind TRAF2, TRAF3, and TRAF5 and initiate canonical NFκB signaling cascades [203]. Experimental models have shown that OX40-deficient mice are able to initiate $CD4^+$ T cell responses normally, but that these cells aren't sustained and there are fewer memory cells present in the OX40KO animals compared to wild-type controls [204]. Other studies have shown that OX40 reduces the suppressive capacity and de novo generation of T_{regs}

[201] while enhancing the formation and reactivation of $CD8^+$ memory T cells by promoting the expression of anti-apoptotic molecules [205].

The therapeutic benefit of manipulating OX40 signaling in T cell responses has been well characterized in animal models of cancer. It was recently shown that the use of agonistic anti-OX40 antibodies reduces the suppressive capacity of Tregs and that therapies combining agonistic anti-OX40 and anti-CTLA-4 promote tumor rejection by increasing IFN-γ production [206]. A great deal of work has also been done to study the role of OX40/OX40L interactions in the context of organ transplantation. Levels of OX40 mRNA transcripts have been shown to be elevated in the urine and blood of patients experiencing acute rejection compared to stable or healthy controls [207, 208]. *In vitro* treatment of PBMCs from renal transplant recipients experiencing rejection with anti-CD3 and anti-OX40 decreases production of IFN-γ and IL-4 [209]. Similarly, allogeneic stimulation of $CD8^+$ T cells in the presence of OX40 blockade increases apoptosis of alloreactive T cells [210]. *In vivo* administration of anti-OX40 in mice prevents skin graft rejection, but does not induce tolerance, as the grafts are eventually rejected if the animals are taken off of anti-OX40 therapy [210]. The role of OX40 in regulating the generation of $CD8^+$ cells that go on to form long-lasting memory was elegantly demonstrated in a series of experiments that showed that when anti-OX40 was administered in conjunction with anti-CD40 and anti-LFA-1 at the time of skin transplantation, there was prolonged survival of subsequently transplanted cardiac allografts from matched donors [211]. Though OX40/OX40-L blockade has been the subject of study for many years now, more work is still necessary to determine how best blockade of this pathway may be clinically utilized in human transplant recipients to inhibit rejection and induce tolerance.

41BB:41BBL

41BB (CD137) is a costimulatory receptor of the TNFR superfamily expressed on $CD4^+$ and $CD8^+$ T cells following activation that exists at the cell surface as either a ~30kDa monomer or a ~55kDa dimer [212]. 41BB is preferentially involved in the activation and regulation of $CD8^+$ rather than $CD4^+$ cells, as it plays an important role in the generation of CTLs [213]. 41BB is bound by 41BBL, which is expressed at low levels on activated APCs during normal immune responses but can be highly expressed in states of chronic inflammation [214].

Upon ligation by 41BBL, TRAF1 and TRAF2 are recruited to phosphorylated sites in its cytoplasmic tail and initiate signaling that results in the activation of NFκB, ERK, JNK, and MAP, culminating in proliferation promoting and survival enhancing activities, including increased accumulation of Bcl-Xl and related anti-apoptotic proteins [214, 215].

Due to its involvement in regulating T cell activation, proliferation, and effector function, work done in the last decade has examined the therapeutic potential of manipulating 41BB signaling. The first evidence supporting this precedence was been established in the cancer literature. It has long been recognized that tumor microenvironments are highly immunosuppressive [216]. Many current immunotherapeutic strategies aim to augment anti-tumor T cell responses to overcome this immunosuppression. Some strategies have been developed to specifically target 41BB including the use of agonistic antibodies and chimeric antigen receptors. Administration of agonistic anti-41BB mAbs is especially useful for controlling effector T cell populations that have downregulated CD28, and has been shown to enhance effector functions by promoting expression of anti-apoptotic molecules, the reversal of anergy, and has been shown to inhibit progressive tumor growth [217-219]. Chimeric antigen receptors have been designed with the extracellular domain of CD19 fused to the cytoplasmic domain of 41BB, and whose expression on T cells resulted in augmented anti-tumor activities and prolonged survival [217]. Based on the successful manipulation of 41BB signaling to improve outcomes in cancer, we now turn our attention to the ways in which manipulation of 41BB may be used to attenuate the function of alloaggressive effector T cells in the context of transplantation. In 2000, the Larsen group showed that 41BB and 41BBL transcripts are detectible in rejecting cardiac allografts following transplantation in mice and that 41BB ligation in this model promoted proliferation of $CD8^+$ T cells as well as the production of IL-2 and IFN-γ [220]. While these findings have augmented interest in the therapeutic potential of 41BB blockade following transplantation, there is still a need for more studies in large animal models to best determine how therapies targeting 41BB may be included in immunosuppressive regimens to reduce rejection following transplantation.

TIM family members and their binding partners

Discovered in 2001, the T cell immunoglobulin mucin domain containing [221] family of cosignaling molecules is a group of type I transmembrane glycoproteins that contain a highly glycosylated mucin motif in the extracellular domain [141]. The TIM family is composed of TIM-1, TIM-2, TIM-3, which will be the focus of this section, and TIM-4.

TIM-3, which binds galectin-9, is perhaps the best studied of the known TIM family members. A number of recent reports have aimed to determine the role of TIM-3 blockade in transplantation. Administration of recombinant galectin-9 attenuates graft-versus-host-disease (GVHD) in mice while TIM-3 blockade conversely worsens GVHD [222]. This effect may result from the apoptosis of donor-specific T cells, as a recent study showed that TIM-3 ligation by galectin-9 inhibits *in vitro* MLR reaction by inducing apoptosis in T cells [223]. Interestingly, TIM-3 blockade also enhances the development of diabetes in mice [224]. In this murine model, alloislets are transplanted and DST plus anti-CD40L are given therapeutically in conjunction with TIM-3 blockade. When the combined therapies are given together one month before transplantation, long-term alloislet survival is achieved and tolerance is induced as subsequent matched grafts are also accepted [224]. Interestingly, if the triple therapy regimen is given at the time of transplantation instead of in advance, tolerance could not be induced and grafts did not survive long-term [224]. Additionally, a subset of donor-specific TIM-3^+ PD-1^+ regulatory T cells (Tregs) has recently been identified in mice following transplantation [225]. These cells infiltrate allografts but are short-lived, and it has been shown that TIM-3^+ Tregs are more suppressive than TIM-3^- Tregs and can prolong graft survival [225]. These studies of TIM manipulation *in vivo* are exciting and suggest that the incorporation of therapies targeting TIM family members into current immunosuppression regimens may promote the induction of transplant tolerance.

2B4:CD48

The role of 2B4 in modulating immune responses is complex in that, due to the presence of an immunotyrosine switch motif (ITSM, TxYxxV/I), in its cytoplasmic tail, it has been shown to have both costimulatory and coinhibitory properties [226-228]. In mice, crosslinking of 2B4 on NK cells induces

proliferation and augments effector function, while signaling via other inhibitory receptors can prevent 2B4 phosphorylation of 2B4 and lead to its exclusion from lipid rafts [229, 230]. Interestingly, 2B4 has also been reported to inhibit lysis of syngeneic tumor cells by NK cells via an MHC-independent pathway and can prevent NK cells from killing each other and inhibit killing of allogeneic cells *in vitro* [231-233]. The dual nature of 2B4 signaling is thought to be due to the context dependent recruitment of a number of adaptor, scaffold, and signaling molecules. The four tyrosine motifs in 2B4's cytoplasmic tail have been shown to bind the adaptors EAT-2A and EAT-2B, which can bind PLCγ1 or Vav-1 and may be costimulatory or coinhibitory [234-236]. Diverse 2B4 signaling may also be mediated by Vav-1, SHIP-1, and c-Cbl [237]. Studies have shown that in human NK cells 2B4 can associate with the linker of activated T cells (LAT), which, when phosphorylated by 2B4, can recruit PLCγ and Grb2 [238]. The classic intracellular adaptor that binds 2B4, is the SLAM-associated protein (SAP), which recruits the Src family kinase FynT to 2B4 [237]. Coinhibitory signaling resulting from 2B4 ligation is primarily mediated by SHP-2, which competes with SAP for binding to 2B4's cytoplasmic ITSM motifs [239].

2B4 is inducibly expressed on $CD8^+$ T cells with activated and memory-like phenotypes [240] and promotes cytotoxicity when ligated by CD48 on adjacent, non-target cells [241]. The signaling cascades downstream of 2B4 on $CD8^+$ T cells is thought to be similar as those described above in NK cells. Importantly, the ability of 2B4 to transmit either inhibitory or stimulatory signals is highly context dependent. In NK cells, the outcome of 2B4 signaling depends on 1) the extent of its expression at the cell surface, 2) the amount of expression of its ligand, CD48, and 3) the degree to which it associates with and recruits a number of intracellular proteins, including SAP, LAT, EAT-2, SHP-2, and ERT [242] [235, 238, 239].

2B4 binds the Ig superfamily member CD48, which is widely expressed on hematopoietic cells [231, 243-245]. CD48 is approximately 40-45 kDa and structurally similar to CD2, but is GPI-anchored without a transmembrane domain [245, 246]. CD48 was first discovered on virally infected B cells in the early 1980s, and a recent report suggests that murine cytomegalovirus (MCMV) can downregulate CD48 expression [247, 248]. CD48 is upregulated by interferons and other inflammatory cytokines [249]. CD48 plays important roles in both costimulation and adhesion, and helps to organize the immune synapse [246, 250]. Binding of CD48 to 2B4 on

NK and T cells stimulates expansion and cytotoxicity and can provide costimulatory signals to neighboring T cells via direct cell-to-cell contact [240, 241, 251].

The signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), is an intracellular adaptor protein encoded by the *SH2D1A* gene, mutations in which cause a number of defects, including X-linked lymphoproliferative disease (XLP) [252]. It has been shown in NK cells that SAP mediates its regulatory functions via a dual mechanism of action: it augments cell activation by recruiting the kinase Fyn, while simultaneously preventing inhibitory signals by uncoupling SLAM receptors from inhibitory phosphatases [252-255]. Given this background, it is possible that a similar mechanism of action influences the costimulatory or coinhibitory nature of 2B4 signaling in $CD8^+$ T cells. Taken together, these data suggest that if CD48 is highly expressed, 2B4 would be more heavily ligated, resulting in increased SAP association and thus increased 2B4 costimulatory function.

2B4 is an important modulator of T cell responses, and its role in pathogen-specific immunity has been explored previously. A study from Arlene Sharpe's group demonstrated that 2B4-deficient mice develop a spontaneous lupus-like disease, and Rafi Ahmed's group recently showed that in the context of chronic infection, 2B4 limits the proliferation and functionality of secondary, but not primary, effector T cells [227, 256]. A recent report indicates that 2B4 is upregulated on $CD8^+$ T cells following HTLV-1 infection, with the highest fraction of 2B4-expressing cells being antigen-specific with a terminally differentiated effector like phenotype [257]. This same study showed that blockade of 2B4 increases effector $CD8^+$ T cell function by augmenting perforin production and degranulation [257]. 2B4 may in part function to regulate these responses via the kinetics of its expression, as concurrent TCR stimulation and 2B4 ligation result in internalization of TCR complexes in virus specific $CD8^+$ T cells [258]. Finally, 2B4 expression on NK cells also plays an important role in regulating anti-pathogen immune responses. Loss of 2B4 on NK cells during LCMV infection can dampen anti-viral CD8 responses and result in lengthened viremia [259].

These findings are of clinical relevance as augmentation of coinhibitory signaling in allospecific $CD8^+$ T cells is critical in maintenance of long-term graft survival [260, 261]. Our recently published study suggests that 2B4 plays a functional role in the inhibition of donor-reactive $CD8^+$ T cell responses following CD28

costimulation blockade *in vivo*, raising the possibility that 2B4 may be a therapeutic target to attenuate allograft rejection [262]. Indeed, 2B4 was specifically upregulated on graft-specific $CD8^+$ T cells following selective costimulation blockade with anti-CD28 domain antibodies (dAbs), which contain an antigen-binding Vk domain but do not express an Fc region [262]. In contrast, 2B4 was expressed at low levels on naïve $CD8^+$ T cells and activated $CD8^+$ T cells in mice that did not receive immune modulation [262]. Additionally, mice lacking 2B4 on antigen-specific $CD8^+$ T cells exhibited increased expansion of alloreactive effector $CD8^+$ T cells compared to WT controls in the setting of a-CD28dAb costimulatory blockade [262]. Furthermore, treatment with a-CD28 dAbs failed to decrease cytokine production by $2B4^{-/-}$ graft-specific $CD8^+$ T cells [262]. A follow up study determined that coinhibitory signals generated by 2B4 on graft-specific $CD8^+$ T cells limit ICOS expression in a cell-intrinsic manner following treatment with CD28 dAbs [263], indicating that 2B4 is a functionally important coinhibitor that attenuates graft-specific T cell responses following transplantation.

These studies highlight the importance of signaling via an array of costimulatory and coinhibitory receptors following renal transplantation in humans. We therefore set out further explore how alloreactive T cell responses following solid organ transplantation may be modulated by additional, less well-studied cosignaling molecules, such as 2B4. Excitingly, 2B4 may be specifically manipulated to attenuate acute graft rejection mediated by alloreactive $CD8^+$ T cells following transplantation, while simultaneously maintaining functional immune responses capable of defending the host from pathogen infection. The role of 2B4 in controlling alloreactive human and mouse T cells will be discussed further in Chapters 2 and 3.

TIGIT/DNAM:CD112/CD155

The recently discovered T cell immunoreceptor with Ig and ITIM domains (TIGIT) molecule is a receptor found on the surface of T cells that can bind to CD155 and CD112 expressed on dendritic cells [264]. Importantly, TIGIT competes with CD226 (also known as DNAM) for binding to its ligands, thus resembling the relationship between the binding of CD28 and CTLA-4 to CD80 and CD86. The TIGIT protein contains an immunotyrosine based inhibitory motif (ITIM) in its cytoplasmic domain which can recruit phosphatases, conferring its inhibitory properties [265]. TIGIT functions in a cell intrinsic fashion, as dysregulation of the

signaling pathway has been associated with the development of autoimmune like disease as a result of T cell hyperproliferation in its absence[266]. Work has also shown that TIGIT can be expressed on NK cells and functions to inhibit cytotoxicity of those cells[267] .

In addition to the direct role that TIGIT signaling has in inhibiting T cell responses as illustrated above, TIGIT signaling also has a net dampening effect on immune activation by promoting the induction and maturation of tolerance-supporting dendritic cells via enhanced production of IL-10 and decreased IL-12 [268]. Importantly, TIGIT is also expressed on $CD4^+$ regulatory T cells (T_{regs}), where it functions to limit immune mediated collateral damage to the host via inhibition of both Th1 and Th17 responses[269].

Recent work has also described a role for TIGIT in modulating tumor microenvironments. TIGIT is highly expressed on tumor-specific $CD8^+$ T cells, and antibody-mediated blockade of TIGIT ligation enhanced the effector function of these cells, restoring anti-tumor immunity [264]. The importance of TIGIT in mediating cancer-specific immune responses via its expression on tumor-infiltrating lymphocytes is further highlighted by the marked increase of in vitro T cell functionality following combined ex vivo blockade of TIGIT and PD-1 on T cell isolated from human melanoma patients [270]. $TIGIT^+$ T_{reg} cells have also been shown to upregulate the coinhibitor TIM-3 following chronic antigen exposure, providing another mechanism by which TIGIT expression is able to suppress the generation of antitumor immune responses[271]. Interestingly, $CD4^+$ human T_{regs} that express TIGIT have also been shown to express FCRL3, both of which have been demonstrated to be enriched on thymically derived $Helios^+$ $Foxp3^+$ Tregs that are frequently identified in patients with autoimmune complications [272, 273]. Though no work has yet addressed the role of the TIGIT in transplantation, the use of antibody modulation of this signaling pathway in cancer suggest that therapies using costimulation blockade following transplantation may be very useful in prolonging allograft survival and inducing tolerance, and thus merit continued investigation.

The cosignaling families discussed here are diverse and can be involved in both costimulatory and coinhibitory signaling in T cells, and thus play complex roles in the subsequent modulation of T cell responses. Given that many cosignaling molecules act as regulatory switches and can promote allograft survival, future

studies should continue to explore the efficacy of manipulation of cosignaling molecules following transplantation.

Part V: Metabolism & Bioenergetics Impact T Cell Phenotype, Function and Fate

Studies over the last five years have described the impact of changes in cellular metabolism during T cell activation on the programmed differentiation of effector and memory T cell populations [274, 275]. Broadly, resting T cells utilize oxidative phosphorylation as their primary source of energy, while effector cells undergoing rapid proliferation switch to aerobic glycolysis in order to meet the energetic needs of an exponentially expanding T cell clonal population [276]. Specifically, while oxidative metabolism transitions glucose-derived pyruvate to the mitochondria for oxidation all the way down to carbon dioxide, glycolysis instead generates several key intermediates that the dividing cell can use for biosynthesis [277]. Moreover, during aerobic glycolysis, some glucose is funneled through the mitochondria and a portion of the tricarboxylic acid (TCA) cycle in order to generate citrate for the synthesis of lipids necessary for construction of daughter cell membranes.

These critical changes are initiated via integration of signals generated by the ligation of the TCR and costimulatory molecules on the T cell surface [278]. Both TCR and costimulatory receptors trigger the activation of key signaling pathways that alter gene expression, including c-Myc and the nuclear hormone receptors ERRα, β, and γ and NR3B1, 2, and 3 [278]. In addition, CD28 signaling functions to activate the PI3K/Akt/mTOR pathway. Both Akt and mTORC1 activation drive the cell toward aerobic glycolysis and promote the growth and function of effector T cells [279]. Recent work from Erika Pearce's group in Freiburg demonstrates that costimulatory signaling also plays an important role in priming the mitochondrial morphology and capacity of T cells via induced Cpt1a expression, which in turn enhances the development of memory T cells [280]. Importantly, ligation of T cell coinhibitory receptors can also impact T cell metabolism. In particular, ligation of PD-1 was shown to result in a shift from a glycolysis-dependent program to one in which T cells rely more heavily on fatty acid oxidation and lipolysis [281]. It is possible that PD-1-derived signals serve to reinforce a memory-like program characterized by phenotypic and functional senescence as a mechanism to reduce activation in the face of ongoing antigen stimulation. This idea is supported by a number of reports from animal

models of lymphocytic choriomeningitis virus (LCMV), where T cell exhaustion was initially defined, that suggest that PD-1 signals attenuate TCR signaling to preserve terminally differentiated cells that may be important for recall responses [282, 283].

Importantly, a number of these coinhibitors are implicated in the exhaustion of T cells, and has raised interest in the connection between these molecules and the loss of functionality in these cells. Recent studies have demonstrated that the T cell dysfunction that has become a hallmark of exhaustion is preceded by number of metabolic changes, including downregulation of the glucose transporter Glut1, and an increase in mitochondrial mass, though it appears that mitochondrial potential is disrupted [284]. A number of these characteristics are shared with $CD8^+$ T cells found in tumor microenvironments, where hypoxia is prevalent and may drive T cell exhaustion. In addition to limitations imposed by hypoxic conditions, T cells in metabolically restrictive environments must compete with other cells, and in some cases, tumors, for access to key nutrients and substrates, which can skew cells towards altered use of glucose, glycogen, and amino acids, changing the long-term ability of cells to survive and execute their effector functions [285].

At this time, more work is needed to fully understand the mechanisms by which T cell metabolism is controlled in response to distinct environmental stimuli, however recent work indicates that mammalian target of rapamycin (mTOR) kinase complexes may serve as a key integration point of extracellular signals that dictate the bioenergetic choices available to a given cell [286]. Generation of memory cells, for instance, may be favored by the translocation of FOXO1 into the nucleus in response to mTOR signaling, and there it may promote transcription of PGC1a and Eomes, two transcription factors that enable survival and bias a cell towards a memory fate [287]. This is an active area of research, and though a number of studies have been undertaken to address the role of PD-1 in determining the metabolic fitness of T cells, other coinhibitory molecules remain less well studies. Recent reports suggest that LAG-3 may upregulate genes associated with aerobic glycolysis [288], and that in samples derived from human patients with gastric cancers, coinhibitory TIGIT signaling on $CD8^+$ T cells inhibits glucose metabolism [289]. Given that combined blockade of TIGIT and PD-1 resulted in enhanced tumor regression compared to either alone [264], it seems probable that TIGIT signaling may alter metabolism in

a similar and complementary way to PD-1. Though further work will be needed to establish whether additional T cell cosignaling molecules such as BTLA, ICOS, 41BB also function to directly regulate metabolism, the work presented in Chapter 3 herein contributes to our understanding of the biology of 2B4 signaling in modulating glucose metabolism, and we propose this pathway merits further attention and experimentation to determine how therapeutic interventions may apply to enhancing the management of T cell-mediated pathology in solid organ transplantation, autoimmunity, and infectious disease.

Part VI: T Cells and Costimulation Blockade in Transplant Tolerance, Graft Acceptance, and Alloaggression

Memory T cells are a Barrier to Allograft Survival

While the retention of quiescent, antigen-experienced cells is critical for long-term protection of the host, memory T cells are also a barrier to allograft survival following transplantation. Many studies have shown that the human T cell repertoire includes memory cells specific for a number of alloantigens [221, 290]. It has been known for sometime that these alloreactive cells may arise following transplantation, blood transfusions, and pregnancy. Recently, however, a growing body of literature has elucidated how, even in individuals without such exposures, alloreactive cells arise from everyday contact with environmental antigens, including allergens, pathogens, and other materials. For example, studies have shown that some Epstein-Barr virus (EBV) specific T cells are also able to recognize certain human leukocyte antigen epitopes [291].

Individuals with high initial frequencies of alloreactive T cells are more prone to experiencing acute rejection episodes following transplantation [292]. Indeed, risk of rejection is correlated with the preoperative frequency of alloreactive T cells, defined by IFN-γ production following allostimulation [290]. This is especially true in the case of renal transplant recipients, where in those cells that recognize donor-derived antigens that are processed by the indirect pathways are highly correlated with the risk for chronic rejection [293]. Additionally, work has shown that naïve, but not primed, alloreactive cells require costimulatory signals via the CD40/CD154 pathway to become activated and, interestingly, treatment with anti-CD154 blocking antibody therapy cannot extend allograft survival in animals that were previously sensitized with a skin graft from a matched donor [294].

Indeed, though tolerance can be induced with a combination of anti-CD154 and the transfusion of donor spleen cells, when donor-reactive memory cells are added to this regimen the ability to induce tolerance is lost [295].

While clinical evidence clearly suggests that in humans the presence of memory T cells impairs the induction of transplant tolerance, and subsequently, long-term graft survival, this has not been easy to measure in standard laboratory animals used to model human transplantation. Laboratory rodents are kept in specific-pathogen free environments for the entirety of their lives. Therefore, their exposure to pathogens is limited, and thus the majority of their T cells are naïve. This is obviously not the case for human beings who are constantly expose to diverse environmental pressures and studies show that by adulthood about 50% of the human T cell compartment is composed of memory T cells [296, 297]. This is important as many strategies that have been designed and developed to induce tolerance via costimulation blockade have been successful in laboratory animals but have not been able to be translated to human transplant recipients. This is most likely due to the fact that memory cells, present in humans but not lab animals, have lower activation and costimulatory requirements compared to the primarily naïve cells found in experimental animals.

Another critical factor to consider in T cell responses to allografts is heterologous immunity. This term refers how to pre-transplant immunity (cellular memory) to previously encountered pathogens can alter responses to subsequent challenges by unrelated pathogens [298]. While the specificity of an individual T cell is dictated by the peptide it can see in the context of MHC, some T cells are able to recognize multiple antigen-derived peptides, a process known as T cell cross-reactivity. This cross-reactivity underlies heterologous autoimmunity and is mediated by a number of mechanisms. First, a T cell may recognize ligands via different binding motifs, for example shared amino acid sequences within disparate epitopes. For example, in C57BL/6 mice K^b-restricted epitopes recognize shorter peptide sequences (~8 amino acids) generally exhibit more cross-reactivity than D^b-restricted ones do (average peptide length ~9 amino acids) [298]. This is also evidenced in cases of molecular mimicry, where it has been observed that peptides with shorter amino acid sequences are more likely to trigger cross-reactive T cell responses [298]. Secondly, conformational changes can occur in the CDR3 loop of the TCR once a cell has matured and is activated, enabling the T cell to recognize different peptides [298]. Finally, it is the inherent nature of TCR molecules to recognize multiple peptide:MHC (pMHC) complexes. One major factor

that influences the primary pMHC specificity of a given TCR is referred to as the complex's immunodominance, which is determined by efficiency of peptide processing, the affinity of the peptide for the MHC complex, the overall number of pMHC complexes presented, and the availability of the TCR repertoire to recognize a given pMHC complex [298]. In the context of transplantation, heterologous immunity is a threat to long-term graft survival if T cells specific for a previously encountered pathogen or environmental antigen cross-react with donor antigens within the allograft, resulting in rejection [299, 300].

As our knowledge of the role of heterologous immunity in transplantation expands, it's interesting to ask what evolutionary pressures could have selected for cross-reactivity. There are a number of arguments to be made for the benefits of having cross-reactive T cells in the lymphocyte repertoire [301]. First, there is a discrepancy between the theoretical high number of pathogen-derived peptides and individual may encounter and the lower diversity of the T cell repertoire [301]. The presence of cross-reactive T cell enables the host immune system to recognize and respond to a larger number of antigens, which is of benefit since there is a limited physical space for lymphocytes to occupy within the host's body. This system may also have evolved to provide a mechanism by which the host adaptive immune system can recognize viral escape variants. What's more, the affinity of a TCR:pMHC receptor-ligand pair is intrinsically low, so the biophysical mechanics that control these interactions may simply not permit for permanent binding exclusivity [301]. It has become clear, however, that heterologous immunity is threat to the induction of transplant tolerance and the maintenance of long-term graft function and survival. When considered together, the factors discussed and evidence presented here illustrate that both the $CD4^+$ and $CD8^+$ T cells that respond to challenges by pathogens have the ability to cross-reactive with donor-specific antigens, inhibiting transplant tolerance, and compromising allograft survival.

Given this background, it is evident that *de novo* and pre-existing donor-reactive T cell responses must be contained following transplantation to prevent allograft rejection. Proposed strategies to inhibit alloreactive responses include the eradication of memory T cells, inhibition of graft infiltration via disruption of adhesion molecules, and/or preventing memory recall responses by using therapies that specifically interfere with those costimulatory receptors that are preferentially utilized by memory T cells [102]. Some lymphocyte depleting strategies have been shown to reduce rejection and promote long-term allograft survival following transplantation

when used in conjunction with costimulation blockade [302, 303]. More investigation is warranted however, as it is known that memory T cells are resistant to radiation [304] and lymphoablation has also been noted to expand T cells populations as a result of homeostatic proliferation [102]. Additionally, depleting T cells in a non-antigen-specific fashion in an attempt to induce transplant tolerance is risky as it may result in undesirable depletion of T cells that are essential for host protection. While blockade of adhesive molecules, such as LFA-1, has had success in promoting T_{reg} formation and prolonging allograft survival in both murine and primate models of transplantation [305-307], inhibition of costimulatory signaling has been at the center of research for the last decade or so. Costimulatory signaling is an attractive therapeutic target for manipulating memory T cells while leaving naïve cells unaffected, as recent studies have demonstrated that some costimulatory pathways may be utilized by memory but not naïve cells [102]. One such pathway in T cells is downstream of the inducible T cell costimulator (ICOS), which binds to the B7-related molecule ICOSL on antigen presenting cells [308]. Studies by Harada and colleagues showed that blockade of ICOS/ICOSL interactions prolong allograft survival, even when the treatment was not administered immediately at the time of transplantation, indicating that it may have an important role in inhibiting the responses of primed alloreactive T cells [148]. The costimulatory molecule OX40 and its ligand, OX40L, have also been recently implicated in playing a role in graft rejection in pre-sensitized murine recipients. Therapeutic blockade of OX40L in tandem with B7 prevents the rejection of cardiac grafts, and prolongs allograft survival even when recipients received primed lymphocytes [309].

With mounting evidence describing the therapeutic benefits of CD28 costimulation blockade with CTLA-4Ig in small animal models, researchers began exploring its efficacy in non-human primate models in the early 2000s. Belatacept (LEA29Y) is a CTLA-4Ig derivative made from a human fusion protein composed of extracellular CTLA-4 domain with the Fc region of human IgG1. Belatacept binds to B7-1 and B7-2, blocking Signal 2 and preventing CD28-mediated T cell activation while promoting anergy and apoptosis [310]. Belatacept is a modified version of the drug abatacept, with the two differing by only 2 amino acid residues. While abatacept has been shown to be successful in attenuating T cell responses in rheumatoid arthritis [311] and plaque psoriasis [312], it failed to prolong allograft survival in a non-human primate model of transplantation [310]. Belatacept, unlike abatacept, has been shown to extend renal allograft survival in NHPs [313]. The clinical study of

belatacept is important as it represents the first-generation of immunosuppression targeted at T cell costimulatory pathways, as opposed to previous standard of care treatments based on the use of calcenurin-inhibitors (CNIs) that are associated with significant side affect profiles that are toxic to the donor-graft and recipient [310]. The Belatacept Study Group began the BENEFIT study, a phase III clinical trial in humans to investigate the ability of belatacept to prevent rejection and graft loss following renal transplantation. In this study it was determined that belatacept was not inferior at prolonging graft survival compared to the standard-of-care CNI-based regimen [310]. Patients treated with a belatacept-based immunosuppression regimen had better graft function as measured by the glomerular filtration rate (GFR) than those treated with standard CNI's alone, even 3 years post-transplantation [314]. While the benefits of belatacept use include reduced toxicity and a lowered long-term risk of cancer and infection [310], use of belatacept in the BENEFIT study was associated with an increased rate of acute rejection after 2-3 years and an increased incidence of post-transplant lymphoproliferative disease (PTLD), when compared to standard of care CNI-based regiments (Figure 1) [315, 316]. Despite these disappointing early findings, continued investigation of the potential clinical use of belatacept has been exciting. Kirk et al. recently demonstrated that use of belatacept following renal transplantation in human recipients, when combined with donor-derived bone marrow infusion, enabled 7/10 allograft recipients to be completely weaned off of calcenurin inhibitors [317]. Taken together, these findings suggest that while costimulation blockade with CTLA-4Ig is a powerful tool that may be used clinically to reduce allograft rejection, additional pathways and mechanisms of costimulation must be studied to enable long-term allograft acceptance.

Clearly, T cell function is tightly regulated by a fine balance of costimulatory and coinhibitory signals [318, 319]. Developing a complete understanding of this equilibrium and how these signals impact T cell responses is of the utmost clinical relevance to transplantation. About 95% of adult patients receive traditional calcenurin inhibitor (CNI) immunosuppression (primarily tacrolimus- based regimens) following solid organ transplantation, and over 60% receive some type of T cell depleting treatment [1]. While only about 5% of renal allografts obtained from deceased donors fail within the first year post-transplant, the most recently available data indicates that over 50% of renal grafts cease to function by ten years post-transplant [1]. The use of calcenurin inhibitors, like tacrolimus, coupled with mycophenolate, often results in nephrotoxicity and the development of metabolic

disease, manifested primarily as Type II diabetes and hyperlipidemia [310, 314, 315, 320, 321]. Therefore, there has been a recent surge of interest in replacing CNI-based regimens with therapies that specifically target costimulatory molecules on T cells, as potential benefits of using T cell-specific immunosuppression include reduced toxicity and thus improved physical outcomes and quality of life for transplant recipients [262]. Though new reagents are being developed to target CD28-mediated costimulation, as described above, there is an additional need to design alternative therapies to target other costimulatory molecules, as some $CD4^+$ and $CD8^+$ T cells have been shown to progressively downregulate CD28 following antigen-stimulation or exposure to inflammatory cytokines, and though these $CD28^{null}$ T cells have been implicated in costimulation blockade-resistant rejection, it is also possible that these cells may assume a more regulatory phenotype [322] (Figure 2*)*. This dichotomy will be addressed in mechanistic detail further in Chapter 2.

Part VII: Conclusions

Taken together, the clinical and experimental background presented here set forth precedence for interfering with costimulatory signaling to prevent recall responses by alloreactive memory T cells. While it is clear that all arms of the immune system are involved in mediating graft rejection following transplantation, T cells are the overwhelming orchestrators of anti-donor responses, and these studies highlight the importance of controlling alloreactive T cells by manipulating costimulatory and coinhibitory molecules, a topic which will be discussed in more depth in the following chapters.

FIGURES

Figure 1.1. Clinical use of belatacept enhances graft function but is associated with increased incidence of early acute rejection following transplantation.

Figure 1.2. Some human T cells downregulate the costimulatory molecule CD28 following antigen exposure.

FIGURE LEGENDS

*Figure 1.1. Clinical use of belatacept enhances graft function but is associated with increased incidence of early acute rejection following transplantation. **A)** Use of belatacept results in superior graft function even six-years after transplantation when compared to traditional cyclosporine-based regiments but B) is accompanied by increased rates of acute rejection early after transplantation. Adapted from Vincenti et al, 2010, Am J Trans & Vincenti et al, 2016, NEJM.*

Figure 1.2. Some human T cells downregulate the costimulatory molecule CD28 following antigen exposure.
As T cells undergo antigen exposure or experience cytokine-mediated bystander activation, they may lose expression of CD28. Loss of CD28 may be associated with the acquisition of a cytotoxic phenotype, which may contribute to allograft rejection. Alternatively, some CD28 null cells appear to assume an immunoregulatory role in modulating the activity of other immune compartments. *Adapted from Mou et al, 2014, AJT.*

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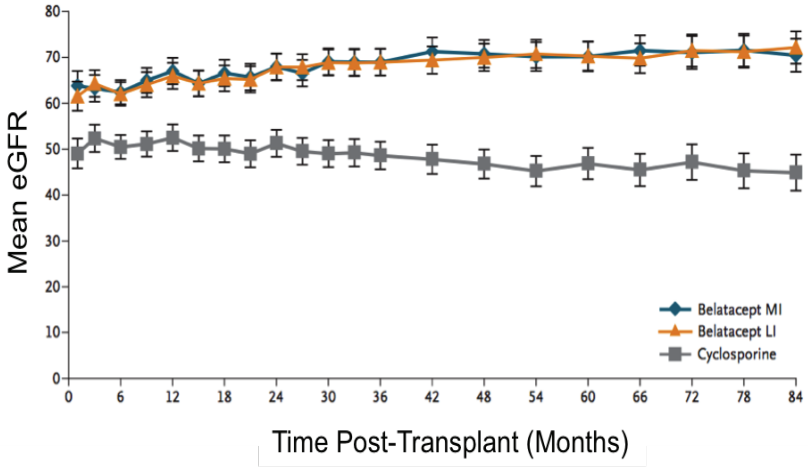
A.



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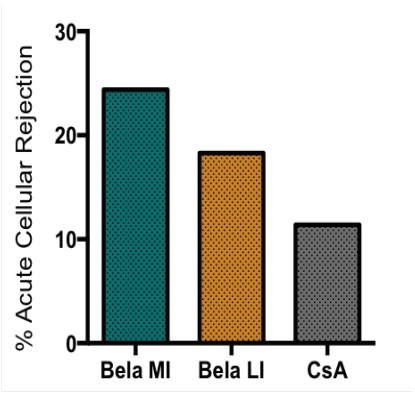
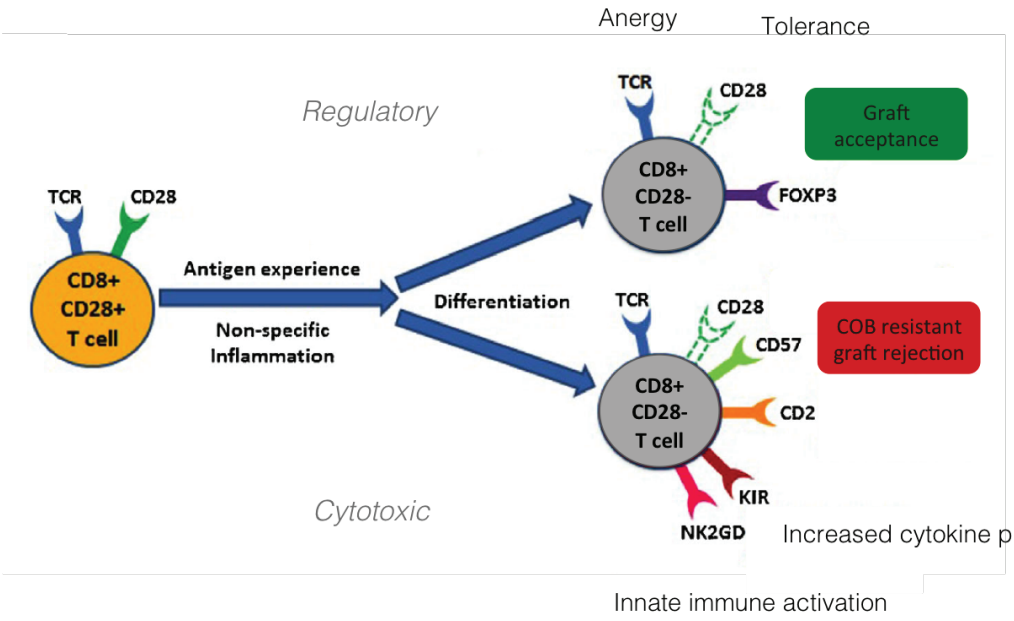


MI = more intensive
LI = less intensive
CsA = Cyclosporine A

Adapted from Vincenti et al, *NEJM*, 2016

Figure 1.1



Adapted from Mou et al., *Am J Trans,* 2014

Figure 1.2

Chapter 2:

2B4 Mediates Inhibition of $CD8^+$ T Cell Responses via Attenuation of Glycolysis and Cell Division

ABSTRACT

We recently showed that 2B4 expression on memory T cells in human renal transplant recipients was associated with reduced rates of rejection. To investigate whether 2B4 functionally underlies graft acceptance during transplantation, we established an experimental model wherein 2B4 was retrogenically expressed on donor-reactive murine $CD8^+$ T cells (2B4rg), which were then transferred into naïve recipients prior to skin transplantation. We found that constitutive 2B4 expression resulted in significantly reduced accumulation of donor-reactive $CD8^+$ T cells following transplantation, and significantly prolonged graft survival following transplantation. This marked reduction in alloreactivity was due to reduced proliferation of $CD8^+$ $Thy1.1^+$ 2B4rg cells as compared to control cells, underpinned by extracellular flux analyses demonstrating that 2B4 deficient (2B4KO) $CD8^+$ cells activated in vitro exhibited increased glycolytic capacity and upregulation of gene expression profiles consistent with enhanced glycolytic machinery as compared to WT controls. Furthermore, 2B4KO $CD8^+$ T cells primed in vivo exhibited significantly enhanced ex vivo uptake of a fluorescent glucose analog. Finally, the proliferative advantage associated with 2B4 deficiency was only observed in the setting of glucose sufficiency; in glucose-poor conditions 2B4KO $CD8^+$ T cells lost their proliferative advantage. Together, these data indicate that 2B4 signals function to alter T cell glucose metabolism, thereby limiting the proliferation and accumulation of $CD8^+$ T cells. Targeting 2B4 may therefore represent a novel therapeutic strategy to attenuate unwanted $CD8^+$ T cell responses.

INTRODUCTION

A fine balance of costimulatory and coinhibitory signals regulates the activation, differentiation, and proliferation of T cells following encounter with cognate allogeneic peptide:major histocompatibility complexes (pMHC). As such, manipulation of these cosignaling molecules may effectively inhibit unwanted T cell responses during autoimmunity and transplantation. 2B4 (SLAMf4, CD244) is an immunoglobulin (Ig) superfamily member expressed on natural killer (NK) cells and induced on some $CD8^+$ T cells (1-3). 2B4 contains an immunoreceptor tyrosine-based switch motif (ITSM) and is known to associate with the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), an intracellular adaptor protein, and bind CD48, a surface Ig molecule widely expressed on hematopoietic cells (4-10). Binding of CD48 to 2B4 can provide costimulatory signals to neighboring T cells via direct cell-to-cell contact (11-13). SAP mediates its function in NK cells via a dual mechanism of action: it augments cell activation by recruiting the kinase Fyn, while simultaneously preventing inhibitory signals by uncoupling SLAM receptors from inhibitory phosphatases (14-17).

Previous studies have shown that 2B4 can be expressed on $CD8^+$ T cells with activated and memory-like phenotypes and the majority of studies suggest that it functions in a coinhibitory capacity to regulate responses on these cells. In particular, 2B4-deficient mice develop a spontaneous lupus-like disease dependent on aberrant T cell activation (18). Further, in mouse models of chronic infection, 2B4 has been shown to limit the expansion and functionality of secondary effector T cells (18, 19). More recently, we found that human transplant recipients that went on to experience stable graft function for at least one year post-transplant exhibited increased frequencies of $2B4^+$ $CD28^{null}$ effector memory T cells (20) at baseline as compared to

patients that experienced acute rejection following transplantation (21). These associative data implied that expression of 2B4 on T cells might dampen alloreactive immune responses; however, this hypothesis has not been formally tested, and potential mechanisms underlying it are unknown.

Studies over the last five years have described the impact of changes in cellular metabolism during T cell activation on the programmed differentiation of effector and memory T cell populations (22, 23). Broadly, resting T cells utilize oxidative phosphorylation as their primary source of energy, while effector cells undergoing rapid proliferation switch to aerobic glycolysis in order to meet the energetic needs of an exponentially expanding T cell clonal population (24). Specifically, while oxidative metabolism transitions glucose-derived pyruvate to the mitochondria for oxidation all the way down to carbon dioxide, glycolysis instead generates several key intermediates that the dividing cell can use for biosynthesis (25). Moreover, during aerobic glycolysis, some glucose is funneled through the mitochondria and a portion of the tricarboxylic acid (TCA) cycle in order to generate citrate for the synthesis of lipids necessary for construction of daughter cell membranes. These critical changes are initiated via integration of signals generated by the ligation of the TCR and costimulatory molecules on the T cell surface (26). Both TCR and costimulatory receptors trigger the activation of key signaling pathways that alter gene expression, including c-Myc and the nuclear hormone receptors ERRα, β, and γ and NR3B1, 2, and 3 (26). In addition, CD28 signaling functions to activate the PI3K/Akt/mTOR pathway. Both Akt and mTORC1 activation drive the cell toward aerobic glycolysis and promote the growth and function of effector T cells (27). Importantly, ligation of T cell coinhibitory receptors can also impact T cell metabolism. In particular, ligation of PD-1 was shown to result

in a shift from a glycolysis-dependent program to one in which T cells rely more heavily on fatty acid oxidation and lipolysis (28). Additionally, a recent report describes that in samples derived from human patients with gastric cancers, coinhibitory TIGIT signaling on $CD8^+$ T cells inhibits glucose metabolism (29). However, the impact of 2B4 coinhibition on T cell metabolism and programmed differentiation has been less well studied. Here, we used a retrogenic approach to express 2B4 on antigen-specific $CD8^+$ T cells, in order to understand the effects of 2B4 signaling on $CD8^+$ T cell programmed differentiation and cellular metabolism.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (30) and OT-II (31) transgenic mice were purchased from Taconic Farms (Germantown, NY) and bred to Thy1.1$^+$ background at Emory University. mOVA mice (C57BL/6 background, H-2b) (32) were a generous gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University (protocol number: DAR-2002050-092815GN). All surgery was performed under general anesthesia with maximum efforts made to minimize suffering. All animals were housed in specific pathogen-free animal facilities at Emory University.

Donor-Reactive T Cell Adoptive Transfers

In order to approximate the precursor frequency of donor-reactive cells in a fully MHC mismatched model of transplantation, we utilized our previously described system in which we adoptively transfer a higher frequency of OVA-specific TCR transgenic cells into naïve hosts prior to transplantation. For adoptive transfer of donor-reactive T cells, spleen and mesenteric lymph nodes (mLN) isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were processed and stained with monoclonal antibodies for CD8 (Invitrogen), CD4, Thy1.1, and Vα2 (all from BD Pharmingen) for flow cytometric analysis. Cells were resuspended in 1X phosphate buffered saline (PBS) and 10^6 of each Thy1.1$^+$ OT-I and OT-II were injected i.v. 48 hours prior to skin transplantation. Where indicated, OT-I T cells were isolated and labeled with 5 μM CellTrace

Violet dye (Life Technologies, Invitrogen) prior to adoptive transfer. Proliferation was measured following sacrifice ten days post-transplantation via flow cytometry on a BD LSR II (BD Biosciences) and data were analyzed with FlowJo (TreeStar) and Prism (GraphPad). Numbers of precursor cells recruited into the anti-donor immune responses were calculated by first multiplying the absolute number of Thy1.1$^+$ CD8$^+$ T cells by the frequency of cells within a given round of division. We then divided the number of cells in each division by 2^n, where n is the number of divisions and the total number of precursors that gave rise to the cells in each division were summed.

Skin Transplantation

Full thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (33). Where indicated in Figure 1, recipients were treated with CTLA-4 Ig (abatacept, Bristol-Myers Squibb) (500 μg i.p. on days 0, 2, 4, and 6). In all cases, grafts with less than 10% viable tissue remaining were scored as rejected

2B4 Plasmid Construction and Transfection

The murine 2B4 gene was derived from mouse cDNA (OriGene: MC209044-113649) and produced by PCR using the primers: (Forward: GCGAATTCGCACCATGTTGGGG CAAGCTGTCCTGTTCACAA, Reverse: CGCTCGAGCTAGGAGTAGACATCAAAGTT CTC). The resulting PCR fragment was cloned into the pMY-IRES-GFP retroviral vector (Cell Biolabs, RV-021) using EcoRI and XhoI cut sites. The Platinum-E retroviral packaging cell line (Cell Biolabs, RV-101. Ecotropic for rat and mouse cells) was used to produce the 2B4-

containing retrovirus. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 μg/ml puromycin, 10 μg/ml blasticidine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2, humidified atmosphere. The packaging cells were incubated in 10-cm plates at 4.5×10^6/plate overnight at 37°C. Transfections were performed with the reagent Lipofectamine LTX (Invitrogen, 15338-100). Cells were transiently transfected with 10 μg DNA (2B4 plasmid DNA or empty-vector control). After 48 hours incubation the culture supernatant was harvested and virus was concentrated per manufacturer's instructions (Cell Biolabs, RV-201).

Retroviral transduction and generation of 2B4rg OT-I T cells

Two days before transduction, bone marrow (BM) cells (BMC) were harvested from 8 to 12 week old OT-I transgenic mice and cultured at $1.5x10^7$ cells per 10 cm plate in 15 ml DMEM supplemented with 15% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin, 10mM HEPES, 20 ng/ml murine interleukin-3 (IL-3), 50 ng/ml human IL-6 and 50 ng/ml murine stem cell factor (SCF) (R&D Systems). Concentrated virus was transduced into the pre-cultured BMCs. After 48 hours incubation bone marrow cells were collected and washed. 4×10^6 bone marrow cells in PBS were injected into sub-lethally irradiated (800 rads) wild type (WT) B6 recipients. Splenocytes from these BM chimeras were harvested 6-8 weeks post-transfer and were enriched by negative selection using a $CD8\alpha^+$ T cell Isolation Kit II (Miltenyi Biotec,). Purity of $CD8\alpha^+$ T cells was between 60 and 85%. Cells were then stained with anti-CD8 (Invitrogen), anti-Thy1.1 (BD Biosciences), and anti-2B4 (BD Biosciences) and GFP^+ $CD8^+$ $Thy1.1^+$ $2B4^+$ cells were purified by FACS sorting on a BD FACS Aria (BD Biosciences). Post-sort 2B4-OT-I T cell populations were over 94% pure. Cells were

resuspended in PBS with 10^6 wild-type $CD4^+$ $Thy1.1^+$ OT-II T cells and injected i.v. 48 hours prior to skin transplantation. Where indicated, retrogenic cells were prepared from the spleen and transferred i.v. without sorting along with wild-type $CD4^+$ $Thy1.1^+$ OT-II T cells 48 hours prior to skin transplantation. Upon sacrifice of the recipients ten days following transplantation, these cells were analyzed separately form bulk splenocytes via flow cytometric gating.

Flow Cytometry and Intracellular Cytokine Staining

Cells isolated from spleens and graft-draining axillary and brachial lymph nodes (dLN) were stained with anti-CD4 (BD Biosciences), anti-CD8 (Invitrogen) and anti-Thy1.1 (BD Biosciences). For phenotypic analysis cells were also surface-stained with anti-PD-1 (BioLegend), anti-2B4 (BD Biosciences or eBioSciences), anti-Thy1.1 (BD Biosciences), anti-LAG-3 (BioLegend), anti-CD127 (BioLegend), anti-KLRG-1 (eBioSciences), anti-CD44 (BioLegend or BD Biosciences), and anti-CD48 (BioLegend). Absolute numbers of lymphocytes from the spleen and draining lymph nodes were calculated using a Cellometer Auto T4 Cell Viability Counter (Nexcelom) according to the manufacturer's instructions. Samples were analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo 9 software (Treestar, San Carlos, CA) and Prism 6 software (GraphPad Software Inc.). For intracellular cytokine staining, lymphocytes were restimulated *ex vivo* with 1 μg/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Life Sciences) and 1 μg/mL ionomycin (Sigma Life Sciences) where indicated, in the presence of 1 μg/mL Brefeldin A (BD Biosciences) for 4 hours. The Fix/Perm intracellular staining kit (BD Pharmingen) was used to detect IL-2 (BD Biosciences), TNF (BioLegend), and IFN-γ (BD Biosciences), according to manufacturer's instructions.

RT-PCR Analysis

Splenocytes were isolated and prepared as a single-cell suspension. $3x10^6$ WT and 2B4KO OT-I T cells, respectively, were resuspended in 1.5 mL of complete media (RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 0.5 mM 2-mercaptoethanol) in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 4 days. After 4 days cells were collected and live cells were isolated via Ficoll gradient separation. $1x10^6$ live cells were plated with $5x10^6$ naïve splenocytes stimulator cells isolated from WT C57BL/6 donors and restimulated with 1 nM SIINFEKL peptide for four additional days in a final volume of 1.5 mL in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. Following restimulation, cells were harvested and incubated with Pacific Orange anti-CD8 (Invitrogen), PerCP anti-Thy1.1 (BD Biosciences), and PE-Cy7 anti-CD244 (eBioSciences) for 30 minutes at 4°C. Following staining, cells were washed with sterile 1x PBS and resuspended in ~1 mL of sorting buffer (sterile 1x PBS with 2% serum and 1 mM EDTA). Cells were sorted on FACSAriaII (BD Biosciences) and 2B4KO and $2B4^+$ OT-I T cells were isolated to at least 90% purity. FACS-purified cells were then resuspended in RLT buffer with β-ME (Qiagen, 350 μl for $>5x10^6$ cells, 600 μl for $<5x10^6$ cells) and flash frozen on dry ice for 10 minutes prior to storage at -80°C. RNA was isolated from previously frozen cells using the RNEasy Mini Kit (Qiagen, Germantown, MD) and quantified using a Nanodrop Microvolume Spectrophotometer (Thermofisher, Waltham, MA). RNA was converted to cDNA using the RT^2 First Strand Kit (Qiagen), and RT-PCR was performed using the Qiagen Glucose Metabolism RT^2 Profiler PCR Array with the RT^2 SYBR Green qPCR Mastermix (Qiagen) on a BioRad CFX384 Touch Real-

Time PCR Detection System. Data was analyzed online via the Qiagen GeneGlobe Data Analysis Center.

Seahorse XF Glycolysis Stress Test

Splenocytes were isolated and stimulated for four days as described above. Following Ficoll separation, $2x10^5$ OT-I live cells were then plated in buffer-free, glucose-free XF assay media (Agilent Technologies) supplemented with 2 mM L-glutamine, pH 7.4+/0.1. Extracellular acidification rate (ECAR) was measured and recorded at basal conditions as well as at indicated time points following the addition of glucose (final concentration 10 mM/well), oligomycin (final concentration 1 μM/well), and 2-deoxyglucose (final concentration 50 mM/well), using a Seahorse XFe96 Analyzer (Agilent Technologies). Basal ECAR readings were generated from the average of five measurements prior to the addition of oligomycin, while maximal ECAR was calculated from the average of three measurements following the addition oligomycin.

In vitro Glucose-limiting Stimulation Assays

Splenocytes were isolated and prepared as a single-cell suspension. $3x10^6$ WT and 2B4KO OT-I T cells, respectively, were resuspended in 1.5 mL of complete media (glucose-free RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 0.5 mM 2-mercaptoethanol) in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. A D-glucose solution of 200g/L (Gibco by Life Technologies) was titered into the glucose-free complete media and diluted on a half-log scale. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 5 days. After 5 days in culture cells were harvested and stained with anti-CD8 (BD Horizon), anti-Thy1.1 (BD

Biosciences or BioLegend), anti-CD244 (eBioSciences), anti-Va2 (BD), anti-CD127 (BioLegend), anti-CD62L (BD), anti-KLRG-1, anti-Vb5 (BD), anti-CD44 (BD), 7AAD (BD) and AnnexinV (BioLegend), anti-IL-2 (BD), anti-IFN-g (BD) as described by the manufacturer. As described above, samples were analyzed on an LSRII flow cytometer (BD Biosciences) and data was analyzed using FlowJo 9 software (Treestar, San Carlos, CA) and Prism 6 software (GraphPad Software Inc.).

2-NBDG Uptake Assay

To assess the ability of 2B4-deficient OT-I T cells to take up glucose, 10^4 WT and 2B4KO OT-I T cells were transferred into naïve C57BL/6 hosts and infected them with 10^4 colony forming units (CFU) of OVA-expressing *Listeria monocytogenes* (LM-OVA) two days. 14 days after infection, the animals were sacrificed and spleens were harvested. The cells were isolated and resuspended in a single cell solution in PBS. $2x10^6$ splenocytes were stained with anti-CD4 (BD Biosciences), anti-CD8 (Invitrogen) and anti-Thy1.1 (BD Biosciences), anti-CD44 (eBioSciences), anti-CD44 (BD Biosciences) for 30 minutes at 4°C. Cells were washed twice in 250 µl of PBS and then resuspended in 200 µl of 50 µM 2-NBDG (Thermofisher) and incubated at 37°C for 30 minutes. Cells were washed with PBS and then analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo 9 software (Treestar) and Prism 6 software (GraphPad Software Inc.).

Statistical Analysis

T cell responses were analyzed using unpaired, non-parametric Mann-Whitney t-tests. Results were considered significant if $p < 0.05$. Survival curves were analyzed by log-rank test and

plotted on Kaplan-Meier curves. All analyses were done using Prism software (GraphPad Software Inc.).

RESULTS

Ectopic expression of 2B4 on donor-reactive CD8$^+$ T cells results in prolongation of allograft survival

Given the observation that increased 2B4 expression was associated with reduced incidence of rejection in renal transplant recipients treated with belatacept (21), we investigated the causal role of 2B4 expression in attenuating donor-reactive CD8$^+$ T cell responses following transplantation. To test this we utilized a retrogenic approach to generate donor-reactive CD8$^+$ T cells that constitutively express 2B4 (2B4rg). Though primary effector cells do not express 2B4 at baseline (Figure 1E), we have previously identified an association between 2B4 expression on T cells and improved graft survival in both murine models and human transplant recipients (21, 34). Thus, we aimed to create a system in which we could isolate and interrogate this effect, and in which we would be able to determine if upregulation of 2B4 can functionally impact alloreactive T cell responses. To this end, we utilized retrovirally-transduced bone marrow derived from OT-I mice with a 2B4-bearing construct or an empty control vector. Transduced cells express GFP under the control of the IRES promoter, allowing us to identify, isolate, and track these cells in both in vivo and in vitro assays. Briefly, CD45.2$^+$ Thy1.1$^+$ OT-I bone marrow was transduced with a construct that expresses 2B4 under a constitutively active viral promoter and contained an IRES-GFP to facilitate tracking the cells. At day 2 post transduction, ~5–10% of Thy1.1$^+$ OT-I BM cells expressed GFP alone (for pMY control vector-transduced cells) or both GFP and 2B4 (for 2B4 vector-transduced cells) (Figure 1A). BM cells were then adoptively transferred into irradiated CD45.1$^+$ Thy1.2$^+$ animals. At 8–10 weeks post-in vivo transfer, GFP labeled Thy1.1$^+$ OT-I T cells were detectable in recipients of 2B4 vector-transduced and pMY control vector-transduced BM cells at similar frequencies (Figure 1B), suggesting that 2B4

expressing OT-I T cells mature normally in these animals. GFP^+ $CD8^+$ $Thy1.1^+$ (for pMY) or GFP^+ $2B4^+$ $CD3^+$ $Thy1.1^+$ (for 2B4rg) OT-I T cells were isolated from the spleen and mesenteric lymph nodes of pMY or 2B4rg chimeric animals and then MACS and FACS sorted to >86% purity (Figure 1C).

To determine the impact of constitutive 2B4 expression on graft-specific alloimmune responses, naïve B6 animals were adoptively transferred with 10^6 congenically labeled 2B4rg $Thy1.1^+$ OT-I T cells (or pMY $Thy1.1^+$ OT-I controls) along with 10^6 $Thy1.1^+$ $CD4^+$ WT OT-II T cells and then challenged with an OVA-expressing skin graft (Figure 1D). Animals were sacrificed at day 10 post-transplant, the magnitude of the $CD8^+$ $Thy1.1^+$ response was assessed. Importantly, expression of 2B4 was maintained in the 2B4rg OT-I T cells during the *in vivo* response (Figure 1E). To determine the impact of donor-reactive $CD8^+$ T cell 2B4 expression on graft survival, recipients of either 2B4rg or control OT-I T cells were monitored for graft survival. Under these conditions, we saw no difference in graft survival (not shown). Likewise, there was also no difference in graft survival when OT-I T cells were WT vs. 2B4 KO (data not shown). To test whether or not expression of 2B4 can prolong graft survival in the context of minimal immunotherapy, we adoptively transferred 2B4rg OT-I T cells into animals that received a low dose of CTLA4Ig in the first week following transplantation in order to extend graft survival enough to allow us to see any potential differences in survival between animals that received the WT vs. 2B4KO cells. Results indicated a significant prolongation in survival in recipients of 2B4rg donor-reactive $CD8^+$ T cells as compared to recipients of control donor-reactive $CD8^+$ T cells (MST 31.5 vs. 23, $p=0.01$) (Figure 1F).

Ectopic expression of 2B4 resulted in reduced accumulation of antigen-specific CD8$^+$ T cells

In order to investigate the mechanisms underlying the observed prolongation in graft survival in CTLA-4Ig-treated graft recipients possessing 2B4rg donor-reactive CD8$^+$ T cells, spleens and draining LN were harvested on day 10 post-transplantation and the magnitude and functionality of the Thy1.1$^+$ CD8$^+$ T cell response was assessed. Results revealed detectable populations of CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells in the spleens and lymph nodes (data not shown) of mice that received either pMY or 2B4rg OT-I T cells, respectively (Figure 2A). Strikingly, constitutive expression of 2B4 resulted in significantly decreased accumulation of donor-specific CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells in the spleen following transplantation in the absence of any further immune modulation, both in terms of frequency and absolute number (Figure 2B). In contrast, retrogenic 2B4 expression did not significantly impair production of IFN-γ on a per cell basis following *ex vivo* restimulation (Figure 2C and 2D), though fewer total cytokine-secreting OT-I T cells could be detected in the spleen 10 days following transplantation (Figure 2E). Taken together, these results indicate that expression of 2B4 on CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells results in the attenuation of graft-specific T cell responses in the spleen 10 days following transplantation.

Failure of 2B4 retrogenic cells to accumulate in the spleen is not due to differences in expression of the 2B4 ligand CD48, T cell activation or exhaustion markers, or T cell death

We next investigated whether or not the reduced accumulation of Thy1.1$^+$ CD8$^+$ T cells in the spleens of 2B4rg recipients was due to differences in the activation, differentiation, or exhaustion status of these cells. In experiments similar to those described above, naïve B6 animals were adoptively transferred with 10^6 congenically labeled 2B4rg Thy1.1$^+$ OT-I T cells or

pMY controls, along with 10^6 Thy1.1$^+$ CD4$^+$ WT OT-II T cells, challenged with an OVA-expressing skin graft, and sacrificed at day 10 post transplant at which time splenic T cells were stained for markers of activation, differentiation, and exhaustion and analyzed by flow cytometry. Analysis of cell surface phenotypes indicates there was no difference in the activation status of the 2B4rg Thy1.1$^+$ OT-I T cells when compared to the pMY Thy1.1$^+$ OT-I controls as measured by CD44 expression (Figure 3A). There was also no detectible difference in the presence of short-lived vs. memory precursor effector cells as distinguished by CD127 and KLRG-1 staining (Figures 3A, 3B and 3C). Additionally, no differences were found in the expression of PD-1 and lymphocytes activation gene 3 (LAG-3) exhaustion markers on 2B4rg vs. pMY Thy1.1$^+$ OT-I T cells (Figures 3A, 3D and 3E). Finally, the expression of the 2B4 ligand CD48 did not differ between the 2B4rg Thy1.1$^+$ OT-I or pMY Thy1.1$^+$ OT-I control cells (Figures 3A and 3F). Overall, these data suggest that the failure of 2B4rg Thy1.1$^+$ OT-I cells to accumulate in the spleen after transplantation was not due to differences in their expression of the 2B4 ligand or T cell activation or exhaustion markers. We therefore next sought to determine whether the paucity of 2B4rg cells observed in the spleen 10 days after transplantation was due to increased cell death. As depicted in Figure 3G, no increase in cell death as measured by Annexin V$^+$ 7-AAD$^+$ double-positivity was observed in the 2B4rg Thy1.1$^+$ OT-I T cells as compared to pMY controls. Instead, 2B4rg OT-I T cells actually exhibited reduced frequencies of Annexin V$^+$ 7-AAD$^+$ cells (Figure 3H). These data therefore demonstrate that the observed reduced accumulation of 2B4rg graft-specific CD8$^+$ T cells was not due to increased cell death within the 2B4rg compartment.

2B4 rg OT-I T cell population exhibits less division compared to pMY control OT-I due to reduced recruitment into the anti-donor immune response

Because the above experiments failed to explain impaired accumulation of 2B4-expressing $CD8^+$ T cells following transplantation, we next asked if the reduced accumulation of 2B4rg graft-specific OT-I T cells following transplantation was the result of differences in the amount of cell division following activation. To test this, GFP^+ $CD8^+$ $Thy1.1^+$ OT-I T cells were isolated from spleen and mLN of 2B4rg chimeric animals and stained with CellTrace Violet prior to being adoptively transferred (10^6 /recipient) into naïve B6 hosts that received 10^6 WT $CD4^+$ $Thy1.1^+$ OT-II T cells and were grafted with an OVA-expressing skin graft. As previously described, animals were sacrificed on day 10 post-transplant 2B4rg chimeric cells were isolated from the spleen analyzed by flow cytometry. Our analyses revealed striking differences between the frequency and number of GFP^+ $CD8^+$ $Thy1.1^+$ OT-I T cells dividing in the 2B4rg and non-retrogenic compartments (Figures 4A, 4B and data not shown). The frequency of OT-I cells that underwent 4 or more rounds of division was significantly lower in the 2B4rg OT-I populations as compared to the GFP single-positive controls (Figures 4C and 4D). Furthermore, we observed a statistically significant increase in the number of 2B4rg OT-I that remained undivided 10 days following transplantation as compared to pMY OT-I controls (Figure 4E). Additional analyses of cell division calculating the number of precursors that were recruited into the response (based on the number of cells present in each CTV peak at day 10 as described in materials and methods) revealed that fewer 2B4rg OT-I precursors were recruited into the response as compared to pMY OT-I precursors (Figure 4F). Taken together, these findings demonstrate that expression of 2B4 impairs expansion of the anti-donor $CD8^+$ effector population by preventing cells from entering the cell cycle.

2B4 expression limits glycolytic capacity of CD8$^+$ T cells

To determine the mechanism by which constitutive expression of 2B4 impairs proliferation of anti-donor CD8$^+$ T cells, we next assessed T cell metabolism. It is known that T cells undergo metabolic reprogramming as they differentiate into effectors, and that glycolysis is the primary method by which these cells derive the energy needed to proliferate during this process (35, 36). As bioenergetic needs of T cells are significantly augmented during this critical time, it is possible to detect a notable increase in the uptake and utilization of glucose soon after activation (37). To address whether or not glycolysis was altered in the absence of 2B4, we utilized a model in which we stimulated WT and 2B4-deficient (2B4KO) OT-I T cells in vitro and then probed their metabolic capacity. To test the hypothesis that 2B4 signaling limits CD8$^+$ T cell proliferation by inhibiting glycolysis, we established a model in which WT and 2B4KO OT-I T cells were stimulated in culture for seven days with their cognate peptide SIINFEKL, purified via Ficoll gradient centrifugation, and then restimulated for four more days in vitro. After restimulation, 2B4$^+$ cells were purified to >90% purity from WT OT-I T cells by FACS sorting, and 2B4KO OT-I T cells were confirmed to be 2B4$^-$ by flow cytometry. Following sorting, we assessed the expression of 84 genes associated with glucose metabolism in 2B4$^+$ WT vs. 2B4 KO cells using the Qiagen Glucose Metabolism RT^2 Profiler PCR Array. Of the 84 genes assessed in this array, we noted that 42 were differentially expressed between 2B4$^+$ OT-I cells isolated from WT animals and 2B4KO OT-I T cells (Figure 5A). Of those, a number were associated with glycolytic shifts in response to activation, the pentose phosphate pathway, the TCA cycle, and in aerobic glycolysis (Figure 5B).

Based on the differential expression of genes in the glycolytic pathways identified via transcriptional profiling, we next endeavored to assess functional changes in T cell metabolism based on 2B4 expression using the Seahorse XFe96 Analyzer. As demonstrated in Figures 5C, we found that the loss of 2B4 on antigen-specific T cells results in enhanced glycolytic metabolism as measured by an increase in extracellular acidification rate (ECAR) during glucose-induced glycolysis (38). Additionally, we observed that the ability of OT-I T cells to perform glucose-induced glycolysis was greater in the absence of 2B4, and that the total glycolytic capacity of the 2B4KO cells was greater than that of the wild type controls (Figure 5D-E). Next, to determine whether 2B4KO T cells achieved increased glycolytic capacity in part via increasing their uptake of glucose from the extracellular environment, we moved to an in vivo model of T cell stimulation followed by ex vivo incubation with the fluorescent glucose analog 2-NBDG (Figure 5E-G). Because WT graft-elicited $CD8^+$ T cell responses did not exhibit high frequencies of 2B4-expressing cells (Figure 2 and (34)), we moved to a model of bacterial infection. Briefly, 14 days following infection with OVA-expressing *Listeria monocytogenes,* $2x10^6$ cells were plated directly ex vivo and incubated with 2-NBDG for 30 minutes, and uptake was determined by assessing fluorescence via flow cytometry. Data indicated that uptake of 2-NBDG was significantly increased in 2B4KO $CD8^+$ T cells as compared to WT $CD8^+$ T cells (Figure 5F-5G).

Proliferative advantage of 2B4 KO T cells is erased under glucose limiting conditions

Our data thus far demonstrated increased proliferation and increased glycolysis in the setting of 2B4 deficiency. In order to identify a causal link between these two observations, we queried whether the proliferative advantage observed in 2B4 KO T cells would persist under glucose-

restrictive conditions. To test this, WT and 2B4KO OT-I T cells were labeled with CellTrace Violet prior to stimulation with SIINFEKL N4 peptide at 1 nM in media containing varying concentrations of glucose as described in Materials and Methods and Figure 6A. Our results demonstrate that 2B4KO OT-I T cells restimulated in glucose-replete media exhibited a statistically significant increase in CTV dilution relative to WT control cells (Fig. 6B-C) as expected, demonstrating that 2B4 deficiency confers a proliferative advantage when glucose is abundant, in these experiments at the level of 3 mM or higher. In contrast, when glucose was present only at low levels (1 mM or less) WT and 2B4KO OT-I T cells exhibited similar levels of CTV dilution (Fig. 6B, 6C). To rule out the possibility that measured proliferation of these cells may have been impacted by increased rates of cell death in the cultures with low glucose, we assessed apoptosis by quantifying the frequency of 7AAD and AnnexinV double-positive cells and show that there were no differences between death of WT and 2B4KO cells in any culture condition (Fig. 6D). These data thus suggest that the observed differential proliferation of 2B4KO T cells is dependent on their ability to undergo increased glycolytic metabolism.

2B4-mediated signals impact CD8$^+$ T cell memory recall potential

To determine whether or not enhanced glycolytic capacity following activation impacts the functionality of 2B4KO T cells, we transferred WT and 2B4KO OT-I T cells into naïve C57BL/6 hosts and then infected them with OVA-expressing *Listeria monocytogenes* (LM-OVA) two days later (Fig. 7A). Ten days later, each animal received an OVA-expressing skin graft (Fig. 7A). Animals were sacrificed five days following transplantation, and spleens as well as axillary and brachial draining lymph nodes were harvested for analysis. In comparison to the wild type controls, graft-specific cells lacking 2B4 express less KLRG-1 (Figure 7B, 6C), which has been suggested to be associated with a senescent-like program (40). Additionally, the 2B4KO cells

express higher levels of CXCR3 and CD69 (Figure 7B, 6C), consistent with a model in which the loss of 2B4 results in a more activated phenotype. CD44 expression was not different between the two cell types (Fig. 7B-C). Strikingly, we found in addition to their activated phenotype, graft-specific 2B4 KO $CD8^+$ $Thy1.1^+$ secondary effectors contained a higher frequency of IFN-γ^+ IL-2^+ double producers upon rechallenge with a skin graft as compared to WT $CD8^+$ $Thy1.1^+$ cells (Fig. 7D, 6E). These data support the conclusion that loss of 2B4 coinhibitory signaling on $CD8^+$ donor-reactive T cells results in enhanced metabolism and altered memory T cell differentiation that leads to more robust secondary recall responses following rechallenge.

DISCUSSION

In this study, we showed that constitutive expression of 2B4 prolongs allograft survival and limits alloreactivity by attenuating the accumulation of donor-specific $CD8^+$ T cells following transplantation (Figs. 1A, B). Our data reveal increased T cell glycolytic metabolism in the absence of 2B4, demonstrated both functionally using the Seahorse extracellular flux assay as well as by analysis of changes in expression of 84 metabolism-associated genes. Of note, genes upregulated in activated 2B4-deficient $CD8^+$ T cells were associated with glycolytic machinery, the pentose phosphate pathway, the TCA cycle, and aerobic glycolysis. For example, *Eno2* and *Eno3*, encode Enolases 2 and 3, metalloenzymes responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the ninth and penultimate step of glycolysis. We also noted an increase in *G6pdx* in cells deficient in 2B4 (Fig 5B). *G6pdx* encodes glucose-6-phosphate dehydrodgenase X-linked, a key enzyme in the pentose phosphate pathway, a mechanism of generating energy that is strongly relied upon by proliferating T cells (43). Additionally, the enhanced presence of transcripts for *Pdk1*, which encodes pyruvate dehydrogenase kinase 1, in the absence of 2B4, suggests that 2B4-mediated signaling may promote aerobic glycolysis by inhibiting the activity of pyruvate dehydrogenases (22). Overall, these findings on the impact of 2B4 on T cell metabolism are consistent with recent studies showing that T cell coinhibitory molecules can impact immunometabolism. For example, signaling via PD-1 on activated T cells was shown to prevent glycolysis (36). Similarly, work from Wherry and colleagues recently showed that PD-1 signaling impacts early glycolytic activity in T cells and represses the transcriptional regulator PCG-1a; forced overexpression of PGC-1a was able to improve the functionality of exhausted T cells (44). Interestingly, PD-1 signaling also augmented lipolysis and β-oxidation of fatty acids, leading to a

marked increase in mitochondrial spare respiratory capacity (SRC) (36). PD-1-mediated reinforcement of FAO may serve to explain long-term preservation of these cells during cancer and persistent viral infection. In support of this, recent work has shown that signaling via PD-1 activates AMP-activated protein kinase (AMPK), which is required for the survival and longevity of T cells in nutrient-compromised microenvironments (45). Together with these published studies on the role of PD-1, our data further highlight the emerging role for coinhibitory molecules in the control of T cell bioenergetics and metabolism.

While our study demonstrates that signaling via 2B4 on antigen-specific $CD8^+$ T cells modulates glycolytic metabolism, further studies are needed to assess the impact of this coinhibitor on alternative mechanisms of energy generation, such as fatty acid oxidation. Our data indicate that *Idh2* and *Idh3a* are increased in the absence of 2B4 (Figure 5B). These genes encode isocitrate dehydrogenases that are critical in the tricarboxylic acid (TCA) cycle, which has been shown to be increased in activated T cells, but not to the same degree as glycolysis (46), more work is warranted to dissect a possible role of oxidative metabolism in contributing to T cell function and fate in the presence or absence of 2B4.

Our data also reveal an impact of 2B4-mediated metabolic alterations on programmed T cell differentiation; specifically, 2B4-deficient OT-I cells exhibited enhanced effector recall potential after rechallenge when compared to the wild-type controls (Fig. 7). Thus, 2B4 deficient cells exhibit increased glycolytic metabolism during primary differentiation but also augmented recall responses. These findings of increased glycolytic function being associated with improved recall responses are seemingly at odds with work from Sukumar and colleagues, which showed that

enhanced glycolytic flux promotes a state of terminal differentiation, while inhibiting it promotes the generation of long-lived memory $CD8^+$ T cells (47). More recent work, however, has shown that the division of effector-like and memory-like $CD8^+$ T cells into populations that rely on glycolysis and oxidative phosphorylation of fatty acids, respectively, is not so clear cut. For example, van der Windt et al. showed that memory $CD8^+$ T cells exhibit both increased glycolysis and oxidative phosphorylation as compared to primary effectors (48), and a more recent study demonstrated that elevated oxidative phosphorylation is dispensable for the ability of T cells to form long-lived stable memory following an acute infection (38). In line with these findings, the results that we present here demonstrate that the enhanced glycolytic capacity and 2-NBDG uptake observed in the absence of 2B4 (Fig. 5) is correlated with enhanced memory recall potential following rechallenge (Fig. 7). These findings further are supported by a recent study demonstrating that the differentiation of $CD8^+$ effector memory T cells during persistent viral infection was supported by consistent glycolytic metabolism (38); the mechanisms by which immunometabolism controls memory T cell differentiation remains an area of intense investigation.

It is interesting to note that ectopic 2B4 expression resulted in a trend toward decreased effector cytokine production by antigen-specific primary $CD8^+$ T cells on a per cell basis (Fig. 2C-E), and a statistically significant decrease in the absolute number of IFN-γ-secreting cells per spleen. Likewise, the absence of 2B4 on secondary effectors conversely enhanced cytokine production following antigenic rechallenge (Fig. 4B and C). We speculate that this attenuation in cytokine effector function by 2B4 signaling is mediated by altered immunometabolism. Indeed, a recent report demonstrated that conversion to glycolysis is required for effective IFN-γ production, and

showed that this requirement is due to the binding of the glycolysis enzyme GAPDH to AU-rich elements within the 3' UTR of IFN-γ mRNA (49). Thus, expression of GAPDH induced by aerobic glycolysis controls effector cytokine production. These data support our hypothesis that 2B4-mediated regulation of glycolytic metabolism in T cells affects effector function.

Finally, the results presented in our study suggest that as a regulator of metabolism, 2B4 may be an important therapeutic target in the design of new strategies to control donor-reactive T cells following transplantation. Indeed, recent work demonstrated that the blockade of both glycolysis and glutamine metabolism results in the prevention of allograft rejection in a model of fully MHC-mismatched skin and cardiac transplantation, suggesting that the manipulation of effector cell metabolism is an important mechanism by which alloimmunity can be controlled (35). In this study we present data suggesting that engagement of 2B4 can negatively regulate donor-reactive T cell responses in the context of transplantation. This is of translational importance, as we have recently published that not all alloreactive T cells are targeted by the CD28 costimulation blocker belatacept following transplantation, and that those that express low levels of CD28 express high levels of 2B4 (20, 50, 51). In sum, our study suggests that agonistic ligation of 2B4 may be a novel target for therapeutic manipulation to control unwanted T cell responses in the setting of transplantation and autoimmunity.

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AUTHOR CONTRIBUTIONS

SJL and MLF conceived the study and wrote the manuscript. CT provided animals. SJL, DL, and MEW performed the research, and SJL analyzed the data.

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FIGURE LEGENDS

Figure 1. Generation of retrogenic graft-specific CD8$^+$ T cells that constitutively express 2B4. A, Frequencies of GFP$^+$ (pMY control vector) or GFP$^+$ 2B4$^+$ (for 2B4 vector) CD45.2$^+$ Thy1.1$^+$ OT-I BM cells at d2. B, BM cells were adoptively transferred into irradiated CD45.1$^+$ Thy1.2$^+$ animals and were detectable at 8–10 weeks. C, GFP$^+$ CD8$^+$ Thy1.1$^+$ (pMY) and GFP$^+$ 2B4$^+$ CD3$^+$ Thy1.1$^+$ (2B4rg) OT-I T cells sorted and D, adoptively transferred (10^6/recipient) into naïve B6 hosts that received two OVA-expressing skin grafts 48 hours later. Data shown in A–D are representative of 11-12 mice/group from 3 independent experiments.
E, 2B4 expression is maintained on the retrovirally transduced retrogenic cells following transplantation. F, 10^6 WT CD8$^+$ Thy1.1$^+$ or GFP$^+$ 2B4$^+$ CD3$^+$ Thy1.1$^+$ (2B4rg) OT-I T cells were adoptively transferred naïve B6 hosts 48 hours prior to receiving two OVA-expressing skin grafts. Animals received 250 μg of CTLA4Ig on post-operative Day 0, 2, 4, and 6. Graft survival was measured, and rejection was scored based on a loss of +90% viable tissue. Data shown in Figure 1 A–E are representative of 11-12 mice/group from 3 independent experiments. ***$p<0.0001$. Data shown in Figure 1F are representative of 12-18 mice/group from 2 independent experiments.

Figure 2. Ectopic expression of 2B4 results in reduced accumulation of antigen-specific CD8$^+$ T cells but does not impact function of antigen-specific CD8$^+$ T cells on a per cell basis. A, Representative flow cytometry plots gated on CD8$^+$ cells isolated from the spleen. B, Frequencies and absolute numbers of CD44^{hi} Thy1.1$^+$ of CD8$^+$ cells. C, IFN-γ$^+$ pMY or 2B4rg cells following *ex vivo* stimulation with PMA and ionomycin. D, Summary of the frequency of IFN-γ-producing Thy1.1$^+$ CD8$^+$ T cells, representative of 2 independent experiments with 7-8

mice/group. E, Summary of the absolute numbers of IFN-γ-producing Thy1.1$^+$ CD8$^+$ T cells, representative of 2-3 independent experiments with 7-12 mice/group. *p<0.05, ** p<0.001, *** p<0.0001.

Figure 3. Failure of 2B4 retrogenic cells to accumulate in the spleen is not due to differences in expression of the 2B4 ligand or of T cell activation or exhaustion markers. Naïve B6 animals received 10^6 2B4rg Thy1.1$^+$ OT-I T cells or pMY Thy1.1$^+$ OT-I controls and were challenged with OVA-expressing skin grafts. A, Representative flow histograms indicating expression intensity of CD44, CD48, CD127, KLRG-1, PD-1, and LAG-3 on CD8$^+$ Thy1.1$^+$ T cells isolated from the spleen 10 days following transplantation. B-G, Summary of the frequencies of CD44^{hi}, CD48$^+$, CD127$^+$, KLRG-1$^+$, PD-1$^+$, and LAG-3$^+$ CD8$^+$ Thy1.1$^+$ T cells isolated from the spleen on day 10. H-I, Representative flow dot plots and summary bar graphs illustrating the frequency of Annexin V and 7AAD double-positive OT-I T cells 10 days after transplantation.

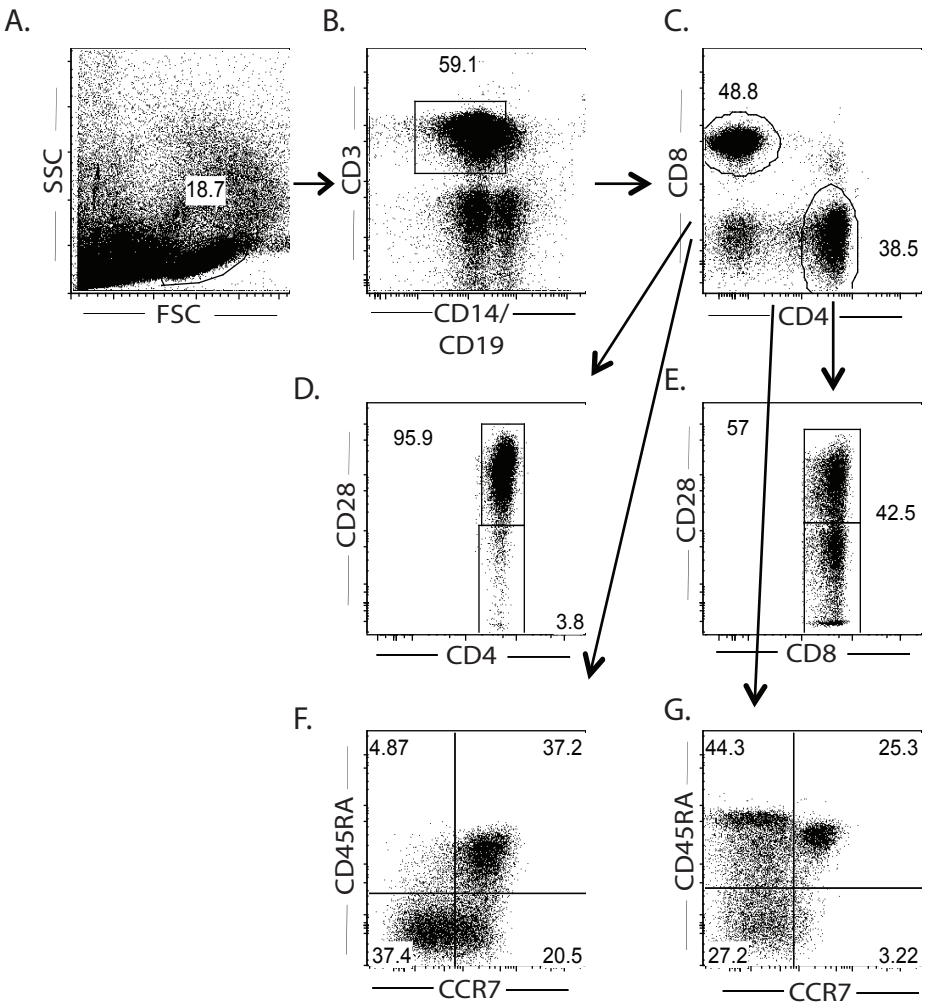
Figure 4. 2B4 rg cells undergo less division compared to their empty-vector pMY control counterparts. A and B, CTV dilution of Thy1.1$^+$ CD8$^+$ 2B4rg and Thy1.1$^+$ CD8$^+$ non-rg OT-I controls on day 10. C-D, Summary of frequency of 2B4rg and control OT-I T cells in each round of division. E, Frequencies of undivided CD8$^+$ T cells in the spleen on d 10. F, Summary of the number of Thy1.1$^-$ CD8$^+$ non-OT-I and Thy1.1$^+$ CD8$^+$ OT-I T precursors recruited into the anti-donor immune response. All data are representative of two independent experiments with a total of 9-11 mice/group. $p < 0.05$.

Figure 5. 2B4 signaling limits glycolytic capacity of CD8$^+$ T cells. A, cDNA was synthesized from 400 ng of RNA isolated from 2B4$^+$ WT or 2B4KO OT-I T cells following *in vitro* activation (7d) and restimulation (4d) and subsequently used to assess changes in glycolytic metabolism between 2B4$^+$ and 2B4KO cells via the Qiagen Glucose Metabolism RT^2 Profiler PCR Array. Representative of 2 independent experiments with a total of 6-8 mice per group. B, Summary derived from the data set described above in A of selected genes that were increased over baseline in OT-I T cells in the absence of 2B4. C, 3x10^6 WT or 2B4KO OT-I T cells were stimulated with 1nM SIINFEKL for 4 d. 2x10^5 OT-I Ficoll-purified cells were then plated as described in Supplemental Methods. D-E, Rate of ECAR during glucose-induced glycolysis was averaged from three measurements following the addition of glucose, while glycolytic capacity was calculated from the average of three measurements following the addition of oligomycin. C-E are representative of two experiments, $p < 0.0001$. F, 10^4 WT or 2B4KO OT-I T cells were transferred into naïve B6 hosts and infected with 10^4 CFU of LM-OVA two days later. After 14 days, recipients were sacrificed, splenocytes were prepared as a single-cell suspension, washed with PBS, and then incubated with 2-NBDG *ex vivo* and analyzed via flow cytometry. G, Summary of expression of 2-NBDG as gated in part F. $p = 0.01$. F-G are representative of 4-5 mice/group.

Figure 6. Proliferative advantage of 2B4 KO T cells is erased under glucose limiting conditions. A, 3x10^6 WT and 2B4KO OT-I T cells, respectively, were resuspended in complete media and a D-glucose solution was titered into the glucose-free complete media and diluted on a half-log scale. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 5 days. After 5 days in culture cells were harvested and stained for flow cytometry and CTV-dilution of

Thy1.1$^+$ CD8$^+$ antigen-reactive T cells was assessed. B-C, Representative flow cytograms and summary data depicting dilution of CTV in Thy1.1$^+$ OT-I T cell compartments under glucose-limiting culture conditions. D, Summary data depicting frequency of 7AAD and AnnexinV double positive apoptotic cells from each culture condition. Data are representative of 2 independent experiments with a total of 6-8 animals per group. p = 0.01

Figure 7. 2B4-mediated signals impact memory recall potential. A, 10^4 WT or 2B4KO OT-I T cells were transferred into naïve B6 hosts, which were infected with 10^4 CFU LM-OVA 2d later. Animals received OVA-expressing skin grafts at day 10 post-transplant and were sacrificed five days later. B-C, Representative flow cytograms and summary data depicting the expression of CD44, KLRG-1, CXCR3, and CD69 on Thy1.1$^+$ CD8$^+$ OT-I T cells isolated from the spleen. D-E, Splenocytes were restimulated *ex vivo* with PMA and ionomycin and analyzed by ICS. Data shown in A–E are representative of 6-11 mice/group from 2 independent experiments. p = 0.0005.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B.
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CD45RA
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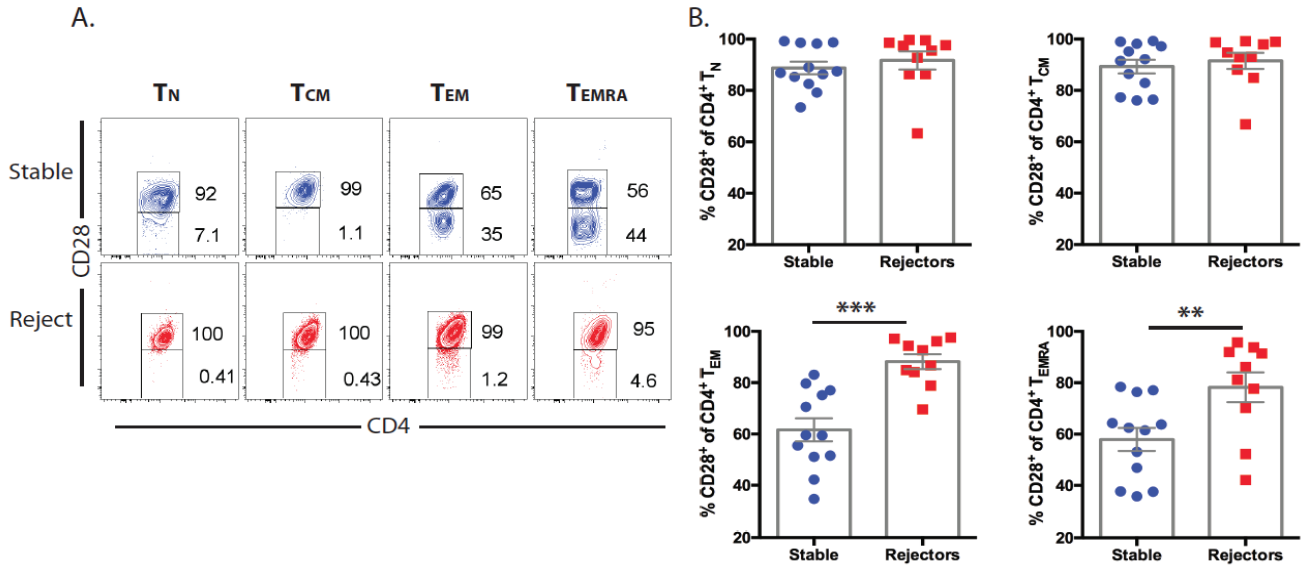
CCR7

Figure 2.1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% CD28+ of CD4+ TN
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Figure 2.2

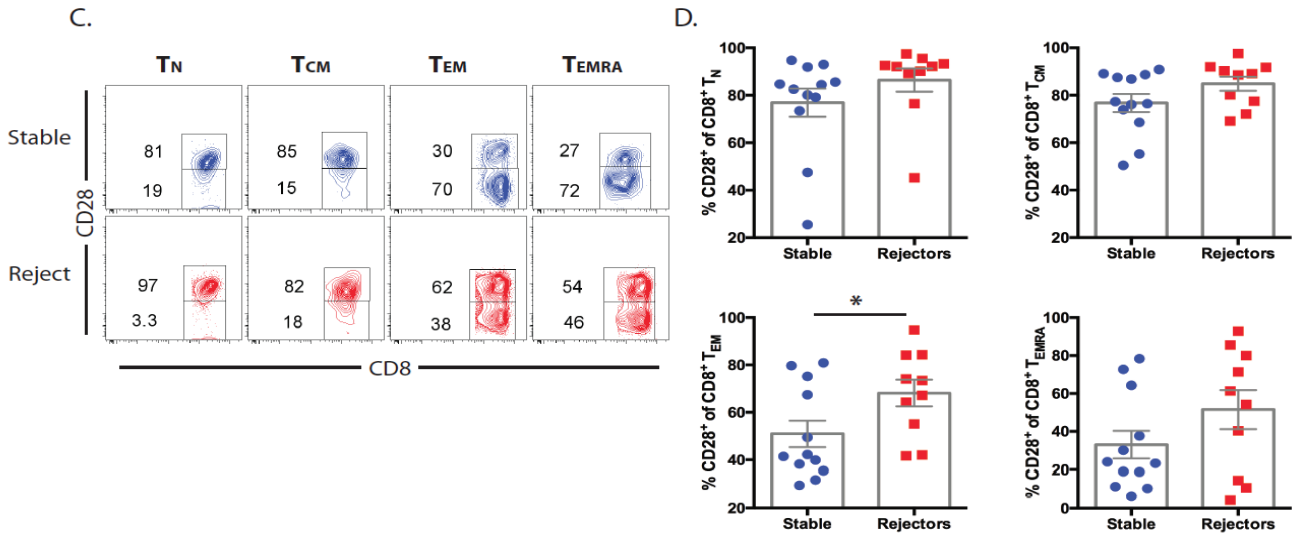
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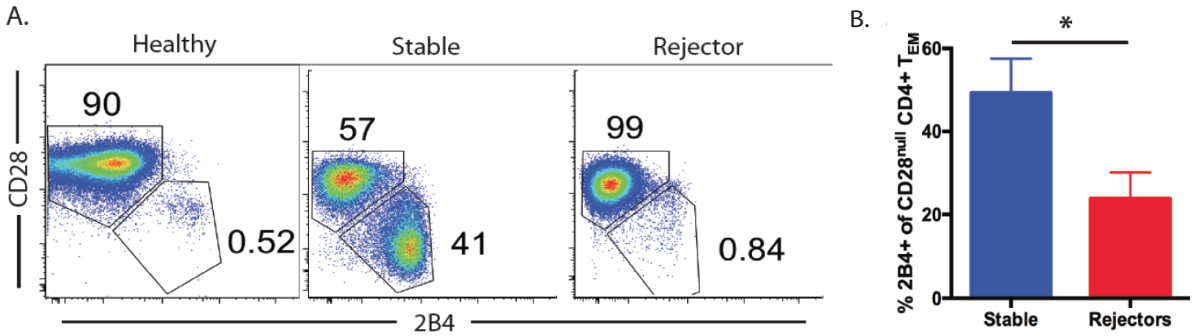
Figure 2.3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Rejector
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99
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CD28
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60
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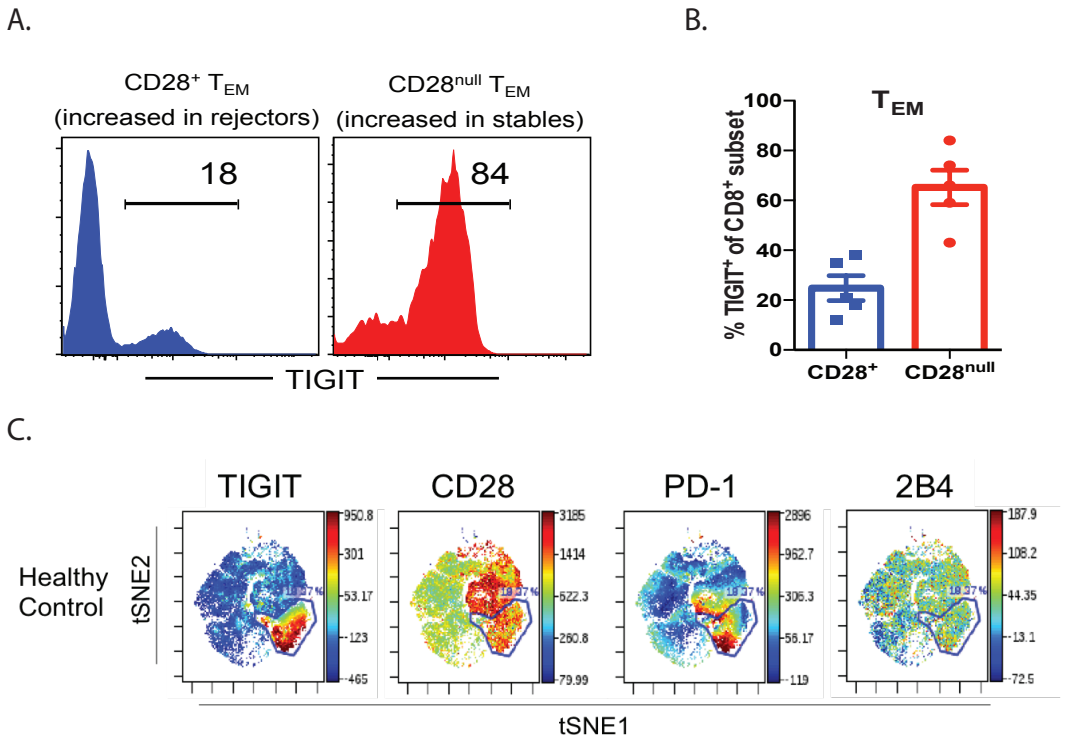
Rejectors

Figure 2.4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CD28+ TEM
(increased in rejectors)
CD28null TEM
(increased in stables)
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84
TIGIT
B.
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100
80
60
40
20
0
CD28+
CD28null
C.
TIGIT
CD28
PD-1
2B4
Healthy
Control
tSNE2
tSNE1

Figure 2.5

Table 1.
Transplant Recipient Characteristics for Belatacept Treatment Regimen

	Stables (n=13)	Rejectors (n=10)	*p value*
Median age (years)	51 (39-72)	55 (25-79)	0.61
Male/female (%)	11 (84.6)/2 (15.4)	7 (70)/3 (30)	0.73
Cause of ESRD			
Hypertensive	4	4	0.98
nephrosclerosis	5	2	0.61
Diabetes	4	3	0.67
Glomerulonephritis	0	1	0.89
PCKD			
CMV recipient status + (%)	11 (84.6)	7 (70)	0.73
Median time on dialysis (years)	5 (0.2-12)	5 (1.4-14)	0.98
Median time to rejection or follow up (months)	1 (1-5)	1.3 (0.5-7)	0.66

*Continuous variables: median, range. Categorical variables: absolute number and frequencies. CMV: cytomegalovirus; ESRD: end stage renal disease; PCKD: polycystic kidney disease

Table 2.1

Cut off of >80 (%$CD28^+$ of $CD4^+$ T_{EM})	Value	C.I.
Sensitivity	81.82%	48.22% to 97.72%
Specificity	92.31%	63.97% to 99.81%
Positive Predictive Value	90.00%	55.50% to 99.75%
Negative Predictive Value	85.71%	57.19% to 98.22%

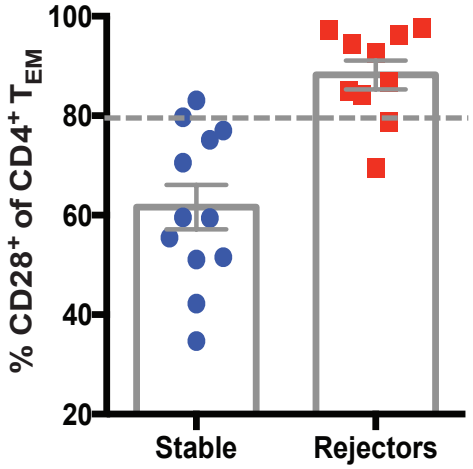

Table 2.2

Chapter 3:

2B4 Mediates Inhibition of $CD8^+$ T Cell Responses via Attenuation of Glycolysis and Cell Division

ABSTRACT

We recently showed that 2B4 expression on memory T cells in human renal transplant recipients was associated with reduced rates of rejection. To investigate whether 2B4 functionally underlies graft acceptance during transplantation, we established an experimental model wherein 2B4 was retrogenically expressed on donor-reactive murine $CD8^+$ T cells (2B4rg), which were then transferred into naïve recipients prior to skin transplantation. We found that constitutive 2B4 expression resulted in significantly reduced accumulation of donor-reactive $CD8^+$ T cells following transplantation, and significantly prolonged graft survival following transplantation. This marked reduction in alloreactivity was due to reduced proliferation of $CD8^+$ $Thy1.1^+$ 2B4rg cells as compared to control cells, underpinned by extracellular flux analyses demonstrating that 2B4 deficient (2B4KO) $CD8^+$ cells activated in vitro exhibited increased glycolytic capacity and upregulation of gene expression profiles consistent with enhanced glycolytic machinery as compared to WT controls. Furthermore, 2B4KO $CD8^+$ T cells primed in vivo exhibited significantly enhanced ex vivo uptake of a fluorescent glucose analog. Finally, the proliferative advantage associated with 2B4 deficiency was only observed in the setting of glucose sufficiency; in glucose-poor conditions 2B4KO $CD8^+$ T cells lost their proliferative advantage. Together, these data indicate that 2B4 signals function to alter T cell glucose metabolism, thereby limiting the proliferation and accumulation of $CD8^+$ T cells. Targeting 2B4 may therefore represent a novel therapeutic strategy to attenuate unwanted $CD8^+$ T cell responses.

INTRODUCTION

A fine balance of costimulatory and coinhibitory signals regulates the activation, differentiation, and proliferation of T cells following encounter with cognate allogeneic peptide:major histocompatibility complexes (pMHC). As such, manipulation of these cosignaling molecules may effectively inhibit unwanted T cell responses during autoimmunity and transplantation. 2B4 (SLAMf4, CD244) is an immunoglobulin (Ig) superfamily member expressed on natural killer (NK) cells and induced on some $CD8^+$ T cells (1-3). 2B4 contains an immunoreceptor tyrosine-based switch motif (ITSM) and is known to associate with the signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), an intracellular adaptor protein, and bind CD48, a surface Ig molecule widely expressed on hematopoietic cells (4-10). Binding of CD48 to 2B4 can provide costimulatory signals to neighboring T cells via direct cell-to-cell contact (11-13). SAP mediates its function in NK cells via a dual mechanism of action: it augments cell activation by recruiting the kinase Fyn, while simultaneously preventing inhibitory signals by uncoupling SLAM receptors from inhibitory phosphatases (14-17).

Previous studies have shown that 2B4 can be expressed on $CD8^+$ T cells with activated and memory-like phenotypes and the majority of studies suggest that it functions in a coinhibitory capacity to regulate responses on these cells. In particular, 2B4-deficient mice develop a spontaneous lupus-like disease dependent on aberrant T cell activation (18). Further, in mouse models of chronic infection, 2B4 has been shown to limit the expansion and functionality of secondary effector T cells (18, 19). More recently, we found that human transplant recipients that went on to experience stable graft function for at least one year post-transplant exhibited increased frequencies of $2B4^+$ $CD28^{null}$ effector memory T cells (20) at baseline as compared to

patients that experienced acute rejection following transplantation (21). These associative data implied that expression of 2B4 on T cells might dampen alloreactive immune responses; however, this hypothesis has not been formally tested, and potential mechanisms underlying it are unknown.

Studies over the last five years have described the impact of changes in cellular metabolism during T cell activation on the programmed differentiation of effector and memory T cell populations (22, 23). Broadly, resting T cells utilize oxidative phosphorylation as their primary source of energy, while effector cells undergoing rapid proliferation switch to aerobic glycolysis in order to meet the energetic needs of an exponentially expanding T cell clonal population (24). Specifically, while oxidative metabolism transitions glucose-derived pyruvate to the mitochondria for oxidation all the way down to carbon dioxide, glycolysis instead generates several key intermediates that the dividing cell can use for biosynthesis (25). Moreover, during aerobic glycolysis, some glucose is funneled through the mitochondria and a portion of the tricarboxylic acid (TCA) cycle in order to generate citrate for the synthesis of lipids necessary for construction of daughter cell membranes. These critical changes are initiated via integration of signals generated by the ligation of the TCR and costimulatory molecules on the T cell surface (26). Both TCR and costimulatory receptors trigger the activation of key signaling pathways that alter gene expression, including c-Myc and the nuclear hormone receptors ERRα, β, and γ and NR3B1, 2, and 3 (26). In addition, CD28 signaling functions to activate the PI3K/Akt/mTOR pathway. Both Akt and mTORC1 activation drive the cell toward aerobic glycolysis and promote the growth and function of effector T cells (27). Importantly, ligation of T cell coinhibitory receptors can also impact T cell metabolism. In particular, ligation of PD-1 was shown to result

in a shift from a glycolysis-dependent program to one in which T cells rely more heavily on fatty acid oxidation and lipolysis (28). Additionally, a recent report describes that in samples derived from human patients with gastric cancers, coinhibitory TIGIT signaling on $CD8^+$ T cells inhibits glucose metabolism (29). However, the impact of 2B4 coinhibition on T cell metabolism and programmed differentiation has been less well studied. Here, we used a retrogenic approach to express 2B4 on antigen-specific $CD8^+$ T cells, in order to understand the effects of 2B4 signaling on $CD8^+$ T cell programmed differentiation and cellular metabolism.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (30) and OT-II (31) transgenic mice were purchased from Taconic Farms (Germantown, NY) and bred to Thy1.1$^+$ background at Emory University. mOVA mice (C57BL/6 background, H-2b) (32) were a generous gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University (protocol number: DAR-2002050-092815GN). All surgery was performed under general anesthesia with maximum efforts made to minimize suffering. All animals were housed in specific pathogen-free animal facilities at Emory University.

Donor-Reactive T Cell Adoptive Transfers

In order to approximate the precursor frequency of donor-reactive cells in a fully MHC mismatched model of transplantation, we utilized our previously described system in which we adoptively transfer a higher frequency of OVA-specific TCR transgenic cells into naïve hosts prior to transplantation. For adoptive transfer of donor-reactive T cells, spleen and mesenteric lymph nodes (mLN) isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were processed and stained with monoclonal antibodies for CD8 (Invitrogen), CD4, Thy1.1, and Vα2 (all from BD Pharmingen) for flow cytometric analysis. Cells were resuspended in 1X phosphate buffered saline (PBS) and 10^6 of each Thy1.1$^+$ OT-I and OT-II were injected i.v. 48 hours prior to skin transplantation. Where indicated, OT-I T cells were isolated and labeled with 5 μM CellTrace

Violet dye (Life Technologies, Invitrogen) prior to adoptive transfer. Proliferation was measured following sacrifice ten days post-transplantation via flow cytometry on a BD LSR II (BD Biosciences) and data were analyzed with FlowJo (TreeStar) and Prism (GraphPad). Numbers of precursor cells recruited into the anti-donor immune responses were calculated by first multiplying the absolute number of $Thy1.1^+$ $CD8^+$ T cells by the frequency of cells within a given round of division. We then divided the number of cells in each division by 2^n, where n is the number of divisions and the total number of precursors that gave rise to the cells in each division were summed.

Skin Transplantation

Full thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (33). Where indicated in Figure 1, recipients were treated with CTLA-4 Ig (abatacept, Bristol-Myers Squibb) (500 μg i.p. on days 0, 2, 4, and 6). In all cases, grafts with less than 10% viable tissue remaining were scored as rejected

2B4 Plasmid Construction and Transfection

The murine 2B4 gene was derived from mouse cDNA (OriGene: MC209044-113649) and produced by PCR using the primers: (Forward: GCGAATTCGCACCATGTTGGGG CAAGCTGTCCTGTTCACAA, Reverse: CGCTCGAGCTAGGAGTAGACATCAAAGTT CTC). The resulting PCR fragment was cloned into the pMY-IRES-GFP retroviral vector (Cell Biolabs, RV-021) using EcoRI and XhoI cut sites. The Platinum-E retroviral packaging cell line (Cell Biolabs, RV-101. Ecotropic for rat and mouse cells) was used to produce the 2B4-

containing retrovirus. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 μg/ml puromycin, 10 μg/ml blasticidine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2, humidified atmosphere. The packaging cells were incubated in 10-cm plates at 4.5×10^6/plate overnight at 37°C. Transfections were performed with the reagent Lipofectamine LTX (Invitrogen, 15338-100). Cells were transiently transfected with 10 μg DNA (2B4 plasmid DNA or empty-vector control). After 48 hours incubation the culture supernatant was harvested and virus was concentrated per manufacturer's instructions (Cell Biolabs, RV-201).

Retroviral transduction and generation of 2B4rg OT-I T cells

Two days before transduction, bone marrow (BM) cells (BMC) were harvested from 8 to 12 week old OT-I transgenic mice and cultured at $1.5x10^7$ cells per 10 cm plate in 15 ml DMEM supplemented with 15% heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin, 10mM HEPES, 20 ng/ml murine interleukin-3 (IL-3), 50 ng/ml human IL-6 and 50 ng/ml murine stem cell factor (SCF) (R&D Systems). Concentrated virus was transduced into the pre-cultured BMCs. After 48 hours incubation bone marrow cells were collected and washed. 4×10^6 bone marrow cells in PBS were injected into sub-lethally irradiated (800 rads) wild type (WT) B6 recipients. Splenocytes from these BM chimeras were harvested 6-8 weeks post-transfer and were enriched by negative selection using a $CD8\alpha^+$ T cell Isolation Kit II (Miltenyi Biotec,). Purity of $CD8\alpha^+$ T cells was between 60 and 85%. Cells were then stained with anti-CD8 (Invitrogen), anti-Thy1.1 (BD Biosciences), and anti-2B4 (BD Biosciences) and GFP^+ $CD8^+$ $Thy1.1^+$ $2B4^+$ cells were purified by FACS sorting on a BD FACS Aria (BD Biosciences). Post-sort 2B4-OT-I T cell populations were over 94% pure. Cells were

resuspended in PBS with 10^6 wild-type $CD4^+$ $Thy1.1^+$ OT-II T cells and injected i.v. 48 hours prior to skin transplantation. Where indicated, retrogenic cells were prepared from the spleen and transferred i.v. without sorting along with wild-type $CD4^+$ $Thy1.1^+$ OT-II T cells 48 hours prior to skin transplantation. Upon sacrifice of the recipients ten days following transplantation, these cells were analyzed separately form bulk splenocytes via flow cytometric gating.

Flow Cytometry and Intracellular Cytokine Staining

Cells isolated from spleens and graft-draining axillary and brachial lymph nodes (dLN) were stained with anti-CD4 (BD Biosciences), anti-CD8 (Invitrogen) and anti-Thy1.1 (BD Biosciences). For phenotypic analysis cells were also surface-stained with anti-PD-1 (BioLegend), anti-2B4 (BD Biosciences or eBioSciences), anti-Thy1.1 (BD Biosciences), anti-LAG-3 (BioLegend), anti-CD127 (BioLegend), anti-KLRG-1 (eBioSciences), anti-CD44 (BioLegend or BD Biosciences), and anti-CD48 (BioLegend). Absolute numbers of lymphocytes from the spleen and draining lymph nodes were calculated using a Cellometer Auto T4 Cell Viability Counter (Nexcelom) according to the manufacturer's instructions. Samples were analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo 9 software (Treestar, San Carlos, CA) and Prism 6 software (GraphPad Software Inc.). For intracellular cytokine staining, lymphocytes were restimulated *ex vivo* with 1 μg/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Life Sciences) and 1 μg/mL ionomycin (Sigma Life Sciences) where indicated, in the presence of 1 μg/mL Brefeldin A (BD Biosciences) for 4 hours. The Fix/Perm intracellular staining kit (BD Pharmingen) was used to detect IL-2 (BD Biosciences), TNF (BioLegend), and IFN-γ (BD Biosciences), according to manufacturer's instructions.

RT-PCR Analysis

Splenocytes were isolated and prepared as a single-cell suspension. $3x10^6$ WT and 2B4KO OT-I T cells, respectively, were resuspended in 1.5 mL of complete media (RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 0.5 mM 2-mercaptoethanol) in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 4 days. After 4 days cells were collected and live cells were isolated via Ficoll gradient separation. $1x10^6$ live cells were plated with $5x10^6$ naïve splenocytes stimulator cells isolated from WT C57BL/6 donors and restimulated with 1 nM SIINFEKL peptide for four additional days in a final volume of 1.5 mL in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. Following restimulation, cells were harvested and incubated with Pacific Orange anti-CD8 (Invitrogen), PerCP anti-Thy1.1 (BD Biosciences), and PE-Cy7 anti-CD244 (eBioSciences) for 30 minutes at 4°C. Following staining, cells were washed with sterile 1x PBS and resuspended in ~1 mL of sorting buffer (sterile 1x PBS with 2% serum and 1 mM EDTA). Cells were sorted on FACSAriaII (BD Biosciences) and 2B4KO and $2B4^+$ OT-I T cells were isolated to at least 90% purity. FACS-purified cells were then resuspended in RLT buffer with β-ME (Qiagen, 350 μl for $>5x10^6$ cells, 600 μl for $<5x10^6$ cells) and flash frozen on dry ice for 10 minutes prior to storage at -80°C. RNA was isolated from previously frozen cells using the RNEasy Mini Kit (Qiagen, Germantown, MD) and quantified using a Nanodrop Microvolume Spectrophotometer (Thermofisher, Waltham, MA). RNA was converted to cDNA using the RT^2 First Strand Kit (Qiagen), and RT-PCR was performed using the Qiagen Glucose Metabolism RT^2 Profiler PCR Array with the RT^2 SYBR Green qPCR Mastermix (Qiagen) on a BioRad CFX384 Touch Real-

Time PCR Detection System. Data was analyzed online via the Qiagen GeneGlobe Data Analysis Center.

Seahorse XF Glycolysis Stress Test

Splenocytes were isolated and stimulated for four days as described above. Following Ficoll separation, $2x10^5$ OT-I live cells were then plated in buffer-free, glucose-free XF assay media (Agilent Technologies) supplemented with 2 mM L-glutamine, pH 7.4+/0.1. Extracellular acidification rate (ECAR) was measured and recorded at basal conditions as well as at indicated time points following the addition of glucose (final concentration 10 mM/well), oligomycin (final concentration 1 μM/well), and 2-deoxyglucose (final concentration 50 mM/well), using a Seahorse XFe96 Analyzer (Agilent Technologies). Basal ECAR readings were generated from the average of five measurements prior to the addition of oligomycin, while maximal ECAR was calculated from the average of three measurements following the addition oligomycin.

In vitro Glucose-limiting Stimulation Assays

Splenocytes were isolated and prepared as a single-cell suspension. $3x10^6$ WT and 2B4KO OT-I T cells, respectively, were resuspended in 1.5 mL of complete media (glucose-free RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 0.5 mM 2-mercaptoethanol) in a 24-well flat-bottomed plate at 37°C in a 5% CO_2, humidified atmosphere. A D-glucose solution of 200g/L (Gibco by Life Technologies) was titered into the glucose-free complete media and diluted on a half-log scale. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 5 days. After 5 days in culture cells were harvested and stained with anti-CD8 (BD Horizon), anti-Thy1.1 (BD

Biosciences or BioLegend), anti-CD244 (eBioSciences), anti-Va2 (BD), anti-CD127 (BioLegend), anti-CD62L (BD), anti-KLRG-1, anti-Vb5 (BD), anti-CD44 (BD), 7AAD (BD) and AnnexinV (BioLegend), anti-IL-2 (BD), anti-IFN-g (BD) as described by the manufacturer. As described above, samples were analyzed on an LSRII flow cytometer (BD Biosciences) and data was analyzed using FlowJo 9 software (Treestar, San Carlos, CA) and Prism 6 software (GraphPad Software Inc.).

2-NBDG Uptake Assay

To assess the ability of 2B4-deficient OT-I T cells to take up glucose, 10^4 WT and 2B4KO OT-I T cells were transferred into naïve C57BL/6 hosts and infected them with 10^4 colony forming units (CFU) of OVA-expressing *Listeria monocytogenes* (LM-OVA) two days. 14 days after infection, the animals were sacrificed and spleens were harvested. The cells were isolated and resuspended in a single cell solution in PBS. $2x10^6$ splenocytes were stained with anti-CD4 (BD Biosciences), anti-CD8 (Invitrogen) and anti-Thy1.1 (BD Biosciences), anti-CD44 (eBioSciences), anti-CD44 (BD Biosciences) for 30 minutes at 4°C. Cells were washed twice in 250 µl of PBS and then resuspended in 200 µl of 50 µM 2-NBDG (Thermofisher) and incubated at 37°C for 30 minutes. Cells were washed with PBS and then analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo 9 software (Treestar) and Prism 6 software (GraphPad Software Inc.).

Statistical Analysis

T cell responses were analyzed using unpaired, non-parametric Mann-Whitney t-tests. Results were considered significant if $p < 0.05$. Survival curves were analyzed by log-rank test and

plotted on Kaplan-Meier curves. All analyses were done using Prism software (GraphPad Software Inc.).

RESULTS

Ectopic expression of 2B4 on donor-reactive CD8$^+$ T cells results in prolongation of allograft survival

Given the observation that increased 2B4 expression was associated with reduced incidence of rejection in renal transplant recipients treated with belatacept (21), we investigated the causal role of 2B4 expression in attenuating donor-reactive CD8$^+$ T cell responses following transplantation. To test this we utilized a retrogenic approach to generate donor-reactive CD8$^+$ T cells that constitutively express 2B4 (2B4rg). Though primary effector cells do not express 2B4 at baseline (Figure 1E), we have previously identified an association between 2B4 expression on T cells and improved graft survival in both murine models and human transplant recipients (21, 34). Thus, we aimed to create a system in which we could isolate and interrogate this effect, and in which we would be able to determine if upregulation of 2B4 can functionally impact alloreactive T cell responses. To this end, we utilized retrovirally-transduced bone marrow derived from OT-I mice with a 2B4-bearing construct or an empty control vector. Transduced cells express GFP under the control of the IRES promoter, allowing us to identify, isolate, and track these cells in both in vivo and in vitro assays. Briefly, CD45.2$^+$ Thy1.1$^+$ OT-I bone marrow was transduced with a construct that expresses 2B4 under a constitutively active viral promoter and contained an IRES-GFP to facilitate tracking the cells. At day 2 post transduction, ~5–10% of Thy1.1$^+$ OT-I BM cells expressed GFP alone (for pMY control vector-transduced cells) or both GFP and 2B4 (for 2B4 vector-transduced cells) (Figure 1A). BM cells were then adoptively transferred into irradiated CD45.1$^+$ Thy1.2$^+$ animals. At 8–10 weeks post-in vivo transfer, GFP labeled Thy1.1$^+$ OT-I T cells were detectable in recipients of 2B4 vector-transduced and pMY control vector-transduced BM cells at similar frequencies (Figure 1B), suggesting that 2B4

expressing OT-I T cells mature normally in these animals. GFP^+ $CD8^+$ $Thy1.1^+$ (for pMY) or GFP^+ $2B4^+$ $CD3^+$ $Thy1.1^+$ (for 2B4rg) OT-I T cells were isolated from the spleen and mesenteric lymph nodes of pMY or 2B4rg chimeric animals and then MACS and FACS sorted to >86% purity (Figure 1C).

To determine the impact of constitutive 2B4 expression on graft-specific alloimmune responses, naïve B6 animals were adoptively transferred with 10^6 congenically labeled 2B4rg $Thy1.1^+$ OT-I T cells (or pMY $Thy1.1^+$ OT-I controls) along with 10^6 $Thy1.1^+$ $CD4^+$ WT OT-II T cells and then challenged with an OVA-expressing skin graft (Figure 1D). Animals were sacrificed at day 10 post-transplant, the magnitude of the $CD8^+$ $Thy1.1^+$ response was assessed. Importantly, expression of 2B4 was maintained in the 2B4rg OT-I T cells during the *in vivo* response (Figure 1E). To determine the impact of donor-reactive $CD8^+$ T cell 2B4 expression on graft survival, recipients of either 2B4rg or control OT-I T cells were monitored for graft survival. Under these conditions, we saw no difference in graft survival (not shown). Likewise, there was also no difference in graft survival when OT-I T cells were WT vs. 2B4 KO (data not shown). To test whether or not expression of 2B4 can prolong graft survival in the context of minimal immunotherapy, we adoptively transferred 2B4rg OT-I T cells into animals that received a low dose of CTLA4Ig in the first week following transplantation in order to extend graft survival enough to allow us to see any potential differences in survival between animals that received the WT vs. 2B4KO cells. Results indicated a significant prolongation in survival in recipients of 2B4rg donor-reactive $CD8^+$ T cells as compared to recipients of control donor-reactive $CD8^+$ T cells (MST 31.5 vs. 23, $p=0.01$) (Figure 1F).

Ectopic expression of 2B4 resulted in reduced accumulation of antigen-specific CD8$^+$ T cells

In order to investigate the mechanisms underlying the observed prolongation in graft survival in CTLA-4Ig-treated graft recipients possessing 2B4rg donor-reactive CD8$^+$ T cells, spleens and draining LN were harvested on day 10 post-transplantation and the magnitude and functionality of the Thy1.1$^+$ CD8$^+$ T cell response was assessed. Results revealed detectable populations of CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells in the spleens and lymph nodes (data not shown) of mice that received either pMY or 2B4rg OT-I T cells, respectively (Figure 2A). Strikingly, constitutive expression of 2B4 resulted in significantly decreased accumulation of donor-specific CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells in the spleen following transplantation in the absence of any further immune modulation, both in terms of frequency and absolute number (Figure 2B). In contrast, retrogenic 2B4 expression did not significantly impair production of IFN-γ on a per cell basis following *ex vivo* restimulation (Figure 2C and 2D), though fewer total cytokine-secreting OT-I T cells could be detected in the spleen 10 days following transplantation (Figure 2E). Taken together, these results indicate that expression of 2B4 on CD44^{hi} Thy1.1$^+$ CD8$^+$ T cells results in the attenuation of graft-specific T cell responses in the spleen 10 days following transplantation.

Failure of 2B4 retrogenic cells to accumulate in the spleen is not due to differences in expression of the 2B4 ligand CD48, T cell activation or exhaustion markers, or T cell death

We next investigated whether or not the reduced accumulation of Thy1.1$^+$ CD8$^+$ T cells in the spleens of 2B4rg recipients was due to differences in the activation, differentiation, or exhaustion status of these cells. In experiments similar to those described above, naïve B6 animals were adoptively transferred with 10^6 congenically labeled 2B4rg Thy1.1$^+$ OT-I T cells or

pMY controls, along with 10^6 Thy1.1$^+$ CD4$^+$ WT OT-II T cells, challenged with an OVA-expressing skin graft, and sacrificed at day 10 post transplant at which time splenic T cells were stained for markers of activation, differentiation, and exhaustion and analyzed by flow cytometry. Analysis of cell surface phenotypes indicates there was no difference in the activation status of the 2B4rg Thy1.1$^+$ OT-I T cells when compared to the pMY Thy1.1$^+$ OT-I controls as measured by CD44 expression (Figure 3A). There was also no detectible difference in the presence of short-lived vs. memory precursor effector cells as distinguished by CD127 and KLRG-1 staining (Figures 3A, 3B and 3C). Additionally, no differences were found in the expression of PD-1 and lymphocytes activation gene 3 (LAG-3) exhaustion markers on 2B4rg vs. pMY Thy1.1$^+$ OT-I T cells (Figures 3A, 3D and 3E). Finally, the expression of the 2B4 ligand CD48 did not differ between the 2B4rg Thy1.1$^+$ OT-I or pMY Thy1.1$^+$ OT-I control cells (Figures 3A and 3F). Overall, these data suggest that the failure of 2B4rg Thy1.1$^+$ OT-I cells to accumulate in the spleen after transplantation was not due to differences in their expression of the 2B4 ligand or T cell activation or exhaustion markers. We therefore next sought to determine whether the paucity of 2B4rg cells observed in the spleen 10 days after transplantation was due to increased cell death. As depicted in Figure 3G, no increase in cell death as measured by Annexin V$^+$ 7-AAD$^+$ double-positivity was observed in the 2B4rg Thy1.1$^+$ OT-I T cells as compared to pMY controls. Instead, 2B4rg OT-I T cells actually exhibited reduced frequencies of Annexin V$^+$ 7-AAD$^+$ cells (Figure 3H). These data therefore demonstrate that the observed reduced accumulation of 2B4rg graft-specific CD8$^+$ T cells was not due to increased cell death within the 2B4rg compartment.

2B4 rg OT-I T cell population exhibits less division compared to pMY control OT-I due to reduced recruitment into the anti-donor immune response

Because the above experiments failed to explain impaired accumulation of 2B4-expressing $CD8^+$ T cells following transplantation, we next asked if the reduced accumulation of 2B4rg graft-specific OT-I T cells following transplantation was the result of differences in the amount of cell division following activation. To test this, GFP^+ $CD8^+$ $Thy1.1^+$ OT-I T cells were isolated from spleen and mLN of 2B4rg chimeric animals and stained with CellTrace Violet prior to being adoptively transferred (10^6 /recipient) into naïve B6 hosts that received 10^6 WT $CD4^+$ $Thy1.1^+$ OT-II T cells and were grafted with an OVA-expressing skin graft. As previously described, animals were sacrificed on day 10 post-transplant 2B4rg chimeric cells were isolated from the spleen analyzed by flow cytometry. Our analyses revealed striking differences between the frequency and number of GFP^+ $CD8^+$ $Thy1.1^+$ OT-I T cells dividing in the 2B4rg and non-retrogenic compartments (Figures 4A, 4B and data not shown). The frequency of OT-I cells that underwent 4 or more rounds of division was significantly lower in the 2B4rg OT-I populations as compared to the GFP single-positive controls (Figures 4C and 4D). Furthermore, we observed a statistically significant increase in the number of 2B4rg OT-I that remained undivided 10 days following transplantation as compared to pMY OT-I controls (Figure 4E). Additional analyses of cell division calculating the number of precursors that were recruited into the response (based on the number of cells present in each CTV peak at day 10 as described in materials and methods) revealed that fewer 2B4rg OT-I precursors were recruited into the response as compared to pMY OT-I precursors (Figure 4F). Taken together, these findings demonstrate that expression of 2B4 impairs expansion of the anti-donor $CD8^+$ effector population by preventing cells from entering the cell cycle.

2B4 expression limits glycolytic capacity of CD8$^+$ T cells

To determine the mechanism by which constitutive expression of 2B4 impairs proliferation of anti-donor CD8$^+$ T cells, we next assessed T cell metabolism. It is known that T cells undergo metabolic reprogramming as they differentiate into effectors, and that glycolysis is the primary method by which these cells derive the energy needed to proliferate during this process (35, 36). As bioenergetic needs of T cells are significantly augmented during this critical time, it is possible to detect a notable increase in the uptake and utilization of glucose soon after activation (37). To address whether or not glycolysis was altered in the absence of 2B4, we utilized a model in which we stimulated WT and 2B4-deficient (2B4KO) OT-I T cells in vitro and then probed their metabolic capacity. To test the hypothesis that 2B4 signaling limits CD8$^+$ T cell proliferation by inhibiting glycolysis, we established a model in which WT and 2B4KO OT-I T cells were stimulated in culture for seven days with their cognate peptide SIINFEKL, purified via Ficoll gradient centrifugation, and then restimulated for four more days in vitro. After restimulation, 2B4$^+$ cells were purified to >90% purity from WT OT-I T cells by FACS sorting, and 2B4KO OT-I T cells were confirmed to be 2B4$^-$ by flow cytometry. Following sorting, we assessed the expression of 84 genes associated with glucose metabolism in 2B4$^+$ WT vs. 2B4 KO cells using the Qiagen Glucose Metabolism RT^2 Profiler PCR Array. Of the 84 genes assessed in this array, we noted that 42 were differentially expressed between 2B4$^+$ OT-I cells isolated from WT animals and 2B4KO OT-I T cells (Figure 5A). Of those, a number were associated with glycolytic shifts in response to activation, the pentose phosphate pathway, the TCA cycle, and in aerobic glycolysis (Figure 5B).

Based on the differential expression of genes in the glycolytic pathways identified via transcriptional profiling, we next endeavored to assess functional changes in T cell metabolism based on 2B4 expression using the Seahorse XFe96 Analyzer. As demonstrated in Figures 5C, we found that the loss of 2B4 on antigen-specific T cells results in enhanced glycolytic metabolism as measured by an increase in extracellular acidification rate (ECAR) during glucose-induced glycolysis (38). Additionally, we observed that the ability of OT-I T cells to perform glucose-induced glycolysis was greater in the absence of 2B4, and that the total glycolytic capacity of the 2B4KO cells was greater than that of the wild type controls (Figure 5D-E). Next, to determine whether 2B4KO T cells achieved increased glycolytic capacity in part via increasing their uptake of glucose from the extracellular environment, we moved to an in vivo model of T cell stimulation followed by ex vivo incubation with the fluorescent glucose analog 2-NBDG (Figure 5E-G). Because WT graft-elicited $CD8^+$ T cell responses did not exhibit high frequencies of 2B4-expressing cells (Figure 2 and (34)), we moved to a model of bacterial infection. Briefly, 14 days following infection with OVA-expressing *Listeria monocytogenes,* $2x10^6$ cells were plated directly ex vivo and incubated with 2-NBDG for 30 minutes, and uptake was determined by assessing fluorescence via flow cytometry. Data indicated that uptake of 2-NBDG was significantly increased in 2B4KO $CD8^+$ T cells as compared to WT $CD8^+$ T cells (Figure 5F-5G).

Proliferative advantage of 2B4 KO T cells is erased under glucose limiting conditions

Our data thus far demonstrated increased proliferation and increased glycolysis in the setting of 2B4 deficiency. In order to identify a causal link between these two observations, we queried whether the proliferative advantage observed in 2B4 KO T cells would persist under glucose-

restrictive conditions. To test this, WT and 2B4KO OT-I T cells were labeled with CellTrace Violet prior to stimulation with SIINFEKL N4 peptide at 1 nM in media containing varying concentrations of glucose as described in Materials and Methods and Figure 6A. Our results demonstrate that 2B4KO OT-I T cells restimulated in glucose-replete media exhibited a statistically significant increase in CTV dilution relative to WT control cells (Fig. 6B-C) as expected, demonstrating that 2B4 deficiency confers a proliferative advantage when glucose is abundant, in these experiments at the level of 3 mM or higher. In contrast, when glucose was present only at low levels (1 mM or less) WT and 2B4KO OT-I T cells exhibited similar levels of CTV dilution (Fig. 6B, 6C). To rule out the possibility that measured proliferation of these cells may have been impacted by increased rates of cell death in the cultures with low glucose, we assessed apoptosis by quantifying the frequency of 7AAD and AnnexinV double-positive cells and show that there were no differences between death of WT and 2B4KO cells in any culture condition (Fig. 6D). These data thus suggest that the observed differential proliferation of 2B4KO T cells is dependent on their ability to undergo increased glycolytic metabolism.

2B4-mediated signals impact CD8$^+$ T cell memory recall potential

To determine whether or not enhanced glycolytic capacity following activation impacts the functionality of 2B4KO T cells, we transferred WT and 2B4KO OT-I T cells into naïve C57BL/6 hosts and then infected them with OVA-expressing *Listeria monocytogenes* (LM-OVA) two days later (Fig. 7A). Ten days later, each animal received an OVA-expressing skin graft (Fig. 7A). Animals were sacrificed five days following transplantation, and spleens as well as axillary and brachial draining lymph nodes were harvested for analysis. In comparison to the wild type controls, graft-specific cells lacking 2B4 express less KLRG-1 (Figure 7B, 6C), which has been suggested to be associated with a senescent-like program (40). Additionally, the 2B4KO cells

express higher levels of CXCR3 and CD69 (Figure 7B, 6C), consistent with a model in which the loss of 2B4 results in a more activated phenotype. CD44 expression was not different between the two cell types (Fig. 7B-C). Strikingly, we found in addition to their activated phenotype, graft-specific 2B4 KO $CD8^+$ $Thy1.1^+$ secondary effectors contained a higher frequency of IFN-γ^+ IL-2^+ double producers upon rechallenge with a skin graft as compared to WT $CD8^+$ $Thy1.1^+$ cells (Fig. 7D, 6E). These data support the conclusion that loss of 2B4 coinhibitory signaling on $CD8^+$ donor-reactive T cells results in enhanced metabolism and altered memory T cell differentiation that leads to more robust secondary recall responses following rechallenge.

DISCUSSION

In this study, we showed that constitutive expression of 2B4 prolongs allograft survival and limits alloreactivity by attenuating the accumulation of donor-specific $CD8^+$ T cells following transplantation (Figs. 1A, B). Our data reveal increased T cell glycolytic metabolism in the absence of 2B4, demonstrated both functionally using the Seahorse extracellular flux assay as well as by analysis of changes in expression of 84 metabolism-associated genes. Of note, genes upregulated in activated 2B4-deficient $CD8^+$ T cells were associated with glycolytic machinery, the pentose phosphate pathway, the TCA cycle, and aerobic glycolysis. For example, *Eno2* and *Eno3*, encode Enolases 2 and 3, metalloenzymes responsible for the catalysis of the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the ninth and penultimate step of glycolysis. We also noted an increase in *G6pdx* in cells deficient in 2B4 (Fig 5B). *G6pdx* encodes glucose-6-phosphate dehydrodgenase X-linked, a key enzyme in the pentose phosphate pathway, a mechanism of generating energy that is strongly relied upon by proliferating T cells (43). Additionally, the enhanced presence of transcripts for *Pdk1*, which encodes pyruvate dehydrogenase kinase 1, in the absence of 2B4, suggests that 2B4-mediated signaling may promote aerobic glycolysis by inhibiting the activity of pyruvate dehydrogenases (22). Overall, these findings on the impact of 2B4 on T cell metabolism are consistent with recent studies showing that T cell coinhibitory molecules can impact immunometabolism. For example, signaling via PD-1 on activated T cells was shown to prevent glycolysis (36). Similarly, work from Wherry and colleagues recently showed that PD-1 signaling impacts early glycolytic activity in T cells and represses the transcriptional regulator PCG-1a; forced overexpression of PGC-1a was able to improve the functionality of exhausted T cells (44). Interestingly, PD-1 signaling also augmented lipolysis and β-oxidation of fatty acids, leading to a

marked increase in mitochondrial spare respiratory capacity (SRC) (36). PD-1-mediated reinforcement of FAO may serve to explain long-term preservation of these cells during cancer and persistent viral infection. In support of this, recent work has shown that signaling via PD-1 activates AMP-activated protein kinase (AMPK), which is required for the survival and longevity of T cells in nutrient-compromised microenvironments (45). Together with these published studies on the role of PD-1, our data further highlight the emerging role for coinhibitory molecules in the control of T cell bioenergetics and metabolism.

While our study demonstrates that signaling via 2B4 on antigen-specific $CD8^+$ T cells modulates glycolytic metabolism, further studies are needed to assess the impact of this coinhibitor on alternative mechanisms of energy generation, such as fatty acid oxidation. Our data indicate that *Idh2* and *Idh3a* are increased in the absence of 2B4 (Figure 5B). These genes encode isocitrate dehydrogenases that are critical in the tricarboxylic acid (TCA) cycle, which has been shown to be increased in activated T cells, but not to the same degree as glycolysis (46), more work is warranted to dissect a possible role of oxidative metabolism in contributing to T cell function and fate in the presence or absence of 2B4.

Our data also reveal an impact of 2B4-mediated metabolic alterations on programmed T cell differentiation; specifically, 2B4-deficient OT-I cells exhibited enhanced effector recall potential after rechallenge when compared to the wild-type controls (Fig. 7). Thus, 2B4 deficient cells exhibit increased glycolytic metabolism during primary differentiation but also augmented recall responses. These findings of increased glycolytic function being associated with improved recall responses are seemingly at odds with work from Sukumar and colleagues, which showed that

enhanced glycolytic flux promotes a state of terminal differentiation, while inhibiting it promotes the generation of long-lived memory $CD8^+$ T cells (47). More recent work, however, has shown that the division of effector-like and memory-like $CD8^+$ T cells into populations that rely on glycolysis and oxidative phosphorylation of fatty acids, respectively, is not so clear cut. For example, van der Windt et al. showed that memory $CD8^+$ T cells exhibit both increased glycolysis and oxidative phosphorylation as compared to primary effectors (48), and a more recent study demonstrated that elevated oxidative phosphorylation is dispensable for the ability of T cells to form long-lived stable memory following an acute infection (38). In line with these findings, the results that we present here demonstrate that the enhanced glycolytic capacity and 2-NBDG uptake observed in the absence of 2B4 (Fig. 5) is correlated with enhanced memory recall potential following rechallenge (Fig. 7). These findings further are supported by a recent study demonstrating that the differentiation of $CD8^+$ effector memory T cells during persistent viral infection was supported by consistent glycolytic metabolism (38); the mechanisms by which immunometabolism controls memory T cell differentiation remains an area of intense investigation.

It is interesting to note that ectopic 2B4 expression resulted in a trend toward decreased effector cytokine production by antigen-specific primary $CD8^+$ T cells on a per cell basis (Fig. 2C-E), and a statistically significant decrease in the absolute number of IFN-γ-secreting cells per spleen. Likewise, the absence of 2B4 on secondary effectors conversely enhanced cytokine production following antigenic rechallenge (Fig. 4B and C). We speculate that this attenuation in cytokine effector function by 2B4 signaling is mediated by altered immunometabolism. Indeed, a recent report demonstrated that conversion to glycolysis is required for effective IFN-γ production, and

showed that this requirement is due to the binding of the glycolysis enzyme GAPDH to AU-rich elements within the 3' UTR of IFN-γ mRNA (49). Thus, expression of GAPDH induced by aerobic glycolysis controls effector cytokine production. These data support our hypothesis that 2B4-mediated regulation of glycolytic metabolism in T cells affects effector function.

Finally, the results presented in our study suggest that as a regulator of metabolism, 2B4 may be an important therapeutic target in the design of new strategies to control donor-reactive T cells following transplantation. Indeed, recent work demonstrated that the blockade of both glycolysis and glutamine metabolism results in the prevention of allograft rejection in a model of fully MHC-mismatched skin and cardiac transplantation, suggesting that the manipulation of effector cell metabolism is an important mechanism by which alloimmunity can be controlled (35). In this study we present data suggesting that engagement of 2B4 can negatively regulate donor-reactive T cell responses in the context of transplantation. This is of translational importance, as we have recently published that not all alloreactive T cells are targeted by the CD28 costimulation blocker belatacept following transplantation, and that those that express low levels of CD28 express high levels of 2B4 (20, 50, 51). In sum, our study suggests that agonistic ligation of 2B4 may be a novel target for therapeutic manipulation to control unwanted T cell responses in the setting of transplantation and autoimmunity.

FIGURE LEGENDS

Figure 1. Generation of retrogenic graft-specific CD8$^+$ T cells that constitutively express 2B4. A, Frequencies of GFP$^+$ (pMY control vector) or GFP$^+$ 2B4$^+$ (for 2B4 vector) CD45.2$^+$ Thy1.1$^+$ OT-I BM cells at d2. B, BM cells were adoptively transferred into irradiated CD45.1$^+$ Thy1.2$^+$ animals and were detectable at 8–10 weeks. C, GFP$^+$ CD8$^+$ Thy1.1$^+$ (pMY) and GFP$^+$ 2B4$^+$ CD3$^+$ Thy1.1$^+$ (2B4rg) OT-I T cells sorted and D, adoptively transferred (10^6/recipient) into naïve B6 hosts that received two OVA-expressing skin grafts 48 hours later. Data shown in A–D are representative of 11-12 mice/group from 3 independent experiments.
E, 2B4 expression is maintained on the retrovirally transduced retrogenic cells following transplantation. F, 10^6 WT CD8$^+$ Thy1.1$^+$ or GFP$^+$ 2B4$^+$ CD3$^+$ Thy1.1$^+$ (2B4rg) OT-I T cells were adoptively transferred naïve B6 hosts 48 hours prior to receiving two OVA-expressing skin grafts. Animals received 250 μg of CTLA4Ig on post-operative Day 0, 2, 4, and 6. Graft survival was measured, and rejection was scored based on a loss of +90% viable tissue. Data shown in Figure 1 A–E are representative of 11-12 mice/group from 3 independent experiments. ***$p<0.0001$. Data shown in Figure 1F are representative of 12-18 mice/group from 2 independent experiments.

Figure 2. Ectopic expression of 2B4 results in reduced accumulation of antigen-specific CD8$^+$ T cells but does not impact function of antigen-specific CD8$^+$ T cells on a per cell basis. A, Representative flow cytometry plots gated on CD8$^+$ cells isolated from the spleen. B, Frequencies and absolute numbers of CD44^{hi} Thy1.1$^+$ of CD8$^+$ cells. C, IFN-γ$^+$ pMY or 2B4rg cells following *ex vivo* stimulation with PMA and ionomycin. D, Summary of the frequency of IFN-γ-producing Thy1.1$^+$ CD8$^+$ T cells, representative of 2 independent experiments with 7-8

mice/group. E, Summary of the absolute numbers of IFN-γ-producing Thy1.1$^+$ CD8$^+$ T cells, representative of 2-3 independent experiments with 7-12 mice/group. *p<0.05, ** p<0.001, *** p<0.0001.

Figure 3. Failure of 2B4 retrogenic cells to accumulate in the spleen is not due to differences in expression of the 2B4 ligand or of T cell activation or exhaustion markers. Naïve B6 animals received 10^6 2B4rg Thy1.1$^+$ OT-I T cells or pMY Thy1.1$^+$ OT-I controls and were challenged with OVA-expressing skin grafts. A, Representative flow histograms indicating expression intensity of CD44, CD48, CD127, KLRG-1, PD-1, and LAG-3 on CD8$^+$ Thy1.1$^+$ T cells isolated from the spleen 10 days following transplantation. B-G, Summary of the frequencies of CD44^{hi}, CD48$^+$, CD127$^+$, KLRG-1$^+$, PD-1$^+$, and LAG-3$^+$ CD8$^+$ Thy1.1$^+$ T cells isolated from the spleen on day 10. H-I, Representative flow dot plots and summary bar graphs illustrating the frequency of Annexin V and 7AAD double-positive OT-I T cells 10 days after transplantation.

Figure 4. 2B4 rg cells undergo less division compared to their empty-vector pMY control counterparts. A and B, CTV dilution of Thy1.1$^+$ CD8$^+$ 2B4rg and Thy1.1$^+$ CD8$^+$ non-rg OT-I controls on day 10. C-D, Summary of frequency of 2B4rg and control OT-I T cells in each round of division. E, Frequencies of undivided CD8$^+$ T cells in the spleen on d 10. F, Summary of the number of Thy1.1$^-$ CD8$^+$ non-OT-I and Thy1.1$^+$ CD8$^+$ OT-I T precursors recruited into the anti-donor immune response. All data are representative of two independent experiments with a total of 9-11 mice/group. $p < 0.05$.

Figure 5. 2B4 signaling limits glycolytic capacity of CD8$^+$ T cells. A, cDNA was synthesized from 400 ng of RNA isolated from 2B4$^+$ WT or 2B4KO OT-I T cells following *in vitro* activation (7d) and restimulation (4d) and subsequently used to assess changes in glycolytic metabolism between 2B4$^+$ and 2B4KO cells via the Qiagen Glucose Metabolism RT^2 Profiler PCR Array. Representative of 2 independent experiments with a total of 6-8 mice per group. B, Summary derived from the data set described above in A of selected genes that were increased over baseline in OT-I T cells in the absence of 2B4. C, 3x10^6 WT or 2B4KO OT-I T cells were stimulated with 1nM SIINFEKL for 4 d. 2x10^5 OT-I Ficoll-purified cells were then plated as described in Supplemental Methods. D-E, Rate of ECAR during glucose-induced glycolysis was averaged from three measurements following the addition of glucose, while glycolytic capacity was calculated from the average of three measurements following the addition of oligomycin. C-E are representative of two experiments, $p < 0.0001$. F, 10^4 WT or 2B4KO OT-I T cells were transferred into naïve B6 hosts and infected with 10^4 CFU of LM-OVA two days later. After 14 days, recipients were sacrificed, splenocytes were prepared as a single-cell suspension, washed with PBS, and then incubated with 2-NBDG *ex vivo* and analyzed via flow cytometry. G, Summary of expression of 2-NBDG as gated in part F. $p = 0.01$. F-G are representative of 4-5 mice/group.

Figure 6. Proliferative advantage of 2B4 KO T cells is erased under glucose limiting conditions. A, 3x10^6 WT and 2B4KO OT-I T cells, respectively, were resuspended in complete media and a D-glucose solution was titered into the glucose-free complete media and diluted on a half-log scale. Cells were then stimulated with SIINFEKL N4 peptide at 1 nM for 5 days. After 5 days in culture cells were harvested and stained for flow cytometry and CTV-dilution of

Thy1.1$^+$ CD8$^+$ antigen-reactive T cells was assessed. B-C, Representative flow cytograms and summary data depicting dilution of CTV in Thy1.1$^+$ OT-I T cell compartments under glucose-limiting culture conditions. D, Summary data depicting frequency of 7AAD and AnnexinV double positive apoptotic cells from each culture condition. Data are representative of 2 independent experiments with a total of 6-8 animals per group. p = 0.01

Figure 7. 2B4-mediated signals impact memory recall potential. A, 10^4 WT or 2B4KO OT-I T cells were transferred into naïve B6 hosts, which were infected with 10^4 CFU LM-OVA 2d later. Animals received OVA-expressing skin grafts at day 10 post-transplant and were sacrificed five days later. B-C, Representative flow cytograms and summary data depicting the expression of CD44, KLRG-1, CXCR3, and CD69 on Thy1.1$^+$ CD8$^+$ OT-I T cells isolated from the spleen. D-E, Splenocytes were restimulated *ex vivo* with PMA and ionomycin and analyzed by ICS. Data shown in A–E are representative of 6-11 mice/group from 2 independent experiments. p = 0.0005.

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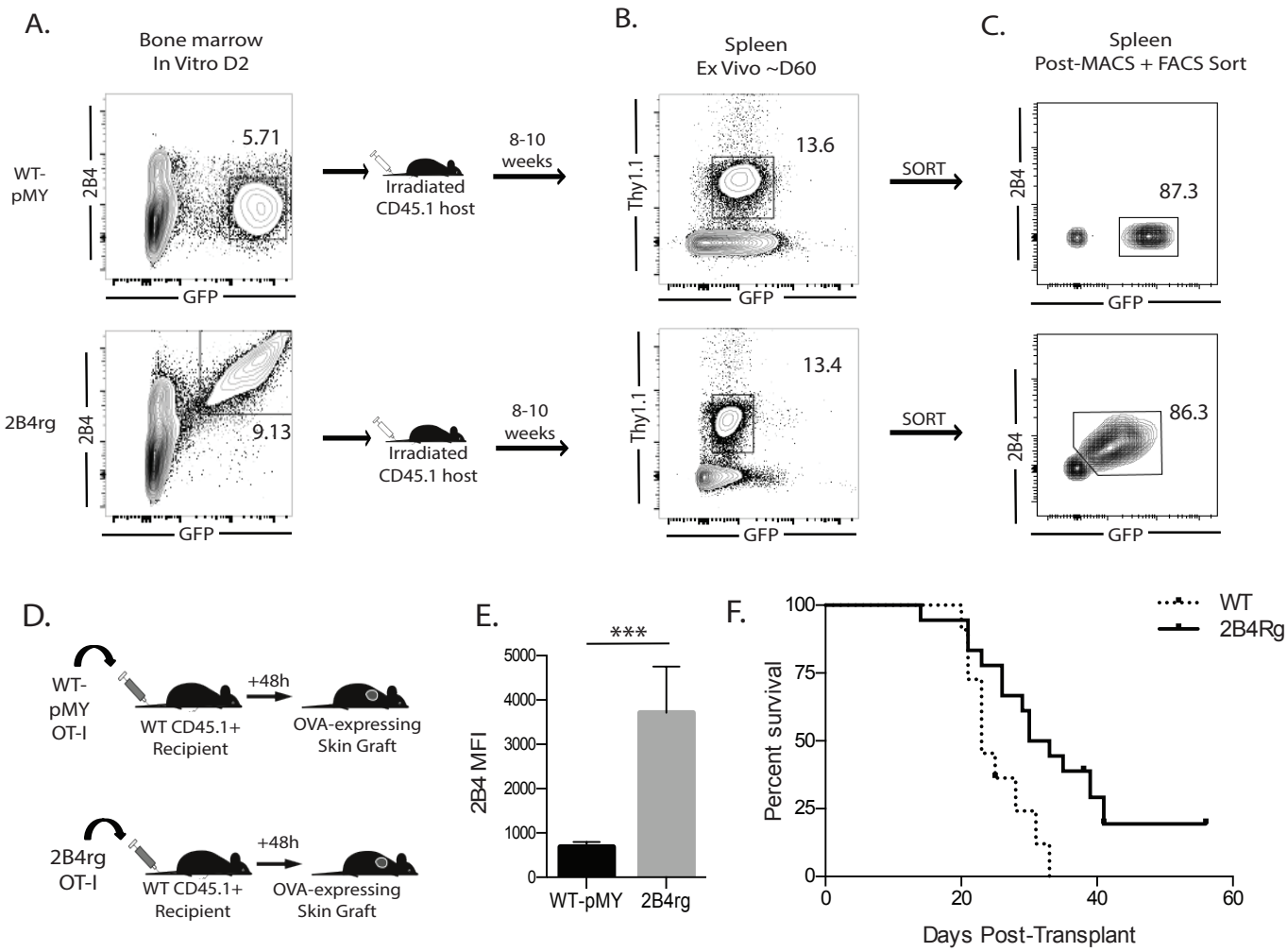
A.
Bone marrow
In Vitro D2
WT-
pMY
2B4
5.71
GFP
Irradiated
CD45.1 host
8-10
weeks
2B4rg
9.13
B.
Spleen
Ex Vivo ~D60
Thy1.1
13.6
13.4
SORT
C.
Spleen
Post-MACS + FACS Sort
87.3
86.3
D.
WT-
pMY
OT-I
+48h
WT CD45.1+
Recipient
OVA-expressing
Skin Graft
2B4rg
OT-I
E.
***
2B4 MFI
5000
4000
3000
2000
1000
0
WT-pMY
2B4rg
F.
WT
2B4Rg
Percent survival
100
75
50
25
0
0
20
40
60
Days Post-Transplant

Figure 3.1

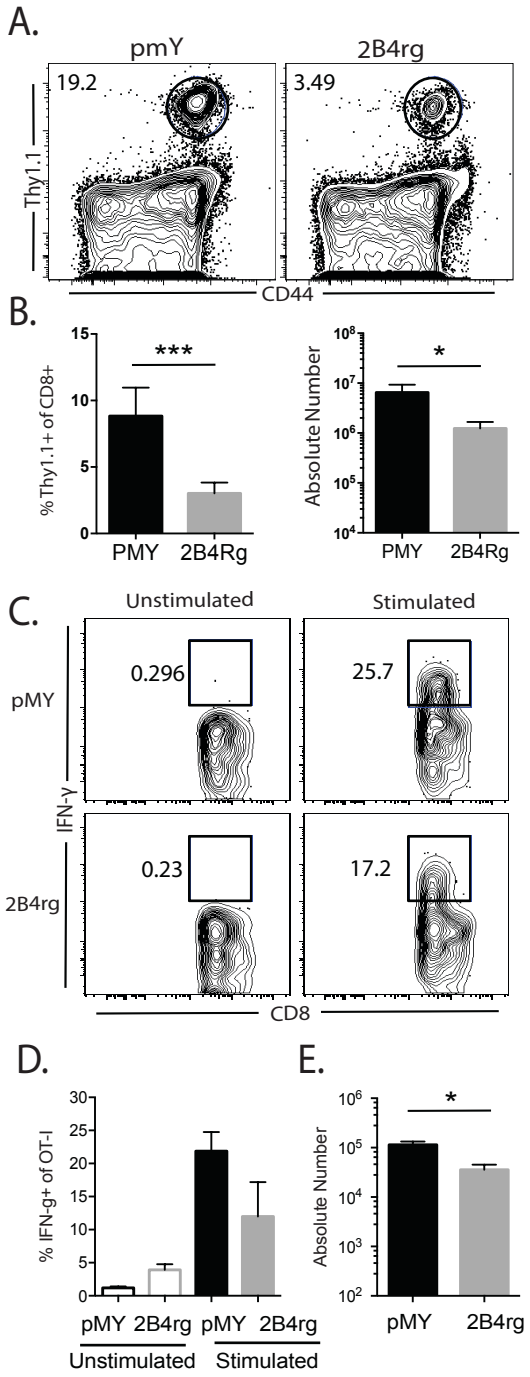
A.
pmY
2B4rg
19.2
3.49
Thy1.1
CD44
B.
***
*
% Thy1.1+ of CD8+
Absolute Number
PMY
2B4Rg
C.
Unstimulated
Stimulated
pMY
2B4rg
IFN-γ
0.296
25.7
0.23
17.2
CD8
D.
E.
% IFN-g+ of OT-I
Absolute Number
pMY 2B4rg pMY 2B4rg
Unstimulated
Stimulated

Figure 2

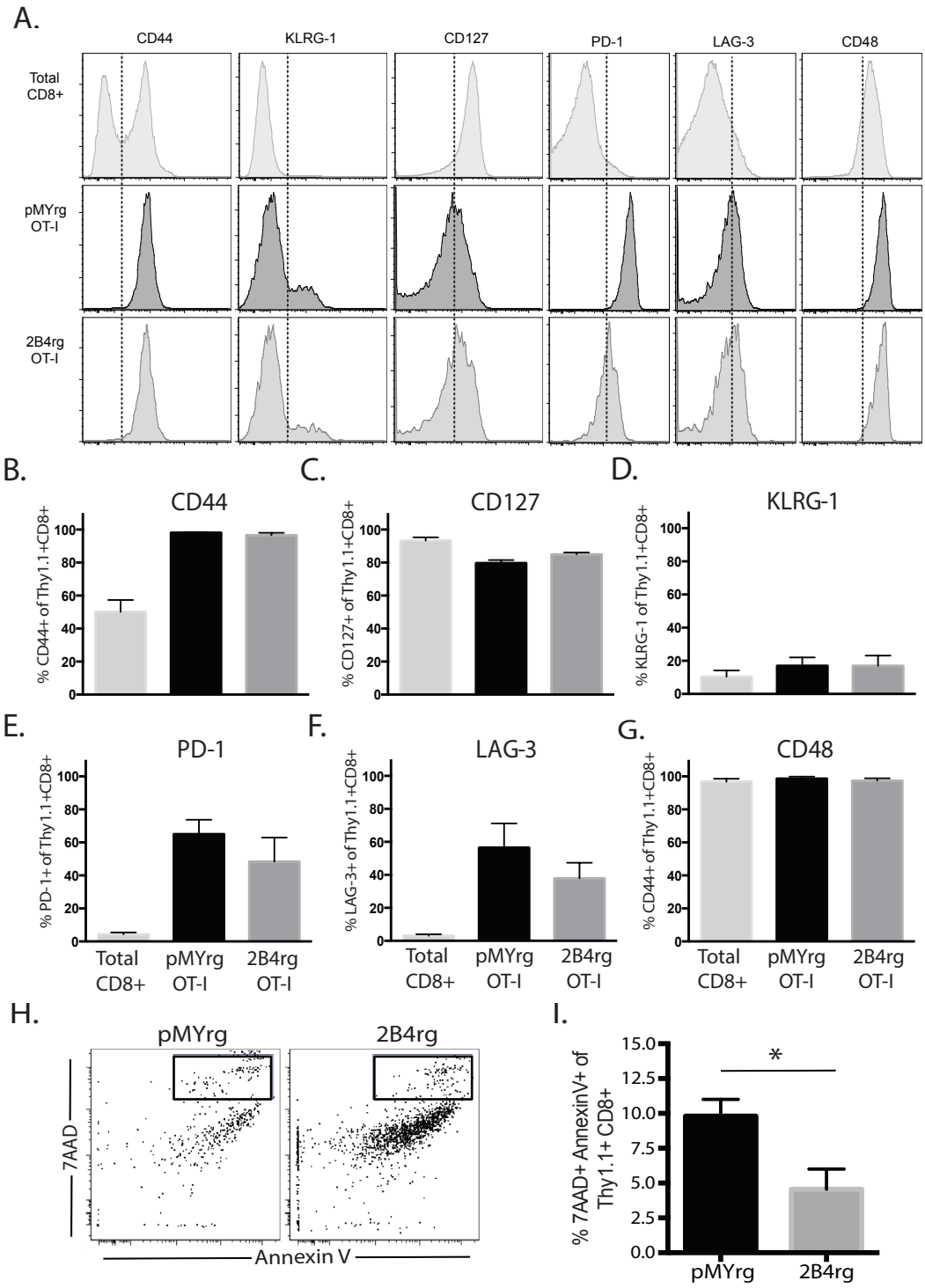
A.
CD44
KLRG-1
CD127
PD-1
LAG-3
CD48
Total CD8+
pMYrg OT-I
2B4rg OT-I
B.
CD44
% CD44+ of Thy1.1+CD8+
C.
CD127
% CD127+ of Thy1.1+CD8+
D.
KLRG-1
% KLRG-1 of Thy1.1+CD8+
E.
PD-1
% PD-1+ of Thy1.1+CD8+
F.
LAG-3
% LAG-3+ of Thy1.1+CD8+
G.
CD48
% CD44+ of Thy1.1+CD8+
Total CD8+
pMYrg OT-I
2B4rg OT-I
H.
pMYrg
2B4rg
7AAD
Annexin V
I.
% 7AAD+ AnnexinV+ of Thy1.1+ CD8+
*
pMYrg
2B4rg

Figure 3.3

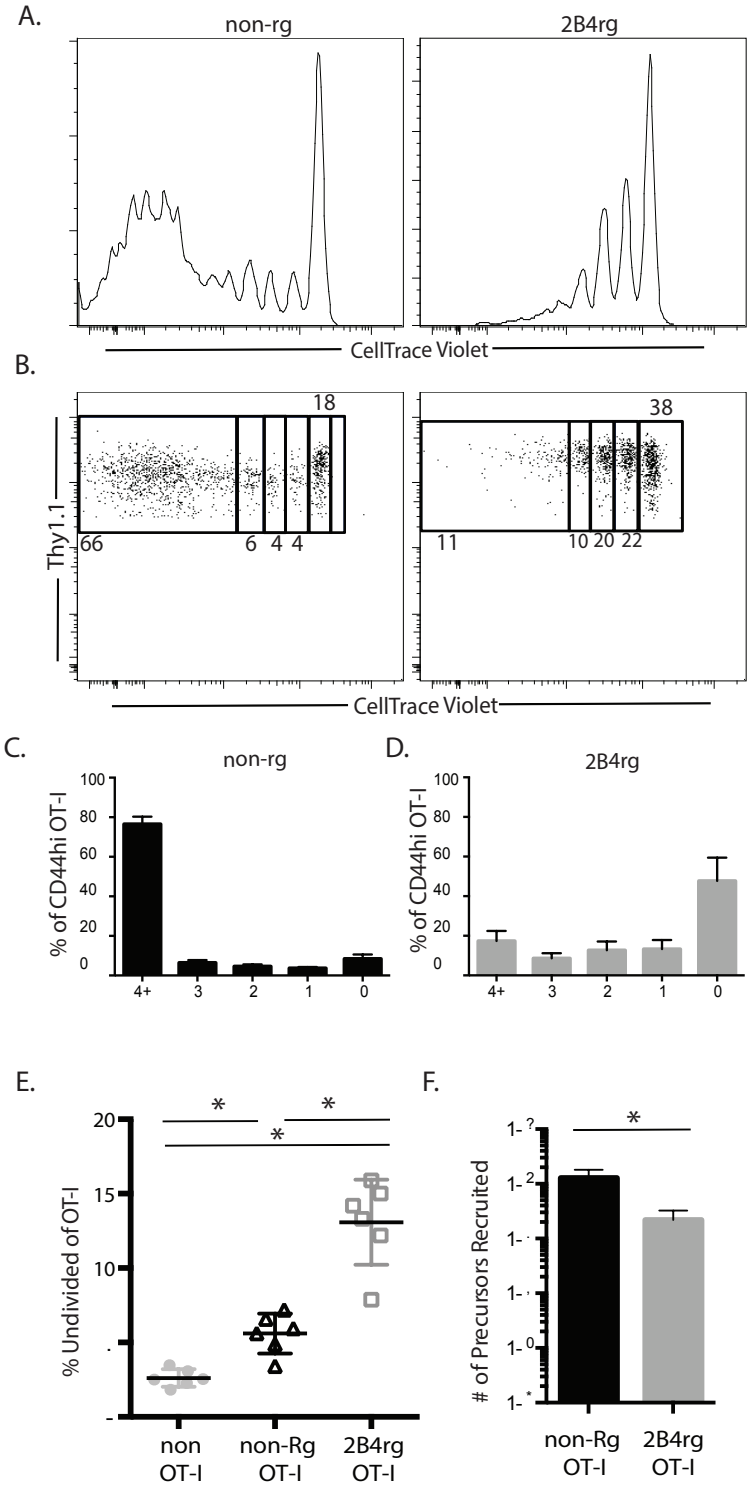
A.
non-rg
2B4rg
CellTrace Violet
B.
18
38
Thy1.1
66
6 4 4
11
10 20 22
CellTrace Violet
C.
non-rg
% of CD44hi OT-I
100
80
60
40
20
0
4+
3
2
1
0
D.
2B4rg
% of CD44hi OT-I
E.
% Undivided of OT-I
20
15
10
non
OT-I
non-Rg
OT-I
2B4rg
OT-I
F.
# of Precursors Recruited
non-Rg
OT-I
2B4rg
OT-I

Figure 3.4

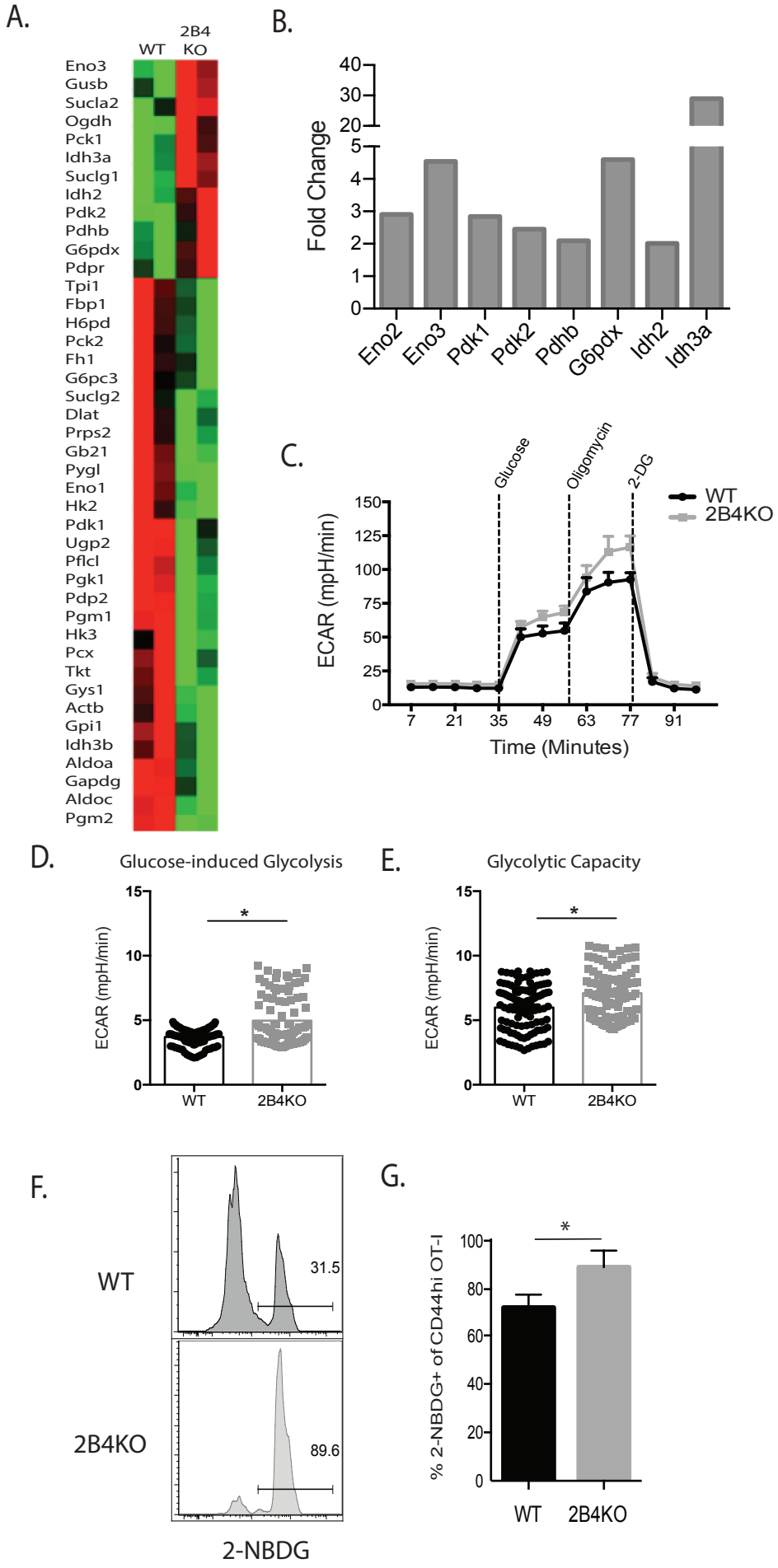
A.
WT
2B4 KO
Eno3
Gusb
Sucla2
Ogdh
Pck1
Idh3a
Suclg1
Idh2
Pdk2
Pdhb
G6pdx
Pdpr
Tpi1
Fbp1
H6pd
Pck2
Fh1
G6pc3
Suclg2
Dlat
Prps2
Gb21
Pygl
Eno1
Hk2
Pdk1
Ugp2
Pflcl
Pgk1
Pdp2
Pgm1
Hk3
Pcx
Tkt
Gys1
Actb
Gpi1
Idh3b
Aldoa
Gapdg
Aldoc
Pgm2
B.
Fold Change
Eno2
Eno3
Pdk1
Pdk2
Pdhb
G6pdx
Idh2
Idh3a
C.
ECAR (mpH/min)
Glucose
Oligomycin
2-DG
WT
2B4KO
Time (Minutes)
D.
Glucose-induced Glycolysis
ECAR (mpH/min)
WT
2B4KO
E.
Glycolytic Capacity
ECAR (mpH/min)
WT
2B4KO
F.
WT
2B4KO
31.5
89.6
2-NBDG
G.
% 2-NBDG+ of CD44hi OT-I
WT
2B4KO

Figure 3.5

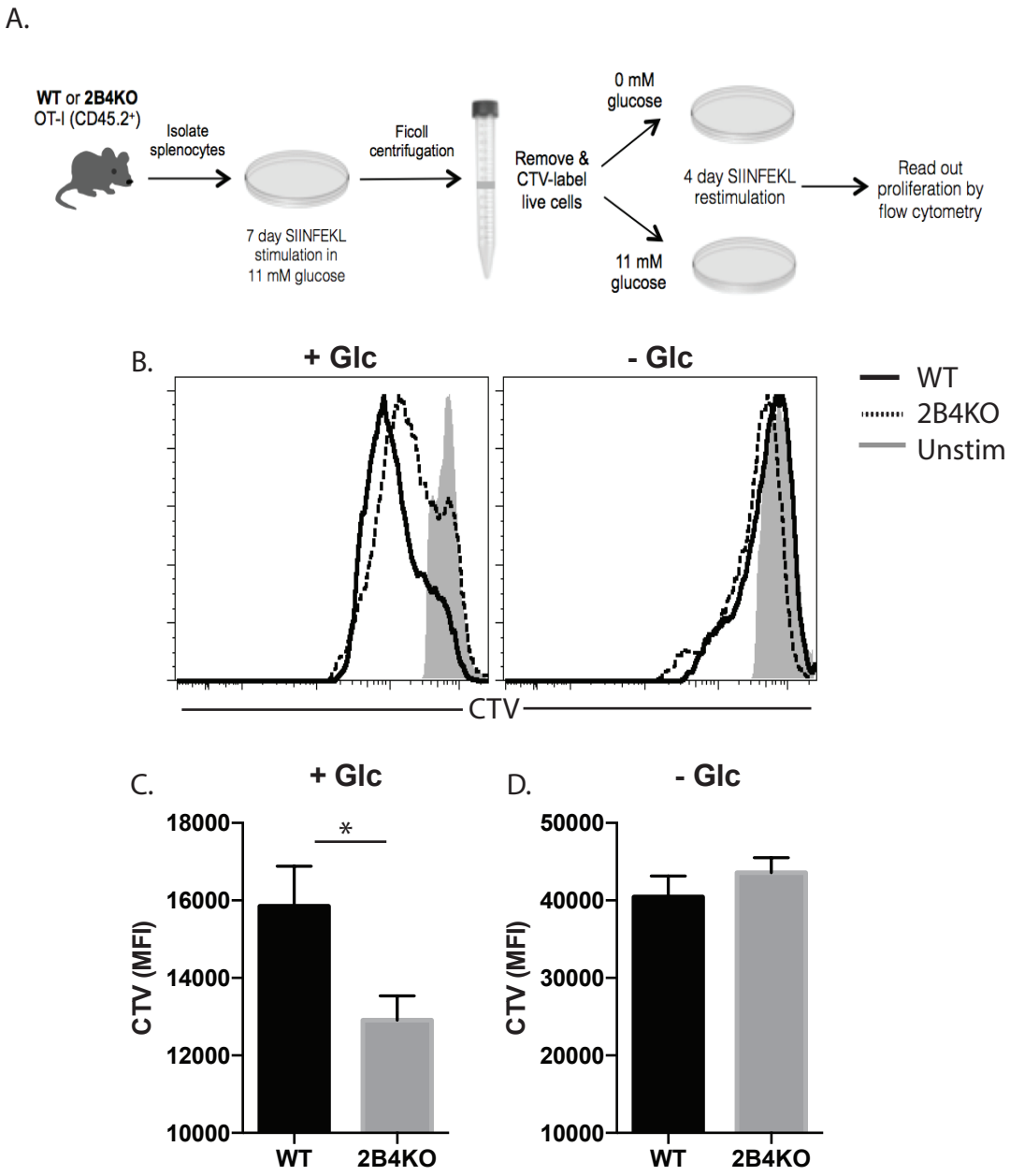
A.
WT or 2B4KO
OT-I (CD45.2+)
Isolate splenocytes
7 day SIINFEKL stimulation in 11 mM glucose
Ficoll centrifugation
Remove & CTV-label live cells
0 mM glucose
11 mM glucose
4 day SIINFEKL restimulation
Read out proliferation by flow cytometry
B.
+ Glc
- Glc
WT
2B4KO
Unstim
CTV
C.
+ Glc
CTV (MFI)
18000
16000
14000
12000
10000
*
WT
2B4KO
D.
- Glc
CTV (MFI)
50000
40000
30000
20000
10000
WT
2B4KO

Figure 3.6

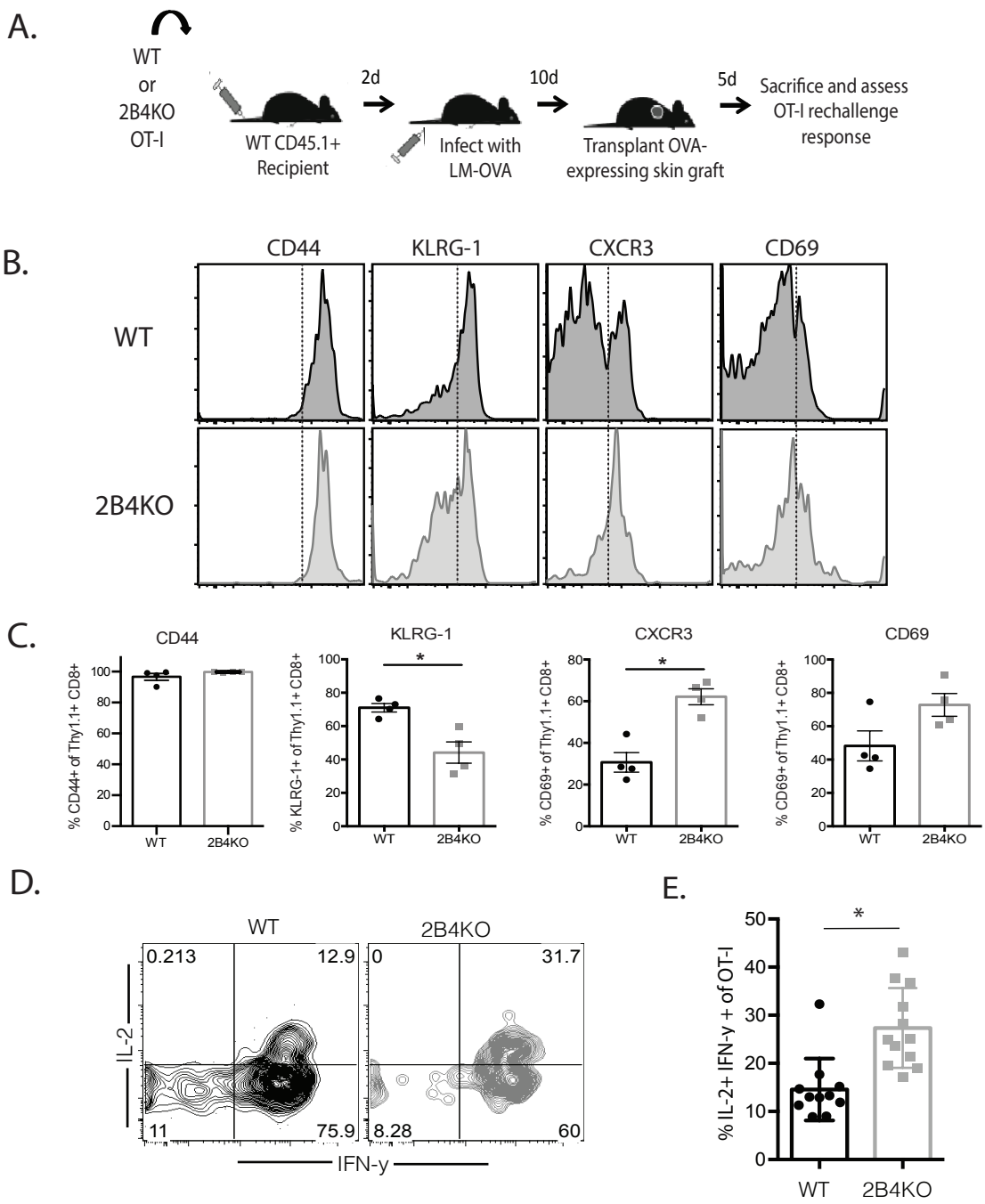
A.
WT
or
2B4KO
OT-I
WT CD45.1+
Recipient
2d
Infect with
LM-OVA
10d
Transplant OVA-
expressing skin graft
5d
Sacrifice and assess
OT-I rechallenge
response
B.
CD44
KLRG-1
CXCR3
CD69
WT
2B4KO
C.
CD44
KLRG-1
CXCR3
CD69
% CD44+ of Thy1.1+ CD8+
% KLRG-1+ of Thy1.1+ CD8+
% CD69+ of Thy1.1+ CD8+
% CD69+ of Thy1.1+ CD8+
WT
2B4KO
D.
WT
2B4KO
0.213
12.9
11
75.9
0
31.7
8.28
60
IL-2
IFN-γ
E.
% IL-2+ IFN-γ + of OT-I
WT
2B4KO

Figure 3.7

Chapter 4

The balance of CD28 and CTLA4 signals controls TIGIT expression on antigen-specific $CD4^+$ effector T cells

ABSTRACT

T cell functionality is tightly regulated by a fine balance of costimulatory and coinhibitory signals. We sought to determine the role of TIGIT-mediated coinhibitory signals on alloreactive T cell populations following transplantation. While TIGIT was not expressed on naïve donor-reactive $CD4^+$ and $CD8^+$ T cells, maximal TIGIT expression was observed 20 days post-transplantation, where it was detected on 26% of antigen-specific $CD4^+$ and 8% of antigen-specific $CD8^+$ T cells. To investigate the role of costimulation in regulating TIGIT expression following T cell activation, we treated skin transplant recipients with a novel anti-CD28 domain antibody that selectively blocks CD28 signals. This resulted in downregulation of TIGIT on $CD4^+$ effector cells in graft draining lymph nodes after 10 days. In contrast, CTLA4Ig, which blocks CD28-mediated costimulation as well as CTLA-4-mediated coinhibition, resulted in intermediate TIGIT expression. The observation that physiologic TIGIT-mediated coinhibitory signals are diminished following CD28-directed costimulation blockade led us to hypothesize that agonism of TIGIT may be synergistic with CD28 costimulation blockade in inhibiting donor-reactive T cell responses and prolonging allograft survival. To test this, we treated skin graft recipients with an agonistic anti-TIGIT antibody along with CTLA-4Ig and noted significant prolongation of allograft survival as compared to CTLA-4 Ig alone. These results identify a role for both CD28- and CTLA-4 mediated signals in calibrating the level of TIGIT expression following T cell activation, and suggest that pharmacologic agonism of TIGIT may synergize with CD28-directed immunotherapy in inhibiting unwanted T cell responses following transplantation.

INTRODUCTION

The recently discovered T cell immunoreceptor with Ig and ITIM domains (TIGIT) molecule is a receptor found on the surface of T cells that can bind to CD155 and CD112 expressed on dendritic cells [1]. Importantly, TIGIT competes with CD226 (also known as DNAM) for binding to its ligands, thus resembling the relationship between the binding of CD28 and CTLA-4 to CD80 and CD86 (Figure 4.1). The TIGIT protein contains an immunotyrosine based inhibitory motif (ITIM) in it's cytoplasmic domain which can recruit phosphatases, conferring its inhibitory properties [2]. TIGIT functions in a cell intrinsic fashion, as dysregulation of the signaling pathway has been associated with the development of autoimmune like disease as a result of T cell hyperproliferation in its absence[3]. Work has also shown that TIGIT can be expressed on NK cells and functions to inhibit cytotoxicity of those cells[4]. In addition to the direct role that TIGIT signaling has in inhibiting T cell responses as illustrated above, TIGIT signaling also has a net dampening effect on immune activation by promoting the induction and maturation of tolerance-supporting dendritic cells via enhanced production of IL-10 and decreased IL-12 [5]. Importantly, TIGIT is also expressed on $CD4^+$ regulatory T cells (T_{regs}), where it functions to limit immune mediated collateral damage to the host via inhibition of both Th1 and Th17 responses[6].

Recent work has also described a role for TIGIT in modulating T cells in a variety of tumor microenvironments. TIGIT is highly expressed on tumor-specific $CD8^+$ T cells, and antibody-mediated blockade of TIGIT ligation enhanced the effector function of these cells, restoring anti-tumor immunity [1]. The importance of TIGIT in mediating

cancer-specific immune responses via its expression on tumor-infiltrating lymphocytes is further highlighted by the marked increase of *in vitro* T cell functionality following combined ex vivo blockade of TIGIT and PD-1 on T cell isolated from human melanoma patients [7]. $TIGIT^+$ T_{reg} cells have also been shown to upregulate the coinhibitor TIM-3 following chronic antigen exposure, providing another mechanism by which TIGIT expression is able to suppress the generation of antitumor immune responses[8]. Interestingly, human T_{regs} that express TIGIT have also been shown to express FCRL3, both of which have been demonstrated to be enriched on thymically derived $Helios^+$ $Foxp3^+$ Tregs that are frequently identified in patients with autoimmune complications [9, 10].

Though no work has yet addressed the role of the TIGIT in transplantation, the use of antibody modulation of this signaling pathway in cancer suggest that therapies using costimulation blockade may be useful in prolonging allograft survival and inducing tolerance, and thus merit continued investigation. We therefore set out to assess the expression of TIGIT on donor-reactive $CD4^+$ and $CD8^+$ T cells, as well as polyclonal $CD4^+$ $Foxp3^+$ regulatory T cells (T_{regs}) in a murine model of minor-mismatched skin transplantation. Strikingly, we observed that TIGIT is upregulated on antigen-specific $CD4^+$ cells and polyclonal T_{reg}s following transplantation, and that its agonism prolongs allograft survival. These results suggest that signaling via TIGIT is an important pathway to modulate alloreactive T cells, and that further work is warranted to uncover the mechanistic basis by which TIGIT controls donor-aggressive cells following transplantation.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I [11] and OT-II [12] transgenic mice were purchased from Taconic Farms (Germantown, NY) and bred to Thy1.1$^+$ background at Emory University. mOVA mice (C57BL/6 background, H-2b) [13] were a generous gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University (protocol number: DAR-2002050-092815GN). All surgery was performed under general anesthesia with maximum efforts made to minimize suffering. All animals were housed in specific pathogen-free animal facilities at Emory University.

Donor-Reactive T Cell Adoptive Transfers

In order to approximate the precursor frequency of donor-reactive cells in a fully MHC mismatched model of transplantation, we utilized our previously described system in which we adoptively transfer a higher frequency of OVA-specific TCR transgenic cells into naïve hosts prior to transplantation. For adoptive transfer of donor-reactive T cells, spleen and mesenteric lymph nodes (mLN) isolated from Thy1.1$^+$ OT-I and Thy1.1$^+$ OT-II mice were processed and stained with monoclonal antibodies for CD8 (Invitrogen), CD4, Thy1.1, and Vα2 (all from BD Pharmingen) for flow cytometric analysis. Cells

were resuspended in 1X phosphate buffered saline (PBS) and 10^6 of each Thy1.1$^+$ OT-I and OT-II were injected i.v. 48 hours prior to skin transplantation.

Skin Transplantation

Full thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described [14]. Where indicated, recipients were treated with CTLA-4 Ig (abatacept, Bristol-Myers Squibb) (250 μg i.p. on days 0, 2, 4, and 6). Grafts with less than 10% viable tissue remaining were scored as rejected. Where indicated, grafted recipients were treated with a short course of 100 μg of anti-CD28dAb on days 0, 2, 4, and 6.

Flow Cytometry and Intracellular Cytokine Staining

Cells isolated from spleens and graft-draining axillary and brachial lymph nodes (dLN) were stained with anti-CD4-Pacific Blue (BD Biosciences), anti-CD8- Pacific Orange or BV786 (BD Biosciences or Invitrogen) and anti-Thy1.1-PerCP (BD Biosciences or BioLegend). For phenotypic analysis cells were also surface-stained with anti-TIGIT-PE (BioLegend), anti-CD62L-PE-Cy7 (eBioSciences), anti-FoxP3-FITC (eBioSciences), anti-CD25-PE-Cy7 (BioLegend), anti-CD226-APC (BioLegend), and anti-CD44-APC-Cy7 (BD Biosciences). Absolute numbers of lymphocytes from the spleen and draining lymph nodes were calculated using a Cellometer Auto T4 Cell Viability Counter (Nexcelom) according to the manufacturer's instructions. Samples were analyzed on an LSRII flow cytometer (BD Biosciences). Data was analyzed using FlowJo 9 software (Treestar, San Carlos, CA) and Prism 6 software (GraphPad Software Inc.). For

intracellular cytokine staining, lymphocytes were restimulated *ex vivo* with 1 μg/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Life Sciences) and 1 μg/mL ionomycin (Sigma Life Sciences) where indicated, in the presence of 1 μg/mL Brefeldin A (BD Biosciences) for 4 hours. The Fix/Perm intracellular staining kit (BD Pharmingen) was used to detect IL-2 (BD Biosciences), TNF (BioLegend), and IFN-γ (BD Biosciences), according to manufacturer's instructions.

Statistical Analysis

T cell responses were analyzed using unpaired, non-parametric Mann-Whitney t-tests. Results were considered significant if $p < 0.05$. All analyses were done using Prism software (GraphPad Software Inc.).

RESULTS

TIGIT and DNAM are expressed on antigen-specific $CD4^+$ and $CD8^+$ T cells following transplantation

Given that the human immunophenotyping data presented in Chapter 2 identified that signaling via the TIGIT pathway contributes to reduced T cell alloreactivity, we set out to assess the expression of TIGIT on donor-reactive $CD4^+$ and $CD8^+$ T cells, as well as polyclonal $CD4^+$ $Foxp3^+$ regulatory T cells (T_{regs}) in a murine model of minor-mismatched skin transplantation (Figure 4.2A). While TIGIT was not expressed on naïve donor-reactive $CD4^+$ and $CD8^+$ T cells, maximal TIGIT was observed 20 days post-transplantation, where it was detected on approximately 20-25% of antigen-specific $CD4^+$ and 5-10% of antigen-specific $CD8^+$ cells (Figure 4.2 B-C). Strikingly, we noted that the highest level of TIGIT expression was on polyclonal T_{reg} cells, where it was upregulated as early as day 8, and was found on roughly 25-30% of $CD4^+$ $Foxp3^+$ cells by day 20 post-transplantation.

Balance of CD28 and CTLA4 coinhibitory signals determine TIGIT expression

To investigate the role of costimulation in regulating TIGIT expression following T cell activation, we treated skin transplant recipients with a novel anti-CD28 domain antibody (aCD28 dAb) that selectively blocks CD28 signals. As depicted in Figure 4.3A-B, this resulted in downregulation of TIGIT on $CD4^+$ effector cells in graft draining lymph nodes after 10 days ($p=0.0079$). In contrast, CTLA4Ig, which blocks CD28-mediated

costimulation as well as CTLA-4-mediated coinhibition, resulted in intermediate TIGIT expression ($p=0.01$ compared to untreated).

Agonism of TIGIT signaling prolongs skin graft survival

The observation that physiologic TIGIT-mediated coinhibitory signals are diminished following CD28-directed costimulation blockade led us to hypothesize that agonism of TIGIT may be synergistic with CD28 costimulation blockade in inhibiting donor-reactive T cell responses and prolonging allograft survival. To test this, we treated skin graft recipients with an agonistic anti-TIGIT antibody along with CTLA-4Ig and noted significant prolongation of allograft survival as compared to CTLA-4 Ig alone (Figure 4.4).

anti-TIGIT antibody treatment does not prolong allograft survival by reducing the accumulation of or cytokine production by $CD4^+$ or $CD8^+$ effector T cells

To assess the mechanism by which agonism of TIGIT prolongs allograft survival, we treated skin graft recipients with an agonistic anti-TIGIT antibody along with low-dose CTLA4-Ig as described above in Figure 4.4 and sacrificed the animals 10 days following transplantation at the peak of the antigen-specific T cell response. Interestingly, agonism of TIGIT did not decrease the accumulation of antigen-specific $CD4^+$ or $CD8^+$ T cells in the spleen or axillary and brachial draining lymph nodes compared to animals treated with CTLA-4Ig alone (Figure 4.5 A-D).

DISCUSSION

Though TIGIT was not expressed on donor-reactive $CD4^+$ and $CD8^+$ T cells at baseline, we were able to detect on $CD4^+$ and $CD8^+$ T cells following transplantation via flow cytometry. TIGIT expression was highest on polyclonal T cells and antigen-specific $CD4^+$ cells, with markedly less expression observed on antigen-specific $CD8^+$ cells. Importnatly, Our data reveal that TIGIT expression is controlled by a fine balance of CD28 and CTLA-4 signals, as selective CD28-specific costimulation blockade with anti-CD28 domain antibodies resulted in striking downregulation of TIGIT on $CD4^+$ effector cells in graft draining lymph nodes after 10 days, while treatment with CTLA4-Ig resulted in only intermediate TIGIT expression. We went on to ask if agonism of TIGIT signaling would result in enhanced suppression of donor-reactive T cell responses following transplantation and thus impact graft survival. We observed that therapeutic intervention with an agonistic anti-TIGIT antibody along with low-dose CTLA-4Ig significantly prolongation of allograft survival as compared to CTLA-4 Ig alone.

Though this work characterizes an important role for inhibitory TIGIT signaling in extending graft survival, we have not yet identified on which cell type TIGIT functions to mediate this effect. We are currently breeding TIGIT flox/flox animals to beta-actin cre and Foxp3 cre animals, respectively, to generate experimental animals where TIGIT is lacking either globally, or specifically on regulatory T cells, which will allow us to further probe whether TIGIT prolongs graft survival via an affect on T cells (Figure 4.6). Additionally, we are particularly interested to determine if TIGIT expression on Tregs antigen-driven, and, it will be important to assess whether or not there is there a

differential requirement for CD28 signals for TIGIT expression of effector versus regulatory T cells.

If further experimentation reveals that TIGIT does not impact allograft survival via altering T cell responses, it is possible the effect that we observed may be mediated by alterations of the response of innate cells to the allograft. A recent publication has shown that the binding of TIGIT to it's receptor on macrophages can skew these phagocytes towards an anti-inflammatory phenotype, characterized by enhanced IL-10 prodution. It is possible that this may contribute to the stability of skin grafts that we observed following treatment with agonistic TIGIT, though more thorough proof of concept and mechanistic studies will be needed to further explore this possibility [15].

Our work in human renal transplant recipients indicates that expression of TIGIT contributes to reduced T cell alloreactivity (Chapter 2). To bridge the gap between mouse and man, and to determine the translational application of manipulating TIGIT signaling in human cells, future studies will be needed to determine whether or expression of TIGIT associated with protection from graft rejection following renal transplantation between patients that receive calcenurin inhibitors versus CD28 blockade with belatacept. Taken together, these findings suggest there may be an important role for both CD28- and CTLA-4-mediated signals in calibrating the level of TIGIT expression following T cell activation, suggesting pharmacologic agonism of TIGIT may inhibit T cell responses following transplantation.

FIGURES

Figure 4.1. The binding of CD155/CD112 to TIGIT and CD226 is analogous to the relationship between CD28/CTLA-4 and CD80/86

Figure 4.2. TIGIT and DNAM are expressed on antigen-specific CD4+ and CD8+ T cells following transplantation

Figure 4.3. Balance of CD28 and CTLA4 coinhibitory signals determine TIGIT expression

Figure 4.4. anti-TIGIT antibody treatment does not prolong allograft survival by reducing the accumulation of or cytokine production by $CD4^+$ or $CD8^+$ effector T cells

Figure 4.5. Agonism of TIGIT signaling prolongs skin graft survival.

Figure 4.6. Breeding TIGIT deficient animals to determine the impact of the loss of TIGIT signaling on T cells in impacting allograft survival.

FIGURE LEGENDS

Figure 4.1. The binding of CD155/CD112 to TIGIT and CD226 is analogous to the relationship between CD28/CTLA-4 and CD80/86. Similar to the competition between B7 ligands and CTLA-4 and CD28, CD155 (also known as the Polio virus receptor, PVR) and CD112 can each bind to TIGIT, eliciting coinhibition, or to DNAM (also known as CD226), which initiates a stimulatory signaling cascade. This figure is adapted from Levin et al, Eur J Immunol, 2011, 41(4): 902–915.

Figure 4.2. TIGIT is expressed on donor-reactive $CD4^+$ and $CD8^+$ T cells following transplantation. A) 10^6 transgenic $Thy1.1^+$ $CD8^+$ OT-I and $CD4^+$ OT-II T cells with T cell receptors specific for fragments of the OVA albumin peptide were adoptively transferred into naïve $CD45.1^+$ B6 recipients 48 hours prior to the receipt of an OVA-expressing skin graft. **B + C)** Animals were sacrificed following transplantation, and flow cytometry was used to assess TIGIT expression antigen-specific $CD4^+$ and $CD8^+$ T cells as well as polyclonal regulatory T cells ($Foxp3^+$ and $CD25^+$).

Figure 4.3. TIGIT expression on Th1 cells is dynamically regulated by CD28 and CTLA-4 signaling. A) 10^6 $Thy1.1CD8^+$ OT-I and $CD4^+$ OT-II T cells were adoptively transferred into naïve $CD45.1^+$ B6 recipients 48 hours prior to the receipt of an OVA-expressing skin graft. Recipients were randomized into three groups and received no treatment, 250 μg CTLA4Ig, or 100 μg αCD28 dAb 0, 2, 4, and 6 days post-transplant. B) CD80 and CD86 blockade caused downregulation of TIGIT on graft-specific $CD4^+$ T cells, with markedly more reduction observed in animals receiving CD28-specific blockade. C) Summary of TIGIT expression on OT-II T cells.

Figure 4.4. Agonism of TIGIT results in prolonged allograft survival following transplantation. A) 10^6 Thy1.1$^+$ CD8$^+$ OT-I and CD4$^+$ OT-II T cells were adoptively transferred into naïve CD45.1$^+$ B6 recipients 48 hours prior to the receipt of an OVA-expressing skin graft. At the time of transplantation, recipients also received a donor specific transfusion composed of $10x10^6$ mOVA splenocytes. Recipients were randomized and received either no treatment, 250 μg CTLA4Ig, or 250 μg CTLA4Ig + 200 μg α-TIGIT 0, 2, 4, and 6 days post-transplant. **B)** Survival of α-TIGIT treated skin graft recipients ($p = 0.0007$).

Figure 4.5..Agonism of TIGIT does not prevent accumulation of alloreactive T cells in the spleen or draining lymph nodes. 10^6 Thy1.1$^+$ CD8$^+$ OT-I and CD4$^+$ OT-II T cells, along with $10x10^6$ splenocytes as DST, were transferred into naïve recipients 48h prior to transplantation. Recipients received no treatment, 250 μg CTLA4Ig, or CTLA4Ig + 200 μg α-TIGIT on days 0, 2, 4, and 6 post-transplant. **A + B)** Accumulation of OT-I and OT-II T cells in the pooled axial and brachial draining lymph nodes 10 days following transplantation. **C + D)** Summary of accumulation of OT-I and OT-II T cells in the spleen 10 days post-transplantation. **E + F)** Cytokine production by splenic T cells following PMA and ionomycin stimulation.

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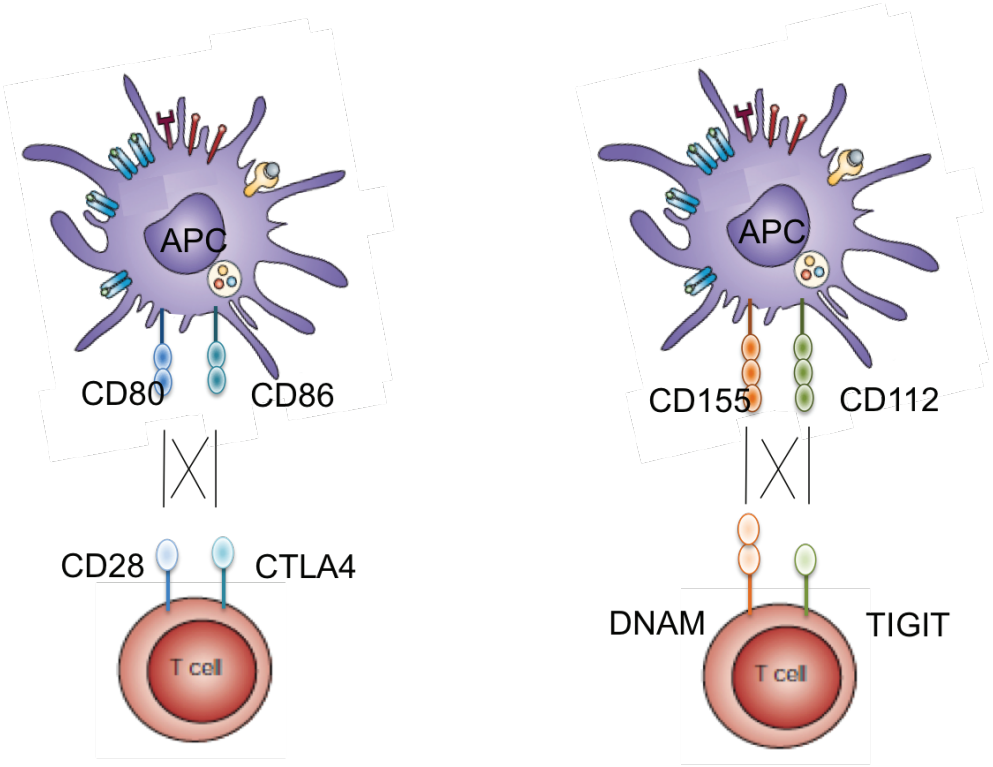
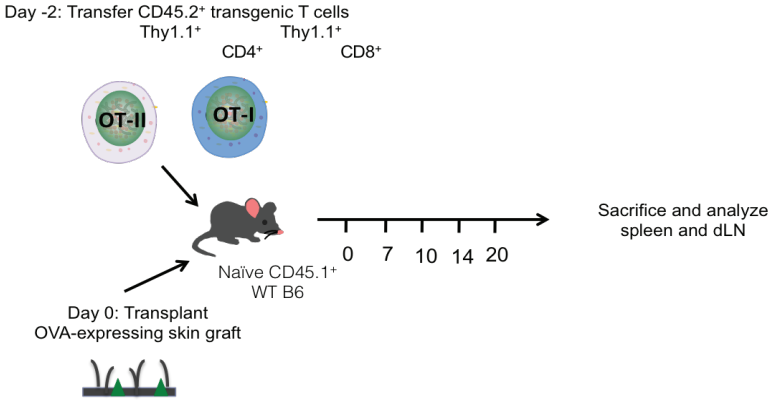


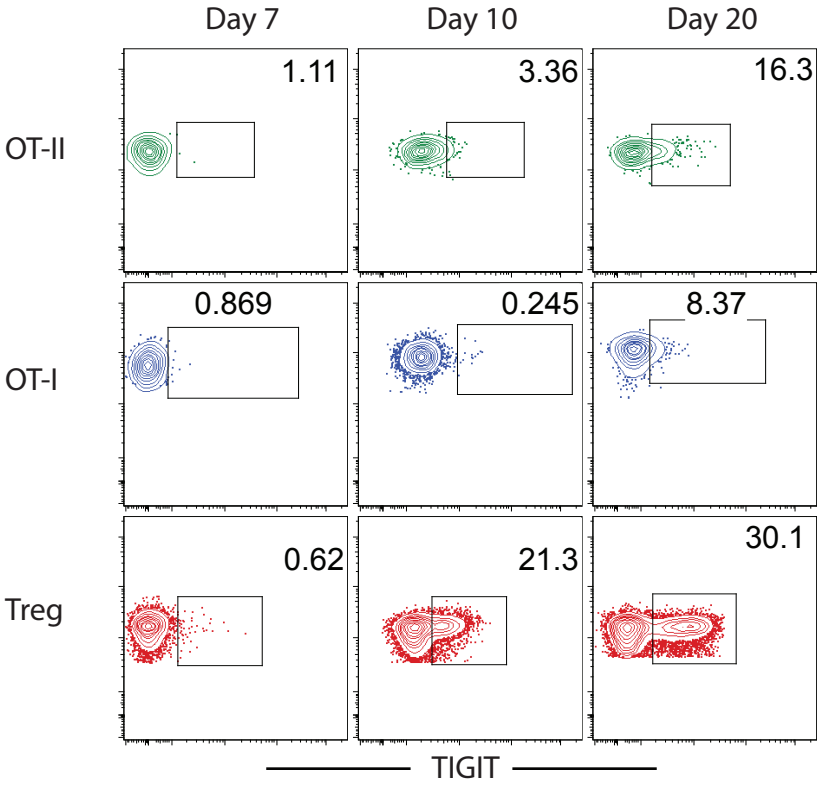
Adapted from Levin et al, *Eur J Immunol*, 2011

Figure 4.1

A.



B.



C.

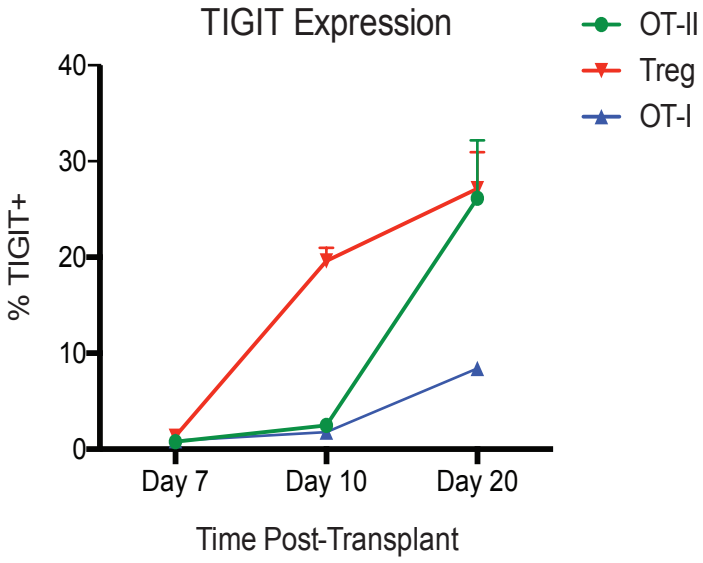
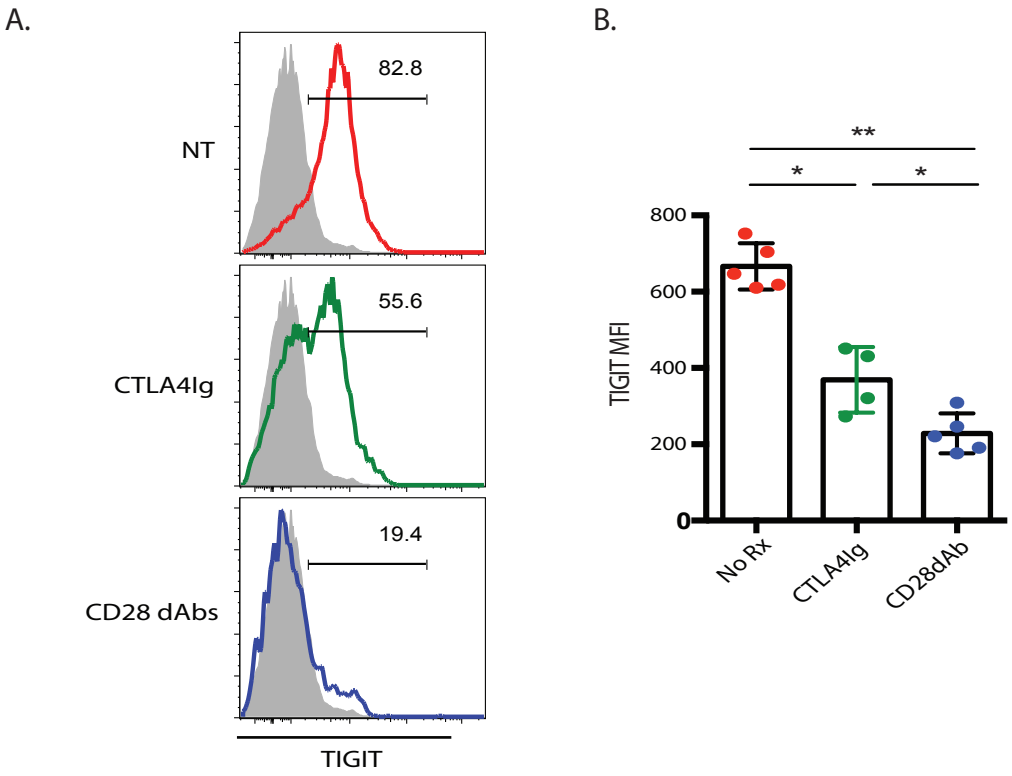


Figure 4.2


A.
82.8
NT
55.6
CTLA4Ig
19.4
CD28 dAbs
TIGIT
B.
**
*
*
800
600
400
200
0
TIGIT MFI
No Rx
CTLA4Ig
CD28dAb

C.

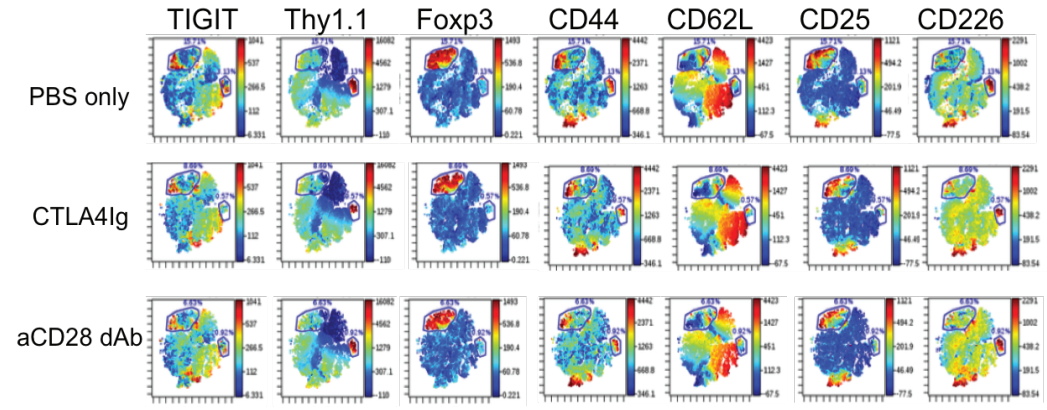
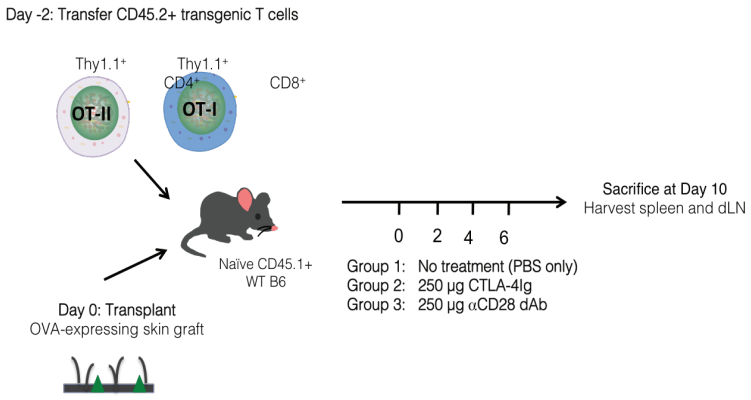

TIGIT
Thy1.1
Foxp3
CD44
CD62L
CD25
CD226
PBS only
CTLA4Ig
aCD28 dAb

Figure 4.3

A.


Day -2: Transfer CD45.2+ transgenic T cells
Thy1.1+
CD4+
OT-II
Thy1.1+
OT-I
CD8+
Naïve CD45.1+
WT B6
Day 0: Transplant
OVA-expressing skin graft
0 2 4 6
Sacrifice at Day 10
Harvest spleen and dLN
Group 1: No treatment (PBS only)
Group 2: 250 µg CTLA-4Ig
Group 3: 250 µg αCD28 dAb

B.

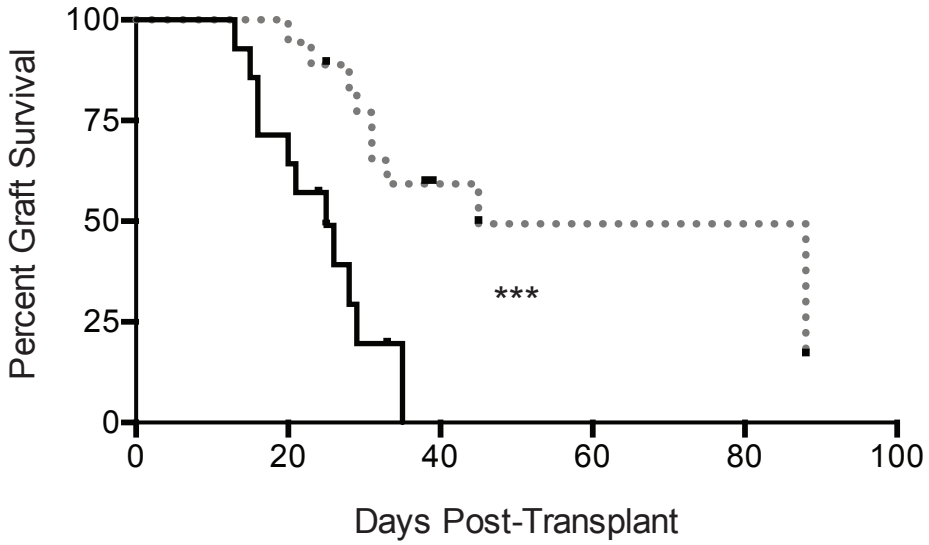

Percent Graft Survival
100
75
50
25
0
0
20
40
60
80
100
***
Days Post-Transplant

Figure 4.4

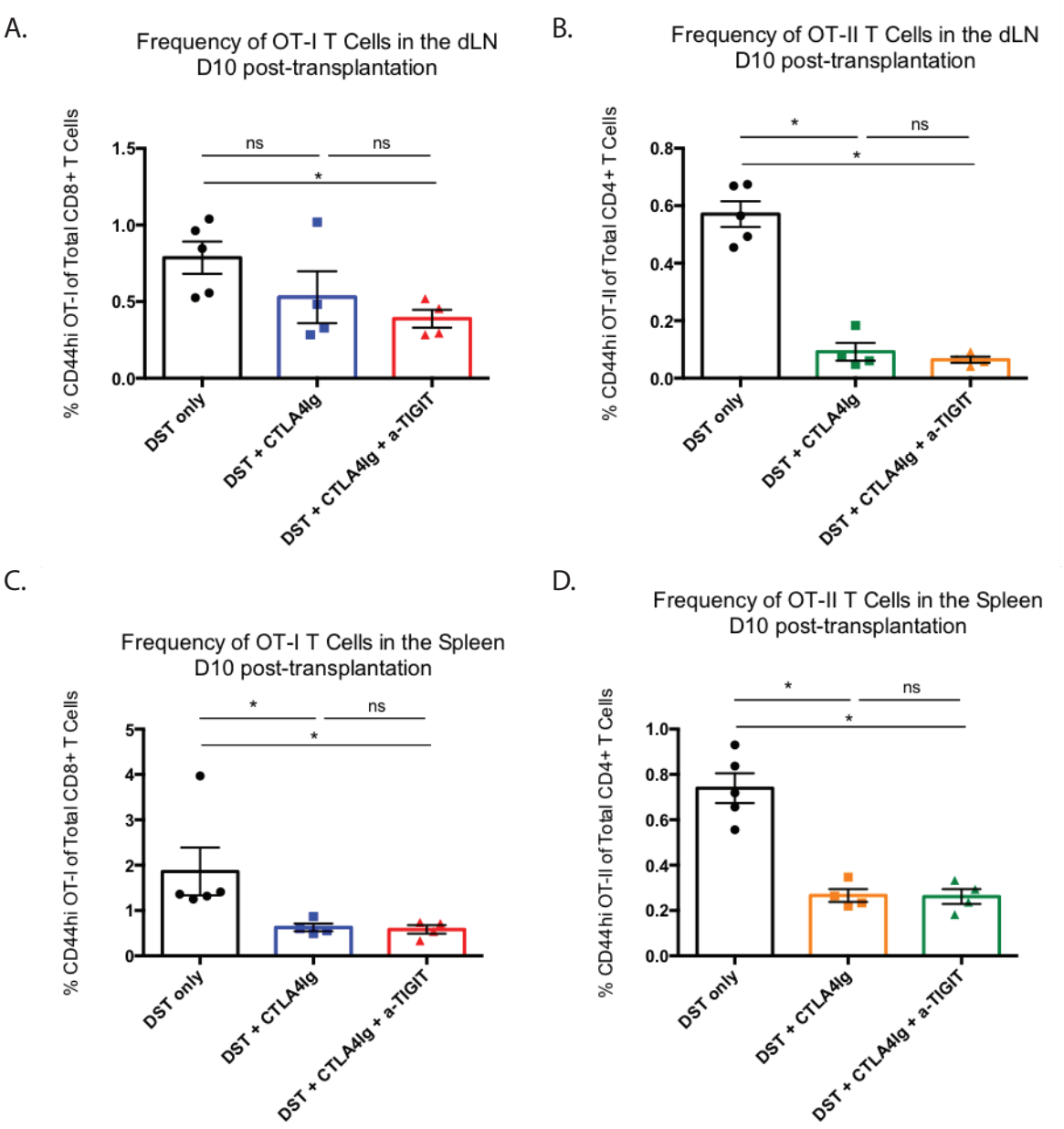

A.
Frequency of OT-I T Cells in the dLN
D10 post-transplantation
% CD44hi OT-I of Total CD8+ T Cells
ns
ns
*
DST only
DST + CTLA4Ig
DST + CTLA4Ig + a-TIGIT
B.
Frequency of OT-II T Cells in the dLN
D10 post-transplantation
% CD44hi OT-II of Total CD4+ T Cells
*
ns
*
C.
Frequency of OT-I T Cells in the Spleen
D10 post-transplantation
% CD44hi OT-I of Total CD8+ T Cells
*
ns
*
D.
Frequency of OT-II T Cells in the Spleen
D10 post-transplantation
% CD44hi OT-II of Total CD4+ T Cells
*
ns
*

Figure 4.5

Chapter 5

Transplantation Preferentially Induces a KLRG-1^{lo} CD127^{hi} Differentiation Program in Antigen-Specific CD8^{+} T Cells

ABSTRACT

Models of infection have shaped our understanding of programmed memory T cell differentiation, yet whether these models apply to memory programming in the context of transplantation has yet to be defined. Previous work has identified differences in the response of antigen-specific $CD8^+$ T cells to cognate antigen based on the environment in which the antigen is presented. Thus, we hypothesized that programming of antigen specific $CD8^+$ T cells responding to graft and pathogen may be dissimilar. Here we find that antigen-specific $CD8^+$ T cells primed by a skin graft contract faster than those primed by gammaherpesvirus (gHV), yet are able to expand more rapidly upon rechallenge. Moreover, graft-primed antigen-specific $CD8^+$ T cells exhibited higher frequencies of cells secreting IL-2 and demonstrate lower expression of KLRG-1, which are qualities suggestive of increased recall potential. Additionally, the expression of CD127 at a memory time point suggests graft-elicited $CD8^+$ antigen specific T cells are maintained in a less terminally-differentiated state compared to gHV-elicited $CD8^+$ antigen specific T cells, despite fewer cells being present at that time point. Taken together, our findings suggest that the surface marker expression and functional profiles of T cells depends on the priming conditions and may be used to predict immunologic risk following transplantation after traditional allosensitization or heterologous immune priming.

INTRODUCTION

Models of infection have shaped understanding of programmed memory T cell differentiation, and were used in the initial characterization of the phenotype and functionality of $CD8^+$ cells responding to cognate antigen. At the peak of a pathogen-stimulated immune response, the majority of antigen-specific $CD8^+$ T cells expand and terminally differentiate to serve the functional purpose of clearing infection, though a small subset of antigen-specific T cells fated to a different developmental potential have the capacity to survive and generate this memory phenotype (1). These memory precursor cells (MPEC) express higher levels of CD127 (IL-7Rα) and lower levels of killer cell lectin-like receptor G-1 (KLRG-1) than their short-lived effector (SLEC) counterparts (2, 3). SLECs uniformly down-regulate CD127 and up-regulate KLRG-1, confirming an inverse relationship between these two surface markers (4). Additionally, these distinct subsets of $CD8^+$ T cells have been defined by their cytokine profile, with interleukin (IL)-2 production being described as a selective property of long-lived antigen-specific $CD8^+$ T cells (4, 5). Therefore, surface marker expression and cytokine profiles have helped to distinguish T cells destined to die during contraction from those that persist and provide long-term immunologic memory, indicating that initial antigenic encounter imprints a developmental program onto $CD8^+$ T cells that persists for the life of that cell (6).

While these phenotypic and functional characteristics have been used to define the fate of $CD8^+$ T cells through contraction and predict the presence of functional memory in viral models, whether these canonical descriptions hold true in models of transplantation is not known. Following infection, presence of immunologic memory is critically important for host protection upon re-encounter with a pathogen, while on the other hand, in transplantation, immunologic memory can pose a significant barrier to allograft tolerance (7). While "traditional" sensitization from previous allograft, pregnancy, or transfusion limits subsequent donor tolerance, so too can microbial-elicited T cell memory (8-10). In this process, known as heterologous immunity, pathogen-elicited memory T cells are cross-reactive with alloantigens and can precipitate and accelerate allograft rejection. Additionally, bystander T cell

activation during an immune challenge can also provide an armamentarium of alloreactive cells. Thus, T cells elicited via different stimuli pose a threat to graft survival, but the question remains whether individual stimuli may elicit distinct differentiation programs that pose differential barriers to graft survival and/or tolerance.

We have observed that exposure to rapamycin paradoxically increases the quantity and quality of antigen-specific $CD8^+$ T cell response when the antigen is presented in the context of a viral infection, but fails to do so when the antigen is presented in the context of a transplant, even when an identical model antigen is used (11). These results highlight differences in $CD8^+$ T cell differentiation programs based on the environment in which the antigen is presented. The programming differences between these two groups have not been defined, and whether the memory fate of graft-elicited T cells can be predicted based on knowledge gleaned from viral models has yet to be determined. Here, we sought to determine whether transplantation results in the differentiation of KLRG-1^{hi} $CD127^{lo}$ SLEC and KLRG-1^{lo} $CD127^{hi}$ MPEC populations, and to define the functional characteristics of these subsets in the context of transplantation as compared to infection. We find that the expression of surface markers and cytokines in $CD8^+$ T cells responding to skin grafts differs from those responding to an infection, and may help predict the strength of subsequent recall development.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2b) mice were obtained from the National Cancer Institute (Frederick, MD). OT-I (12) and OT-II (13) transgenic mice were purchased from Taconic Farms (Germantown, NY) and bred to Thy1.1$^+$ background at Emory University. mOVA mice (C57BL/6 background, H-2b) (14) were a generous gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University (protocol number: DAR-2002050-092815GN). All surgery was performed under general anesthesia with maximum efforts made to minimize suffering. All animals were housed in specific pathogen-free animal facilities at Emory University.

T cell adoptive transfers

OT-I Thy1.1$^+$ TCR transgenic T cells were harvested from spleen and mLN of naïve animals. Flow cytometry was used to determine the frequency of OT-I T cells prior to adoptive transfer by staining with anti-Vα2 (used by both TCRs) and anti-CD8 (BD Pharmingen, San Diego, CA). Cells were counted with a Nexcelom Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA), and 10^6 WT OT-I T cells with our without the same number of OT-II T cells were transferred into naive C57BL/6 mice i.v. 24 to 48 hours prior to infection or transplantation.

Infections

Mice were infected with 10^5 PFU gHV68-OVA i.p. on day 0 as has been previously described (15). The virus was a gift of Dr. Sam Speck (Emory University, Atlanta, GA). For *Listeria monocytogenes* infections, mice were infected with 10^4 CFU LM-OVA i.p. on day 0 (16).

Skin grafting

Full thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (Trambley et al., 1999). Grafts with less than 10% viable tissue remaining were scored as rejected. Where indicated, mice were treated on days 0, 2, 4, and 6 with 500 ug CTLA-4 Ig (abatacept, Bristol Myers-Squibb), 250ug anti-CD154 (clone MR-1, BioXCell, West Lebanon, NJ), and 250 ug anti-CD127 (clone A7R34, BioXCell.)

Secondary T cell effector generation

Mice footpad immunizations have been previously described (17). In brief, SIINFEKL stock **[**$OVA_{257-264}$**]** (GenScript) was diluted in PBS and combined with Incomplete Freund's Adjuvant (LifeTechnologies) to create an emulsion (final peptide concentration 0.1 mM). 50 µl of the prepared emulsion was injected subcutaneously into each hind foot (100 µl/mouse; 10 µg peptide/mouse) at four weeks post priming. Five days subsequent to footpad injection, popliteal lymph nodes were harvested for analysis.

Flow cytometric analyses

Splenocytes were stained according to the manufacturer's guidelines with CD4-BUV395, CD8-BV786, CD44-APC-Cy7, KLRG-1-V450, IL-2-PE (BD Pharmingen), LIVE/DEAD Fixable Aqua Dead Cell Stain (ThermoFisher Scientific), and CD-127-PE-Cy7, CD3-BV711, Thy1.1-A700, IFN-γ-PE/Dazzle594 (BioLegend). Flow cytometry was performed using a BD LSR Fortessa Cell Analyzer (BD Biosciences, San Jose, CA). Data collected were analyzed using FlowJo Software (Tree Star, San Carlos, CA) and analyzed with Prism 6 software (GraphPad Software Inc).

Intracellular cytokine staining

Cells were cultured for 4 hours in 96-well round-bottomed plates at a concentration of 1-2.5 × 10^6 cells/well in 0.2 mL of complete medium and restimulated *ex vivo* with 1 μg/mL PMA (Sigma Life Sciences) and 1 μg/mL Ionomycin (Sigma Life Sciences) where indicated, in the presence of 1 μg/mL Brefeldin A (BD Biosciences) for 4 hours. The Fix/Perm intracellular staining kit (BD Pharmingen) was used to detect IL-2 (BD Biosciences), TNF (BioLegend), and IFN-γ (BD Biosciences), according to manufacturer's instructions.

Statistical analyses

Groups were compared by nonparametric t-test or ANOVA (GraphPad Prism Software, GraphPad, La Jolla, CA). Survival curves were compared by log-rank test.

RESULTS

Graft-elicited CD8$^+$ T cell responses exhibit more rapid contraction as compared to gHV-elicited CD8$^+$ T cell responses

To interrogate the differences T cell programming between graft- and pathogen-elicited antigen specific CD8$^+$ T cell responses, monoclonal TCR-transgenic Thy 1.1$^+$ CD8$^+$ T cells specific for chicken ovalbumin (OVA) were adoptively transferred into naïve C57BL/6 (B6) mice. Twenty-four hours later, mice were either infected with OVA-expressing gammaherpesvirus-68 (gHV-OVA) or received an OVA-expressing skin graft (OVA-SG). Gammaherpesvirus 68 was selected in this model given its functional similarity to and genetic homology with human Epstein Barr Virus (EBV). EBV has been shown to elicit T cells that exert allo-HLA cross reactivity (9, 18, 19). Thus, EBV in transplant poses potential risk for rejection by heterologous immunity. To evaluate subsequent differences in the kinetics of the immune response, frequencies and numbers of OVA-specific Thy 1.1$^+$ CD8$^+$ T cells were determined at the peak of the immune response (day 9-10), during contraction (day 16), and at day 30 (Fig. 5.1A). Spleens were harvested at 9, 16, and 30 days post-infection. The frequency of Thy1.1$^+$ CD8$^+$ antigen-specific T cells harvested from the spleens of mice that were transplanted was significantly lower than the frequency of Thy1.1$^+$ CD8$^+$ antigen specific T cells from the mice that were infected with gHV at all time points (day 9 $p < 0.05$, day 16 $p < 0.05$, day 30 $p < 0.0001$) (Fig. 5.1B,C). The number of Thy1.1$^+$ CD8$^+$ antigen-specific T cells harvested from the spleens of mice that received a skin graft was also significantly lower than the number of Thy 1.1$^+$ CD8$^+$ antigen specific T cells harvested from the mice that were infected with gHV-OVA at all time points ($p < 0.01$ at Days 9, 16, and 30 post-challenge (Figure 5.1D).

In order to assess the relative amount of contraction in each of the immune responses, the difference in the numbers of Thy1.1$^+$ CD8$^+$ antigen specific T cells present from day 9 (peak) to day 16 was calculated, and no differences were detected. However, the percent reduction in Thy1.1$^+$ CD8$^+$ antigen specific T cell number between day 9 and day 30 was statistically greater for graft-elicited T cells relative to gHV-elicited T cells (Figure 5.1E). Specifically, there was a nearly 89% reduction in Thy1.1$^+$ CD8$^+$ antigen specific T cell number on day 30 relative to the peak on day 9 after skin graft priming, as

compared to an approximate 64% reduction in Thy1.1$^+$ CD8$^+$ antigen specific T cell number on day 30 relative to the peak on day 9 after infectious priming. These differences in frequency, number, and percent reduction from peak could not be explained by a difference in activation given that the expression of CD44 remained similar between the two groups (Figure 5.1F).

Skin-graft-elicited CD8$^+$ antigen-specific T cells express lower levels of KLRG-1 than those generated following infection

Previous models of infection have described memory precursor effector cell (MPEC) phenotype as low KLRG-1 expression and high CD127 expression. Based on the reduced degree of contraction after infection as compared to skin grafting, one would expect a higher frequency of MPECs after infection during the effector phase of the response. In order to test this, the effector phase of the response was examined and surface marker expression studied by flow cytometry. At day 9, KLRG-1 expression in skin graft-primed Thy 1.1$^+$ CD8$^+$ antigen-specific T cells was significantly lower than that in infection-primed Thy 1.1$^+$ CD8$^+$ antigen-specific T cells. These differences were also observed at 16 and 30 days post-infection (Figure 5.2A-C). Conversely, there was no difference in CD127 expression at day 9, though day 30 expression of CD127 in skin graft-primed Thy 1.1$^+$ CD8$^+$ antigen-specific T cells was significantly higher than that of gHV-OVA-primed cells (Figure 5.2D). In order to exclude the possibility that these differences in KLRG-1 expression were specific to gHV, another infection was used as a comparator. After adoptively transferring Thy1.1$^+$ CD8$^+$ antigen specific T cells into B6 mice, cells were introduced to the cognate OVA antigen expressed by either *Listeria monocytogenes* (LM) or a skin graft. Similar to what was previously observed with gHV, skin graft-primed Thy 1.1$^+$ CD8$^+$ antigen-specific T cells showed lower KLRG-1 expression than LM-primed Thy 1.1$^+$ CD8$^+$ antigen-specific T cells (Fig. 5.2E-F).

Cytokine profiles of skin graft- versus gHV-elicited antigen-specific CD8$^+$ T cells differ at the peak of the response

Given the phenotypic differences of Thy 1.1^+ $CD8^+$ antigen-specific cells primed in different environments, cytokine profile was assessed to determine whether the functionality of these cells mirrored the phenotypic profile as would be predicted based on viral models. After adoptively transferring antigen specific $CD8^+$ T cells and priming with either gHV-expressing OVA or skin graft-expressing OVA, $Thy1.1^+$ $CD8^+$ antigen specific cells were harvested from the spleen at day 9 post-infection. After four hours of in vitro stimulation using PMA and ionomycin, cytokine production was evaluated. Skin graft-elicited $Thy1.1^+$ $CD8^+$ antigen-specific T cells produced more IL-2 and less IFN-γ than the gHV-elicited $Thy1.1^+$ $CD8^+$ antigen-specific T cells (Fig. 5.3A-B). Thus, the cytokine profile of graft-elicited cells was consistent with that of KLRG-1^{lo} cells (MPECs), which exhibit enhanced IL-2 expression as compared to KLRG-1^{hi} cells (SLECs).

The context in which antigen is presented may affect recall potential of Thy1.1$^+$ CD8$^+$ antigen specific T cells

While the reduced frequency and number and accelerated contraction of $Thy1.1^+$ $CD8^+$ antigen specific T cells primed by skin graft suggested a potentially less robust antigen-specific memory T cell population, the phenotypic and functional characteristics of antigen-specific $CD8^+$ T cells generated by a graft were consistent with the classically-defined MPEC phenotype (2, 3, 6). To interrogate recall potential of cells primed in different environments, cells were primed with either gHV-OVA or SG-OVA as previously described. Four weeks after priming, mesenteric lymph nodes were harvested to assess the memory status of $Thy1.1^+$ $CD8^+$ antigen specific T cells primed under these different conditions. A second cohort of mice received footpad immunization with SIINFEKL ($OVA_{257-264}$) to generate secondary effectors. Draining popliteal nodes were harvested five days after rechallenge (Figure 5.4A).

$Thy1.1^+$ $CD8^+$ antigen specific T cells primed under both conditions showed a trend toward expansion after recall stimulus as evidenced by an increase in frequency and number of $Thy1.1^+$ $CD8^+$ antigen specific T cells, however, under these conditions only cells primed via a skin graft reached statistical significance ($p<0.05$) (Figure 5.4B-D). In comparing the fold change of $Thy1.1^+$ $CD8^+$antigen

specific T cells numbers found after rechallenge to the average number of Thy1.1$^+$ CD8$^+$antigen T cells found four weeks after priming, the number of Thy1.1$^+$ CD8$^+$antigen specific T cells primed by skin graft and rechallenged with footpad peptide increased by 6.03 fold, while the number of Thy1.1$^+$ CD8$^+$antigen specific T cells primed by gHV and rechallenged with footpad peptide increased by only 2.5 fold. These results suggest that skin graft-elicited memory T cells are capable of more robust recall responses as compared to pathogen-elicited memory T cells.

CD127 blockade differentially impacts graft survival in recipients possessing graft vs. gHV-primed CD8$^+$ memory T cells

Based on the observation that donor-reactive CD8$^+$ memory T cells elicited via a prior transplant exhibited a more MPEC-like phenotype with greater expression of CD127, we posited that graft-elicited CD8$^+$ memory T cell population may be more reliant on CD127-mediated signals for survival and/or recall responses, and therefore more susceptible to CD127 blockade. To test this, recipients containing either graft- or gHV-elicited CD8$^+$ T cells were rechallenged with an OVA-expressing skin graft and treated with costimulation blockade (CTLA-4 Ig and anti-CD154 (MR-1) along with anti-CD127. As is depicted in Figure 5.5, while animals containing gHV-primed CD8$^+$ T cells rapidly rejected their skin grafts with a median survival time of 16 days, the majority of recipients possessing graft-elicited CD8$^+$ memory T cells maintained their grafts for the duration of the observation period (45 days). These data suggest that graft-elicited memory CD8$^+$ T cell populations may be more reliant on IL-7-mediated signals in order to persist and carry out secondary recall responses as compared to gHV-elicited CD8$^+$ memory T cells.

DISCUSSION

We have identified a difference in the response of Thy1.1^{+} CD8^{+} antigen-specific T cells to cognate antigen based on the environment in which the antigen is presented. Antigen specific CD8^{+} T cells, when primed by a skin graft, contract faster than antigen specific CD8^{+} T cells primed by gHV, yet expand more rapidly upon restimulation. This increased recall potential is consistent with their KLRG-1^{lo} CD127^{hi} phenotype (as originally defined in viral models (4)), and higher frequencies of IL-secreting cells. Taken together, this study suggests that KLRG-1/CD127 surface marker expression and IL-2 production depend on the priming conditions, and reveal that the T cell differentiation program induced in the context of transplantation is distinct from those induced in the context of exposure to a viral pathogen.

What are the mechanisms mediating this effect? It is possible that differences in the degree of antigen exposure underlie the observed phenotypic and functional differences, because in previously published work, limiting the amount and duration of antigen exposure during priming resulted in reduced expression of KLRG-1 (20). However, our data do not support this possibility, because it is established that the clearance of *Listeria monocytogenes* and gammaherpesvirs-68 is different, as antigen clearance after inoculation with *Listeria monocytogenes* in low dose (10^5 CFU) is 5 days (21), while completion of antigen clearance and establishment of latency for acute gamma herpesvirus is around 15 days (22). In comparing the response of Thy1.1^{+} CD8^{+} antigen-specific T cells to gammaherpesvirus-68, *Listeria monocytogenes*, and skin graft, we demonstrate that in the face of variable antigen clearance, the phenotypic expression of KLRG-1 on the surface of pathogen-elicited Thy1.1^{+} CD8^{+} antigen-specific T cells remains similar to one another, and yet divergent from the expression seen on skin graft-elicited Thy1.1^{+} CD8^{+} antigen-specific T cells. Thus, the data presented here do not support the conclusion that variability in the degree of antigen exposure underlies the observed differences in KLRG-1 expression.

Alternatively, the difference in KLRG-1 expression may relate to a difference in the inflammatory milieu associated with infection versus grafting. Data have emerged regarding the role of inflammatory molecules in regulating CD8^{+} T cell potential (23). For example, IL-12 has been shown to influence the expression of KLRG-1 (24, 25), and type I interferons and IL-6 have potent

immunomodulatory effects that can potentiate allograft rejection even after graft tolerance (26). Thus, differences in inflammatory environment associated with infection versus grafting are likely to influence T cell differentiation programs. It is also important to note that differences in memory T cell precursor frequency could impact the relative resistance of the population to CD127 blockade. Nonetheless, our data demonstrate that differential priming conditions result in different frequencies of graft-specific memory T cells and that these differences in numbers of graft-reactive $CD8^+$ T cells could impact susceptibility to CD127 blockade at the population level.

Importantly, identifying distinguishing characteristics of T cells primed by graft versus infection may enable the definition of cellular recall potential within a memory repertoire and ultimately help to stratify risk of rejection. Memory T cells can be a barrier to successful transplantation, but which memory cells pose the greatest risk to the establishment of tolerance remains to be fully elucidated (7). We have observed that while accelerated contraction kinetics resulted in skin graft-primed $Thy1.1^+$ $CD8^+$ T cells forming a smaller memory pool, the surface marker and cytokine expression profile of these cells at the peak of the response bear functional and phenotypic markers of a long lived memory precursors rather than short lived effectors. Specifically, increased expression of CD127 on skin graft-elicited $CD8^+$ T cells may suggest they are less terminally differentiated and better able to serve as secondary effectors on rechallenge relative to pathogen-primed $Thy1.1^+CD8^+$ T cells (despite fewer numbers). In support of this, subsequent evaluation of recall potential by peptide rechallenge revealed a trend toward increased expansion by skin graft-primed $Thy1.1^+CD8^+$ antigen specific T cells. These data further support the idea that surface marker expression and cytokine profile may be able to predict recall potential during transplantation. Moreover, this finding also suggests that "traditional" sensitization may pose a greater barrier to transplant tolerance than heterologous immunity because it may generate cells that are less differentiated and therefore have better recall potential.

While it is uncertain whether the differences in KLRG-1 expression on human $CD8^+$ T cells would predict recall potential as it pertains to graft rejection, KLRG-1 expression as a marker of terminal differentiation and senescence has been described in humans (27). Emerging data in both non-human

primates and humans have shown that presence of memory T cells with less evidence of senescence (i.e., those expressing high levels of CD28 and CD127 and secreting IL-2) may predict propensity toward belatacept (costimulation)-resistant rejection (28, 29). These findings are in line with in our murine model, where we describe lower expression of KLRG-1 at the peak of the response and higher expression of CD127 at memory time points on $CD8^+$ antigen specific T cells that may have a more robust recall potential.

Most importantly, more granular understanding of the cell subsets that are most capable of precipitating transplant rejection will facilitate development of immunosuppression to more specifically target these subsets. Our data indicate that at four weeks post priming, the antigen specific $CD8^+$ T cells that are associated with the greatest potential for recall are those expressing high levels of CD127, and that blocking this receptor may be one way to overcome a graft-elicited memory barrier. Indeed, the addition of anti-CD127 to costimulation blockade-based immunosuppression in murine models has been previously been shown to prevent the development of allograft rejection and lead to indefinite survival (30), but further investigations will be needed to determine the utility in non-human primates and humans.

In sum, our data suggest that by understanding how phenotypic differences in memory $CD8^+$ T cells impacts functionality during recall, it may be possible to characterize those cells that put patients at increased risk for allograft rejection, and may allow for more personalized immunosuppression following transplantation. Further, increased understanding of the cellular and molecular pathways by which stimulation history affects memory differentiation may lead to the identification of novel targets for therapeutic intervention in order to improve outcomes in patients at increased immunologic risk following transplantation.

FIGURE LEGENDS

Figure 5.1. Magnitude and kinetics of antigen specific T cell responses following skin grafting or gHV infection. A) 10^6 WT $CD45.2^+$ OVA-specific monoclonal TCR-transgenic $Thy1.1^+$ CD8+ T cells were transferred into naïve $CD45.1^+$ C57BL/6 mice at day -1. Twenty-four hours later these animals were

infected with 10^5 PFU gHV-OVA or received two OVA-expressing full thickness dorsal skin grafts. Spleens were harvested and processed at multiple time points from day 9 onward and cells were collected for surface and intracellular cytokine analyses. **B)** Representative flow cytometric staining of Thy1.1$^+$ CD8$^+$ antigen-specific T cells at days 9, 16, and 30. **C)** The frequency of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells harvested after gHV and skin graft priming was different at days 9, 16 , and 30. **D)** The total number of Thy1.1$^+$ CD8$^+$ antigen-specific T cells was different at day 9, 16, 30. **E)** Peak numbers were characterized as mean number of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells harvested at day 9 after gHV and skin graft priming. The percent reduction in Thy 1.1$^+$ CD8$^+$ antigen-specific T cell number from day 9 to day 16 was not statistically different, but was statistically different at day 30. **F)** CD44 expression among Thy 1.1$^+$ CD8$^+$ antigen-specific T cells stimulated by graft and pathogen is similar across all time points. Representative of 3 independent experiments with 5-10 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

Figure 5.2. Skin-graft-elicited Thy1.1$^+$ CD8$^+$ cells can be distinguished from those elicited by based on KLRG-1 expression. A) Representative flow cytometric staining of Thy1.1$^+$ CD8$^+$ antigen-specific T cells indicating the increased expression of KLRG-1 expression in gHV-primed Thy1.1$^+$ CD8$^+$ antigen-specific T cells as compared to similar skin graft-primed cells at day 9. **B)** Histogram representation of the increased expression KLRG-1 in gHV-primed Thy1.1$^+$ CD8$^+$ antigen-specific T cells as compared to skin graft -primed cells at day 9, 16, and 30. **C)** Based on the gMFI, gHV-primed Thy1.1$^+$ CD8$^+$ antigen-specific T cells show increased expression of KLRG-1 as compared to skin graft -primed cells at day 9, day 16, and day 30. **D)** Based on gMFI, there is no difference in expression in CD127 at day 9 between gHV- and skin graft -primed antigen-specific Thy1.1$^+$ CD8$^+$ T cells, however skin graft -primed Thy1.1$^+$ CD8$^+$ antigen-specific T cells show increased expression of CD127 at memory. **E)** Histogram representation of increased KLRG-1 expression on *Listeria monocytogenes (LM)*-primed Thy1.1$^+$ CD8$^+$ antigen-specific T cells as compared to skin graft -primed cells at day 10. **F)** Graphical depiction of KLRG-1 expression by gMFI showing increased expression of KLRG-1 in LM-primed Thy1.1$^+$ CD8$^+$

antigen-specific T cells as compared to similar skin graft-primed cells at day. Representative of 3 independent experiments with 5-10 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

Figure 5.3. Cytokine profiles of skin graft- versus gHV-elicited antigen-specific Thy1.1$^+$ CD8$^+$ T cells are different at the peak of the response. A) Splenocytes were harvested at day 9 and stimulated with PMA/Ionomycin. The frequency of IFN-γ and IL-2 producing cells was determined by flow cytometric staining. **B)** The results from figure 4A were quantitated. Skin graft-elicited antigen-specific Thy1.1$^+$ CD8$^+$ T cells show increased frequency of IL-2 producing cells and decreased frequency of IFNγ producing cells at day 9. Representative of 3 independent experiments with 5-10 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

Figure 5.4. The context in which antigen is presented may affect recall potential of Thy1.1$^+$ CD8$^+$ antigen specific T cells. A) Experimental design for generation of secondary effectors and assessments. OVA-specific monoclonal TCR-transgenic Thy1.1$^+$ T cells were transferred into C57BL/6 mice at day -1. The cells were then exposed OVA antigen in vivo by OVA expressing gHV or skin grafts at day zero. Mesenteric lymph nodes were harvested four weeks after primary challenge for baseline assessments and draining popliteal lymph nodes were harvested 5 days post footpad rechallenge. **B)** Representative flow cytometric staining of Thy1.1$^+$ CD8$^+$ antigen-specific T cells at four weeks post priming and five days after rechallenge. C) The frequency of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells harvested after gHV and skin graft priming was not statistically different between priming environments. D) The number of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells harvested after gHV and skin graft priming was not statistically different between priming environments. E) The fold change in number from average numbers of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells was not statistically different, but there appears to be a trend toward a greater fold change in the number of Thy 1.1$^+$ CD8$^+$ antigen-specific T cells primed by skin graft.

Representative of 3 independent experiments with 5-10 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

Figure 5.5. CD127 blockade differentially impacts graft survival in recipients possessing graft vs. gHV-primed $CD8^+$ memory T cells. A) 10^6 WT $CD45.2^+$ OVA-specific monoclonal TCR-transgenic $Thy1.1^+$ $CD8^+$ T cells were transferred into naïve $CD45.1^+$ C57BL/6 mice at day -1. Twenty-four hours later these animals were infected with 10^5 PFU gHV-OVA or received an OVA-expressing full thickness dorsal skin grafts. At day 30 post-grafting or post-infection, all animals were rechallenged with an OVA-expressing skin graft. Animals were treated on days 0, 2, 4, and 6 post-challenge with CTLA-4 Ig and anti-CD154 costimulation blockade along with 250 ug/dose of anti-CD127 as described in materials and methods and were monitored for graft survival. While animals possessing gHV-primed T cells rejected their grafts with an MST of 16 days (n=24), the majority of animals possessing SG-primed donor-reactive T cells (n=13) went on to accept their allografts (MST undefined, p=0.0139, 3 independent experiments were performed).

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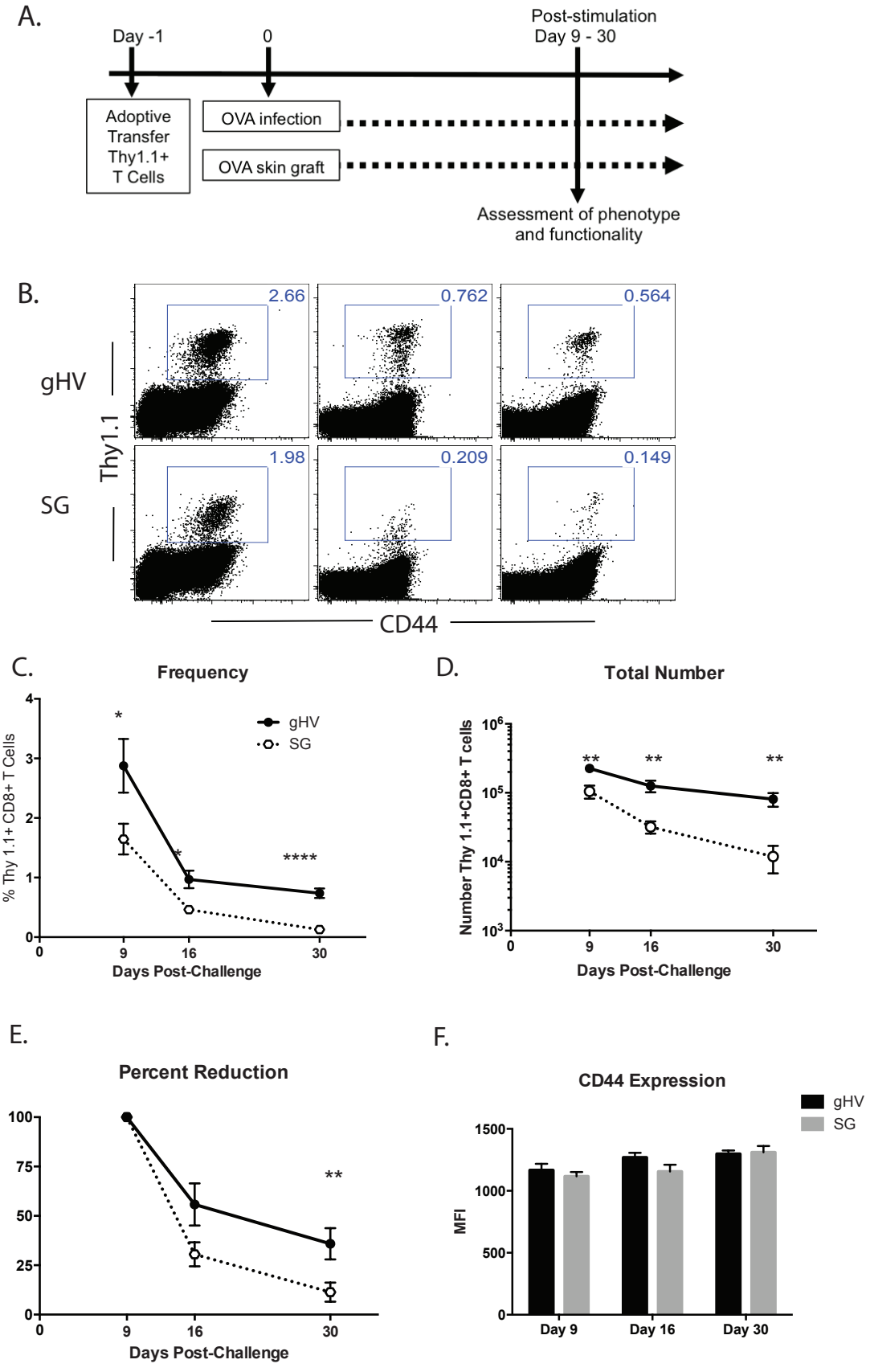

A.
Day -1
0
Post-stimulation
Day 9 - 30
Adoptive Transfer Thy1.1+ T Cells
OVA infection
OVA skin graft
Assessment of phenotype and functionality
B.
gHV
SG
Thy1.1
CD44
2.66
0.762
0.564
1.98
0.209
0.149
C.
Frequency
% Thy 1.1+ CD8+ T Cells
Days Post-Challenge
gHV
SG
D.
Total Number
Number Thy 1.1+CD8+ T cells
Days Post-Challenge
E.
Percent Reduction
% of Peak
Days Post-Challenge
F.
CD44 Expression
MFI
gHV
SG
Day 9
Day 16
Day 30

Figure 5.1

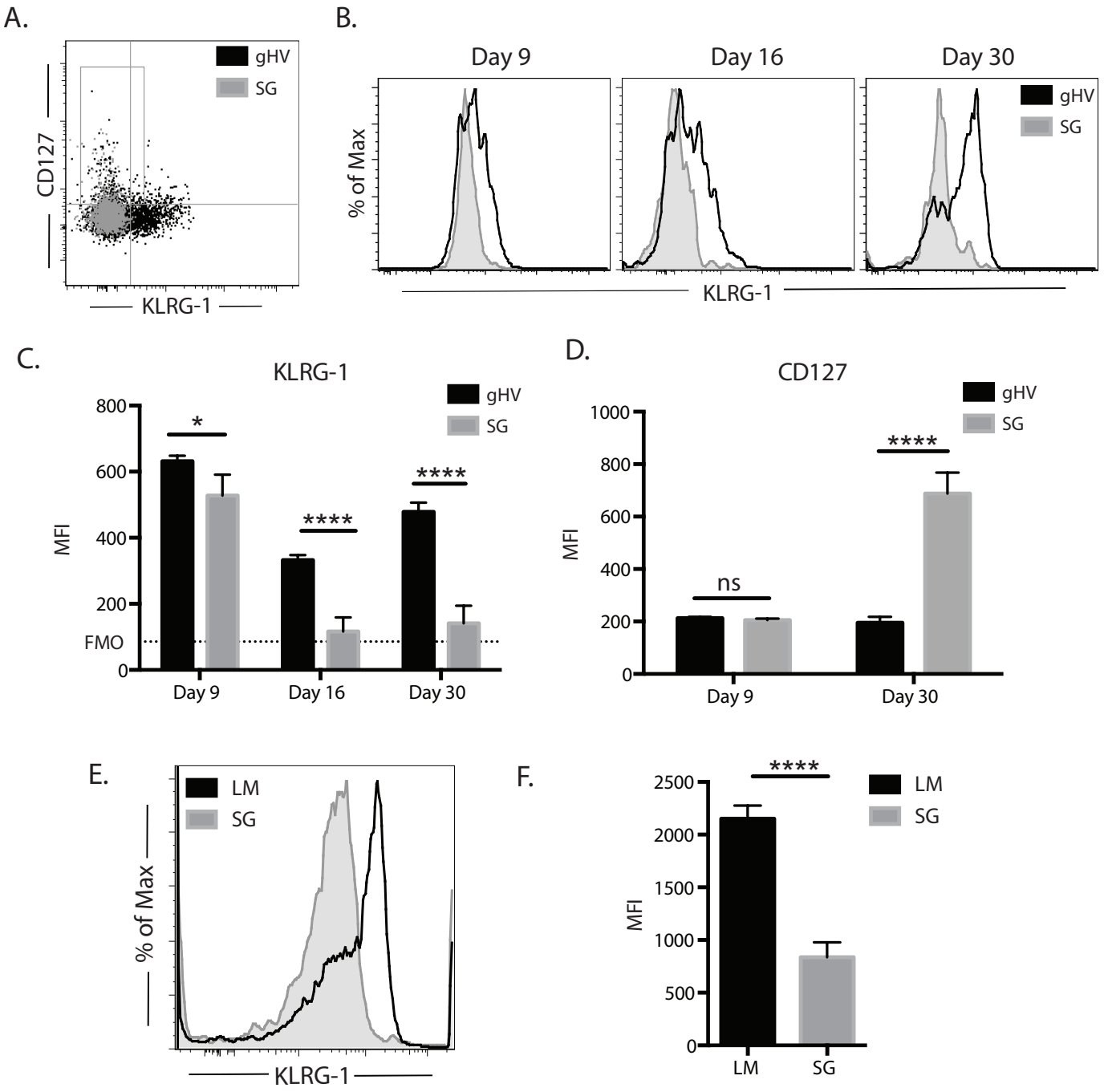
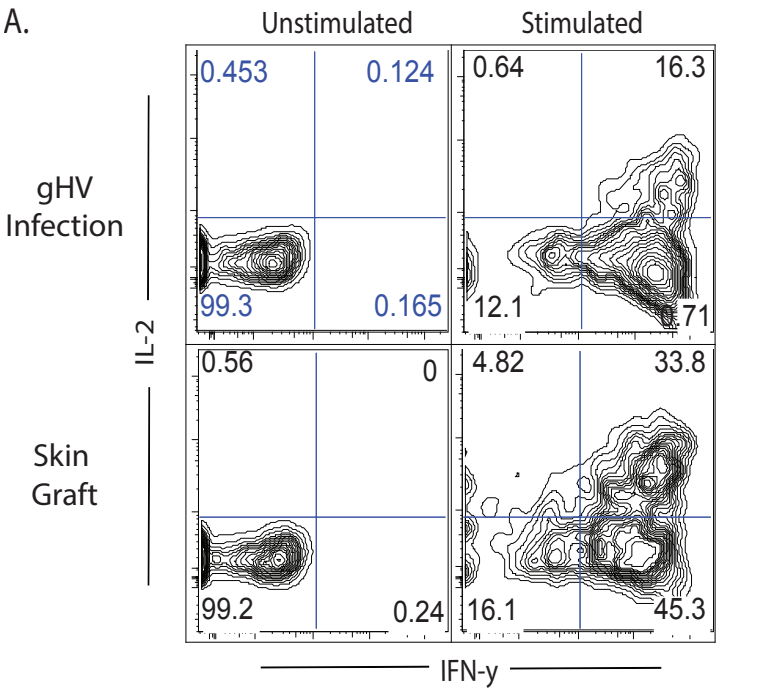

A.
gHV
SG
CD127
KLRG-1
B.
Day 9
Day 16
Day 30
gHV
SG
% of Max
KLRG-1
C.
KLRG-1
gHV
SG
800
600
400
200
FMO
0
MFI
*
****
****
Day 9
Day 16
Day 30
D.
CD127
gHV
SG
1000
800
600
400
200
0
MFI
ns
****
Day 9
Day 30
E.
LM
SG
% of Max
KLRG-1
F.
****
LM
SG
2500
2000
1500
1000
500
0
MFI
LM
SG

Figure 5.2


A.
Unstimulated
Stimulated
gHV Infection
Skin Graft
IL-2
IFN-y
0.453
0.124
99.3
0.165
0.64
16.3
12.1
71
0.56
0
99.2
0.24
4.82
33.8
16.1
45.3

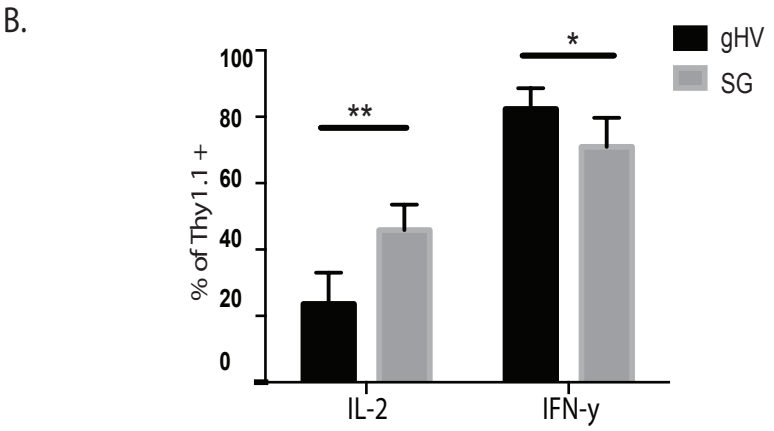

B.
gHV
SG
% of Thy1.1 +
100
80
60
40
20
0
**
*
IL-2
IFN-y

Figure 5.3

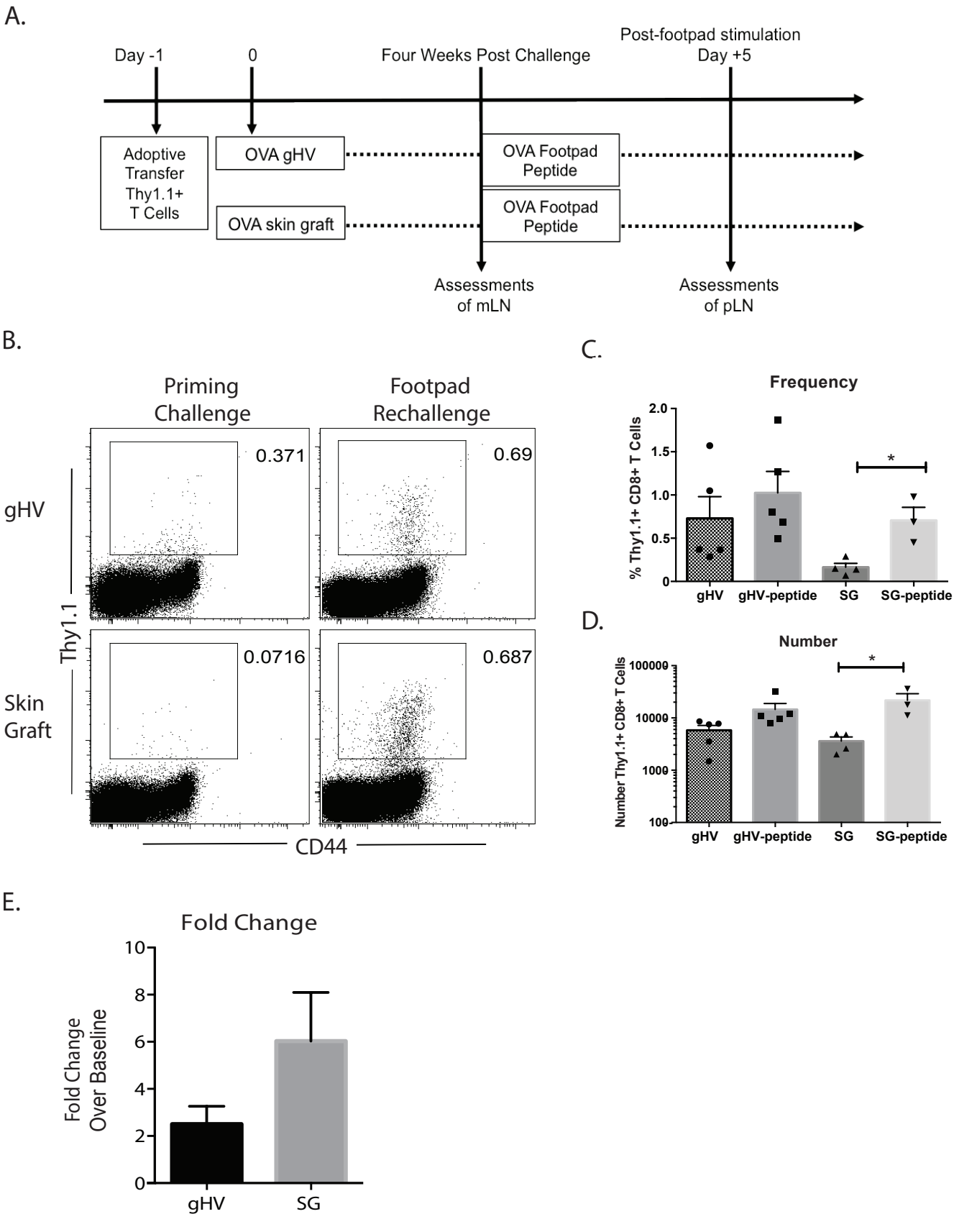

A.
Day -1
0
Four Weeks Post Challenge
Post-footpad stimulation
Day +5
Adoptive Transfer Thy1.1+ T Cells
OVA gHV
OVA skin graft
OVA Footpad Peptide
OVA Footpad Peptide
Assessments of mLN
Assessments of pLN
B.
Priming Challenge
Footpad Rechallenge
gHV
Skin Graft
Thy1.1
CD44
0.371
0.69
0.0716
0.687
C.
Frequency
% Thy1.1+ CD8+ T Cells
gHV
gHV-peptide
SG
SG-peptide
D.
Number
Number Thy1.1+ CD8+ T Cells
E.
Fold Change
Fold Change Over Baseline

Figure 5.4

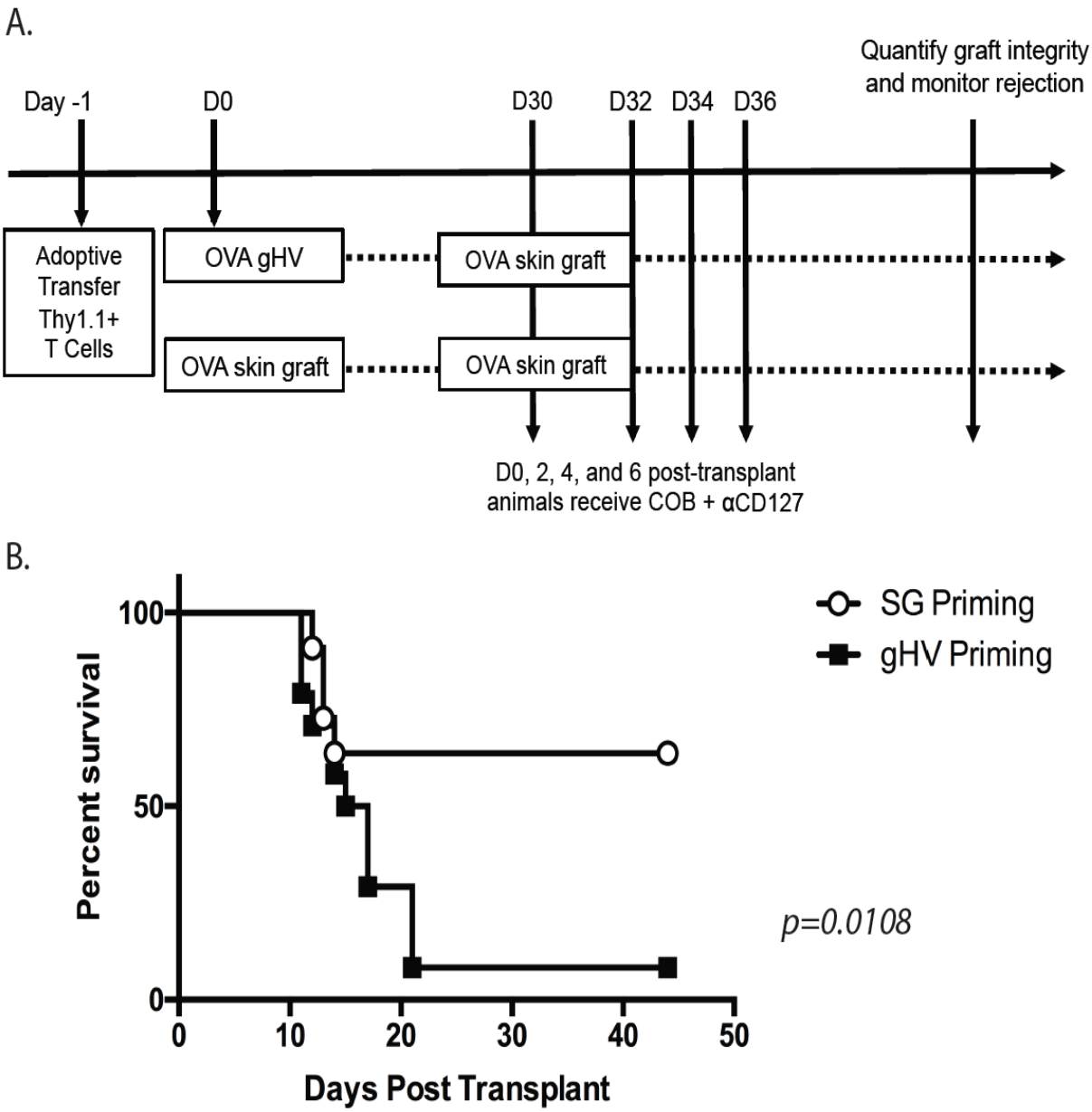

A.
Quantify graft integrity
and monitor rejection
Day -1
D0
D30
D32
D34
D36
Adoptive
Transfer
Thy1.1+
T Cells
OVA gHV
OVA skin graft
OVA skin graft
OVA skin graft
D0, 2, 4, and 6 post-transplant
animals receive COB + αCD127
B.
SG Priming
gHV Priming
Percent survival
100
50
0
0
10
20
30
40
50
Days Post Transplant
p=0.0108

Figure 5.5

Chapter 6

Discussion

Part I: Coinhibitory Receptors on Antigen-experienced Memory T Cells Represent a New Class of Therapeutic Targets to Control T Cells Following Transplantation

As has been discussed in other places in this work, a potent barrier to successful control of T cell responses following allotransplantation is heterologous immunity, or pre-existing immune memory to previously encountered pathogens that can alter responses to subsequent challenges by unrelated antigens [1, 2]. Recent work has described how current methods used to block costimulatory signaling in T cells following transplantation, including the blockade of CD28-mediated costimulation, has had clinical success, as is exemplified by the development and clinical use of belatacept, a CTLA-4Ig derivative that binds to B7-1 and B7-2. Patients treated with a belatacept-based immunosuppression regimen had better graft function as measured by the glomerular filtration rate (GFR) than those treated with standard CNI's alone, even 3 years post-transplantation [3]. While the benefits of belatacept use include reduced toxicity and a lowered long-term risk of cancer and infection [4], use of belatacept in the BENEFIT study was associated with an increased rate of acute rejection after 2-3 years when compared to standard of care CNI-based regiments (Refer to Figure 1.1) [4-6]. Thus, though these therapies are clearly superior to immunosuppression using traditional calcenurin inhibitors, new targets are needed to develop therapies that can be used in conjunction with CD28 blockade.

The work we present in Chapter Two challenges the widely held, yet unproven notion, that $CD28^{null}$ cells mediate costimulation blockade. Interestingly, though antigen-experienced T cells do downregulate CD28, possibly rendering them less susceptible to control by CD28-directed immunosuppressive strategies, our data clearly challenge the

paradigm that CD28 null cells are *more* alloreactive. Instead, we show that patients possessing higher frequency of $CD28^{null}$ $CD4^{+}$ T_{EM} prior to transplant were actually less likely to experience acute rejection following treatment with a belatacept-based immunosuppressive regimen. Importantly, our data also show that as cells lose surface expression of CD28 following activation, they upregulate coinhibitory molecules to introduce another layer of control to prevent aberrant pathology. We show an association between high frequencies of $CD28^{+}$ $CD4^{+}$ T_{EM} and rejection, and have established that the $CD28^{null}$ cells associated with stability express high levels of the coinhibitors 2B4 and TIGIT. These data suggest that perhaps a $CD28^{+}$ $CD57^{+}$ $2B4^{-}$ $TIGIT^{null}$ T cell may be most capable of mediating belatacept-resistant rejection, a hypothesis that warrants further investigation. Taken together, these data suggest that the mechanisms of rejection at play in recipients containing high pre-transplant frequencies of $CD28^{+}$ $CD4^{+}$ T_{EM} are likely effectively controlled by calcineurin inhibition. Identifying other immunologic pathways critical for recall responses in these CD28-independent populations remains an important step toward the goal of developing CNI-free treatment regimens for these patient populations. In sum, our data offer insight into the immunologic environments associated with belatacept-resistant rejection in human renal transplant recipients and demonstrate an example of how pre-transplant immune profiling may allow for personalized immunotherapy to improve outcomes following transplantation.

In Chapter Three, we established an experimental model wherein 2B4 was retrogenically expressed on donor-reactive murine $CD8^{+}$ T cells in order to investigate whether 2B4 functionally underlies graft acceptance during transplantation. We found that constitutive 2B4 expression resulted in significantly reduced accumulation of donor-

reactive $CD8^+$ T cells following transplantation, and significantly prolonged graft survival following transplantation. This marked reduction in alloreactivity was due to reduced proliferation of 2B4 expressing OT-I cells as compared to control cells, underpinned by increased glycolytic capacity and upregulation of gene expression profiles consistent with enhanced glycolytic machinery in animals where 2B4 was absent on antigen-specific $CD8^+$ cells. Finally, the proliferative advantage associated with 2B4 deficiency was only observed in the setting of glucose sufficiency; in glucose-poor conditions 2B4KO $CD8^+$ T cells lost their proliferative advantage. The results presented in our study suggest that as a regulator of metabolism, 2B4 may be an important therapeutic target in the design of new strategies to control donor-reactive T cells following transplantation. Indeed, recent work demonstrated that the blockade of both glycolysis and glutamine metabolism results in the prevention of allograft rejection in a model of fully MHC-mismatched skin and cardiac transplantation, suggesting that the manipulation of effector cell metabolism is an important mechanism by which alloimmunity can be controlled [7]. In this study we present data suggesting that engagement of 2B4 can negatively regulate donor-reactive T cell responses in the context of transplantation. This is of translational importance, as we have recently published that not all alloreactive T cells are targeted by the CD28 costimulation blocker belatacept following transplantation, and that those that express low levels of CD28 express high levels of 2B4 [8-10]. In sum, our study suggests that agonistic ligation of 2B4 may be a novel target for therapeutic manipulation to control unwanted T cell responses in the setting of transplantation and autoimmunity.

In addition to determining the mechanistic basis by which 2B4 limits T cell proliferation following transplantation, the work we present in Chapter Four identifies a role for both CD28- and CTLA-4 mediated signals in calibrating the level of TIGIT expression following T cell activation. Our data suggest that pharmacologic agonism of TIGIT may synergize with CD28-directed immunotherapy in inhibiting unwanted T cell responses following transplantation. Therefore, targeting coinhibitory molecules may serve to limit the restimulation and subsequent effector function of donor-specific T cells, prolonging allograft survival.

Importantly, the data presented here indicate that modulation of coinhibitory signaling may serve as a "tunable" approach as the work presented in Chapter Five demonstrates that the phenotypic profile and differentiation status of cells that are generated and primed in response to a graft versus an infectious stimulus are fundamentally different. We have described that antigen-specific $CD8^+$ T cells primed by a skin graft contract faster than those primed by gammaherpesvirus (gHV), yet are able to expand more rapidly upon rechallenge. Moreover, graft-primed antigen-specific $CD8^+$ T cells have qualities suggestive of increased recall potential. Additionally, the expression of CD127 at a memory time point suggests graft-elicited $CD8^+$ antigen specific T cells are maintained in a less terminally-differentiated state compared to gHV-elicited $CD8^+$ antigen specific T cells, despite fewer cells being present at that time point. Identifying distinguishing characteristics of T cells primed by graft versus infection may enable the definition of cellular recall potential within a memory repertoire and ultimately help to stratify risk of rejection. Memory T cells can be a barrier to successful transplantation, but which memory cells pose the greatest risk to the establishment of tolerance remains to

be fully elucidated [11]. We have observed that while accelerated contraction kinetics resulted in skin graft-primed donor-specific $CD8^+$ T cells forming a smaller memory pool, the surface marker and cytokine expression profile of these cells at the peak of the response bear functional and phenotypic markers of a long lived memory precursors rather than short lived effectors. Specifically, increased expression of CD127 on skin graft-elicited $CD8^+$ T cells may suggest they are less terminally differentiated and better able to serve as secondary effectors on rechallenge relative to pathogen-primed cells, despite fewer numbers. In support of this, subsequent evaluation of recall potential by peptide rechallenge revealed a trend toward increased expansion by skin graft-primed antigen specific T cells. These data further support the idea that surface marker expression and cytokine profile may be able to predict recall potential during transplantation and suggest that "traditional" sensitization may pose a greater barrier to transplant tolerance than heterologous immunity because it may generate cells that are less differentiated and therefore have better recall potential.

Part II: Conclusions and Perspectives

The findings presented here serve to provide a unifying framework by which we can understand the distinct environmental needs and programing paradigms of alloreactive T cells, and suggest new mechanisms by which we can develop therapeutic targets to attenuate the undesirable T cell responses. Taken together, these data describe how CD28 loss, the presence or absence of 2B4 and TIGIT, metabolic fitness, and phenotypic differences involved in T cell differentiation affect the function and fate of $CD8^+$ memory T cells following transplantation in mice and humans alike. Using these

parameters, we suggest that it may be possible to characterize those cells that put patients at increased risk for allograft rejection, and may allow for more personalized immunosuppression following transplantation.

Though this exciting work increases our understanding of the cellular and molecular pathways by which the expression of coinhibitory molecules, acquisition of bioenergetic profiles, and stimulation history affects memory differentiation may lead to the identification of novel targets for therapeutic intervention in order to improve patient outcomes, many barriers still exist in identifying the immunologic risks that follow transplantation. First, our data indicate that 2B4 and TIGIT may be used as biomarkers to stratify the patients who are most likely to respond to belatacept-based immunosuppressive regiments, challenges remain in determining how best to assess the expression of these molecules in real time. For many years, diagnosis of rejection has relied on protocol biopsies, however these cannot be utilized repeatedly in at risk patients [12]. Therefore, collection of peripheral blood is a frequently used approach, however this remains a relatively invasive procedure, though it is useful, as Heeger and colleagues have demonstrated that alloreactive memory cells that produce IFN-g upon stimulation can be detected in the peripheral blood of renal transplant recipients [13]. Ideally, next generation approaches will involve the assessment of biomarkers via minimally invasive methods, and work has begun to explore RNA signatures in the urine of transplant recipients, and initial reports have identified neutrophil gelatinase-associated lipocalin (NGAL), IL-18, and CXCL9 as potential factors associated with elevated risk of rejection [14, 15].

These proposed approaches to non-invasively measuring indicators of rejection represent an important next step in detecting allograft damage and provide opportunities for clinical intervention, they also point to a deeper issue that runs at the core of modern transplant immunobiology. Each of these strategies is being investigated as a method to control breakthrough rejection that occurs even though patients are being treated on tightly regulated immunosuppressive regiments, raising doubts as to if we will ever be able to achieve true transplant tolerance in humans. Though many groups have called the induction of tolerance the "holy grail" of transplant immunobiology, others question whether or tolerance is even really necessary, should we be able to improve immunosuppressive strategies to the point where rejection and infection no longer post potent risks to transplant recipients. Work from pioneers and leaders in the field suggest that if tolerance is in fact achievable, it will most likely be induced via mixed chimerism, and mediated by tolerogenic dendritic cells, regulatory T cells, and perhaps by B lymphocytes [16-21]. This will continue to be a hotly debated area within transplantation immunology, and for now we must continue to explore all the mechanisms by which we can provide our patients with the best post-operative care possible.

An additional area of interest that must be more fully in light of the work presented here is the affect of patient health and metabolism on the fitness and responsiveness of T cells. The findings presented here in Chapter Three indicate that the bioenergetic profile of a T cell is a key factor in determining its function and fate. We therefore propose that further work must be undertaken to understand how the systemic health, or lack there of, of patients who present for transplantation impacts the immune response. This is of particular concern because immunosuppressive drugs are commonly

associated with the development of obesity, hypertension, hyperglycemia, and dyslipidemia, which are consistent with metabolic syndrome, which was diagnosed in less than 6% of patients prior to transplantation, but upwards of 50% after [22, 23]. What's more, calcineurin inhibitors such as cyclosporine A (CsA) are known to affect glucose metabolism, as there is an elevated risk of the development diabetes following transplantation in patients treated with CsA [24]. Finally, the risk of atherosclerotic events was elevated in patients that developed metabolic disease following transplantation compared to those who did not [25]. Taken together, these data suggest that more work is necessary to understand how these metabolic changes, and especially those related to glucose utilization, affect the priming and functionality of T cells in patients following transplantation.

In summary, the original body of work presented here illustrates the key role that coinhibitory receptors play in controlling donor-reactive T cell responses following solid organ transplantation. These data indicate that signaling downstream of these molecules impacts the differentiation and metabolism of both $CD4^+$ and $CD8^+$ T cells that can mount de novo attacks or be involved in potent memory recall responses against the transplanted allograft tissue. Though a number of barriers remain to completely comprehending how clinical interventions can prolong graft and patient survival, we believe that these data contribute to novel aspects of our understanding of the role of T cells in mediating rejection following transplantation.

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