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DURATION OF ALLOANTIGEN EXPRESSION AFFECTS THE INDUCTION OF TRANSPLANTATION TOLERANCE

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ABSTRACT

Although transplantation is the treatment of choice for end stage organ failure, transplanted organs are vulnerable to immune-mediated rejection. To prevent rejection, patients are maintained on a strict immunosuppressive regimen; however, immunosuppression is costly, and the side effects associated with continuing life-long immunosuppression are numerous and unpleasant, leading to patient noncompliance. Current research efforts aim to replace conventional immunosuppression with short-term treatments inducing donor-specific immune tolerance, which would mean a patient is only nonresponsive to transplant-derived antigen and would retain their ability to mount immune responses to other antigens, such as those from infections and malignancies. Although possible to achieve in patients, donor-specific tolerance is rare and remains vulnerable to inflammatory challenges capable of precipitating transplant rejection. In this dissertation, we investigated vulnerabilities to donor-specific transplantation tolerance during both the induction and maintenance phases of tolerance. We identified functional heterogeneity within the transplant-reactive T cell compartment, dependent upon the length of their cognate antigen exposure in the context of immunosuppression: persistent antigen promoted T cell dysfunction whereas T cells specific for non-persistent antigens retained function. By forcing antigen exposure, we promoted widespread T cell dysfunction and protected the transplant recipients from infection-mediated rejection. We also investigated the memory T cell response following treatment with persistent antigen and immunosuppression and identified that memory T cells can develop a degree of dysfunction, although not to the same extent as naïve T cells. As robust allospecific T cell dysfunction is a hallmark of tolerance and desirable in patients, these findings may inform future strategies to promote clinical transplantation tolerance.

Chapter 1: Introduction

1.01 Hypofunctional T cells in models of persistent antigen

Much is known about the acquisition of hypofunction by T cells specific to persistent antigen in the context of tumors or chronic infections; however, additional models of repeated antigen exposure, including autoimmune disorders and transplantation, can also promote T cell dysfunction. To understand the T cell response to persistent antigen, the nuances associated with each model can help elucidate the broad hallmarks underpinning the programming of T cells upon exposure to persistent antigen.

1.02 T cell activation, differentiation, and memory

Naïve T cells first encounter antigen in the secondary lymphoid organs, where antigen is presented to them in the context of major histocompatibility complex (MHC) along with various cytokines and costimulatory or coinhibitory signals. T cell activation is a tightly controlled process to prevent immunopathology and autoimmunity. Not only are T cells activated in an antigen-specific manner through their T cell receptor (TCR), but T cells also require context for their activation, provided through costimulatory and coinhibitory receptor/ligand crosstalk between the T cell and the antigen-presenting cell (APC). Should the T cell bind the antigen/MHC complex, the T cell's fate and functional capacity are determined by the sum of direct and environmental signals it receives at this stage[1,2]. Co-signaling receptors cluster at the immune synapse during antigen presentation to the T cell, and are expressed in a "tidal" manner, in which stimulatory vs inhibitory receptor expression is fluid along the immune response, rather than an "all or none" reaction to antigen[3]. Importantly, co-signaling is bidirectional, allowing the

T cell to provide maturation signals to APCs as well as receive signals from them. This crosstalk allows for dendritic cell (DC) licensing, which is essential for downstream effector and memory T cell responses [4]. With enough stimulation and the formation of an immune synapse, T cells will initiate effector responses, which vary based on T cell differentiation, but largely include cytokine production, proliferation, metabolic changes, and, for $CD8^+$ T cells, granzyme/perforin production over the course of a few weeks[2].

Environmental cytokines also provide context during T cell activation, leading to downstream differentiation. These signals are important for $CD4^+$ T cells in particular, as they instruct their future identity. IL-12 promotes T helper 1 (Th1) differentiation whereas IL-25 and IL-4 promote Th2[5,6]. IL-6 can either synergize with TGFβ to promote Th17 cells, or with IL-21 to promote T follicular helper (Tfh), and the combination of TGFβ and IL-2 promotes formation of regulatory T cells (Tregs)[5,7]. These Th subsets are also characterized by unique intracellular signaling networks and regulatory elements that respond to environmental signals to help finalize their differentiation[5]. Similar to $CD4^+$ T cells, $CD8^+$ T cells are also susceptible to environmental influence. $CD8^+$ T cells respond similarly to the cytokines listed above, although instead of Th cells, they form cytotoxic T (Tc) cells[8].

There are additional environmental factors distinct from cytokines that impact T cell differentiation. The clearest example of this is in the gut, which is primed to form either Treg or Th17 phenotypes in responses to food antigens[9,10] and microbes[11,12]. Computational modeling identified that cell surface receptor PPARγ signaling drove Th17 cells to differentiate into induced Tregs[13]. More recently, signaling via PPARγ has been identified to modulate a cell's metabolic profile, promoting fatty acid oxidation, which is preferentially used by Tregs[14]. Environmental vitamins can also impact naïve T cell differentiation, with vitamin D as the most

notable example[15]. Vitamin D promotes Treg accumulation by acting both on DCs[16] to promote Treg differentiation and directly on T cells via the vitamin D receptor to induce FoxP3 expression[17,18]. Retinoic acid, a vitamin A derivative, can also influence T cell differentiation. Retinoic acid produced by DCs in the small intestine lamina propria synergizes with TGFβ to promote peripheral Treg conversion and oral tolerance[19]. This effect is dependent upon signaling via T cell retinoic receptor α, preventing accumulation of Th17 cells even in the presence of IL-6 [20,21].

During an infection, or other immunogenic event, T cell effector expansion peaks around day 7 post-antigen introduction and then begins to contract around day 20[22]. Contraction can last up to a year and is important to prevent off-target side effects and tissue damage by immunopathology[22]. Contraction is mediated either through activation-induced cell death (AICD) or by growth factor withdrawal[23]. Tregs also control effector responses by suppressing Teffector cytokine production and proliferation. Tregs can suppress in either antigen-specific or antigen-independent mechanisms. Antigen-specific Treg suppression is mediated through the APC, which Tregs can influence to become tolerogenic, for example by removing pMHCII availability through trans-endocytosis or by inducing coinhibitory receptor upregulation[24]. Antigen-independent suppression includes acting as an IL-2 sink by expressing the high affinity CD25 receptor, expressing high levels of CD73 to degrade adenosine, and producing the cytokines TGFβ, IL-10, and IL-35[24].

T cell functional avidity plays a crucial role in the immune response. Functional avidity is a result of the sum of TCR affinity for pMHC, numbers of TCRs engaged, coreceptor availability, and intracellular signaling. Functional avidity is a measure of the concentration of peptide necessary to mediate half-maximal outcome in a functional assay (EC50), such as

cytotoxicity or a cytokine production assays [25,26]. In vivo, functional avidity is associated with enhanced T cell function and host protection[25,27]. As a population, T cells undergo avidity maturation over the course of an immune response, where higher avidity T cell clones preferentially expand compared to lower avidity clones[26,28,29]. While TCR affinity for pMHC contributes to T cell functional avidity, it seems to be irrelevant for disease pathogenesis[30]. This can be seen in models of type 1 diabetes (T1D), where T cells with a broad array of affinities remain capable of driving autoimmune insulitis[31]. Similarly, in a mouse model of myelin oligodendroglial glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), disease onset did not correlate with MOG-specific TCR affinity[32]. The kinetics of disease progression are likely impacted by TCR functional avidity; however, these examples provide proof that lower affinity T cell clones are also capable of contributing to disease[30,31].

Memory formation is a hallmark of the adaptive immune system and an evolutionary advantage in terms of survival in the face of pathogens. Memory cells are self-renewing and capable of surviving for long periods without pMHC stimulation[22]. Indeed, by repeatedly activating T cells via prime-boost-boost vaccinations and transferring them to new mice over the course of a ten-year period, it was discovered that memory $CD8^+$ T cells have a seemingly intrinsic and unlimited proliferative potential, which likely evolved to benefit adaptive immune memory: short periods of intense antigen exposure and T cell proliferation, followed by reduction of most of the memory cells[33].

Not all T cells become memory cells. It was previously believed that TCR affinity alone drove memory development; however, newer research indicates that memory T cells form even after weak TCR/pMHC interactions[34]. Rather than affinity alone, the length of the TCR/pMHC interaction promotes memory formation[35]. Additionally, clonal precursor frequency has an

impact on memory cell fate, although it is possible that this is a result of reduced TCR signaling because of increased competition between cells[22,36–38].

The memory T cell population can be further divided into central memory T (Tcm) cells, effector memory T (Tem) cells, and resident memory T (Trm) cells. Tcm cells are mostly restricted to immune tissues, including secondary lymphoid organs and mucosal tissue, by their expression of the CD62L and CCR7 homing receptors[22]. In contrast, $CD69^+$ Trm cells reside within non-lymphoid tissues, and Tem cells can migrate between the lymph nodes and non-lymphoid tissues, depending on their chemokine receptor expression[39].

Memory cell reactivation is largely determined by location as, unlike naïve T cells, memory T cells spend more time in peripheral tissues. For tissue-restricted inflammatory events, Trm cells are the first responders due to their proximity. Tem cells must be recruited to inflamed tissue and Tcm cells circulate in the secondary lymphoid organs, thereby delaying antigen encounter. Additionally, memory T cells remember their previous lineage commitment, and reacquire specific effector function upon antigen reencounter[40]. Memory T cells, both $CD4^+$ and $CD8^+$, also require CD28 costimulation for secondary responses[41]. In fact, recent genomic approaches have elucidated that human memory T cells are more sensitive to CD28 costimulation than their naïve counterparts[42]. Interestingly, memory $CD4^+$ T cells were less proliferative than naïve T cells when presented with moderate levels anti-CD3, although the memory cells were much more sensitive to low doses of anti-CD3 than the naïve cells[42]. These data reaffirm memory T cells as being poised to respond to minute levels of antigen, and additionally suggest that the memory population is more responsive to general activation than antigen-specific activation signaling through the TCR.

1.03 Models of T cell dysfunction

Whereas effector and memory T cell formation is a canonical fate after stimulation, T cells exposed to persistent antigens do not follow this path, but rather differentiate to a hypofunctional state. The settings in which T cells develop hypofunctional phenotypes vary widely depending on the biological context associated with exposure to persistent antigen; however, it is true that sterile persistent antigen is sufficient for Th1 hypofunctional phenotype[43]. Therefore, we will refer to T cells responses in these contexts as "hypofunctional" or "dysfunctional", for use of a general term, with "exhaustion" as a sub-header for T cells chronically seeing antigen in the context of inflammation. Additional distinctions include "anergy", for T cells that are primed in the absence of costimulation, and "tolerance," which can be used for either T cells unresponsive to self-antigens, or T cells exposed to immunosuppressive drugs aimed at inducing transplant acceptance. These states, rather than discrete and mutually exclusive, likely exist within a continuum of acquired hypofunction.

1.04 Exhaustion

Of all the states of T cell hypofunction, the most is known about exhaustion. T cells become exhausted following integration of a variety of inhibitory signals, including coinhibitory receptors, cytokines, and Treg suppression[44]. Exposure to persistent antigen is also necessary for exhaustion[45,46]. Importantly, the development of exhaustion is tied closely with the length of TCR stimulation, as exhausted T cells are incapable of forming memory even after their cognate antigen is removed/cleared[47]. T cell exhaustion was first characterized in models of chronic viral infection. Exhaustion following chronic infection is triggered by an overwhelming antigenic load during the onset of the infection, followed by persistence of antigen [48,49]. Anti-viral T cells in

mice infected with chronic lymphocytic choriomeningitis virus (LCMV) were either deleted in response to the large antigenic load or were unresponsive to the ongoing infection[50,51]. HIV-infected patients also harbor exhausted, virus-specific T cell populations [52].

Exhaustion is also a hallmark of tumor-reactive T cells. Exhausted T cells can be found both in the tumor microenvironment and in circulation as a result of persistent antigen exposure[53]. Exhausted T cells are unable to clear tumors, allowing for immune escape. As with exhausted T cells in models of chronic infection, exhausted T cells in tumor models are PD-1^{hi} and have impaired cell function, such as reduced cytokine production [54–56]. In an effort to reinvigorate exhausted T cells in tumors, checkpoint blockade therapy uses antibodies to target the coinhibitory regulators of T cell activation, thereby limiting the inhibitory signals a T cell receives during activation [57]. Common checkpoint targets include CTLA4, PD-1 or PD-L1. Although checkpoint blockade is not effective against terminally exhausted T cells, it has the potential to reinvigorate partially exhausted cells, and promote tumor clearance [58,59]. While not effective for all tumor types or all patients, combination therapies using multiple checkpoint blockade regimens, along with chimeric antigen receptor (CAR)-T cells or other immunotherapies may synergize to provide a more robust approach in the future [60].

Autoreactive CD8$^+$ T cells have also been identified to present an exhausted phenotype[61]. In patients with T1D, the rate of disease progression correlated with autoreactive T cell exhaustion: the longer it took for the disease to progress, the more T cell exhaustion was observed [62]. This is likely because of antigen availability, as T cells that take longer to destroy the islets will have longer exposure to antigen, which in turn promotes dysfunction. However, this is not to say that autoimmunity is resolved in these patients, as a subset of the autoreactive cells retain a stem-like phenotype, reminiscent of other exhaustion models [63,64]. Importantly, not

all autoreactive cells become dysfunctional. In a mouse model of T1D, a stem-like population of autoreactive $CD8^+$ T cells populates the pancreas-draining lymph nodes, allowing for continuous repopulation of the pancreas and subsequent autoimmunity [65]. Transfer of as few as 20 of these stem-like progenitors was sufficient to drive T1D, whereas transfer of 100,000 pancreas derived terminally differentiated cells was not, due to their short-lived nature [65]. Thus, autoimmunity represents a heterogenous model in terms of development of T cell dysfunction.

The inhibitory receptor Lag3 has been recently identified as a potential target for immunotherapy, as Lag3-knockout T cells in a mouse model of autoimmune diabetes showed increased function and movement toward the pancreatic islets [66]. Lag3, in combination with anti-PD-1 therapy, has also been described previously as a target for reversing exhaustion and promoting anti-tumor immune responses [67,68].

Exhausted $CD8^+$ T cells are characterized as proliferative or terminally exhausted, which can be distinguished as T-bet^{hi} or $Eomes^{hi}$ T cells, respectively [69,70]. Blimp-1 expression by exhausted T cells also contributes to high expression of coinhibitory receptors [71,72]. TCF-1 expression helps define exhaustion, where TCF-1^+ T cells retain their proliferative capacity and maintain a stem-like phenotype similar to what is seen with memory T cells [66,73,74]. This contrasts with the TCF-1^- population, which is terminally exhausted [66,73,74]. Similar to $CD8^+$ T cells, exhausted $CD4^+$ T cells are also characterized by Bimp-1, T-bet, and Eomes expression [72]. Additionally, exhausted $CD4^+$ T cells express Helios, likely impacting expression of downstream cytokines including IFN-γ, TNF-α, and IL-2 [72]. Interestingly, the exhausted $CD4^+$ T cells showed a high level of diversity in transcription factor expression, indicating a heterogenous population and likely distinct lineages or fates [72].

Since their initial discovery, the unresponsive, exhausted T cells have been found to express high levels of coinhibitory markers such as PD-1 [56]. As T cells move toward exhaustion, they exhibit progressive loss of function and eventual terminal exhaustion. In tumor models of exhaustion, T cells become dysfunctional rapidly following tumorigenesis [46]. This heterogeneity in the exhausted T cell pool informs various responses to immune checkpoint blockade, with less exhausted cells responding to the treatment and regaining function, and more exhausted cells failing to do so [75,76]. For example, patients retaining higher peripheral cytotoxicity also responded better to anti-PD-1 treatment [77].

Interestingly, TCR signal strength contributes to the acquisition of T cell exhaustion. In a model of altered peptide ligands of the model antigen SV40, TCRs with high signal strength had a dysfunctional phenotype, including coinhibitory receptor upregulation and a reduction in effector functions, resulting in an inability to kill the tumor [78]. Interestingly, the TCRs with more intermediate signal strength had the best tumor clearance, because although TCRs with low signal strength retained their functional capacity, they were unable to kill the tumor and were considered functionally inert [78]. These data correspond with tetramer-based studies of TCR affinity in the tumor microenvironment, where tumor-specific TCRs that bound tetramer with low MFI were slower to progress to dysfunction yet were unable to eliminate the tumor [79]. In this model, it was the T cell clones that were tetramer bright, a proxy for high affinity, that produced more cytokines, even though they progressed more rapidly towards exhaustion [79]. It's important to consider that the higher affinity cells may be more sensitive to the antigenic load in the tumor microenvironment, thereby promoting this exhaustion. Overall, these findings suggest a "Goldilocks" phenomenon, where T cell progression toward exhaustion versus effector functions must be balanced to promote tumor clearance.

Phenotypically, intermediately exhausted $CD8^+$ T cells, characterized as T-$bet^{hi}PD^{mid}$, maintain their proliferative capacity, and retain the ability to respond to checkpoint blockade, whereas $EOMES^{hi}PD^{hi}$ exhausted $CD8^+$ T cells have very high co-inhibitory receptor expression and are less proliferative [70]. Importantly, exhausted T cells can reach a terminal state of exhaustion, where they fail to develop characteristic memory or effector functions even after being removed from the inflammatory and antigen-rich environment [80,81]. In contrast, cells terminally exhausted following chronic infection are "antigen addicted", requiring constant antigenic stimulation in order to survive [80].

Exhausted T cells are also distinct metabolically. Exhausted T cells are characterized by their reduced glucose uptake, reduced glycolysis, reduced mitochondrial mass, and increased mitochondrial depolarization [82–84]. The altered T cell metabolism is partially attributed to the expression of PD-1, whose early ligation represses the transcriptional coactivator PGC-1α, resulting in decreased glycolysis and amino acid metabolism, key components of effector metabolism, and instead switching the cell to rely on fatty acid oxidation as a primary metabolic program [83,85]. Importantly, a distinction must be made between PD-1 driving the metabolic phenotype of exhaustion and PD-1 driving exhaustion, as PD-1 is not necessary for the accumulation of exhausted $CD8^+$ T cells [86].

Exhausted T cells originating from infected mice and exhausted T cells originating from exposure to a tumor microenvironment are both hypofunctional; however, there are key environmental factors distinguishing the two groups. As exhaustion refers to a specific developmental program following overstimulation by persistent antigen in a variety of contexts, it must also be considered that environmental nutrient availability impacts the exhausted metabolic phenotype [87]. For example, there are differences in nutrient availability in the tumor

microenvironment, in the circulation, and in tissues such as the kidneys that could contribute to nuanced metabolic phenotypes in both effector and exhausted T cell subsets [88–91]. Therefore, exhausted T cells arising from tumors are likely to have different metabolic pressures than those arising from infected mice. As altered metabolism is considered a hallmark of the exhausted T cell phenotype, understanding the upstream environments between these two models may provide additional clarity for understanding the developmental progression of T cell hypofunction.

Exhaustion can also be induced in $CD4^+$ T cells exposed to sterile persistent antigen, which resulted in loss of Th1 functions and upregulation of anergic surface markers [43]. This phenotypic change was dependent upon both the duration and quantity of antigen, with long periods of time and intermediate antigen levels promoting dysfunction, low antigen levels proving insufficient for driving dysfunction, and high antigen levels inducing cell death. Furthermore, loss of T cell function was transient, as T cells began recovering function and losing anergic surface markers following removal of antigen [43]. Interestingly, in this model, about half of the genes that responded to antigen persistence did so in a dose-dependent manner, which could inform strategies to reactivate cells in the future.

One area of interest is whether memory T cells can become exhausted. During commitment to the exhausted phenotype, T cells progressively lose their potential to form memory [81]. The working understanding of exhaustion is that it is a terminal differentiation state; thus, if an exhausted T cell regains function and establishes a memory phenotype, it would indicate that the cell was not yet terminally exhausted, as has been described in virus-specific $CD8^+$ T cells [81]. Recently, following 10 years of iterative *in vivo* immunizations and antigen encounters, memory T cells repeatedly exposed to antigen were found to progressively

acquire the hallmarks of exhaustion, including PD-1, TIM3, and TOX expression [33]. However, these were not terminally exhausted cells, as they retained their functional capacity [33]. It must be considered, that in this model, antigen was not strictly persistent, as immunizations were spaced out with 60+ days between heterologous immunizations. Thus, whether memory T cells can fully differentiate into exhausted cells following persistent exposure to antigen is still unclear. These questions have implications for both chronic infections and tumor immunity, as well as clinical potential for informing future vaccine designs or strategies to induce transplantation tolerance, as memory T cells pose the major barrier to the induction of tolerance to allografts.

1.05 Anergy

Classically, anergy is defined as immune unresponsiveness to antigen. Anergy is induced in response to incomplete activation of naïve T cells: low IL-2 availability, low costimulation, or high coinhibition. Thus, anergic T cells differ from exhausted T cells, as exhausted cells are initially primed optimally and then lose function, whereas anergic cells are sub-optimally primed. Anergy is thought to be an adaptive mechanism of self-tolerance designed to protect against autoimmunity: T cells capable of initiating robust effector responses to self-antigen in the absence of an infection must be controlled [92–94].

Exposure of T cells to their cognate antigen in the absence of costimulation can promote either deletion or anergy [95]. In a mouse model where hepatocytes expressed the model tumor antigen Gag, antigen-specific, transgenic $CD8^+$ T cells proliferated and expressed activation markers; however, they failed to accumulate as a majority of the cells underwent cell death, with those surviving losing their ability to proliferate [95]. Interestingly, cell-intrinsic anergy alone is

sufficient to prevent autoimmunity, as antigen-specific, $Bim^{-/-}$ T cells incapable of apoptosis maintained peripheral tolerance and their inability to proliferate or produce cytokines [96]. These findings reinforce the evolutionary need to control responses to self-antigen and prevent autoimmunity.

Anergic $CD4^+$ T cells have also been identified *in vivo* in a model of fetal antigen-specific tolerance: anergic cells were $FoxP3^-CD44^{hi}FR4^{hi}CD73^{hi}$ and were enriched for TCRs specific for self-antigen [97]. This population was also identified to generate precursors for eventual differentiation of peripheral Tregs [97]. Anergic T cells have also been studied in an adoptive transfer model of ovalbumin (OVA)-specific $CD4^+$ T cells, clone DO11, into OVA-expressing mice [98]. Upon transfer, the T cells experience their cognate antigen in the absence of inflammation. In this model, anergic, self-reactive T cells expressed cytokine mRNA, but were unable to produce cytokine protein, indicating posttranscriptional mechanisms for maintaining T cell dysfunction [98].

As determined by gene set enrichment analysis, anergic T cells have a distinct gene expression profile from exhausted T cells [69]. The anergic T cell phenotype develops if NFAT1 is prevented from interacting with its binding partner AP-1 [99]. Genes involved in anergy were associated with transcription, signaling, metabolism, proteolysis, and more [99]. Interestingly, few of the anergy-associated genes were found in exhaustion, reinforcing that anergy and exhaustion are distinct fates with unique transcriptional profiles [69].

Infectious tolerance was first discussed in 1971 by Gershon and Kondo, where they identified an immunosuppressive environment sufficient to promote T cell dysfunction [100]. This immunosuppressive environment is characterized as Treg dense, hypoxic, IL-2 deficient, and rich in the inhibitory cytokines IL-10 and TGFβ [101]. The suppressive cytokine IL-35, which,

when produced by Tregs can also promote IL-35 expression in Tconvs, dubbed "iT$_R$35" or, when produced in B regulatory cells (Bregs), has also been associated with infectious tolerance [102,103]. It was recently discovered that the mechanism of Treg-mediated IL-35 spread is through IL-35-coated extracellular vesicles, which fuse to nearby Tconvs or Bregs, coating them with IL-35 and promoting their dysfunction and secondary suppression [104]. In this way, infectious tolerance mimics an anergic pathway, as environmental cues are sufficient to inhibit complete T cell function.

1.06 Transplantation tolerance

Tolerance is often used as a catch all term, referring not only to central tolerance, but also to other general models of immune non-responsiveness, including oral and transplantation tolerance. Central tolerance is the developmental process in which lymphocytes too strongly reactive for self-antigen are eliminated in the thymus during T cell development. In contrast, oral tolerance is an active suppression of peripheral immune responses against orally introduced antigen, particularly food antigens.

In transplantation, a goal of the field is to develop donor-specific immune tolerance, where patients are only nonresponsive to graft-derived antigens and maintain the ability to mount immune responses to other antigens, such as those from infections or cancer. Allospecific T cells are a major threat to the allograft, as both CD4$^+$ and CD8$^+$ T cells participate in graft rejection, with both subsets acquiring effector function and proliferating with comparable kinetics [105]. Interestingly, only CD4$^+$ T cells are sufficient for transplant rejection in wildtype mice, as determined using an anti-CD4 depletion antibody, whereas depletion of CD8$^+$ T cells failed to prevent graft rejection [53,106,107]. However, in a transgenic mouse model where most T cells are

allospecific $CD8^+$ T cells, rejection is also possible in the absence of CD4s [108]. Although this model relies on an artificially high number of allospecific $CD8^+$ T cells for rejection, these findings reinforce that immunosuppressive strategies must target both $CD4^+$ and $CD8^+$ T cells to promote graft acceptance. Studying $CD8^+$ T cells following costimulation blockade is unreliable, as in models of skin, heart, and islet allografts, adoptively transferred allospecific $CD8^+$ T cells were eliminated during the induction of tolerance [109]. This is also true in our hands (unpublished) and further indicates that $CD8^+$ T cells are not necessary for transplantation rejection or tolerance. Therefore, my work focuses on characterizing $CD4^+$ allospecific T cells.

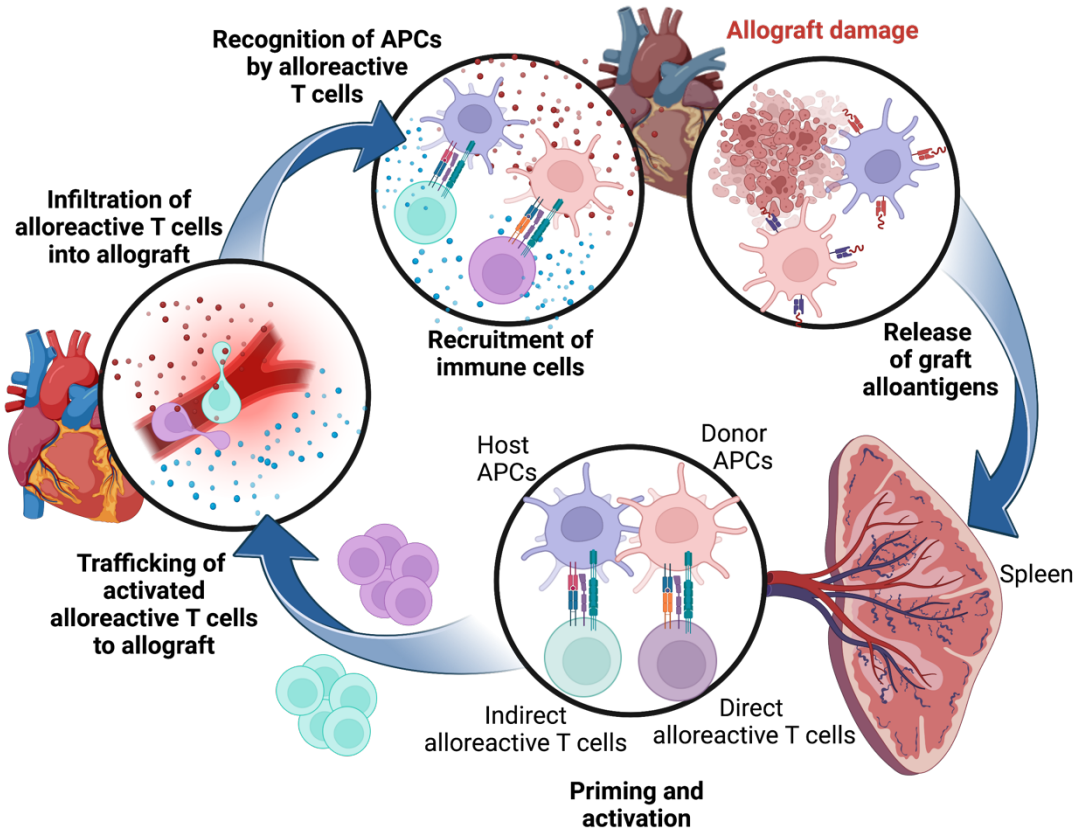

Figure 1.1 Cyclic model of T cell mediated heart allograft rejection.
Following transplantation of a heart allograft, graft alloantigens will be released and drain to the spleen. Once there, APCs, either donor or host derived, can present these alloantigens to T cells indirectly or directly, respectively. This results in T cell priming and activation, and activated T cells can then migrate back to the allograft and infiltrate into the allograft by following a chemokine gradient. Once there, these activated T cells can encounter additional APCs and recruit additional immune cells into the graft, resulting in allograft damage and further release of alloantigens.

Among other hallmarks, tolerance is characterized by an increased Treg to Tconv ratio, in part due to the abortive proliferation of conventional T cells due to the immunosuppressive drugs used [110,111]. Unique to transplantation models, robust dysfunction is induced by persistent antigen in the context of immunosuppression, such as costimulation blockade, which is necessary to prevent rejection of the genetically distinct transplant [112]. Although costimulation blockade prevents T cell maturation by blocking the context necessary for DC licensing, we believe costimulation blockade-induced T cell hypofunction is distinct from normal anergic models due to the requirement of persistent antigen expression for robust T cell dysfunction [112]. Additionally, transplantation is unique from tumor and infection models of exhaustion, as the source of inflammation, the surgery and ischemia/reperfusion injury, heals with time. As such, transplantation tolerance is distinct from classical models of anergy or exhaustion, and further work is needed to understand if it is a terminal differentiation state.

Importantly, the immunosuppression favored in mouse models of transplantation tolerance is costimulation blockade, such as anti-CD40 or anti-CD40L (CD154), supplemented with an injection of donor splenocytes to provide a source of circulating alloantigen that can reach alloreactive T cells that may not be in the secondary lymphoid organ draining the graft. In contrast, patients are treated with combination immunosuppression, including steroids, calcineurin inhibitors, mTOR inhibitors, and other immunosuppressive agents [113]. Although some patients can spontaneously develop donor-specific tolerance [114], the costimulation blockade used in naïve transplanted mice is more reliably able to induce donor-specific tolerance than clinical immunosuppressive regimens. Attempts to use anti-CD40 or anti-CD40L failed in the clinic after it was discovered that human platelets express CD40L, resulting in crosslinking and thrombosis in patients [115,116]. Following this issue, the anti-CD40L antibody has been modified to

be Fc silent [117], and is undergoing clinical trials [118]. Anti-CD40 clinical trials are also ongoing [119–121]; however, whereas anti-CD40L prevents not only CD40/CD40L interactions but also CD154/CD11b interactions that reduce local $CD8^+$ T cell activation and allograft infiltration, anti-CD40 does not prevent the latter, resulting in worse graft outcomes than anti-CD40L [122]. Thus, the Fc silent version of anti-CD154 seems to have better clinical potential for inducing transplantation tolerance.

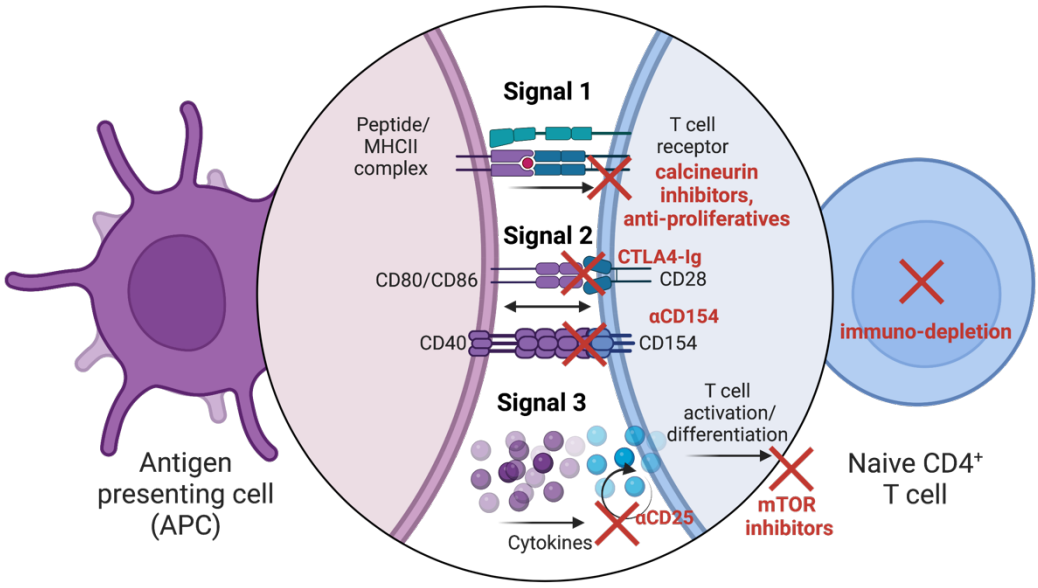

Figure 2.2 Targets of immunosuppression to prevent alloreactive T cell activation.
During T cell activation, T cells require a variety of signals for robust activation, including pMHC-TCR interactions (signal 1), costimulation (signal 2), and cytokines derived from both the antigen presenting cell and from the T cell itself (signal 3). All three of these pathways have been targeted to prevent T cell activation in transplantation, and T cells have also been targeted for deletion in order to prevent rejection.

In major MCH mismatch transplantation, there are a variety of ways in which antigen can be presented to T cells due to the mismatch between the donor and host MHC. Direct allorecognition refers to T cells that identify peptides presented by donor MHC, whereas indirect allorecognition refers to T cells recognizing processed allopeptides presented by host MHC [123]. These subsets contribute differentially to allograft rejection, as chronic rejection is driven mostly by indirect $CD4^+$ T cell responses, whereas acute rejection has contributions from both directly

and indirectly recognizing $CD4^+$ T cells [123]. Semi-direct allorecognition refers to donor pMHC complex acquisition and presentation by host APCs, which occurs during DC migration and is sufficient to promote T cell proliferation [124]. The transfer of the donor pMHC complex may either be mediated via direct cell to cell contacts, or by fusion of extracellular vesicles [123]. Although challenging to distinguish semi-direct recognition from direct recognition, the necessity of recipient DCs for promoting $CD8^+$ T cell cytotoxicity was demonstrated in a transplant model where donor mice lacked APCs [125]. These findings outline a distinct functional role for semi-direct recognition in transplantation. Recently, the "inverted direct" pathway has also been proposed, in which early formation of donor-specific antibodies (DSA) is driven by donor-derived passenger $CD4^+$ T cells that enter the circulation and find/provide help to recipient allospecific B cells [126]. In this way, the inverted direct pathway functions similarly to the direct recognition pathway; however, the origins of the T cells and APCs are inverted. Further research is needed to elucidate the role of the inverted direct pathway in allograft rejection.

Persistent antigen exposure is necessary for T cell hypofunction in transplantation [112]; however, not all antigens in transplantation are persistent [127,128]. The most striking example is donor derived MHC Class II. In an allograft, donor derived MHCII can either be expressed on endothelial cells as a stress response to ischemia/reperfusion injury, or on passenger APCs that came along with the transplanted allograft. These passenger APCs are rapidly cleared, and adoptively transferred naive T cells that indirectly recognize peptide fragments of donor-derived MHCII by host APCs are unable to proliferate due to insufficient antigen availability [127]. Furthermore, these indirectly recognizing T cells were not hypofunctional, as they failed to develop an exhausted gene signature and maintained cytokine production, further underscoring the need for persistent alloantigens in transplantation [128]. Loss of donor APCs over time also has

implications for $CD4^+$ T cells with direct allorecognition, as their corresponding pMHC is eliminated, resulting in short lived effector responses [127]. Understanding how T cells specific for transient alloantigens are maintained during homeostasis and during rejection are important for promoting long-term graft survival in patients and will be a focus of this thesis.

Infectious tolerance, described above, is responsible for tolerizing new thymic emigrants and maintaining long-term transplant survival [129]. Transplantation tolerance can also be enhanced by a phenomenon known as linked suppression. In linked suppression, if tolerance is established, new antigens that have linked expression to alloantigens that have been previously tolerized, such as on an F1 graft, will also be tolerized [130,131]. Importantly, the opposite is also true, and naïve $CD4^+$ T cells can undergo "linked sensitization," promoting transplant rejection after sensitization and pre-transplant memory formation to a single donor antigen [132]. These findings underscore the necessity for a tolerogenic environment when establishing transplantation tolerance and promoting graft survival.

Prolonged pre-exposure of transplant recipients to oral alloantigen in the absence of immunosuppression is sufficient to prolong skin transplant survival[133]. In this model, recipient mice were treated continuously with oral antigen to provide persistent stimulation of antigen-reactive T cells, with omeprazole to inhibit oral antigen cleavage, and olive oil, to promote lymphatic absorption. Alloreactive T cells responding to the transplant and gavaged antigen adopted an anergic phenotype, characterized as $FR4^{hi}CD73^{hi}$ and having poor capacity to produce the cytokines TNFα and IFNγ[133]. Interestingly, punctual oral exposure to alloantigen was insufficient to trigger prolonged graft survival, reinforcing the necessity for persistent antigen in various mechanisms of tolerance[133].

Although persistent antigen exposure combined with costimulation blockade induces robust tolerance, that tolerance remains vulnerable to inflammatory events. During tolerance induction, the transplant is particularly vulnerable. Toll-like receptor (TLR) signaling is particularly dangerous for the graft, as both infections and TLR agonists can precipitate rejection at this stage[134–136]. Interestingly, these conditions are insufficient to initiate rejection during the maintenance phase of tolerance induced by anti-CD154/DST, indicating this tolerance is more stable[137]. However, during the maintenance phase of tolerance, infection with the intracellular bacterium Listeria monocytogenes (Lm) is capable of precipitating rejection or eroding tolerance[138,139]. Not only was Lm-mediated rejection dependent upon IL-6 and type I IFN, but also sterile inflammation using IL-6 and IFNβ plasmids was sufficient to promote rejection[138], indicating that the infection-mediated rejection is not by pathogen-specific cross-reactive T cells, and other infections promoting the production of these cytokines could promote loss of tolerance. Furthermore, infection-mediated rejection is T cell dependent, as depleting either $CD4^+$ or $CD8^+$ T cells prevented rejection[138]. Importantly, infections in tolerant kidney transplant patients (with or without concurrent hematopoietic stem cell or bone marrow transplantation) also correlated with rejection events [114,140,141], indicating that even the most robust clinical tolerance remains vulnerable to infection. Overall, infection poses a threat to transplant recipients, and this thesis will further investigate the mechanisms underlying this vulnerability to tolerance.

1.07 Type 2 immunity and antigen persistence

So far, I have mainly focused on models of type 1 immunity, where major readouts for T cell function include cytotoxicity or production of the cytokines TNFα and IFNγ. However, in models of type 2 immunity, antigen can also reoccur or persist. In contrast from type 1

immunity, type 2 immunity is often driven by parasites, allergens, or venoms and sometimes by viruses or bacteria [6]. Although antigen presentation by DCs is required for type 2 immunity, depletion of basophils, which can also present antigen to initiate type 2 immunity, impaired type 2 responses [142–144]. Basophils, along with mast cells, are key producers of IL-4, which is necessary for type 2 differentiation in T cells [6]. Th2 cells are characterized by their expression of the transcription factor GATA3 and their secretion of IL-4, IL-5, IL-9, and IL-13, with pro-inflammatory Th2 cells also expressing TNFα, and non-inflammatory Th2 cells secreting IL-10[6].

Seasonal allergies are driven by a specific cohort of memory Th2 cells, called Th2A. These cells were identified in patients with allergies by using pMHCII tetramers and are characterized as $CRT_H2^+CD161^+$ [145]. Th2A T cells from patients with allergic rhinitis continuously expressed PD-1 and expressed CTLA4 in a seasonal manner [146]. While PD-1 expression is associated with exhaustion in Th1 cells, PD-1 expression is associated with worse allergic airway inflammation in Th2 settings [147–149]. In this way, the hallmarks of Th1 exhaustion cannot be directly translated to Th2 conditions.

Interestingly, Th2A T cells were deleted following allergen-specific desensitizing oral immunotherapy in patients with peanut allergies. In this therapy, patients received repeated doses of peanut proteins, which eventually promoted tolerance in the patients [145]. Allergen desensitization represents a model of reoccurring antigen exposure where antigen is presented repeatedly to allergy patients to promote unresponsiveness. Patients who ingested low doses of oral allergens daily saw a robust reduction in their responsiveness to these allergens, with hyporesponses persisting 4 years following the cessation of treatment [150–153]. This is not exclusive to oral antigens, as patients exposed to grass-pollen allergens subcutaneously weekly

over the course of three years also showed prolonged clinical remission following the cessation of treatment [154,155].

Patients with seasonal allergic responses, by definition, have long periods of rest between episodes of antigenic stimulation. It is interesting to consider what impact this would have on responding immune cells, as most other models of antigenic encounter are either intense antigen exposure for a brief period, such as during an infection, or continuous antigen exposure, such as in the tumor microenvironment. Interestingly, this idea of long periods of antigenic stimulation followed by periods of rest to restore function was addressed using a tumor-infiltrating CAR-T cell model. In this model, CAR-T cells experienced transient rest from TCR signaling, either following administration of the tyrosine kinase inhibitor desatinib or by using a stabilizing drug, where the presence of the drug was necessary for CAR expression on the engineered T cells [156]. Rest from TCR signaling reversed the exhaustion phenotype of these cells both transcriptionally and functionally [156]. Although the CAR-T cell experiments represent a model of type 1 immunity, it would be interesting to investigate if the similar rest that happens organically restores T cell function in patients with seasonal allergies.

1.08 Th17 plasticity

While most models of persistent antigen responses are models that drive Th1 immunity, there are examples where Th17 cells also see persistent antigen. While imperative for gut barrier homeostasis, Th17 cells are also commonly associated with a variety of autoimmune disorders, including lupus and inflammatory bowel disease [157]. Th17 cells demonstrate functional plasticity, allowing them to behave more like Th1 cells, such as by production of TNF and IFNγ, which can exacerbate autoimmunity [158,159]. If Th17 cells can function similarly to Th1 cells, can they also

develop a dysfunctional phenotype in response to persistent antigen? In a tumor microenvironment, Th17 cells adopt a more Treg phenotype [157]. Indeed, tumor-infiltrating FoxP3$^-$ Th17 cells eventually transdifferentiate, losing their capacity to produce IL-17 and upregulating FoxP3 [160]. These cells are referred to as "ex-Th17," and are suppressive. Although not beneficial in the tumor microenvironment, ex-Th17 cells have also been described in an allogeneic heart transplant model, where tolerance was induced using mesenchymal stem cell-based combinatorial therapy, and in which Tregs are optimal for transplant survival [161]. In this way, it appears that Th17 may be more resistant to developing dysfunction as a side effect of their inherent plasticity, favoring loss of the Th17 phenotype instead.

Th17 cells can also exacerbate disease outcomes in the face of persistent antigen. This is most clear with Th17-driven autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, psoriasis, and more [157]. Th17 cells can also worsen anti-viral responses. Virus-specific Th17 cells in mice infected with Theiler's murine encephalomyelitis virus exacerbated infection by promoting viral persistence, both by inhibiting cytotoxic T cell viral clearance, as well as preventing apoptosis of infected cells [162]. In what is undoubtedly an evolutionary advantage to the virus, infected DCs polarized T cell differentiation toward Th17 cells via IL-6 which, although resistant to developing exhaustion in the face of persistent antigen exposure, promoted the chronic infection [162]. These findings underscore the importance of generating the correct type of immunity for specific pathogens.

1.09 Tfh and germinal center responses

Tfh cells are also sensitive to persistent antigens, but distinctly from Th1 cells. In the light zone of the germinal center (GC), Tfh cells are presented antigen by GC B cells. The Tfh

population is transient and its maintenance requires sustained antigen exposure in addition to GC B cells via ICOS:ICOS-L and CD40:CD40L interactions [163]. Additionally, the amount of antigen presented to Tfh cells drives the GC B cell response in both size and duration [163]. Interestingly, during chronic viral infection, instead of canonical development of exhaustion, virus-specific Th1 cells can be redirected to the Tfh fate and are necessary for promoting antiviral antibody production [164]. Importantly, the observed conversion of Th1 to Tfh cells occurred within 30 days of infection, and it would be interesting to see if this phenotype persisted, or if more canonical exhaustion developed further into the infection. Similar to what has been seen with the Th17 population, these findings lend weight to the idea that Tfh cells represent a plastic, transient differentiation state, rather than a distinct T cell lineage. This idea has been addressed thoroughly, and today it is viewed as a "fluid subpopulation," requiring multiple steps and layers for differentiation [165].

Whether Tfh cells are a distinct T cell lineage, or whether they are a sub-population of Th1 cells, preventing T cell exhaustion/dysfunction by promoting Tfh development has potential therapeutic implications for cancer. In human breast cancer patients, PD-$1^{+}$$CXCL13^{+}$ Tfh cells infiltrated the tumor and helped form tertiary lymphoid structures, and their infiltration correlated with better outcomes[166,167]. A similar population of Tfh cells was found in murine pancreatic cancer, where tumor-infiltrating Tfh cells secreting CXCL13 and IL-21 correlated with better outcomes [168]. The tumor-infiltrating Tfh population has been described as "Th1-oriented" cells, as they not only promote $CXCR5^{+}$ lymphocyte migration to the tumor microenvironment by producing a CXCL13 gradient but are also capable of secreting the Th1 cytokine IFNγ when stimulated ex vivo[169]. As immune checkpoint inhibitors become more common clinically, "Th1-oriented" Tfh cells may be a key target for promoting tumor clearance.

In transplantation, Tfh help drives the formation of DSA that can lead to antibody-mediated rejection. Crucially, the immunosuppressive protocol given to transplant recipients in mouse models of transplantation, anti-CD154 and donor splenocytes can robustly prevent rejection [170]. This is in large part due to anti-CD154 controlling B cell responses. In this case, alloreactive B cells do not form GCs and further suppress new bone marrow emigrants from precipitating antibody-mediated rejection in an antigen-dependent manner[171]. Furthermore, B cells do not exert linked suppression on naïve B cells, as Tfh cells accumulate and remain capable of generating antibody responses against an F1 allograft, as determined using mice with partially distinct antibody allophenotypes[171]. Interestingly, Tfh cells still differentiate during transplantation tolerance, suggesting that the allospecific B cells are still capable of presenting antigen to them[171]. Th1 cells that develop dysfunction normally experience antigen presented by canonical APCs. As Tfh preferentially see antigen presented by B cells, it is possible that the context of antigen presentation in conjunction with the duration of antigen presentation are important for T cell dysfunction. Although this has yet to be investigated, any potential signal is unlikely to be B cell derived, as Tfh cells are able to differentiate in the absence of B cells [172].

Since anti-CD154 isn't yet used clinically, and antibody-mediated rejection is a major problem for patients who otherwise have control of their anti-graft cellular responses [170], understanding the potential to tolerize Tfh cells or understanding how to prevent Tfh or B cell function is a potentially beneficial therapeutic avenue of investigation. As Tfh cells maintain their function in the face of persistent antigen and costimulation blockade, specifically targeting Tfh cells early post-transplantation, such as by using IL-2 to inhibit differentiation and promote Treg formation, may prove beneficial [173]. Additionally, although Bregs are not preventing Tfh responses during tolerance[171], they have been previously associated with regulating T cell

responses [174,175]. Promoting Breg accumulation to prevent antibody-mediated rejection is another potential area of investigation [176].

1.10 Summary

Recurrent exposure to antigen suppresses effector function of T cells specific to that antigen in a variety of biological contexts. This phenomenon can be harnessed for desired outcomes: either to promote dysfunction, such as in transplantation, or to combat dysfunction, such as in cancer. Importantly, in transplantation and cancer, not all antigens are equally persistent. While this concept has been introduced previously [127,128], the focus and novelty of this thesis is understanding the effect that non-persistent antigens have on T cell function, how this impacts transplant vulnerability, and how we can prevent this vulnerability by promoting more robust T cell dysfunction.

In this thesis, I will address the differential functions of T cells responding to antigen of variable persistence in the context of costimulation blockade. We will advocate for persistent antigen exposure and costimulation blockade as a tool for promoting transplantation tolerance and protection against infection-mediated rejection. Furthermore, we will investigate how memory T cells respond to persisting antigen in the context of costimulation blockade, in an effort to promote graft acceptance in previously sensitized patients. The following chapters describe findings that uncover vulnerabilities to the robustness of transplantation tolerance and the ways in which we can target these vulnerabilities by taking advantage of persistent antigen-induced hypofunction. Our findings have the potential to inform clinical attempts to improve graft outcomes in patients, particularly for patients with living donors.

Chapter 2: Materials and Methods

2.01 Mice

C57Bl/6 (B6) and BALB/c (B/c) mice were purchased from Envigo RMS. TCR75 TCR-Tg mice obtained from R. Pat Bucy (University of Alabama) are specific for a peptide derived from K^d (donor MHC Class I) presented on I-A^b (host MHC Class II) and TEa TCR-Tg mice obtained from Alexander Rudensky (when at the University of Washington) are specific for a peptide derived from I-E^d (donor MHC Class II) presented on I-A^b (host MHC Class II). TCR75 and TEa mice were crossed with $Rag^{-/-}$ mice and $CD45.1^+$ mice to generate TCR75/$Rag^{-/-}$/$CD45.1^+$ (TCR75) and TEa/$Rag^{-/-}$/$CD45.1^+$ (TEa) mice, respectively. OTII mice were obtained from the Jackson Laboratory and bred in-house. TGO mice [expressing a fusion protein comprising transferrin receptor transmembrane domain (T), GFP (G) and $OVA_{230-259}$ (O) under the control of a tetracycline response element] were obtained from M. Rosenblum (University of California at San Francisco) and bred to R26-M2rtTA mice [encoding a mutant reverse tetracycline-controlled transactivator (rtTA) with low background activity in the absence of doxycycline (Dox)] obtained from the Jackson Laboratory. Exposure of the resulting mice to Dox results in membrane-bound OVA upregulation in all tissues, while cessation of Dox downregulates OVA expression. Mice were age- and gender-matched when possible and housed under specific pathogen-free conditions. Experiments were performed in agreement with the University of Chicago's Institutional Animal Care and Use Committee, and according to the NIH guidelines for animal use.

2.02 Heart transplantation and tolerance induction

Cardiac transplantation was performed using a technique adapted from Corry et al [177]. For induction of tolerance, mice were treated with 500-600µg of anti-CD154 (MR1, BioXCell) on days 0 (i.v.), 7 and 14 (i.p.) post-transplantation and DST (i.v.) on day 0. B/c DST was prepared by homogenizing a single cell suspension of splenocytes through a 40µm filter. Each injection contained splenocytes from one-quarter to one-sixth spleen in 200µl PBS. In all experiments where mice were treated with DST, day 0 injection was i.v. In mice treated with repeated DST injections every 48 hours, all injections after day 0 were i.p..

2.03 *In vivo* Lm infection

An overnight culture of Lm engineered to express GFP (Lm) [178] was diluted 1:50 in brain heart infusion broth (BD Biosciences) supplemented with 10µl/ml chloramphenicol (Sigma) and grown until OD_{600} was within the log-phase of the Lm growth curve (~2hr). OD_{600} was used to calculate colony-forming units (CFU)/ml. Mice were infected i.p. with Lm ($8x10^5$-$1x10^6$ CFUs in 400µl per mouse) as this dose resulted in the highest rejection rate with minimal lethality. The pre- and post-infection inoculum was plated to confirm dosage. Graft survival was monitored twice per week after infection. A subset of mice received ampicillin (25mg/100µl in 1xPBS i.p.) 48-72hr post-infection to prevent Lm morbidity.

2.04 Adoptive cell transfer

In experiments where TCR-Tg tracer cells were seeded before transplantation or immunization, cells were isolated from the spleen and lymph nodes (inguinal, axillary, brachial, cervical, and

mesenteric) of naïve TCR75, TEa, or OTII mice and counted with an Accuri C6 or Fortessa flow cytometer (BD Biosciences). $5x10^4$ cells were injected i.v. in 200µl of PBS up to 1 day before transplantation or before the first DST injection.

2.05 Isolation of tracer TCR-Tg cells from adoptive transfer hosts

Spleen and lymph nodes from primary hosts were harvested and homogenized ≥30 days following transplantation or first DST injection. Single cell isolates were stained with anti-CD45.1-biotin (eBioscience) and incubated with streptavidin magnetic beads (Miltenyi) for magnetic enrichment with LS columns (Miltenyi) or AutoMACS (Miltenyi). In some experiments, cells from mice within the same experimental group were pooled after magnetic enrichment. Magnetically enriched cells isolated from positive selection were then stained with fluorophore conjugated anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD4 (L3T4), anti-CD8 (Ly2), and anti-CD44 (IM7) and sorted for $CD45.1^+CD4^+CD44^{hi}$ cells on a FACSAria cell sorter (BD Biosciences). Cells were sorted into fetal bovine serum (FBS), then washed and re-suspended in PBS and subjected to further staining or functional analyses *in vitro* or *in vivo*.

2.06 Bulk RNA sequencing (RNAseq) acquisition and analysis

Using the SMART-Seq v4 Ultra Low Input RNAseq kit from Takara Biosciences, we sorted 1,000 TCR75 cells directly into 1x Lysis Buffer with RNase inhibitor. We followed manufacturer instructions, amplifying cDNA for 18 PCR cycles after first strand synthesis. We then used the Nextera XT DNA Library Preparation Kit in combination with IDT DNA Unique Dual Indexes to create libraries for sequencing as per manufacturer's instructions. The samples

were sequenced using the Illumina NovaSeq platform with a 100 bp cassette and SP Flowcell. FAST-QC was performed on the resulting Fastq files to ensure the quality of the samples before moving on to alignment using the STAR splice-aware mapper and the GRCm39 mouse genome from Ensembl. A counts matrix was generated from the resultant BAM files using FeatureCounts. These raw counts were analyzed using the DESeq2 package on R to identify differentially expressed genes and normalized gene counts.

2.07 Cell preparation and staining for flow cytometry

Flow cytometry was performed in the CAT Facility (RRID: SCR_017760) at the University of Chicago, which receives financial support from a Cancer Center Support Grant (P30CA014599). Sorted TCR-Tg cells or unenriched spleen and lymph node cells were homogenized, filtered, and stained with a fixable live/dead dye (Invitrogen). Cells were then stained with fluorophore-conjugated anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD44 (IM7), anti-PD-1 (J43), anti-CD127 (A7R34), anti-FR4 (12A5), anti-CD73 (TY/11.8) and anti-B220 (RA3-6B2). Surface-stained cells were then fixed with the FoxP3 fixation permeabilization buffer kit (eBioscience) for 15-30 minutes at room temperature (RT) and washed with 1x permeabilization buffer. Some samples were intracellularly stained with anti-Ki67 (SolA15) or anti-FoxP3 (FJK-16s) for 30 minutes at RT, washed with permeabilization buffer, and analyzed by flow cytometry. All monoclonal antibodies (mAbs) were from BD Biosciences, eBioscience, or Invitrogen.

To identify dendritic cells (DCs), $5x10^6$ unenriched splenocytes were stained with 1:1000 fixable Live Aqua live/dead dye (Invitrogen) for 20-30min at RT in the dark and surface antibodies to CD45.2 (104), CD19 (6D5), CD11c (N418), I-A/I-E (M5/144.15.2), YAe (eBioY-Ae) for 10min

at RT in the dark, washed, resuspended in FACs buffer and analyzed with the LSR Fortessa. DCs/APCs were isolated by gating on live, $CD45.2^{+}CD19^{-}CD11c^{+}$I-A/I-$E^{+}$ events.

Cardiac grafts were isolated from recipient mice and washed with heparin/1x Hank's balanced salt solution (HBSS, ThermoFisher). Cardiac tissue was then cut to small pieces and digested with 400U/ml of collagenase IV (Sigma), 0.01% DNase I (MP Biomedicals) and 10mM HEPES (ThermoFisher) in HBSS for 40min while incubated at 37°C. The tissue solution was homogenized by passing through a 40μm filter cup, washed, and quenched with an excess of complete DMEM (5% FBS, 1% HEPES, 1% nonessential amino acids, 1% Penicillin-Streptomycin, 1% L-Glutamine, 0.0004% 14M βMercaptoethanol). Cells were washed with 1x PBS, then stained with 1:1000 fixable Live Aqua live/dead stain (Invitrogen) for 20-30min at RT in the dark, followed by surface antibodies to CD45.2 (104), H2:K^{d} (SF1-1.1.1), CD31 (MEC13.3), and I-A/I-E (M5/144.15.2) for 10min at RT in the dark, washed, and resuspended in FACs buffer and analyzed with the LSR Fortessa. Donor-derived endothelial cells were isolated by gating on live, $CD45.2^{-}CD31^{+}$H2:K^{d+} events.

2.08 CFSE dilution assay of proliferation

TCR-Tg cells were stained with a fixable CFSE dye (Invitrogen) as previously described [179]. Briefly, no more than $50x10^{6}$ T cells were resuspended in 1ml PBS and 5% FBS, vortexed, and a 1ml solution of 8-10μM CFSE was added drop by drop to the cell solution, for a final concentration of 4-5μM CFSE. Cells were incubated for 5min at RT prior to being quenched in 5ml 5% FBS medium. Unstimulated CFSE-labeled cells were cultured at 37°C with 1ng/ml human IL-7

(PeproTech) as flow cytometry single stains. Cells were adoptively transferred to mice on day 0 and recovered on day 4 post-injection.

2.09 *In vitro* stimulation for cytokine production

U-bottom tissue culture plates were coated for 90min at 37℃ with 5μg/ml anti-CD3 (2C11) and 1μg/ml anti-CD28 (PV.1) (Fitch Monoclonal Facility). 500-2000 tracer TCR-Tg cells sorted from primary hosts were plated per well and incubated for 16-24h at 37℃ in 5% CO_2. Within each individual experiment, all wells were plated with the same number of cells. In some experiments, $1x10^5$ splenocytes from a naïve B6 mouse were co-cultured as filler cells with TCR-Tg cells to promote viability. Unstimulated controls were plated in uncoated wells with 1ng/ml human IL-7 (PeproTech). Two hours after plating, brefeldin A (BioLegend) was added to all wells. After stimulation, cells were stained with fixable viability dye (Invitrogen) and then surface-stained with fluorophore-conjugated anti-CD4, anti-CD8, anti-CD45.1, anti-CD45.2 and anti-CD44. Cells were then fixed and permeabilized with the FoxP3 fixation permeabilization buffer kit (eBioscience) and stained with fluorophore-conjugated anti-IFNγ (XMG1.2) and anti-TNF (MP6-XT22) for 30min at RT or overnight at 4℃ before washing with permeabilization buffer and analyzed by flow cytometry.

2.10 Evaluation of recall expansion

TCR-Tg cells ($5x10^2$-$6x10^3$) were injected i.v. into secondary naïve B6 hosts immunized 24h later with B/c DST i.v.. Within each experiment, the number of transferred cells was similar between mice. Cell concentration was confirmed by counting on either an Accuri C6 flow cytometer or an

LSR 4-12 flow cytometer using CountBright Plus Absolute Counting Beads (Invitrogen) prior to injection. Five days after DST, $5x10^6$ splenocytes were isolated from the secondary hosts then stained with a viability dye and fluorophore-conjugated anti-CD4, anti-CD8, anti-CD45.1 and anti-CD45.2. The number of $CD45.1^+CD4^+$ cells was calculated per mouse.

2.11 Histology

Heart allografts were removed, weighed, halved laterally, and fixed in 10% neutral buffered formalin at RT for 36-48h. Tissues were sectioned, then stained with haematoxylin and eosin. Slides were imaged at 10x or 20x magnification with an infinity HD camera mounted on an Olympus microscope (model# BX45TF). Myocardial tissue was examined and scored by an independent pathologist in a single-blinded manner using the International Society for Heart and Lung Transplantation Acute Cellular Rejection (ISHLT ACR) grading scale, only analyzing the myocardium tissue and ignoring tissue near where the graft was stitched and anastomosed [180]. Myocardial tissue was also examined for the extent of interstitial infiltrate (mild, moderate, severe), the absence/presence of perivascular infiltrate, and myocyte damage/necrosis (focal, diffuse, or absent), and quantified on a 4-point scale (0-1pt interstitial infiltrate, 0-1pt perivascular infiltrate, 0-2pt myocyte damage/necrosis).

2.12 Data and statistical analysis

Flow cytometry data were analyzed using FlowJo (Tree Star, Ashland, Oregon). Flow cytometry samples were gated on live single cells before analysis. Statistical analyses were performed where appropriate using GraphPad Prism (GraphPad, La Jolla, California). Each statistical test is listed

in the figure legends. Quantitative variables with a gaussian distribution are presented with mean (+/- standard error of the mean). Quantitative variables with a non-gaussian distribution are presented with median (P25-P75). A p-value of 0.05 was considered statistically significant.

2.13 Study approval

All animal experiments were approved by the University of Chicago's Institutional Animal Care and Use Committee. The University of Chicago's animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (National Academies Press, 2011).

Chapter 3: Duration of alloantigen expression promotes heterogeneity in allospecific T cell function in transplant tolerant hosts, resulting in susceptibility to rejection following infection.

3.01 Abstract

Even when successfully induced, solid organ transplant tolerance remains vulnerable to inflammatory insults, which can trigger rejection. In a mouse model of cardiac allograft tolerance in which infection with *Listeria monocytogenes* (Lm) precipitates rejection of previously accepted grafts, we showed that recipient $CD4^+$ TCR75 cells reactive to a donor MHC Class I-derived peptide become hypofunctional if the allograft is accepted for > 3 weeks. Paradoxically, infection-induced transplant rejection is not associated with transcriptional or functional reinvigoration of TCR75 cells. We hypothesized that there is heterogeneity in the level of dysfunction of different allospecific T cells, depending on duration of their cognate antigen expression. Unlike $CD4^+$ TCR75 cells, $CD4^+$ TEa cells specific for a peptide derived from donor MHC Class II, an alloantigen whose expression declines post-transplantation but remains inducible in settings of inflammation, retained function in tolerant mice and expanded during Lm-induced rejection. Repeated injections of alloantigens drove hypofunction in a broader range of allospecific T cells and rendered grafts resistant to Lm-dependent rejection. Our results uncover a functional heterogeneity in allospecific T cells of distinct specificities post-tolerance induction and reveal a strategy to defunctionalize a greater repertoire of allospecific T cells, thereby mitigating a critical vulnerability of tolerance.

3.02 Introduction

Achieving stable donor-specific transplantation tolerance holds the potential to vastly improve the quality of life for transplant recipients by eliminating the life-long exposure to drug toxicity, higher risk of malignancy and infection, and development of chronic rejection associated with conventional immunosuppression. In mice, treatment with anti-CD154 and donor splenocyte transfusion (DST) produces a robust state of graft-specific tolerance to fully MHC-mismatched cardiac allografts [181] that, once established, resists many late inflammatory challenges such as Toll-like receptor (TLR) agonists or infections, including *lymphocytic choriomeningitis virus* [182] and *Staphylococcus aureus* [183]. That these inflammatory challenges at the time of transplantation prevent the induction of tolerance [137] but cannot break established tolerance supports that anti-CD154/DST-induced tolerance is somewhat robust. However, we have shown that late infection of tolerant mice with the intracellular bacterium Lm precipitates T cell-dependent cardiac allograft rejection [138,139]. Lm-induced rejection was dependent on IL-6 and type I IFN, and the combination of IL-6 + IFN-β was sufficient to break established tolerance in the absence of infection, ruling out cross-reactivity to graft antigens by Lm-reactive T cells, and suggesting that tolerance may be vulnerable to any inflammatory settings eliciting these cytokines [138]. There is also circumstantial evidence that infection poses a threat to clinical transplantation tolerance. A subset of patients who spontaneously developed operational tolerance following cessation of conventional immunosuppression rejected their grafts after years of stability, often after an infection [114]. Furthermore, reports of patients made tolerant to renal allografts with concurrent hematopoietic stem cell or bone marrow transplantation who later rejected their allografts following an infection suggest that even the most effective strategies

currently available for clinical tolerance induction leave grafts vulnerable to inflammatory challenges [140,141].

Understanding the mechanisms of rejection after successful establishment of tolerance is important to develop strategies for improving the robustness of tolerance. While many studies have focused on the mechanisms of acute allograft rejection in untreated mice, little is known about rejection in previously tolerant mice. In this study we aim to understand the mechanisms by which Lm-infection was able to precipitate T cell-dependent allograft rejection in tolerant mice [138]. Anti-CD154/DST-mediated tolerance is associated with induction of allospecific regulatory T cells (Tregs) [184] and abortive proliferation of alloreactive conventional T cells (Tconvs), resulting in a high Treg:Tconv ratio [185]. Using adoptively transferred allospecific TCR-transgenic T cells as a tracer of the endogenous alloresponse, our group previously found that TCR75 cells, TCR-transgenic $CD4^+$ Tconvs specific for a donor MHC Class I-derived peptide indirectly presented by host MHC Class II, become antigen experienced and persist in tolerant graft recipients. However, in contrast to TCR75 cells in rejecting mice which develop a phenotype and functional capacity consistent with memory T cells, TCR75 cells in tolerant mice take on a phenotype and hypofunctional profile more consistent with exhausted or anergic T cells [112]. Importantly, TCR75 cells did not appear to regain function after Lm infection when assessed a month after infection [112], despite Lm precipitating rejection [186] or eroding tolerance [139] in all previously tolerant hosts. Thus, it is unlikely that these profoundly hypofunctional allospecific Tconvs mediate rejection after infection of tolerant hosts. We therefore hypothesized that there are allospecific Tconv clones of other specificities that retain functionality and are thus more poised to reject the graft when a stably tolerant host becomes infected.

Parameters of T cell hypofunction have been identified in tolerant recipients with monoclonal tracer TCR75 cells as well as with endogenous allospecific T cells detected with allogeneic peptide:MHC (pMHC) multimers [112]. However, the T cells tracked thus far are unlikely to be representative of the entire allospecific T cell population, which is estimated to contain over 1% of mature T cells [187]. Variables including avidity for alloantigen and specificity for a particular alloantigen may impact the fate of allospecific T cells during tolerance. Duration of antigen presentation has recently become appreciated as a key variable modulating allospecific T cell responses during rejection [127,128]. Having determined that chronic alloantigen exposure is necessary for the development of hypofunction in allospecific TCR75 cells during anti-CD154/DST-induced tolerance [112], we investigated whether allospecific T cells specific for a less persistent alloantigen retain more functionality than TCR75 cells.

Previous findings from our group indicated that the graft must persist for ~3 weeks for tracer TCR75 cells to acquire hypofunction, as TCR75 cells were impaired functionally if grafts were surgically removed 3 weeks, but not 1 week after transplantation in hosts treated with anti-CD154/DST [112]. However, some alloantigens are not expressed persistently in the graft. Unlike donor MHC Class I (the source of cognate peptide for TCR75 cells) which is constitutively expressed by all nucleated cells and thus has the potential to drive persistent stimulation of direct and indirect alloreactive T cells, direct alloresponses to donor MHC Class II and indirect alloresponses to peptides derived from donor MHC Class II and presented by host MHC appear to be transient [127]. Expression of donor MHC Class II antigens is thought to be short-lived in the graft because donor APCs accompanying the graft die shortly after transplantation [127]. Endothelial cells also up-regulate expression of MHC Class II in response to IFNγ, providing a potential source of donor MHC Class II antigen late after transplantation in settings of

inflammation. Any polymorphic inflammation-induced molecules may similarly act as transient sources of alloantigen, with re-expression later during inflammation for example during an infection. Thus, there is a potentially large population of alloreactive T cells specific for transiently expressed alloantigens whose fate during tolerance remains to be explored.

Here, we investigated the functionality of TEa cells, transgenic for a TCR recognizing a donor MHC Class II-derived peptide presented indirectly by recipient MHC Class II, following the induction of transplantation tolerance. We found that these cells retained greater functionality than donor MHC Class I-derived peptide-reactive TCR75 cells. Notably, TEa cells could be made profoundly hypofunctional with repeated antigen stimulation over ~4 weeks. Furthermore, this treatment rendered grafts resistant to Lm-triggered rejection. Prolonging exposure to alloantigens through repeated alloantigen administration during tolerance induction represents a therapeutic approach to improve the robustness of tolerance by achieving hypofunction in a broader array of allospecific T cells. Our study reveals that heterogeneity in functional loss of distinct allospecific T cells is an important vulnerability to durable transplantation tolerance, and that prolonged alloantigen exposure is a possible solution to ensure greater graft stability in the face of inflammatory challenges.

3.03 Donor MHC Class-I peptide-reactive monoclonal T cells are not re-functionalized post-Lm infection

Lm-mediated cardiac allograft rejection following establishment of transplantation tolerance was T cell-dependent and correlated with transiently detectable alloreactivity [138,186], although the T cells responsible for this IFNγ production in response to donor alloantigen stimulation remained to be identified. We had previously shown that allospecific $CD4^+$ TCR75

cells, which are specific for a donor MHC Class I-derived peptide presented indirectly on host MHC Class II, became hypofunctional in tolerant mice and did not show recovered function when tested at day 30 post-Lm infection of tolerant hosts [112]. It remained possible that these T cells were reinvigorated early following infection, but then returned to their hypofunctional phenotype over time following Lm clearance. Alternatively, alloreactive T cells of a differing specificity might retain function despite the tolerance induction regimen and be responsible for Lm-dependent graft loss. To distinguish between these possibilities, we first assessed if TCR75 cells regained function early following Lm-infection. TCR75 cells ($CD45.1^+$ and on a $Rag^{-/-}$ background) were adoptively transferred into congenic B6 recipients ($CD45.2^+$) one day before transplantation with a B/c heart and induction of tolerance with anti-CD154+DST (CoB). Thirty days post-transplantation, some recipient mice were infected with Lm, and TCR75 cell function was assessed 7 days post-infection (Figure 3.1A). As controls, TCR75 cells were injected into B6 mice one day prior to immunization with B/c DST to generate memory TCR75 cells, and these cells were harvested 37 days later. TCR75 cell numbers recovered from the tolerant hosts were lower than from DST-immunized hosts, reflecting the abortive proliferation resulting from the anti-CD154 treatment (Figure 3.1B). Similarly to our prior findings when TCR75 cells were analyzed at 30 days post-Lm infection of tolerant hosts [112], we did not find increased numbers of TCR75 cells at 7 days post-Lm infection of tolerant hosts, when compared to uninfected hosts (Figure 3.1B). Additionally, TCR75 cells from infected tolerant mice did not regain the ability to proliferate upon donor splenocyte rechallenge in naïve secondary hosts, away from the tolerant environment of the primary host (Figure 3.1C), substantiating a lack of refunctionalization post-infection. We further compared gene expression by bulk RNAseq between TCR75 cells from naïve mice, memory TCR75 cells from mice immunized with B/c splenocytes, and TCR75 cells from tolerant mice, either uninfected

or infected with Lm 5 days prior. TCR75 cells from tolerant and Lm-infected mice clustered tightly together by principal component analysis (PCA) and displayed extremely similar gene expression profiles (Figure 3.1D), with only 10 genes differentially expressed (not shown). It thus appeared unlikely that Lm-mediated rejection, which occurs on day 10-20 post-infection [138], results from reinvigoration of hypofunctional, chronically stimulated Tconvs.

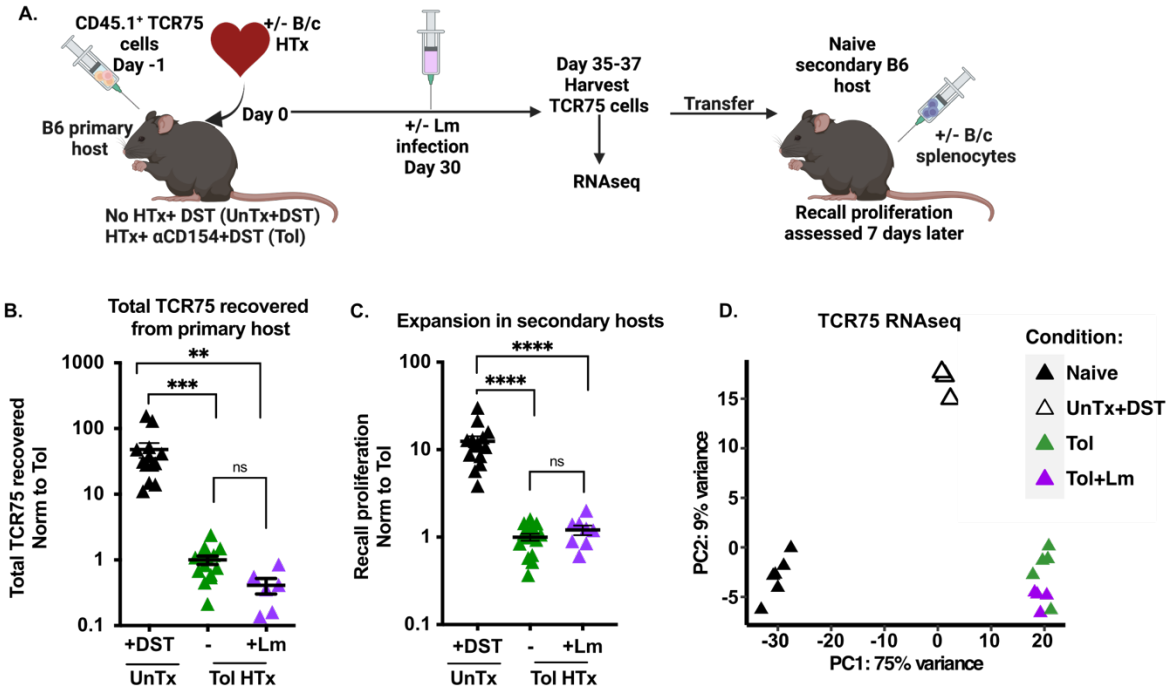

Figure 3.1 Donor MHC Class I-specific T cells remain hypofunctional following Lm infection.

(A) Experimental design. $CD4^{+}CD45.1^{+}$ TCR75/$Rag^{-/-}$ T cells (TCR75) were adoptively transferred into $CD45.2^{+}$ B6 hosts either untransplanted and immunized with B/c donor splenocytes i.p. (UnTx+DST) to induce memory, or prior to transplantation of a B/c heart (HTx) in the presence of anti-CD154 (day 0, day 7, day 14) and DST (day 0) to induce tolerance (Tol). A group of tolerant mice were infected with Lm on d30 post-transplantation (Tol+Lm). $CD45.1^{+}$ TCR75 cells were harvested from the spleen and lymph nodes on d35-37 post-transplantation in all groups, enumerated and subjected to bulk RNAseq. For recall proliferation, an equal number of harvested, sorted TCR75 cells were adoptively transferred into new congenic naïve B6 hosts immunized with B/c splenocytes. Cells were enumerated from the spleen 7 days post-*in vivo* rechallenge. **(B)** Fold change of TCR75 cells recovered from UnTx+DST (n=13), Tol (n=14) and Tol+Lm (n=6, day 7 post-Lm) mice, normalized to the number recovered in uninfected Tol animals. **(C)** Expansion in secondary hosts. Fold change of total TCR75 cells recovered from spleens of secondary hosts normalized to the cells recovered when TCR75 cells originated from Tol hosts prior to adoptive transfer. UnTx+DST (n=14), Tol (n=15), Tol+Lm (n=8). **(D)** Principal component (PC) analysis of

Figure 3.1, continued. RNAseq. Gene expression comparison between naïve TCR75 cells (d0, n=6), and memory TCR75 (UnTx+DST d35, n=3) or tolerant TCR75 cells from uninfected (Tol d35, n=5) or Tol+Lm analyzed d5 post-infection (n=4). Statistical comparisons were performed with one-way ANOVA with multiple comparisons.**p<0.01, ***p<0.001, ****p<0.0001, ns, not significant.

3.04 Presentation of donor MHC Class II-derived peptide decreases with time

In mice transplanted with fully MHC mismatched heart allografts, it has been shown that host T cells specific for donor MHC Class I or their derived peptides have the capacity to engage alloantigen long-term as MHC Class I is persistently expressed by most graft cells [127]. In contrast, responses to donor MHC Class II-derived antigens are more transient, as passenger APCs, a large source of donor MHC Class II, die shortly after transplantation [127]. Graft endothelial cells can up-regulate expression of MHC Class II in response to IFNγ [188,189], but endothelial cells may not provide sufficient donor MHC Class II to persistently stimulate T cells, as expression of MHC Class II is downregulated following resolution of inflammation secondary to ischemia-reperfusion injury. To assess the potential for donor MHC Class I or Class II-derived alloantigens to activate allospecific T cells during the maintenance phase of tolerance, we adoptively transferred CFSE-labeled naïve TCR-Tg T cells >30 days after B/c heart transplantation into B6 recipients that had been treated at the time of transplantation with CoB to induce allograft tolerance. CFSE dilution was assessed 4 days after naïve TCR-Tg cell transfer as a readout of cell proliferation in response to alloantigen expressed by the host. To detect the presence of donor MHC Class I and II, we used TCR75 TCR-Tg cells and TEa TCR-Tg cells (both CD45.1^{+} on a Rag$^{-/-}$ background) that recognize a donor K^{d}-derived peptide and a donor I-E^{d}-derived peptide, respectively, both presented indirectly on host I-A^{b} (

Figure 3.2A). As expected, almost all naïve CFSE-labeled TCR75 cells proliferated sufficiently to fully dilute CFSE (

Figure 3.2B, C), reflecting the persistence of donor MHC Class I expression in the graft of tolerant hosts. Conversely, many CFSE-labeled TEa cells failed to proliferate after adoptive transfer into similarly tolerant mice (

Figure 3.2B, D), suggesting lower expression and/or presentation of donor MHC Class II-derived peptide in the tolerant hosts at the maintenance phase of tolerance. Importantly, TEa cells proliferated to a similar extent as TCR75 cells after transfer into naïve B6 mice immunized with B/c splenocytes, indicating that naïve TEa cells can proliferate in the presence of sufficient alloantigen. It was also possible that even fresh heart allografts do not contain sufficient MHC Class II to stimulate TEa cell proliferation. This was not the case, as both TEa and TCR75 cells fully diluted CFSE when transferred into non-immunosuppressed mice transplanted with a B/c heart 3 days prior and undergoing acute rejection

Figure 3.2B-D). Indeed, proliferation of TEa cells in mice undergoing heart allograft rejection correlated with higher expression of donor MHC Class II on graft endothelial cells when compared to expression on endothelial cells from established tolerant grafts (

Figure 3.2E). Finally, provision of additional antigen in the form of B/c splenocytes could rescue naïve TEa proliferation after transfer into stably tolerant heart recipients (

Figure 3.2B, D), suggesting that the limited TEa response to heart allografts at the maintenance phase of tolerance is due to low donor MHC Class II-derived peptide antigen

availability and not to dominant suppression of these cells. Overall, antigen was limiting for TEa cells, but not for TCR75 cells, in tolerant mice.

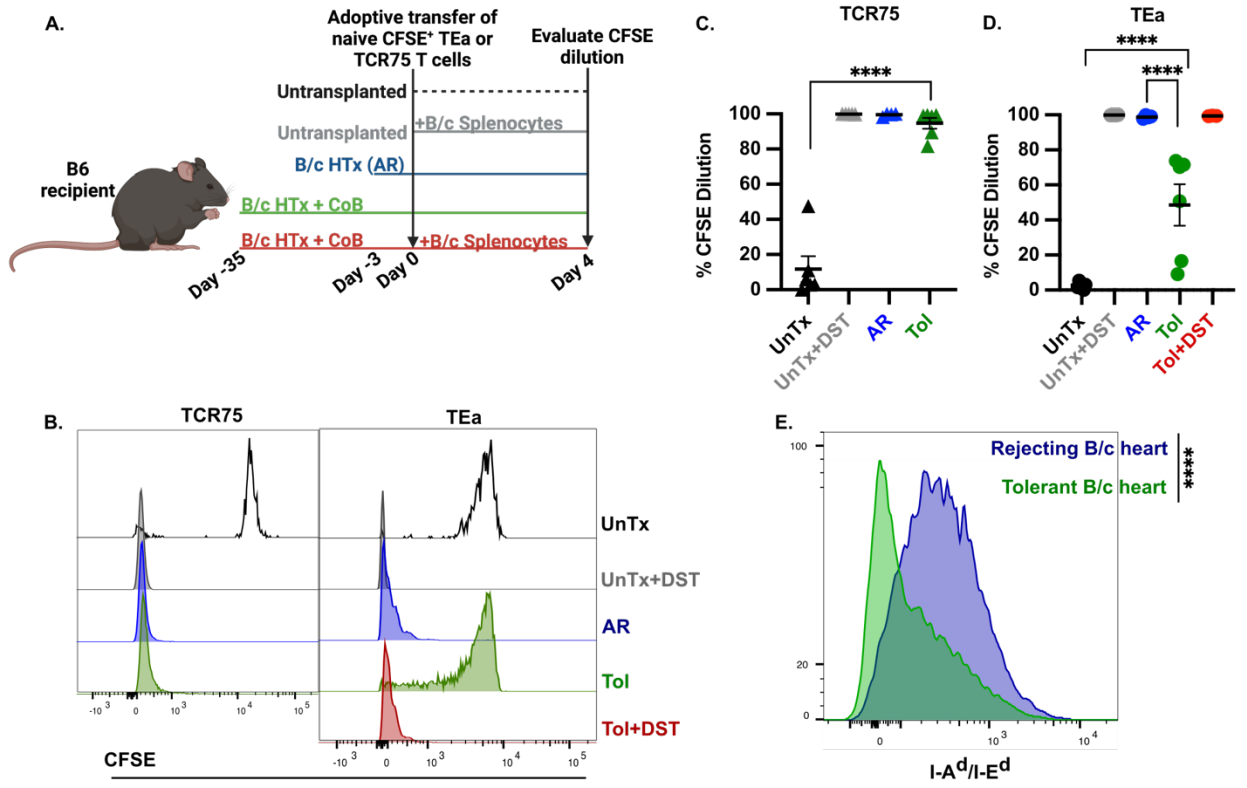

Figure 3.2 The presentation of donor MHC Class II-derived peptide declines during tolerance.

(A) Experimental design for (B-D). Mice were transplanted with a B/c heart and tolerance was induced with anti-CD154/DST (CoB). Thirty-five days post-transplantation, CFSE-labeled CD45.1^{+}TCR75/Rag$^{-/-}$ (TCR75) or CD45.1^{+}TEa/Rag$^{-/-}$ (TEa) cells were adoptively transferred (Tol). Some control mice received a B/c heart without CoB 3 days prior to TCR-Tg cell transfer (acute rejection, AR). Other control mice were immunized with B/c splenocytes the same day as the TCR-Tg adoptive transfer (UnTx+DST). One subset of tolerant mice received extra alloantigen in the form of B/c splenocytes on the day of TEa transfer (Tol+DST). In all cases, TCR-Tg T cells were recovered 4 days post adoptive transfer and evaluated for CFSE dilution. **(B)** Representative histograms showing CFSE dilution in TCR75 or TEa cells 4 days after adoptive transfer into the groups described in A. **(C, D)** Summary data of CFSE dilution in TCR75 (C) or TEa (D) cells 4 days after adoptive transfer into the groups described in A. Results plotted as Mean +/- SEM. For C: UnTx (n=6), UnTx+DST (n=5), AR (n=6), Tol (n=6). For D: UnTx (n=6), UnTx+DST (n=4), AR (n=5), Tol (n=6), Tol+DST (n=3). Significance not pictured: UnTx vs UnTx+DST (****), UnTx+DST vs Tol (****), Tol vs Tol+DST (***). **(E)** Representative histograms of donor MHC II (I-A^{d}/I-E^{d}) expression on graft-derived CD45^{-}CD31^{+}H2K^{d+} endothelial cells. Representative of summary data in Figure 1.7B. Data were compared by unpaired t test. ****p<0.0001.

3.05 T cells specific for donor MHC Class II-derived peptide retain more functionality than T cells specific for donor MHC Class I-derived peptide during the maintenance phase of tolerance

Donor MHC Class II expression during tolerance was insufficient to stimulate the proliferation of naïve TEa cells (

Figure 3.2B), and we knew that persistent antigen stimulation is required for acquisition of hypofunction in TCR75 cells exposed to CoB therapy at the induction of tolerance [112]. Thus, we hypothesized that insufficient chronic stimulation may allow TEa cells to retain functionality during tolerance induction [128]. To test this hypothesis, we compared the phenotype and function of tracer TCR75 versus tracer TEa cells, seeded at the time of transplantation into untreated or CoB-treated recipients of B/c hearts. TCR-Tg cells were isolated ≥35 days after transplantation and T cell transfer (Figure 3.3A), and the phenotype of $CD44^{hi}$ $FoxP3^{-}$ Tconvs was analyzed to compare expression of the anergy markers FR4 and CD73 [190]. While TCR75 cells from tolerant mice displayed a significantly higher percentage of double positive $FR4^{hi}$ $CD73^{hi}$ cells, the percentage on TEa cells was more variable in tolerant mice and not significantly different from that in rejected mice (Figure 3.3B). Similarly, expression of PD-1, a marker of recent T cell activation and of exhaustion, was significantly higher on TCR75 cells from tolerant than rejected mice, but not on TEa cells (Figure 3.3C). These results indicate that the anergy phenotype was more variable and less established in TEa than TCR75 cells from tolerant mice.

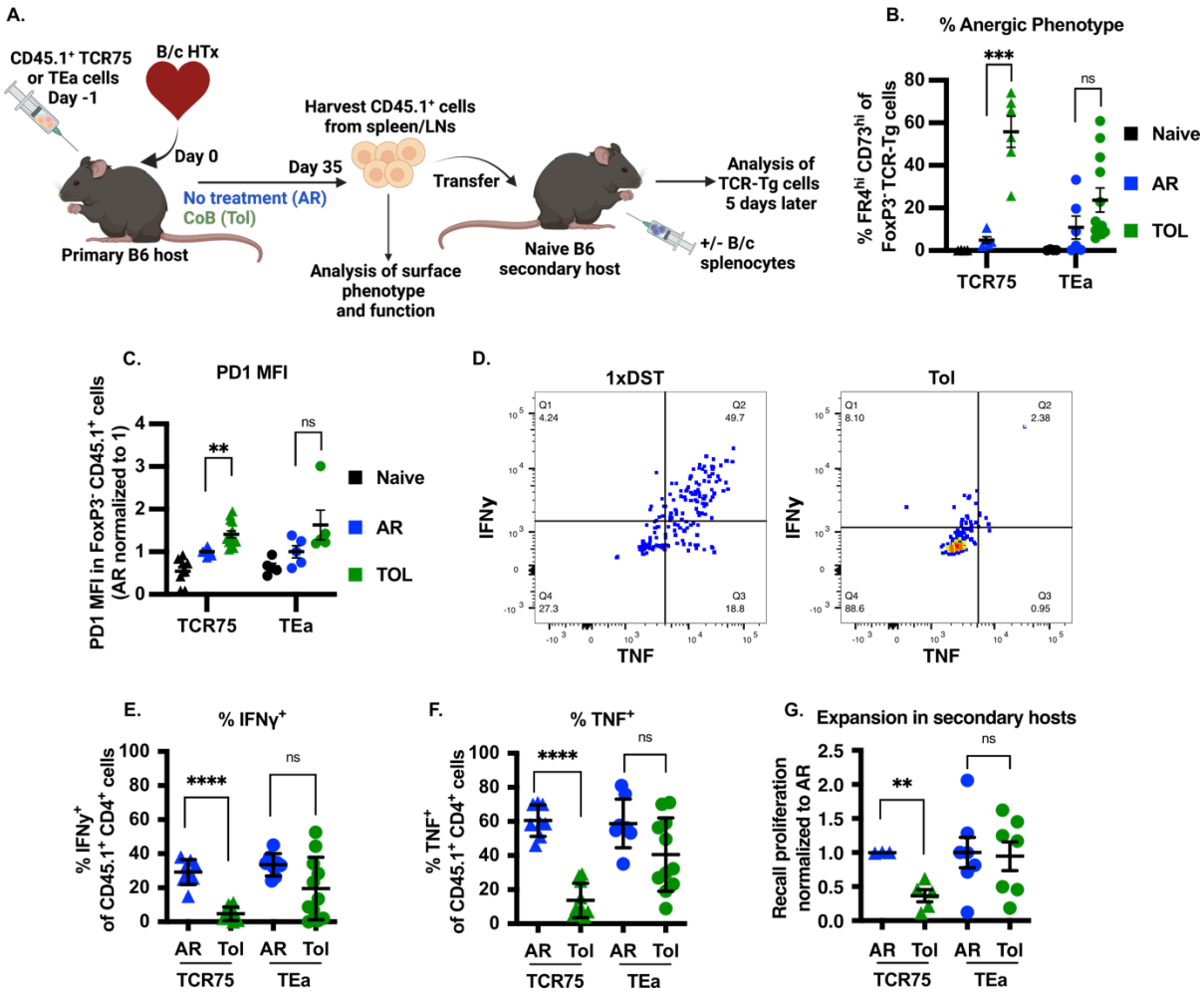

Figure 3.3 T cells specific for donor MHC Class II-derived peptide retain function following induction of transplantation tolerance.

(A) Experimental design. TCR75 or TEa cells were adoptively transferred into B6 hosts prior to transplantation of a B/c heart. Mice were left untreated and allowed to reject the graft (AR) or were treated with CoB (Tol). After 35+ days, cells from spleen and lymph nodes were recovered and assessed for functionality either via overnight stimulation with anti-CD3/CD28 in the presence of brefeldin A, or $CD45.1^+CD4^+CD44^{hi}$ TCR-Tg cells were sorted and transferred in equal numbers to naïve secondary hosts immunized with B/c splenocytes to assess their recall proliferation 5 days later. **(B, C)** TCR75 and TEa cells seeded at the time of transplantation as in Figure 3A were harvested at day >35 post-B/c heart transplantation +/- CoB (acute rejection, AR; tolerance, Tol), analyzed by flow cytometry and compared to naïve TCR-Tg counterparts. $CD45.1^+CD4^+FoxP3^-$-gated events were assessed for the percentage of cells expressing high levels of CD73 and FR4 (A) or for PD1 mean fluorescence intensity (MFI, B). Results were pooled from 2-4 independent experiments. For panel A: Naïve TCR75 (n=4), AR TCR75 (n=5), Tol TCR75 (n=6), Naïve TEa (n=5), AR TEa (n=6), Tol TEa (n=12). For panel B: Naïve TCR75 (n=8), AR TCR75 (n=11), Tol TCR75 (n=14), Naïve TEa (n=5), AR TEa (n=5), Tol TEa (n=5). Results depicted as mean +/-SEM. Data analyzed by one-way ANOVA with multiple comparisons. Significance not depicted in panel A: Naïve TCR75 vs Tol TCR75 (****), Naive TEa vs Tol TEa (*). Significance not depicted in panel B: Naive TCR75 vs Tol TCR75 (**), Naïve TEa vs Tol TEa (*), Naïve TEa vs AR Tea (**). **p<0.01, ***p<0.001, ns, not significant. **(D)** Representative flow plots of cytokine expression.

Figure 3.3, continued. TCR75 cells were seeded at the time of B/c splenocyte immunization (DST) or B/c heart transplantation + CoB treatment (Tol). Splenocytes were harvested on day >30 and restimulated *in vitro* overnight with anti-CD3/CD28 in the presence of brefeldin A. $CD4^+CD45.1^+$-gated events were analyzed for the percentage of cells producing IFNγ and/or TNF. **(E, F)** Percentage of TCR-Tg T cells producing IFNγ and TNF at d35+ post-transplantation following *in vitro* restimulation. Results were pooled from 3 independent experiments. Each data point represents a sample pooled from 1-5 mice with lines indicating mean +/- SEM. AR TCR75 (n=8), Tol TCR75 (n=10), AR TEa (n=8), Tol TEa (n=11). **(G)** Expansion in secondary hosts. TCR75 and TEa cells were enumerated 5 days post-immunization of secondary hosts with B/c splenocytes. Results were normalized to the average cell recovery obtained with TCR-Tg T cells that originated from AR primary hosts, set to 1 for each independent experiment with lines indicating mean +/- SEM. AR TCR75 (n=3), Tol TCR75 (n=5), AR TEa (n=7), Tol TEa (n=7). Data comparing TCR75 or TEa cells in AR versus Tol were analyzed by unpaired t-test **p<0.01, ****p<0.0001, ns, not significant.

To investigate function, TEa and TCR75 cells isolated from tolerant and rejected heart allograft recipients 35 days after transplantation were and subjected to restimulation *in vitro* or *in vivo* (Figure 3.3A,D). As memory cells, both TCR75 and TEa cells from rejected mice were able to produce IFNγ and TNF upon *in vitro* restimulation (Figure 3.3E, F). TCR75 cells from tolerant mice were significantly impaired in their production of both IFNγ and TNF (Figure 3.3E, F). In contrast, TEa cells from tolerant mice retained partial IFNγ and TNF production (Figure 3.3E, F). Furthermore, in the recall proliferation assay in secondary hosts restimulated with B/c splenocytes *in vivo*, TEa cells from tolerant mice accumulated similarly as TEa cells from rejected mice, whereas we confirmed the impaired recall proliferation of TCR75 cells from tolerant mice (Figure 3.3G). These data demonstrate that unlike TCR75 cells, TEa cells retain functionality in tolerant hosts, raising the possibility that T cells specific for alloantigens whose expression decreases over time retain more function following tolerance induction than allospecific T cells recognizing persistently expressed antigens.

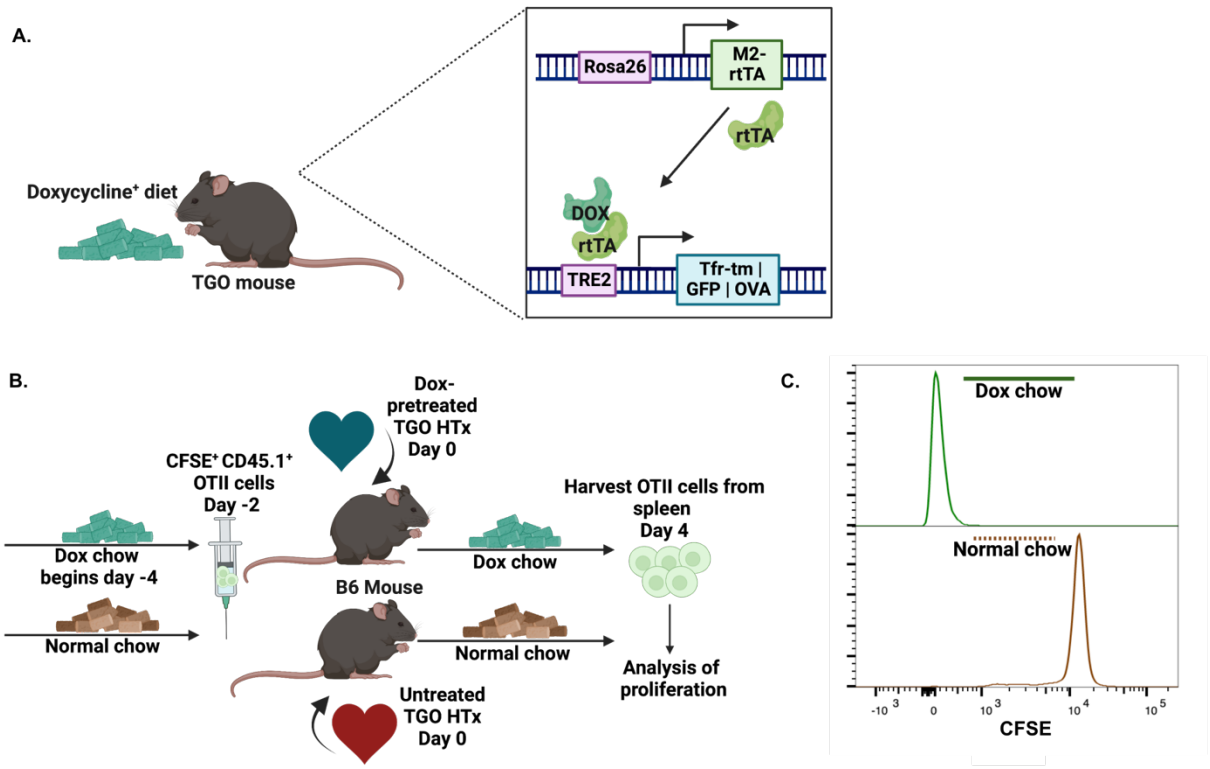

Figure 3.4 Inducible model of OVA expression in heart allografts.
(A) Mouse model. Mice expressing a transferrin transmembrane domain-GFP-OVA fusion protein under the control of a tetracycline-response element (TRE2) were crossed to mice expressing the reverse tetracycline transactivator M2-rtTA under control of the Rosa promoter to enable rtTA expression in all cells. **(B)** Experimental model. B6 recipients were adoptively transferred with CFSE-labeled $CD45.1^{+}CD4^{+}Rag^{-/-}$ OTII T cells (OTII) and either given OVA-expressing or negative control TGO heart transplants. Recipients of the $OVA^{+}TGO^{+}$ hearts received doxycycline (Dox)-containing diet whereas control TGO^{+} hearts were kept on normal chow. OTII cell proliferation was evaluated in the spleen 4 days after transplantation. **(C)** Representative plots of CFSE dilution of OTII cells confirm OVA expression and presentation in hosts of TGO^{+} heart allografts when donors and recipients are kept on Dox chow.

Alternatively, differences in functionality between TEa and TCR75 cells from tolerant hosts may reflect differences in TCR-Tg-intrinsic properties. To address this potential confounder, we used T cells of a single specificity, OVA-reactive OTII TCR-Tg $CD4^{+}$ T cells (also on a $Rag^{-/-}$ and $CD45.1^{+}$ background) and varied the duration of expression of their cognate antigen within the graft. To this end, we obtained TGO mice as transplant donors in which temporal expression of the antigen OVA can be controlled by ingestion of doxycycline (Dox)-containing diet [191]. These mice were crossed to M2-rtTA-expressing mice to ensure all tissues

can express OVA (Figure 3.4A), allowing us to control duration of OVA expression in the heart (Figure 3.4B,C) and skin (Figure 3.5) transplants. We confirmed control of OVA expression upon administration (Figure 3.4B, Figure 3.5A) or cessation (Figure 3.5C) of Dox chow by evaluating CFSE dilution of OTII TCR-Tg $CD4^+$ T cells. Two days of Dox chow prior to OTII transfer were sufficient to drive OTII proliferation (Figure 3.4C, Figure 3.5B) whereas cessation of Dox chow 3 days prior to OTII transfer was sufficient to extinguish OVA expression, preventing proliferation of OTII cells (Figure 3.5D).

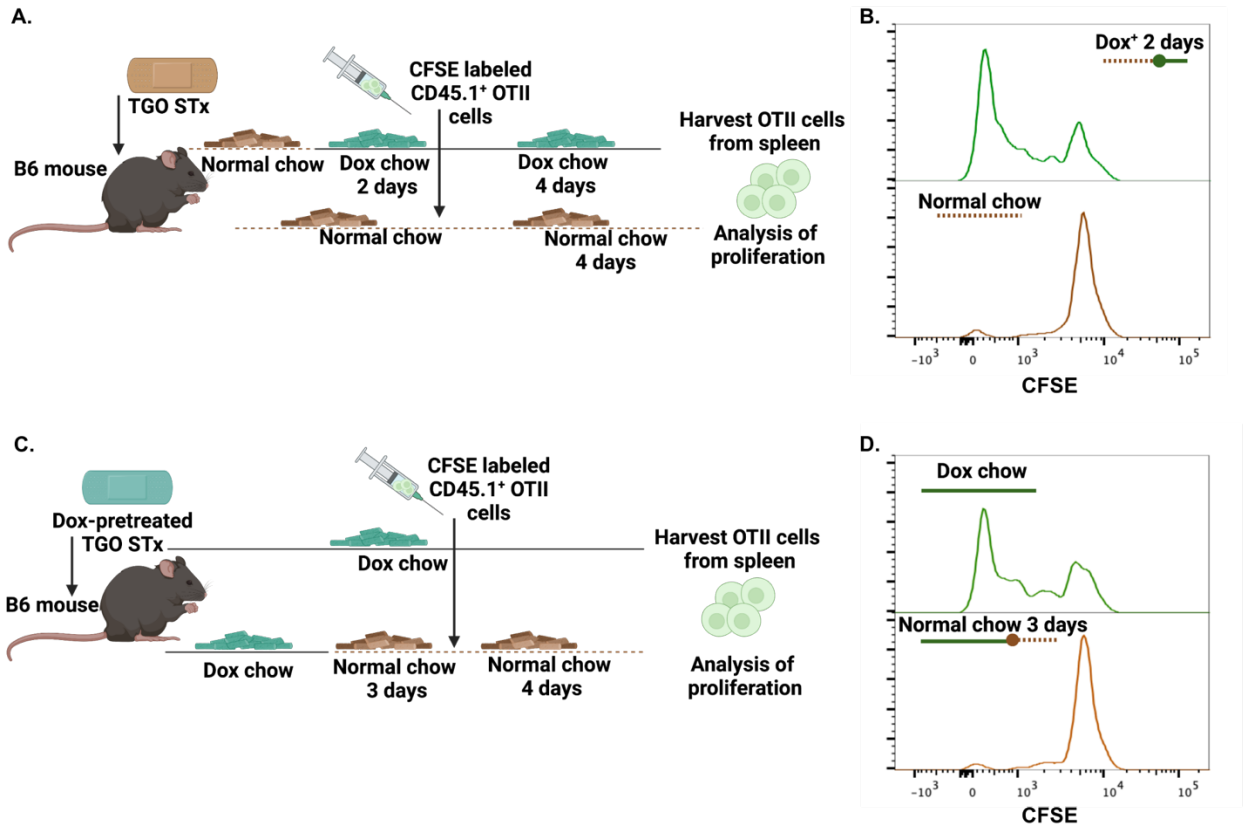

Figure 3.5 OVA expression in TGO^+ skin grafts is tightly controlled by dietary Dox.
(A) Experimental model. B6 recipients were given a TGO^+ skin transplant. Some mice were exposed to a Dox-containing diet two days prior to adoptive transfer of CFSE-labeled OTII cells. Control mice were never exposed to a Dox-containing diet. OTII cells were reisolated from skin-draining lymph nodes 4 days post adoptive transfer. **(B)** Representative plots confirming that a Dox-containing diet results in OTII proliferation, evaluated by CFSE dilution in $CD45.1^+CD4^+$OTII cells. **(C)** Experimental model. B6 recipients were given a TGO skin transplant pre-exposed to a Dox-containing diet. Some mice were

Figure 3.5, continued. returned to a normal chow diet three days prior to adoptive transfer of CFSE-labeled OTII cells. Control mice were continuously exposed to a Dox-containing diet. OTII cells were reisolated from skin-draining lymph nodes 4 days post adoptive transfer. **(D)** Representative plots confirming that stopping a Dox-containing diet prevents OTII proliferation, evaluated by CFSE dilution in $CD45.1^+CD4^+$OTII cells.

To determine whether durable versus transient expression of the same cognate antigen on an allograft would ensure OTII hypofunction versus retained functionality, OTII cells were transferred into congenic B6 hosts one day prior to transplantation with TGO hearts from donors on a Dox chow. All hosts were treated with CoB at the time of transplantation. Hosts received Dox chow from the day of transplantation for either 10 days (transient alloantigen exposure) or 30 days (persistent alloantigen exposure) and all animals were analyzed at 30 days post-transplantation (Figure 3.6A). As a result of CoB treatment, there was no difference in the number of $CD45.1^+$ OTII cells recovered following transient or persistent alloantigen exposure (Figure 3.6B). Upon overnight *in vitro* restimulation, OTII cells exposed to persistent Dox chow displayed reduced production of IFNγ and TNF compared with OTII cells from mice exposed to transient Dox chow (Figure 3.6C-E). These findings are consistent with our observations of greater loss of function in TCR75 than TEa cells in tolerant hosts and support that hypofunction of allospecific $CD4^+$ T cells following a tolerogenic regimen depends on persistent expression of the cognate antigen.

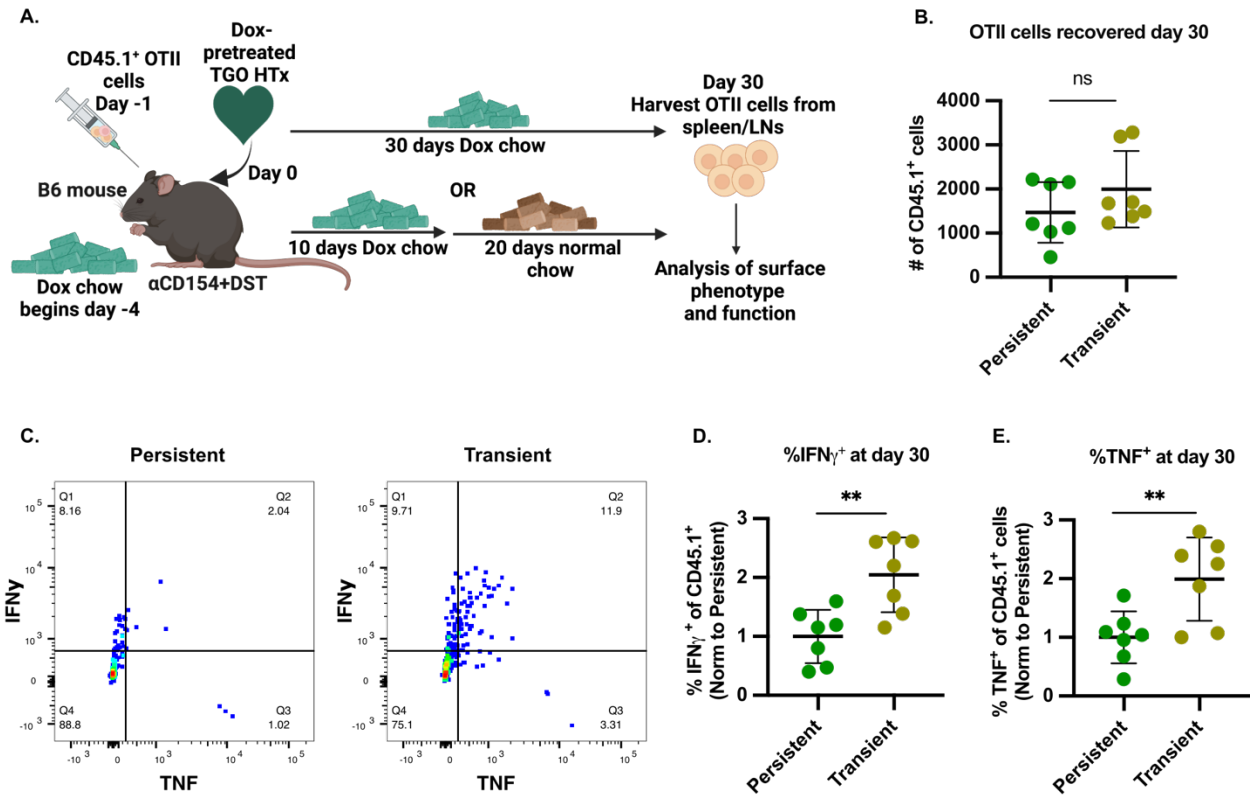

Figure 3.6 Prolonged expression of OVA on tolerant heart grafts results in greater OTII cell hypofunction than hearts with transient OVA expression.

(A) Experimental design. CD45.2$^+$ B6 recipients were pre-treated with a Dox-containing diet 4 days prior to transplantation. Recipients were also adoptively transferred with CD45.1$^+$ OTII cells (on a Rag$^{-/-}$ background) 1 day prior to transplantation with TGO hearts harvested from Dox chow-fed donors. All hosts were treated with CoB. Post-transplantation, mice were maintained on a Dox diet for either 30 days (Persistent), or only 10 days, returning to a normal chow for the remaining 20 days of the experiment (Transient). **(B)** Total CD4$^+$CD45.1$^+$ OTII cells recovered from spleens and lymph nodes on day >30 from mice described in A. Each data point represents a sample from 1 mouse with lines indicating mean +/- SEM. Persistent OVA (n=7), Transient OVA (n=7). **(C)** Representative flow plots of cytokine expression. B6 mice were prepared as described in A. Spleens and lymph nodes were harvested on day >30 and restimulated *in vitro* overnight with anti-CD3/CD28 in the presence of brefeldin A. CD4$^+$CD45.1$^+$-gated events were analyzed for the percentage of cells producing IFNγ and/or TNF. **(D, E)** Percentage of OTII cells producing IFNγ or TNF normalized to the average percentage in the Persistent OVA group in each independent experiment. Results were pooled from 2 independent experiments. Each data point represents a sample from 1 mouse with lines indicating mean +/- SEM. Persistent OVA (n=7), Transient OVA (n=7). Data were compared by unpaired t-test **p<0.01, ns, not significant.

3.06 Lm infection at the maintenance phase of tolerance upregulates donor MHC Class II expression and induces expansion of alloreactive, cytokine producing T cells

Alloreactive T cells specific for transiently expressed alloantigens that retain functionality following tolerance induction may pose a threat to the graft if their cognate antigen becomes re-expressed. Although levels of donor MHC Class II-derived alloantigen were insufficient to fully stimulate the proliferation of naïve TEa cells transferred at the maintenance phase of transplantation tolerance (

Figure 3.2B,D), an inflammatory event leading to an increase in IFNγ production, such as Lm infection, might be able to trigger upregulation of donor MHC Class II on the previously tolerated allograft [188,189]. To determine if Lm infection results in upregulation of donor MHC Class II on endothelial cells of tolerant grafts, tolerant heart recipients were infected with Lm at day 30+ post-transplantation and CoB treatment and $CD45^{-}CD31^{+}$ donor-derived endothelial cells from the graft were evaluated 4 to 8 days post-infection for expression of $I\text{-}A^{d}/I\text{-}E^{d}$ (Figure 3.7A). When compared with donor endothelial cells from uninfected tolerant recipients, Lm infection led to a marked increase in $I\text{-}A^{d}/I\text{-}E^{d}$ expression, with donor MHC Class II levels similar to those in donor endothelial cells from actively rejecting allografts (Figure 3.7B). Moreover, host splenic dendritic cells (DCs) displayed increased presentation of donor MHC Class II-derived peptide (Eα) presented on $I\text{-}A^{b}$ at day 4-8 post-Lm infection of tolerant hosts, as detected by staining with the YAe antibody (Figure 3.7C). To verify if Lm infection-dependent upregulation of donor-derived MHC Class II was sufficient to be detected by donor MHC Class II-reactive T cells, naïve TEa cells were CFSE labeled and adoptively transferred into tolerant heart graft recipients 4 days post-Lm infection (Figure 3.7D). Indeed, these naïve TEa cells

experienced significantly greater CFSE dilution when transferred into Lm-infected than uninfected tolerant mice (Figure 3.7E). Together, these data suggest that host T cells recognizing donor MHC class II indirectly have the potential to be reactivated by their cognate alloantigen during an infection.

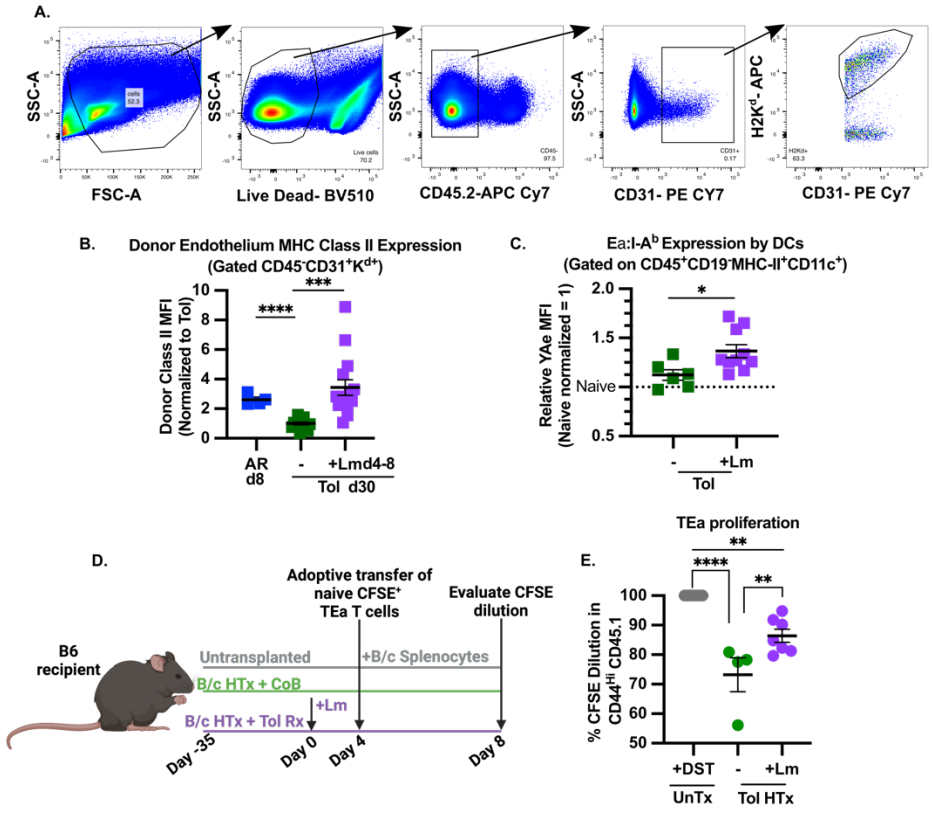

Figure 3.7 Increased presentation of donor MHC II-derived peptides can be induced in settings of Lm-infection during the maintenance phase of tolerance.
(A) Gating strategy for endothelial cells from heart allografts. **(B)** I-A^d/I-E^d expression on graft-isolated CD45$^-$CD31$^+$K^{d+} endothelial cells. AR represents untreated mice at day 8 post-transplantation. Tol mice were observed at day 30 post-transplantation + CoB, Tol+Lm mice were infected day 30 post-transplantation + CoB and evaluated days 4-8 post-infection AR (n=4), Tol (n=14), Tol+Lm (n=15). **(C)** B6 mice transplanted with B/c hearts and treated with CoB were infected or not with Lm on d30 post-transplantation. Splenocytes were analyzed 4-8 days later for expression of Ea:I-A^b on CD11c$^+$-gated events and normalized to the average expression on dendritic cells (DCs) from naïve mice in each independent experiment. Tol (n=6), Tol+Lm (n=9). Comparisons were made by unpaired t-test. *p<0.05. **(D)** Experimental model. Some B6 mice received a B/c heart graft and tolerance was induced with CoB (Tol HTx). Thirty-five days post-transplantation, a subset of these mice was infected with Lm (Tol HTx+Lm). Four days post-infection, all mice were adoptively transferred with CFSE-labeled naïve TEa cells. Control untransplanted mice received TEa cells at the same time as B/c splenocyte immunization

(UnTx+DST). All TEa cells were recovered day 4 post-adoptive transfer. **(E)** Percent of CD45.1$^+$ TEa cells that proliferated on day 4 post-adoptive transfer. UnTx+DST (n=5), Tol (n=4), Tol+Lm (n=7).

To determine if T cells that have been subjected to tolerance induction do indeed become reactivated following infection, we adoptively transferred TCR75 or TEa cells at the time of transplantation and CoB treatment. Tolerant heart graft recipients were infected with Lm at day 30+ post-transplantation, and the congenic T cells were recovered between days 4 and 8 post-infection (Figure 3.8A). Following infection, we saw an increase in CD4+ T cells infiltrating the graft (Figure 3.8B). Adoptive transfer of TEa or TCR75 cells did not affect the induction of tolerance as revealed by similar graft rejection scores in mice with or without transferred T cells, and Lm infection worsened the graft rejection score (Figure 3.8C), in keeping with our previous studies demonstrating that Lm can break established tolerance [138]. Consistent with the increased expression of donor MHC Class II induced by Lm infection of graft endothelial cells (Figure 3.7B), more TEa cells were recovered from infected than uninfected hosts, whereas Lm infection had no impact on the number of TCR75 cells (Figure 3.8D). Although there was not an increased volume of cytokine production by flow cytometry, there was an expansion in the number of cytokine producing TEa T cells following Lm infection (Figure 3.8E), indicating that threat to the graft is associated with an expansion of weakly cytokine producing cells. Thus T cells specific for donor MHC Class II-derived peptide but not donor MHC Class I-derived peptide significantly expand following Lm-infection of tolerant hosts. Combined with their maintained ability to produce cytokines following induction of transplantation tolerance (Figure 3.3E, F, Figure 3.8E), TEa cells, and presumably endogenous T cells of both direct and indirect specificities to donor MHC Class II, may represent allospecific T cells that participate in allograft rejection during Lm infection of tolerant hosts.

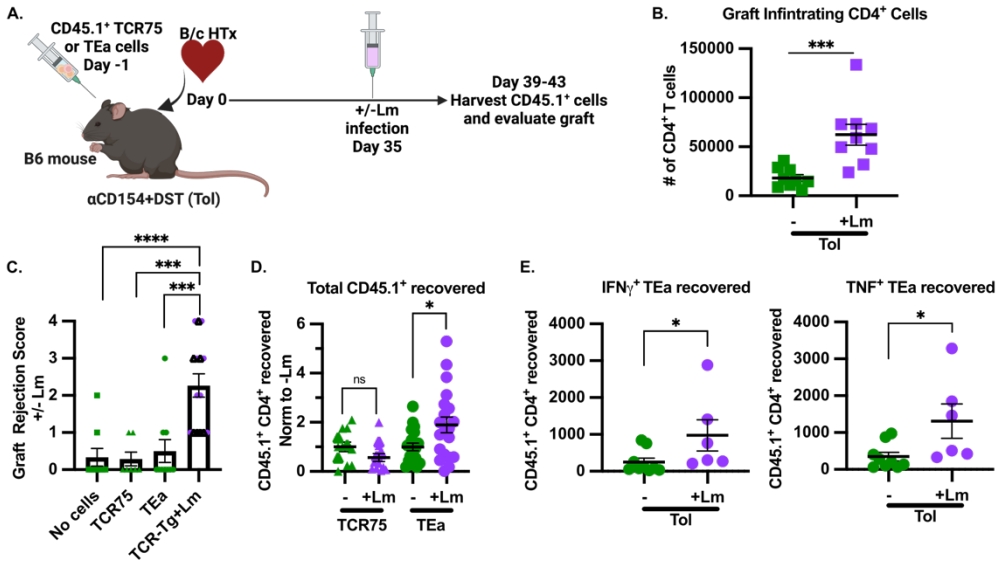

Figure 3.8 Lm infection in tolerant hosts induces upregulation of MHC Class II on donor endothelium and results in expansion of cytokine producing alloreactive T cells.
(A) Experimental design for D-E. TCR75 or TEa cells were adoptively transferred into congenic B6 recipients prior to transplantation with a B/c heart allograft and treatment with CoB to induce tolerance in all hosts. After 35+ days, a subset of mice was infected with Lm. 4-8 days post-infection, heart grafts were palpated and CD45.1$^+$ T cells were recovered, counted, and analyzed by flow cytometry. **(B)** Total CD4$^+$ T cells recovered from transplanted heart at day 39-43 post-transplantation. Tol (n=9), Tol+Lm (n=9). **(C)** Heart graft palpation score day 39-43. Hearts were scored, with a perfect score as 0 and a rejected heart as 4, on the following criteria: presence of heartbeat (absent=1 point), graft size (enlarged=1 point), heartbeat speed (slow=1 point), strength of heartbeat (weak= 1 point). No cells (n=9), TCR75 (n=7), TEa (n=10), TCR-Tg (either TCR75 or TEa) + Lm (n=15). **(D)** Total CD45.1$^+$ T cells recovered from spleen and lymph nodes at day 39-43 post-transplantation. TCR75 (n=12), TCR75+Lm (n=13), TEa (n=20), TEa+Lm (n=20). **(E)** The total number of CD45.1$^+$ TEa T cells recovered by FACS was multiplied to the percentage of the cells capable of producing cytokines upon restimulation with CD3 and CD28 in vitro. Value represents the number of CD45.1$^+$ TEa T cells per mouse capable of producing cytokines. Tol (n=9), Tol+Lm (n=6). Each data point represents a sample pooled from 1-2 mice with lines indicating mean +/- SEM. Data were analyzed by unpaired t-test (E, comparing +/- Lm for each TCR-Tg group) or one-way ANOVA with multiple comparisons (D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns, not significant. Error bars represent mean +/- SEM.

3.07 Repeated injections of donor splenocytes provides persistent antigen and promotes widespread T cell hypofunction in CoB-treated mice, conferring resistance to Lm-dependent rejection

Our combined results from TCR75, TEa, and OTII cells from tolerant mice suggested that persistently expressed cognate antigens drive more loss of function than more transiently expressed antigens. Thus, we investigated whether TEa cells could be made more hypofunctional upon CoB if exposure to their alloantigen was extended. To address this, naïve untransplanted B6 mice seeded with tracer TCR75 or TEa cells were immunized with one injection of B/c splenocytes (1xDST) or were treated with anti-CD154 (d0, 7, and 14) and repeated injections of B/c splenocytes every 48 hours for 35 days (Multi-DST+αCD154) prior to functional analysis of the persisting tracer TCR-Tg cells (Figure 3.9A). Multi-DST+αCD154 successfully drove dysfunction not only of TCR75 but also of TEa cells, as determined by their impaired production of both IFNγ and TNF (Figure 3.9B, C). Furthermore, Multi-DST+αCD154 led to a profound impairment in recall proliferation of both TCR75 and TEa cells when transferred and rechallenged in naïve secondary hosts (Figure 3.9D). These results indicate that increasing the persistence of alloantigen can lead to a loss of function in a wider array of allospecific T cells and show that TEa cells are not intrinsically resistant to developing hypofunction. These data also suggest a therapeutic avenue to increase the robustness of donor-specific T cell dysfunction and potentially transplantation tolerance.

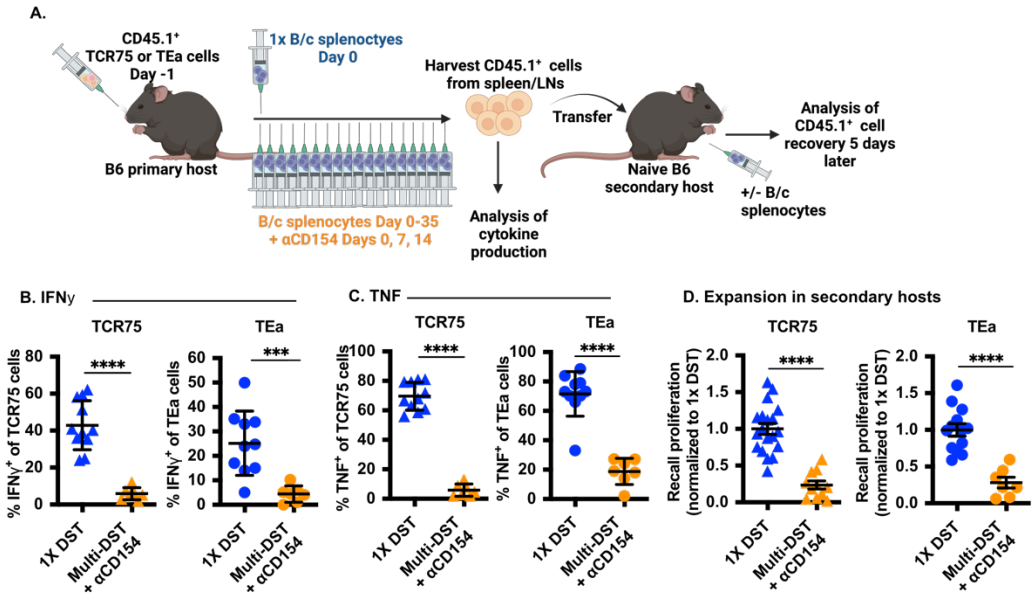

Figure 3.9 T cells reactive to peptides derived from donor MHC Class I- and Class II-molecules become dysfunctional with prolonged exposure to their cognate alloantigen. **(A)** Experimental Design. B6 mice were adoptively transferred with TCR75 or TEa cells then either immunized with a single injection of B/c splenocytes (1xDST) or given anti-CD154 (days 0, 7 and 14) and repeated injections of B/c splenocytes every 48 hours until sacrifice on day 35 (Multi-DST+aCD154). **(B, C)** Percentage of TCR-Tg cells producing IFNγ (B) and TNF (C) following cell isolation from spleen and lymph nodes of primary hosts on day 35 and restimulation overnight with anti-CD3/CD28 in the presence of brefeldin A. For TCR75 cells, 1xDST (n=11), Multi-DST+aCD154 (n=7); for TEa cells, 1xDST (n=10), Multi-DST+aCD154 (n=7). **(D)** $CD44^{hi}CD45.1^{+}$ tracer TCR-Tg cells were sorted from spleens and lymph nodes on day 35 and adoptively transferred into naïve B6 secondary hosts. One day later, secondary hosts were immunized with B/c splenocytes and TCR-Tg cell expansion was measured in the spleen 5 days later, normalized to the average cell recovery from 1xDST set to 1 for each experiment. For TCR75 cells: 1xDST (n=19), Multi-DST+aCD154 (n=11); for TEa cells: 1xDST (n=13), Multi-DST+aCD154 (n=7). All data points represent a sample pooled from 1-2 mice with lines indicating mean +/- SEM. Data were analyzed by unpaired t-test. ***p<0.001, ****p<0.0001.

Lm infection is capable of breaking or eroding stable heart allograft tolerance in infected tolerant hosts [138,140]. We hypothesized that Multi-DST+αCD154 may increase the host's protection from Lm-associated graft rejection, as a wider array of endogenous T cells would persistently encounter their cognate alloantigens, facilitating greater functional loss. To test this hypothesis, mice were transplanted with B/c heart allografts and tolerance was induced with 1xDST+αCD154 or with Multi-DST+αCD154 (Figure 3.10A). Mice were subsequently infected with Lm 35 days post-transplantation and heart grafts were evaluated one-month post-infection.

Mice exposed to Multi-DST+αCD154 showed reduced rejection scores of their heart grafts after Lm infection when compared to mice exposed to 1xDST+αCD154, as well as reduced immune cell infiltration, interstitial, perivascular inflammation, and a noticeable reduction in tissue damage upon histological analysis (Figure 3.10B-D). Thus, with prolonged exposure to a broader array of cognate alloantigens during anti-CD154 therapy, heart allografts are more protected from rejection following Lm infection.

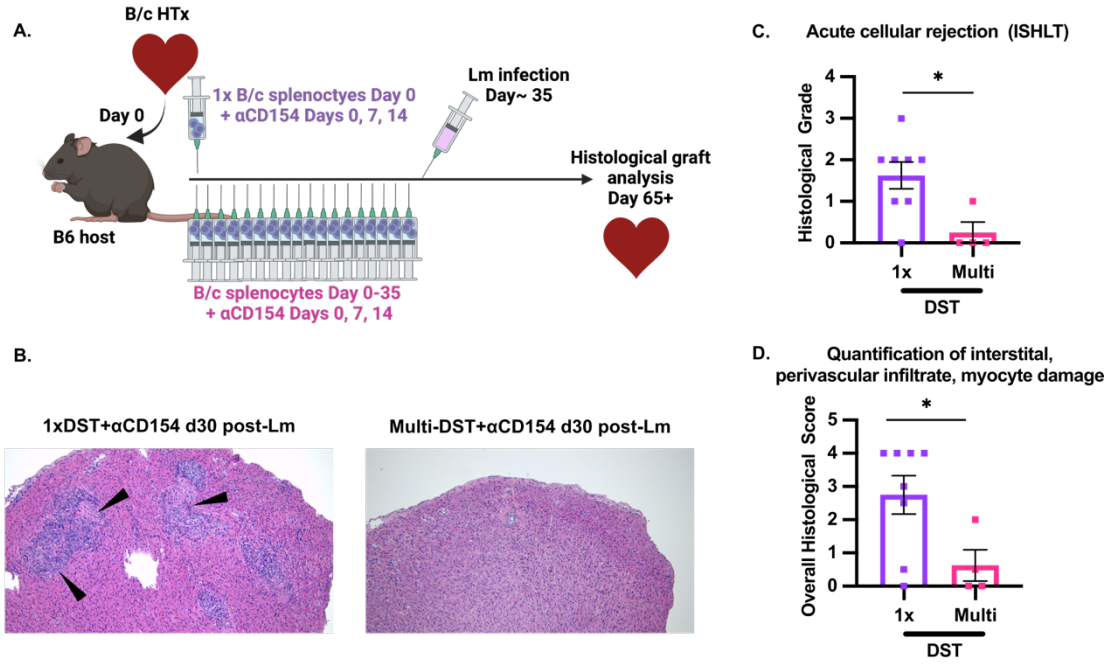

Figure 3.10 Prolonged exposure to alloantigen in the context of costimulation blockade protects heart allografts against post-infection rejection.
(A) Experimental design. B6 mice were transplanted with a B/c heart and tolerance was induced with anti-CD154 (days 0, 7, 14) in all hosts and either a single injection of B/c splenocytes (1x DST) or repeated injections of B/c splenocytes every 48 hours until day 35 (Multi-DST). Transplant hosts were then all infected with Lm on day 35 and grafts were analyzed a month post-infection. **(B)** Representative histological images from mice described in A. Tissues were sectioned and stained with hematoxylin and eosin. Slides were imaged at 10x magnification with an infinity HD camera mounted on an Olympus microscope. **(C, D)** Myocardial tissue was examined and scored by an independent pathologist in a single-blinded manner using the International Society for Heart and Lung Transplantation (ISHLT) acute cellular rejection grading scale [180]. 1x DST+aCD154+Lm (n=8); Multi-DST+aCD154+Lm (n=4). Data were compared by Mann-Whitney non-parametric one-sided t-test with lines indicating mean +/- SEM. *p<0.05.

3.08 Discussion

In this study, we show that alloreactive T_{convs} of different donor antigen specificities develop varied levels of dysfunction during CoB-induced transplantation tolerance, and that the duration of expression of the different alloantigens following transplantation contributes to this heterogeneity. Allospecific T cells experiencing persistent antigen stimulation during tolerance induction, modeled by TCR75 cells, are more hypofunctional than T cells specific for transiently expressed alloantigens, such as TEa cells or OTII cells transiently exposed to their model antigen. Cells that retain more function pose a greater threat to graft survival when tolerant hosts are exposed to inflammatory challenges, such as infections, that can upregulate alloantigen expression. However, these allospecific T cells can be made hypofunctional upon repeated exposure to alloantigen in the form of Multi-DST+αCD154. Most notably, this treatment was sufficient to protect tolerant grafts against Lm-mediated rejection.

Cells specific for donor MHC Class II-derived peptides are likely not the only endogenous T cells exposed to transient alloantigens during tolerance induction. T cells recognizing intact donor MHC Class II directly on donor cells are anticipated to experience similar transient kinetics of stimulation, as the cellular sources of intact donor MHC Class II are eliminated relatively shortly after transplantation [127]. It is also possible that inflammation-induced polymorphic minor histocompatibility antigens, such as MHC class I–related chain A (MICA) antigens, are similarly expressed transiently in the graft. The expression of such molecules is expected to decline after resolution of ischemia-reperfusion injury. Additionally, T cells with low avidity for alloantigens, irrespective of their persistence, or T cells specific for low density alloantigens, may not be sufficiently stimulated to lead to hypofunction during CoB

treatment. Consequently, there is potentially a large population of allospecific T cells that do not experience the chronic stimulation needed to program hypofunction during tolerance. These cells may not reject the graft during stable tolerance due to ignorance in the absence of sufficient cognate antigen and/or to control by Tregs but may escape suppression during inflammatory events. Indeed, in addition to inflammation potentially upregulating expression of certain alloantigens in the graft, IL-6 has been shown to reduce susceptibility of Tconvs to Treg suppression [192] and TNF has been reported to impair the suppressive capacity of Tregs [193].

T cells specific for transiently expressed alloantigens that retain function pose a threat to the allograft when they are re-exposed to their cognate alloantigen at a later time, while T cells specific for alloantigens irreversibly downregulated shortly after transplantation are likely to be harmless. For T cells recognizing donor MHC Class II, we found that secondary alloantigen exposure can occur during an inflammatory challenge, such as Lm infection. We reason that these findings would translate to all vascularized grafts harboring donor-derived endothelial cells that survive long-term after transplantation, as donor MHC Class II is upregulated on graft endothelial cells in response to IFNγ, for example, during infection [194] or autoimmunity [195]. We also hypothesize expression of transiently expressed alloantigens derived from minor histocompatibility antigens such as MICA would also be upregulated during injury or inflammation.

The functional allospecific T cells we have identified may play an important role in the chronic rejection that occurs late after transplantation in patients on conventional immunosuppression and is observed in some grafts with T and/or B cell infiltrates [196–199]. Prior work from our lab showed that TCR75 cells also developed hypofunction in transplanted mice

treated with conventional immunosuppression and do not develop transplantation tolerance [112]. In this setting, alloimmune responses still occurred late after transplantation, resulting in acute and chronic rejection possibly carried out by alloreactive T cells that retained functionality in these mice and in patients. Indeed, alloantibodies produced *de novo* after transplantation are often directed towards alloantigens predicted to exhibit downregulated expression following transplantation but that can be induced later by inflammation. For example, de novo alloantibody production is more frequently directed towards donor MHC Class II than MHC Class I, and patients with class II DSA have worse outcome [197,198]. Additionally, alloantibodies specific for the polymorphic stress-induced molecule MICA have been detected in transplant recipients, and are associated with poor graft outcomes [199].

The concept of tolerizing the immune system with repeated exposure to antigen is reminiscent of clinical methods used to treat allergy patients. Patients who ingested low doses of oral allergens daily saw improved tolerance to their allergens [151,200]. This unresponsiveness was maintained 4 years after the cessation of treatment [152,201]. Similarly, patients exposed weekly to grass-pollen allergens subcutaneously over the course of three years maintained prolonged clinical remission following the cessation of treatment [154,202], implying repeated exposure to antigen does not need to be maintained indefinitely for long-term disease-modifying effects. These data could inform more clinical approaches for patient exposure to alloantigen, where patients could be given small doses of alloantigen to promote robust tolerance.

Our findings have additional implications for autoimmune diseases known for relapse and remittance. In Lewis rat models of experimental autoimmune uveitis, relapsing or monophasic disease onset is determined by the autoantigen used for induction [203]. $CD4^+$ T cells

driving relapsing disease retain greater functionality when compared to $CD4^+$ T cells in monophasic disease [204], perhaps because their cognate antigen is only transiently expressed. It may be possible to ameliorate relapsing disease by targeting these autoreactive T cells with repeated injections of antigen in combination with costimulation blockade. It is unclear how or if persistent antigen exposure would treat or exacerbate models of remitting/relapsing autoimmunity that rely on epitope spreading, such as in autoimmune blistering diseases [205].

As efforts proceed within clinical transplantation towards inducing tolerance therapeutically and predicting which patients can safely undergo immunosuppression weaning without experiencing rejection, a key concern will remain whether the transplant acceptance in these patients is durable. Reports in patients who have developed spontaneous operational tolerance or undergone concurrent renal and bone marrow transplantation indicate that we cannot yet guarantee permanent graft acceptance free from immunosuppression, even in patients who initially experience years of stable function. Understanding the mechanisms by which rejection can occur late after transplantation, both in tolerized patients and those undergoing conventional immunosuppression, is therefore key to improving clinical outcomes for transplant recipients. We have identified a previously unappreciated source of persistent reactivity in the allospecific T cell repertoire that, during tolerance, poses a significant risk to graft survival. By reducing the effector function of these cells, we were able to prevent rejection of cardiac allografts after infection of tolerant hosts. Further work is needed to identify transiently expressed alloantigens in humans, and the fate of T cells responding to those antigens late after transplantation. Our results suggest that prolonging exposure to alloantigen panels under the cover of costimulation blockade, or matching donor and recipient alleles of key inflammation-induced alloantigens, may

increase the robustness of donor-specific tolerance and improve long-term graft survival in transplant recipients.

Chapter 4: Promoting memory T cell dysfunction in transplantation tolerance through persistent exposure to cognate antigen in the context of costimulation blockade.

4.01 Abstract

Memory allospecific T cells represent a threat to transplanted organs, as they are less restrained by immunosuppressive regimens. Finding ways to target memory T cells and promoting hypofunction has the potential to prolong graft survival in patients and enhance the robustness of tolerance. We hypothesized that memory T cells may achieve some level of dysfunction when exposed to a combination of costimulation blockade (CoB) with persistent alloantigen in the form of multiple injections of donor splenocytes (Multi-DST). While this treatment promoted robust T cell dysfunction in naïve T cells, memory T cells were somewhat more resistant, producing fewer cytokines upon restimulation but remaining resistant to CoB when adoptively transferred into transplanted mice. RNA sequencing analysis confirmed that Tmem+Multi-DST+CoB did not phenocopy tolerant T cells; however, they also remained distinct from Tmem, suggesting a potentially intermediate functional phenotype. Our findings further underscore the difficulty in inducing memory T cell hypofunction.

4.02 Introduction

Transplantation represents the treatment of choice for end-stage organ failure. In 2021, over 40,000 life-saving transplants were performed, a new record (Young 2022). However, there remains over 100,000 patients currently on the national transplant waiting list (Young 2022). Excitingly, treatments for patients have improved, and 1 year survival rates for transplanted organs have improved to 95% [207]. Importantly, this is largely dependent upon which organ is

transplanted [208–210], in part due to environmental factors such as microbial colonization[211,212]. While these improvements are impressive, 10-15% of patients will reject their organs and either pass away or will return to the transplant wait list [207]. Additionally, patients who reject their transplanted organ will develop immunological memory [213], making it more difficult for them to receive a transplant in the future. It is therefore imperative that patients who receive a transplanted organ maintain their transplant for life.

Immunological memory is a hallmark of the adaptive immune system, allowing T and B cells who have previously encountered their cognate antigen to initiate rapid and robust effector responses. Compared to naïve T cells, memory T cells (Tmems) have a lower activation threshold [42], and can reside in either peripheral non-lymphoid tissues (Trm), lymphoid tissues (Tcm), or both (Tem), depending on their surface homing marker expression [22,39]. Tmems are long lived and, due to stem-like progenitors, are capable of repeated expansion [33,214,215]. Recently, by repeatedly activating T cells with prime-boost-boost vaccinations and adoptively transferring them to new hosts over a ten-year period, $CD8^+$ Tmems were found to have a seemingly intrinsic and unlimited proliferative potential [33]. In part, the rapidity of Tmems expansion is a result of altered metabolism in Tmem. Tmems rely largely on fatty acid oxidation for energy [216] and have mitochondria with enhanced mass and altered cristae formation for improved oxidative phosphorylation and subsequent improved secondary activation [217,218]. Additionally, $CD8^+$ Tmems generated after an acute infection are capable of antigen-independent self-renewal, driven by IL-7 and IL-15 [80], whereas $CD4^+$ asymmetrical self-renewal is driven by expression of the transcription factor TCF1 and repressed by $CD4^+$ differentiation[219]. The maintenance of a stem-like memory population helps ensure robust memory responses[214,215].

Memory alloreactive cells pose a threat to transplanted organs as they are primed to respond to the foreign tissue and can mediate rapid rejection. Memory T cells do not require as much costimulation as their naïve counterparts [220], which contributes to their resistance to immunosuppressive agents and CoB [221–225]. Priming alloreactive T cells is sufficient to eliminate the effects of CoB on allograft survival [223]. Indeed, memory $CD4^+$ T cells are resistant to CoB and retain their ability to provide help to $CD8^+$ T cells [222]. Additionally, both $CD4^+$ and $CD8^+$ Tmems are more resistant to suppression by regulatory T cells (Tregs), and while the presence of Tregs prevented naïve T cell-mediated graft rejection in RagKO mice, they were insufficient to protect against Tmems-mediated graft rejection [226]. In this way, allogeneic memory T cells pose a clear threat to transplanted organs, and strategies to subdue their alloreactivity are clinically necessary.

Patients can develop allogeneic memory following pregnancy, blood transfusions, or previous transplants. Memory can take the form of donor-specific-alloantibodies (DSA) that can precipitate rejection [227,228] or IFNγ -producing Tmems which have also been found to negatively impact graft outcome [229–231]. Additionally, B cells are sensitized following semiallogeneic pregnancy and could act as a barrier for transplantation in multigravid patients[232].

Even in the absence of prior transplantation, memory alloreactive T cells may arise following infections due to heterologous immunity, where immunogenic molecules expressed by the pathogen mimic allogeneic pMHC complexes [221,233,234]. Heterologous immunity can be driven either by antibodies or by cross-reactive T cells [235]. As much as 80% of T cell lines and 45% of virus-specific T cell clones have been previously shown to cross-react against a panel of allo-HLA^+ target cells [236]. In this way, environmental pathogen exposure poses an unavoidable risk for future transplant patients. To help presensitized patients accept and maintain their

transplanted organs, strategies targeting and repressing memory function are necessary and must be explored.

We have previously demonstrated that repeated exposure to donor-derived antigens in the context of CoB promotes dysfunction in a wide array of alloreactive T cells [112]. We therefore hypothesized that we could use this method of repeated antigen exposure as a pre-treatment prior to transplantation to promote non-responsiveness in Tmems. We found that exposing Tmems to repeated injections of cognate alloantigens along with CoB promoted hypofunction, as Tmems were no longer able to produce the cytokines IFNγ or TNF upon restimulation. Although we tried to assess if persistent antigen exposure promoted robust enough dysfunction to prevent transplant rejection, our limited cell transfer makes these results hard to interpret. We performed RNA sequencing (RNA-seq) analysis on Tmems exposed to multi-DST (Tmem+Multi-DST+CoB) and found that although incapable of producing cytokines, Tmem+Multi-DST+CoB did not exhibit a transcriptional profile similar to tolerant T cells, where T cells are known to be hypofunctional[112]. Our findings suggest that chronic antigen stimulation is an approach that is partially effective at reducing the function of Tmems, but insufficient to promote graft acceptance in presensitized hosts. It also underscores the difficulty in inducing dysfunction in Tmems.

4.03 Memory T cells' effector function is reduced only following repeated exposure to donor splenocytes in the context of CoB

Having found that naïve T cells become profoundly dysfunctional when exposed to Multi-DST (Chapter 3), we hypothesized that the same regimen may also induce dysfunction in Tmems. Tmems represent a problem for transplantation as they are not susceptible to

conventional tolerance protocols[213,222]. If we can induce dysfunction in Tmems prior to transplantation, it would be easier to match patients with potential organs.

We generated alloreactive Tmems by adoptively transferring CD45.1$^+$ TCR75 cells, TCR-transgenic CD4$^+$ T_{convs} specific for a donor MHC Class I-derived peptide indirectly presented by host MHC Class II, into B6 hosts one day prior to transplantation with a BALB/c (B/C) skin transplant. Skin transplants were rejected, and 35 days later, a separate group of naïve mice were adoptively transferred with naïve CD45.1$^+$ TCR75. Naïve and presensitized B6 recipients were given either 1 injection of B/c splenocytes (1xDST), 1xDST + costimulation blockade (CoB), or repeated injections of B/c splenocytes (Multi-DST) + CoB. On day 60+ post-initial transplantation, TCR75 T cells were reisolated and evaluated for cytokine production (Figure 4.1A). TCR75 T cells from presensitized, Multi-DST+CoB-treated mice showed impaired production of IFNγ and TNF compared to TCR75 Tmems (Figure 4.1B,C). As expected, 1xDST+CoB was insufficient for promoting loss of Tmems cytokine production (Figure 4.1B,C). Although we would normally expect 1xDST+CoB to be sufficient for naïve T cell dysfunction when a transplant is present [112], this model lacks a graft and therefore has no persistent antigen, unless supplied through Multi-DST injection. It is therefore expected that naïve T cells exposed to 1xDST+CoB do not develop as robust dysfunction as the T cells exposed to Multi-DST (Figure 4.1B,C). Thus, Tmems are capable of developing hallmarks of the dysfunctional T cell phenotype *in vivo*.

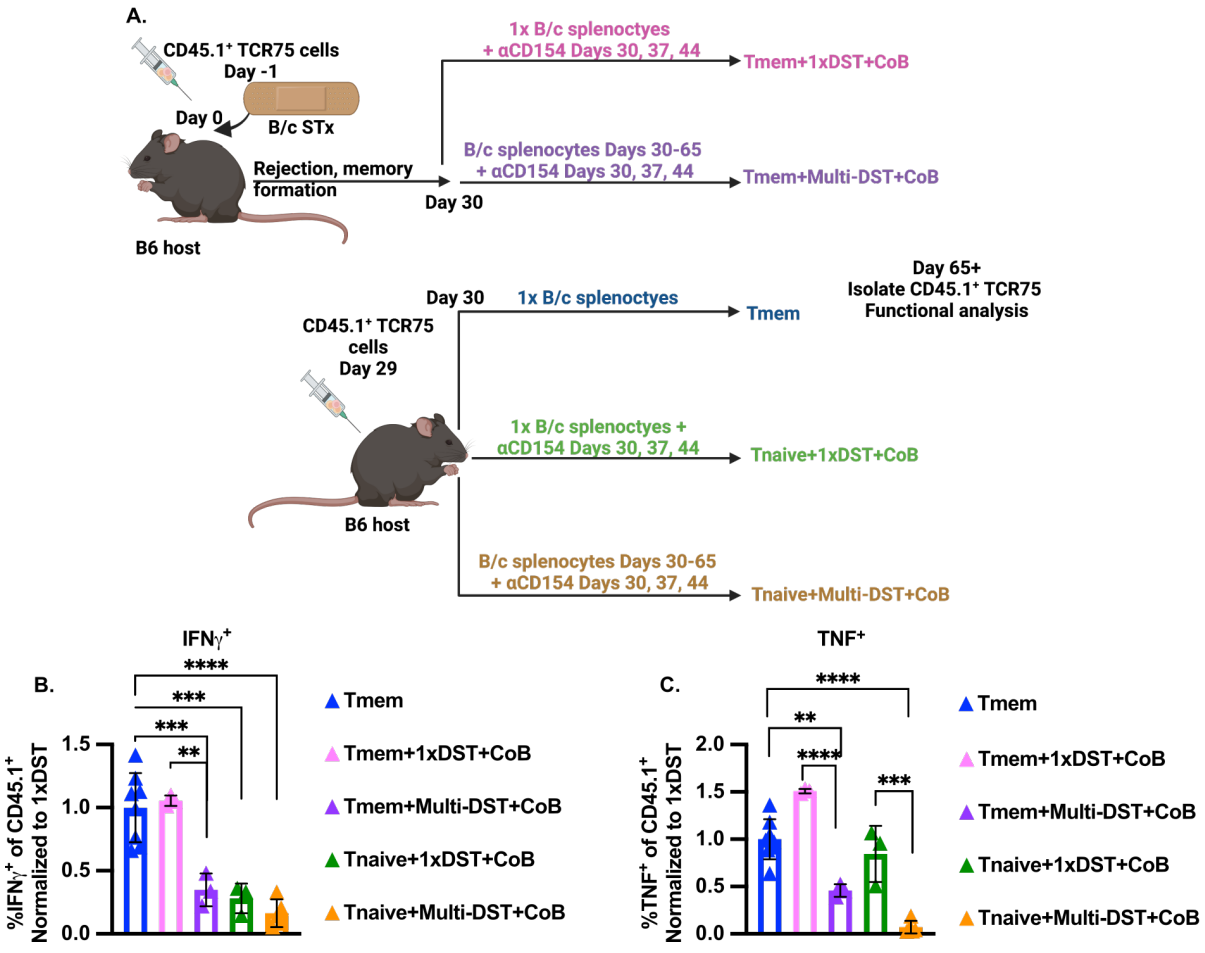

Figure 4.1 Multi-DST + CoB inhibits the cytokine production capacity of memory T cells. **(A)** Experimental design. $CD4^{+}CD45.1^{+}$ TCR75/$Rag^{-/-}$ T cells (TCR75) were adoptively transferred into $CD45.2^{+}$ B6 hosts who were then transplanted with B/c skin to induce memory. Day 30, mice with memory TCR75 cells received anti-CD154 (days 30, day 37, day 44) with either 1xDST or Multi-DST. A separate group of naive mice were adoptively transferred TCR75. Some of these mice received an injection of DST alone, some received 1xDST with anti-CD154 (days 30, day 37, day 44), and some mice received Multi-DST with anti-CD154 (days 30, day 37, day 44). $CD45.1^{+}$ TCR75 cells were harvested from the spleen and lymph nodes on d65+ and restimulated for cytokine production *in vitro.* Tmem (n=8), Tmem+1xDST+CoB (n=3), Tmem+Multi-DST+CoB (n=3), Tnaive+1xDST+CoB (n=3), Tnaive+Multi-DST+CoB (n=5)**. (B)** Percentage of TCR-Tg T cells producing IFNγ at d65+ post-transplantation or day 35+ post-adoptive transfer and following *in vitro* restimulation. Results were pooled from 2 independent experiments. Each data point represents a sample pooled from 1-5 mice with lines indicating mean +/- SD. Significance not pictured Tmem+1xDST+CoB vs Tnaive+Multi-DST+CoB (****), Tmem+1xDST+CoB vs Tnaive+1xDST+CoB (**). **(C)** Percentage of TCR-Tg T cells producing TNF at d65+ post-transplantation or day 35+ post-adoptive transfer and following *in vitro* restimulation. Results were pooled from 2 independent experiments. Each data point represents a sample pooled from 1-5 mice with lines indicating mean +/- SD. Significance not pictured: Tmem vs Tmem+1xDST+CoB (**),Tmem+1xDST+CoB vs Tnaive+Multi-DST+CoB (****), Tnaive+1xDST+CoB vs Tnaive+Multi-DST+CoB (***), Tmem+1xDST+CoB vs Tnaive+1xDST+CoB (**). Statistical comparisons were

Figure 4.1, continued. performed with one-way ANOVA with multiple comparisons.**p<0.01, ***p<0.001, ****p<0.0001, ns, not significant.

4.04 Pre-treatment of memory TCR75 cells with Multi-DST+CoB prior to transplantation improves graft outcomes

To determine if pre-treatment of Tmems with Multi-DST+CoB resulted in profound enough TCR75 Tmem dysfunction to prevent heart graft rejection, we generated TCR75 Tmems by adoptively transferring TCR75 cells into B6 recipients one day prior to transplantation with a B/c skin graft. The skin graft was allowed to reject, and 30 days post transplantation half of the mice were given Multi-DST+CoB, whereas the other half were given an additional injection of donor splenocytes. Around day 65, TCR75 T cells were isolated from the spleens of treated mice and, due to low recovery and anticipating strong anti-graft immune responses, 3,000-7,500 T cells were adoptively transferred into new, naïve B6 hosts. These hosts were then transplanted with a B/c heart graft and treated with CoB (Figure 4.2A). The quality of the heart grafts was tracked over time (Figure 4.2B). One mouse that received memory TCR75 T cells rejected its heart graft, whereas mice that received Multi-DST+CoB-treated memory TCR75 T cells did not, and indeed there was significant difference in the overall graft quality between the two groups when evaluated over time (Figure 4.2B). Ten weeks after heart transplantation, there were no significant differences in the numbers of TCR75 T cells recovered, suggesting that the Tmems exposed to Multi-DST+CoB were not primed for apoptosis (Figure 4.2C). The quality of the heart grafts was also assessed by an independent pathologist (Figure 4.2D). While there were no significant differences in histological scores between mice that received Tmems and Multi-DST+CoB-treated Tmems (Figure 4.2D,E), the pathologist noted that there was overall more background damage in both conditions than typically seen in mice receiving naïve TCR75 cells.

This is not due to differences in surgeons, and likely reflects worse pathology inflicted by Tmems than naïve T cells (Figure 4.2D). Although we only found differences in palpation score but no differences in graft survival (Figure 4.2B,E), we may be below the threshold of Tmems needed to promote transplant rejection, and future experiments will adoptively transfer a larger number of pretreated TCR75 cells.

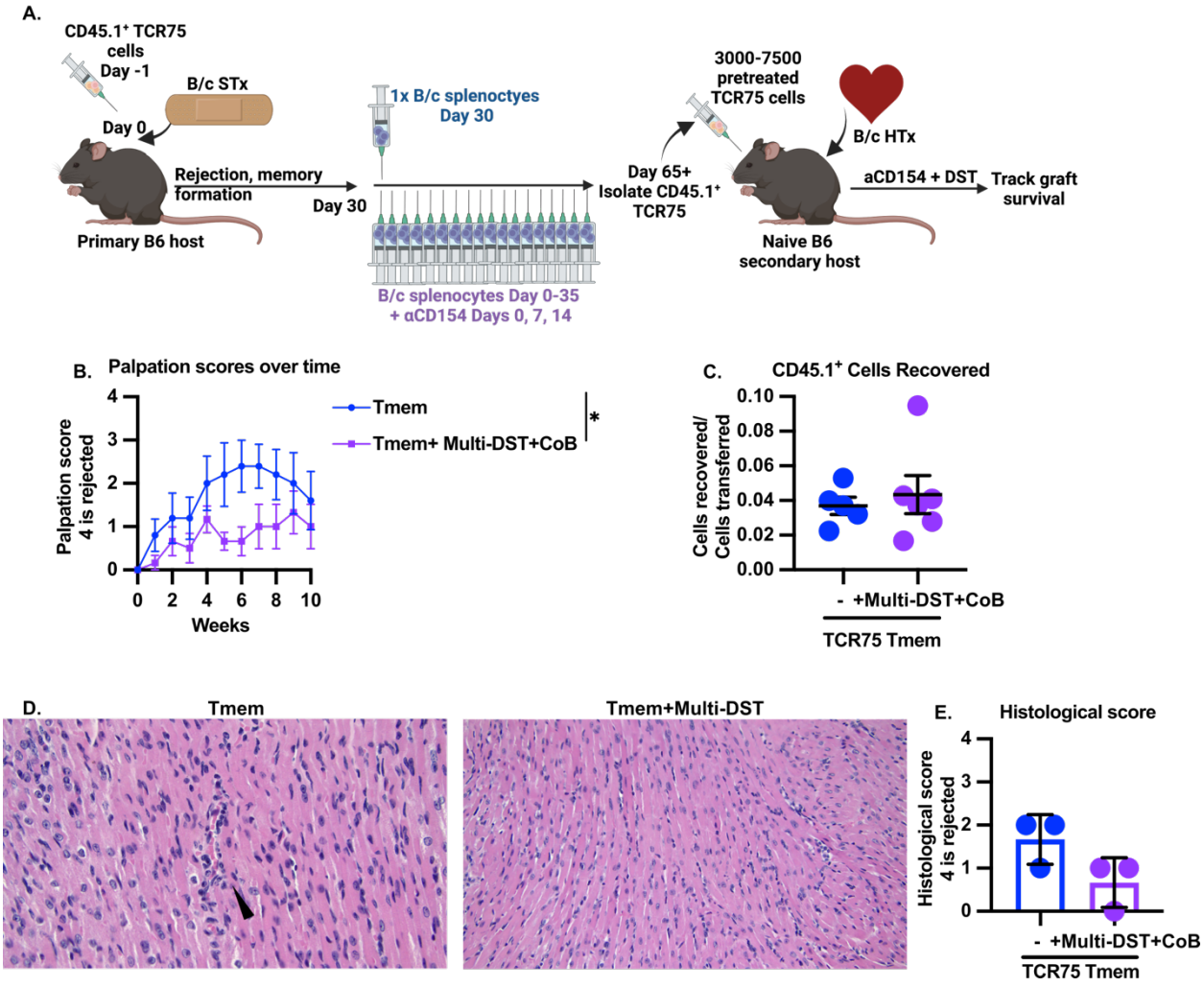

Figure 4.2 Pretreating memory T cells with Multi-DST+CoB protects against graft damage. **(A)** Experimental design. TCR75 cells were adoptively transferred into CD45.2^{+} B6 hosts who were then transplanted with B/c skin to induce memory. Day 30, mice with memory TCR75 cells received anti-CD154 (days 30, day 37, day 44) with either 1xDST or Multi-DST. 65+ days post-transplantation, 3000-7500 presensitized TCR75 cells were isolated and adoptively transferred to new hosts, which were then transplanted with B/c hearts and given CoB. Heart grafts were monitored over time for survival. **(B)** Heart palpation scores over time. Hearts were scored, with a perfect score as 0 and a rejected heart as 4, on the following criteria: presence of heartbeat (absent=1 point), graft size (enlarged=1 point), heartbeat speed

Figure 4.2, continued. (slow=1 point), strength of heartbeat (weak= 1 point). Significance was determined by area under the curve. Tmem (n=5); Tmem+Multi-DST+CoB (n=6).**(C)** Total CD45.1+ TCR75 T cells recovered post-heart transplantation. Tmem (n=5); Tmem+Multi-DST+CoB (n=6). **(D)** Representative histology from mice described in A. Tissues were sectioned and stained with hematoxylin and eosin. Slides were imaged at 20x magnification with an infinity HD camera mounted on an Olympus microscope. **(E)** Myocardial tissue was examined and scored by an independent pathologist in a single-blinded manner using the International Society for Heart and Lung Transplantation (ISHLT) acute cellular rejection grading scale. Tmem (n=5); Tmem+Multi-DST+CoB (n=6). Data were compared by Mann-Whitney non-parametric one-sided t-test with lines indicating mean +/- SD or SEM.

4.05 Repeated antigen exposure alters naïve and memory TCR75 T cell gene expression in the context of CoB

To further understand how Multi-DST+CoB treatment impacts memory TCR75 T cells, we performed RNA-seq comparing memory TCR75 Tmems generated after one injection of B/c splenocytes (Tmem), TCR75 T cells from mice receiving B/c heart grafts and CoB (Tnaive+HTx+CoB), naïve T cells in untransplanted mice exposed to Multi-DST+CoB (Tnaive+Multi-DST+CoB), and Tmems in untransplanted mice exposed to Multi-DST+CoB Tmem+Multi-DST+CoB). Principal component (PC) analysis depicted the largest transcriptional differences between Tmems and Tnaive+Multi-DST+CoB, as expected (Figure 4.3). Interestingly, Tmem+Multi-DST+CoB clustered more closely to Tmem than to Tnaive+Multi-DST+CoB, with Tnaive+HTx+CoB TCR75 T cells clustering in-between the two groups (Figure 4.3). Knowing that Tnaive+Multi-DST+CoB TCR75 cells are the most dysfunctional, it suggests that the Multi-DST+CoB protocol is insufficient to drive complete dysfunction in the Tmem+Multi-DST+CoB. However, as Tmem+Multi-DST+CoB are beginning to cluster more closely to the Tnaive+HTx+CoB TCR75 group, it is possible that, should antigen persist longer, it might drive robust Tmem+Multi-DST+CoB dysfunction. Whether this could or would be a distinct differentiation state instead of canonical dysfunction is unclear.

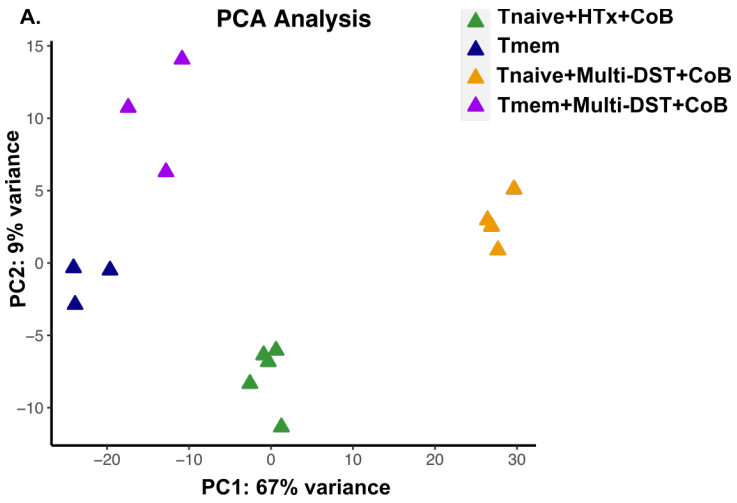

Figure 4.3 Relative level of hypofunction accounts for the majority of transcriptional variance in naïve and memory TCR75 T cells following Multi-DST+CoB treatment.
(A) Principal component (PC) analysis of RNAsequencing data. Tnaive+HTx+CoB (n=5), Tmem (n=3), Tnaive+Multi-DST+CoB(n=4), Tmem+Multi-DST+CoB(n=3).

To further assess the transcriptional differences between experimental groups, we directly compared significantly differentially expressed genes using volcano plots (Figure 4.4). Of particular interest to us was the special AT-rich sequence-binding protein 1 (Satb1). We have previously published that tolerant $CD4^+$ T cells have reduced Satb1 expression, resulting in their increased susceptibility to Treg suppression [237]. Satb1 expression was higher in Tnaive+HTx+CoB TCR75 cells compared to naïve T cells that received the Multi-DST+CoB protocol (Figure 4.4B). Satb1 expression was also higher in Tmem+Multi-DST+CoB cells compared Tnaive+Multi-DST+CoB (Figure 4.4C). As Tmem+Multi-DST+CoB cells are less dysfunctional than Tnaive+HTx+CoB TCR75 cells which are less dysfunctional than Tnaive+Multi-DST+CoB (Figure 4.3), these data suggest that susceptibility to Treg suppression may be particularly important for the gradation of dysfunction of these cells.

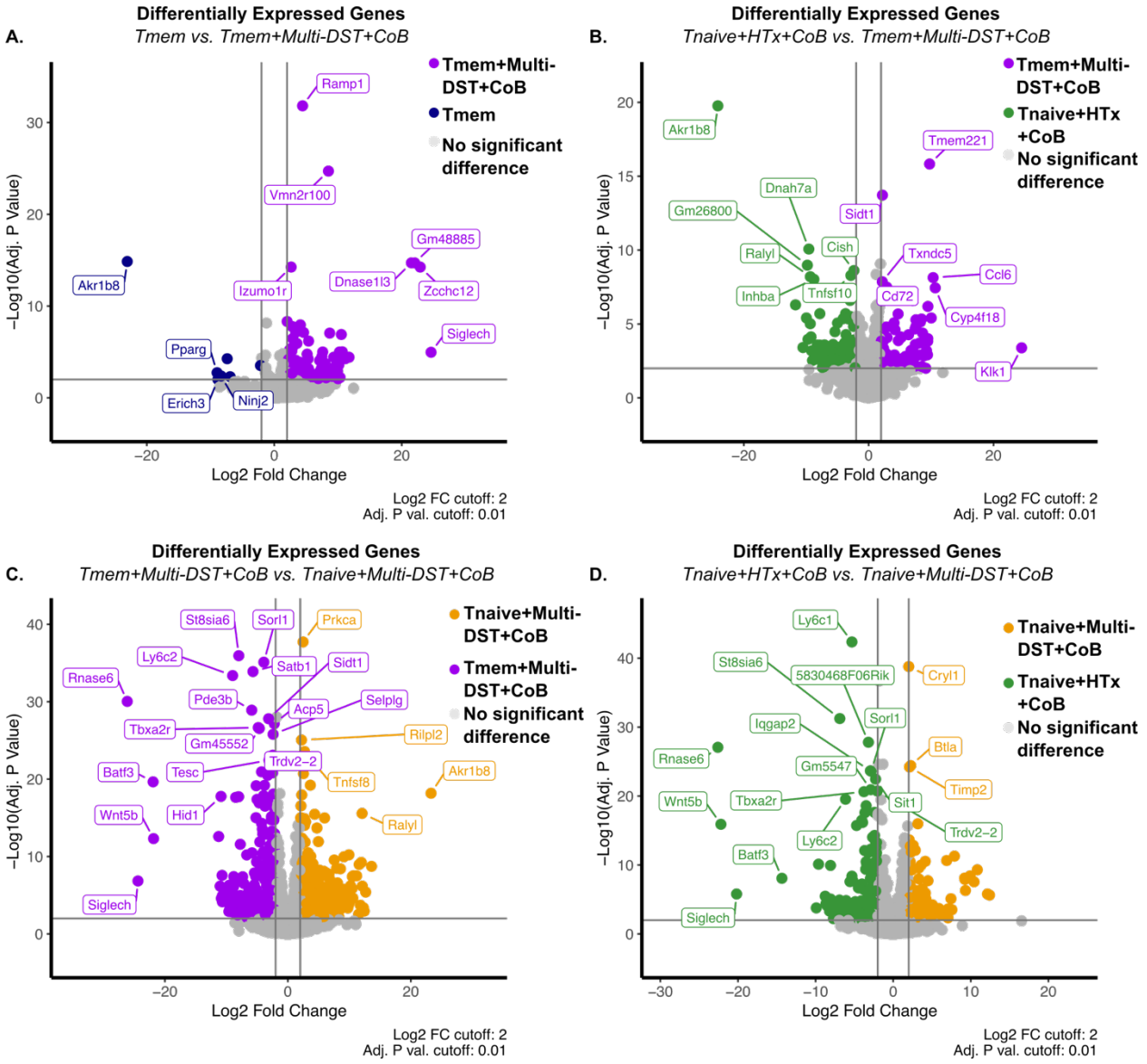

Figure 4.4 Naïve and memory TCR75 T cells treated with Multi-DST+CoB acquire distinct transcriptional profiles.

(A) Volcano plot depicting significantly differentially expressed genes between Tmem (n=3) and Tmem+Multi-DST+CoB (n=3). Log2-fold-change threshold = 3, adjusted p-value threshold = 0.005. **(B)** Volcano plot depicting significantly differentially expressed genes between Tnaive+HTx+CoB (n=5) and

Figure 4.4, continued. Tmem+Multi-DST+CoB (n=3). Log2-fold-change threshold = 3, adjusted p-value threshold = 0.005. **(C)** Volcano plot depicting significantly differentially expressed genes between Tmem+Multi-DST+CoB (n=3) and Tnaive+Multi-DST+CoB (n=4). Log2-fold-change threshold = 3, adjusted p-value threshold = 0.005. **(D)** Volcano plot depicting significantly differentially expressed genes between Tnaive+HTx+CoB (n=5) and Tnaive+Multi-DST+CoB (n=4). Log2-fold-change threshold = 2, adjusted p-value threshold = 0.01. Diagrams generated by Lexi Cassano.

In addition to Satb1, PParγ and Wnt5b were all significantly enriched in multiple of our comparisons of interest. PParγ is the peroxisome proliferator activated receptor gamma, a ligand-induced transcription factor known to regulate adipose tissue [238]. In T cells, PParγ expression is associated with lipid metabolism [238]. Interestingly, Tregs and Tmems are the T cell subsets most famous for their lipid metabolism [87], and PParγ was increased in both Tnaive+HTx+CoB and Tmem cells relative to Tmem+Multi-DST+CoB cells (Figure 4.4A, B). Although in our hands TCR75 cells are resistant to differentiating into Tregs in vivo, it is interesting to consider if they could still exhibit characteristic Treg phenotypes in tolerance. PParγ is also associated with transrepression of NFAT and IL-2 secretion [239,240]. In this way, PParγ limits T cell proliferation. Although we expect Tmem+Multi-DST+CoB cells to be less proliferative than Tmems, Tmem+Multi-DST+CoB cells were more recently exposed to antigen than either Tnaive+HTx+CoB or Tmems, which may explain their differential PParγ phenotype (Figure 4.4A, B).

Wnt5b is a member of the Wingless-related integration site (Wnt) gene family and a member of the non-canonical Wnt signaling pathway, that does not require β-catenin for signaling [241]. In T cells, Wnt5b is most associated with thymic development, with maximum expression in double positive thymocytes and subsequent reduction by 75–95% in later stages of T cell development [242]. Wnt5b is believed to regulate lymphocyte differentiation through control of the transcription factor FoxN1 [242]. While the Wnt5b pathway in T cells warrants further investigation, we found enriched Wnt5b expression in both Tmem+Multi-DST+CoB and Tnaive+HTx+CoB TCR75 T cells compared to Tnaive+Multi-DST+CoB cells (Figure 4.4C, D). Wnt5b and the non-canonical Wnt signaling pathway is upstream of NFAT [241], further

underscoring that naïve T cells exposed to Multi-DST+CoB maintain a hypofunctional phenotype.

4.06 Multi-DST in the context of CoB alters the metabolic and proliferative transcriptional profile of memory T cells

To get a more holistic view of the pathways differentially expressed in TCR75 cells exposed to Multi-DST, we performed gene set enrichment analysis. The gene ontology (GO) pathways significantly differentially expressed between Tmems and Tmem+Multi-DST+CoB TCR75 cells involved a variety of genes associated with metabolic processes and lymphocyte activation (Figure 4.5A). We further investigated these gene candidates by comparing their expression between Tmems and Tmem+Multi-DST+CoB TCR75 cells. Polyketides are a rare subset of lipids, more commonly referred to as "secondary metabolites" with immunosuppressive and anti-inflammatory properties [243]. There were only 3 genes driving the differential GO phenotype associated with polyketide metabolic processes in Tmems and Tmem+Multi-DST+CoB TCR75 cells (Figure 4.5B). Of these genes, two were aldo-keto reductases enriched in the Tmems population (Figure 4.5B). Although aldo-keto reductases have not been previously associated with T cell function, it is possible that their enrichment in Tmems is a side effect of Tmems' reliance on lipid catabolism as a primary metabolic pathway [87].

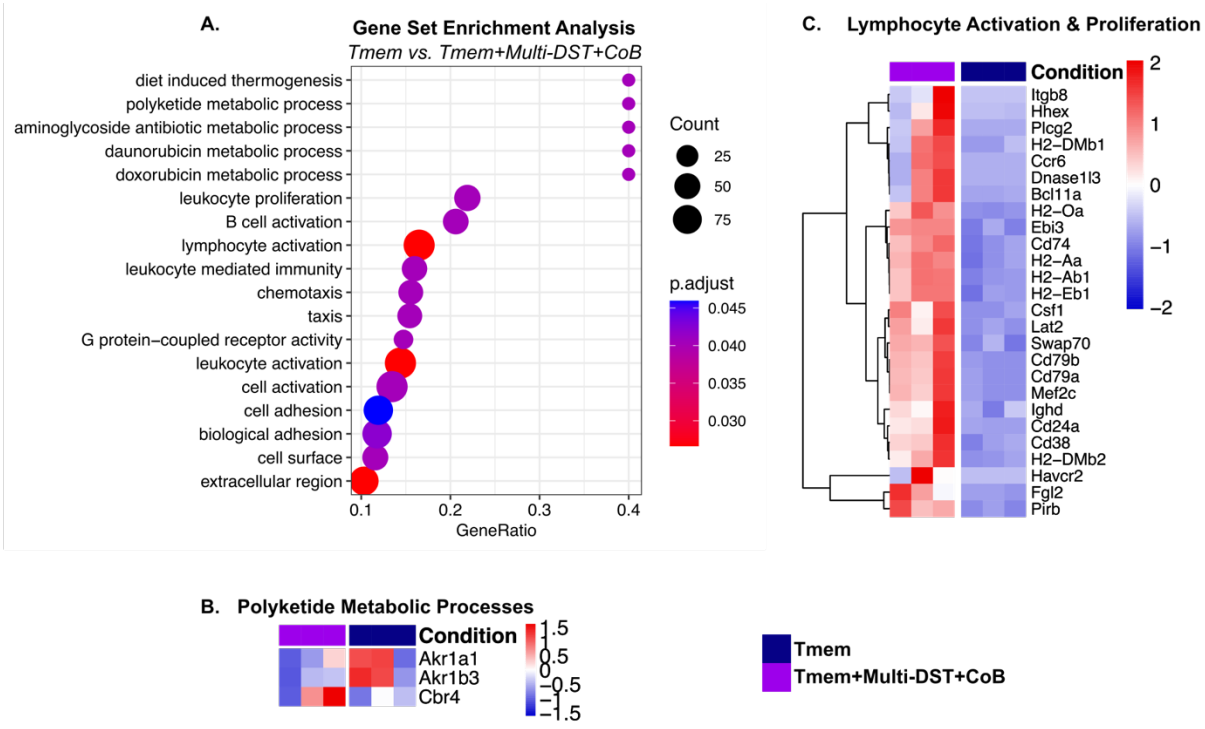

Figure 4.5 Tmem+Multi-DST+CoB acquire a unique metabolic and a more proliferative transcriptome following treatment.
(A) GO (Gene ontology) classifications of differentially expressed genes between Tmem (n=3) and Tmem+Multi-DST+CoB (n=3) TCR75 T cells, determined using hte gseGO function from the clusterProfiler R package. **(B)** Heatmap of differentially expressed genes found in the gene ontology term 'metabolic processes'. **(C)** Heatmap of differentially expressed genes found in the gene ontology term 'lymphocyte activation & proliferation'. Significantly differentially expressed threshold is adjusted p val ≤ 0.01, log2 fold change threshold ≥ 1. Colors for heatmaps determined by z score based on the long-normalized counts**.** Diagrams generated by Lexi Cassano.

Tmems and Tmem+Multi-DST+CoB TCR75 cells also showed differential gene sets for lymphocyte activation (Figure 4.5A). Interestingly, despite their reduced capacity to produce cytokines (Figure 4.1), Tmem+Multi-DST+CoB TCR75 cells had higher transcription for genes associated with lymphocyte activation (Figure 4.5C). However, upon closer inspection, it becomes clear that some of the genes enriched in Tmem+Multi-DST+CoB cells are byproducts of activation rather than responsible for Teffector function, such as the chemokine receptor CCR6, immune regulator CD38, and marker of apoptosis Mef2c. Additionally, Tmem+Multi-

DST+CoB expressed the $CD8^+$ T cell master regulator of exhaustion, Tox. These findings illuminate the reduced function of the Tmem+Multi-DST+CoB cells. Additionally, we hypothesize that this phenotype is due to recent antigen exposure of the Tmem+Multi-DST+CoB TCR75 cells, whereas, at the time of RNA collection, it had been over 30 days since Tmems experienced antigen, and they would therefore not have an activated phenotype but rather a resting memory phenotype.

4.07 Memory T cells retain an activated transcriptional signature following Multi-DST treatment in the context of CoB compared to T cells from tolerant, heart transplanted mice

Transcriptional differences between Tnaive+HTx+CoB and Tmem+Multi-DST+CoB TCR75 cells may elucidate the differentiation state of Tmems exposed to persistent antigen. The GO pathways significantly differentially expressed between Tmem+Multi-DST+CoB and Tnaive+HTx+CoB TCR75 T cells involved antigen processing and lymphocyte activation (Figure 4.6A). The genes associated with antigen processing were enriched in Tmem+Multi-DST+CoB TCR75 cells compared to Tnaive+HTx+CoB cells and involved a variety of MHC II-like genes, including H2-DMb1, H2-DMb2, H2-Ob, H2-Eb1, and H2-Q2 (Figure 4.6B). These genes all map to chromosome 17, indicating that their transcriptional control may be linked [244]. Furthermore, there was overlap with these MHC II-like genes on both the antigen processing and lymphocyte activation gene lists, with the later also showing enrichment in the Tmem+Multi-DST+CoB cells (Figure 4.6C). Unlike what was seen with Tmem+Multi-DST+CoB vs Tmem, the genes enriched in Tmem+Multi-DST+CoB cells compared to Tnaive+HTx+CoB cells were associated with a more functional phenotype, including the MHC II-like genes previously

addressed, Satb1, and, interestingly, Cxcr5, perhaps suggesting differentiation towards a T follicular helper phenotype (Figure 4.6C). These findings underscore the idea that Tmem+Multi-DST+CoB cells have an distinct functional phenotype falling between tolerance and memory, and indicate a potential benefit for the multi-DST protocol on memory cells.

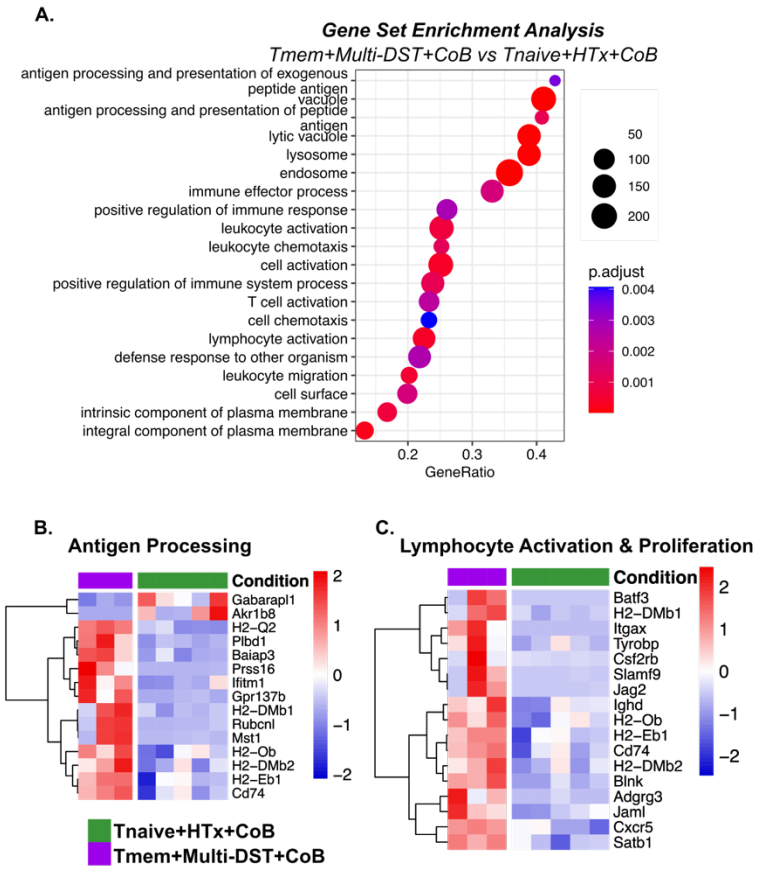

Figure 4.6 Tmem+Multi-DST+CoB demonstrate an enhanced transcriptional profile of antigen processing and activation compared to Tnaive+HTx+CoB.
(A) GO (Gene ontology) classifications of differentially expressed genes between Tmem+Multi-DST+CoB (n=3) and Tnaive+HTx+CoB (n=5) TCR75 T cells, determined using hte gseGO function from the clusterProfiler R package. **(B)** Heatmap of differentially expressed genes found in the gene ontology term 'antigen processing'. **(C)** Heatmap of differentially expressed genes found in the gene ontology term ‘lymphocyte activation & proliferation’. Significantly differentially expressed threshold is adjusted p val ≤ 0.01, log2 fold change threshold ≥ 1. Colors for heatmaps determined by z score based on the long-normalized counts. Diagrams generated by Lexi Cassano.

4.08 The Multi-DST+CoB protocol differentially inhibits the transcriptional profile of activation in naïve and memory T cells

To determine how the Multi-DST+CoB protocol differentially impacted naïve versus memory T cells, we compared gene sets between these two groups. Like in our other comparisons, the gene ontology (GO) pathways significantly differentially expressed between Tmem+Multi-DST+CoB and Tnaive+Multi-DST+CoB TCR75 T cells were associated with lymphocyte activation (Figure 4.7A). Interestingly, about a third of these genes were enriched in Tnaive+Multi-DST+CoB cells, whereas two thirds were enriched in Tmem+Multi-DST+CoB cells. (Figure 4.7B). Of those genes enriched in Tnaive+Multi-DST+CoB cells, we were surprised to find the activation marker CD83 and growth factor CSF-1 (Figure 4.7B). However, we are comparing two T cell subsets that have been persistently and recently exposed to antigen, so it stands to reason that recent recognition of antigen and progressive formation of dysfunction would be associated with activation markers. Indeed, Tnaive+Multi-DST+CoB cells showed high expression of the exhaustion marker Lag3 (Figure 4.7B). By comparison, Tmem+Multi-DST+CoB cells had fewer inhibitory receptor- or exhaustion-associated genes expressed; however, taken into consideration with the comparisons between Tmem+Multi-DST+CoB cells and Tnaive+HTx+CoB cells (Figure 4.6) and Tmems (Figure 4.5), these findings highlight a progressive loss of function such as cytokine production (Figure 4.1) with an increased transcriptional signature of suppression (Figure 4.7).

Interestingly, Pirb and Fgl2 were both low in Tmem (Figure 4.5) and Tnaive+Multi-DST+CoB in comparison to Tmem+Multi-DST+CoB. This was exciting, as these genes may represent a transcriptional signature of Tmem+Multi-DST+CoB, one distinct from both memory and recent stimulation. Pirb expression has previously been described on memory CD4+

interleukin IL-17a^{+} cells, where it promoted Th17 immunity [245], although most literature on Pirb describes its expression on B cells and myeloid cells rather than T cells. Fgl2 was also interesting to us as it is a known inhibitory cytokine, capable of inducing FcγRIIB-mediated apoptosis in CD8^{+} T cells [246]. Fgl2 is also known to be secreted by Tregs and important for Treg suppressive capacity, inhibiting DC maturation and promoting B cell apoptosis [247]. In this way, both Prib and Fgl2 may contribute to the dysfunctional Tmem+Multi-DST+CoB phenotype.

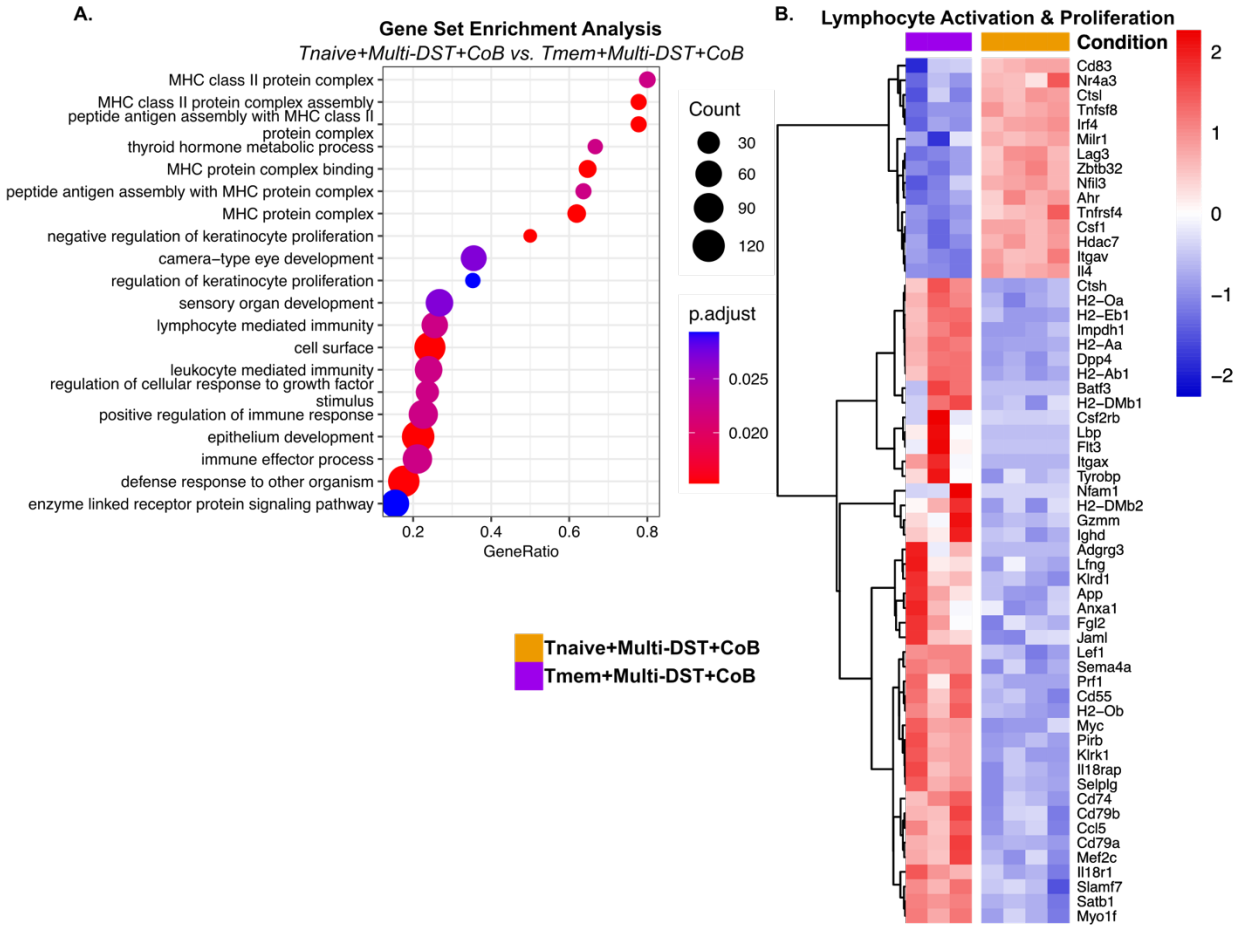

Figure 4.7 The Multi-DST+CoB treatment preferentially limits the Tnaive transcriptional proliferative signature compared to Tmem.
(A) GO (Gene ontology) classifications of differentially expressed genes between Tnaive+Multi-DST+CoB (n=4) and Tmem+Multi-DST+CoB (n=3) TCR75 T cells, determined using hte gseGO function from the clusterProfiler R package. **(B)** Heatmap of differentially expressed genes found in the gene ontology term 'lymphocyte activation & proliferation'. Significantly differentially expressed threshold is adjusted p val ≤ 0.01, log2 fold change threshold ≥ 1. Colors for heatmaps determined by z score based on the long-normalized counts**.** Diagrams generated by Lexi Cassano.

4.09 Naïve T cells exposed to Multi-DST+CoB demonstrate an enhanced transcriptional signature of dysfunction compared to T cells from tolerant, heart transplanted mice

To determine the genes enriched in the most robust tolerance, we compared the GO pathways significantly differentially expressed between Tnaive+HTx+CoB and Tnaive+Multi-DST+CoB TCR75 T cells. Interestingly, we found a large number of GO terms associated with ribosomes; however, upon further inspection, the enrichment in these pathways was due to two genes: Srbd1 and Gm2000 (Figure 4.8A, B). Although the role of these genes in T cells has not been studied, Srbd1 is associated with cell growth and inhibiting apoptosis in cancer cells [248]. As Srbd1 is enriched in Tnaive+Multi-DST+CoB, perhaps the expression of this gene is due to recent TCR stimulation.

We also investigated differentially expressed genes between Tnaive+HTx+CoB and Tnaive+Multi-DST+CoB TCR75 T cells involved in lymphocyte activation and proliferation. Similar to our other comparisons, we found that our most dysfunctional Tnaive+Multi-DST+CoB TCR75 T cells showed enhanced expression of the inhibitory receptorLag3, as well as the aryl hydrocarbon receptor (AhR) in comparison to Tnaive+HTx+CoB cells (Figure 4.8C). These findings further correlate robust dysfunction and a characteristic transcriptional profile of dysfunction, as has been published previously in exhaustion [67,249–251].

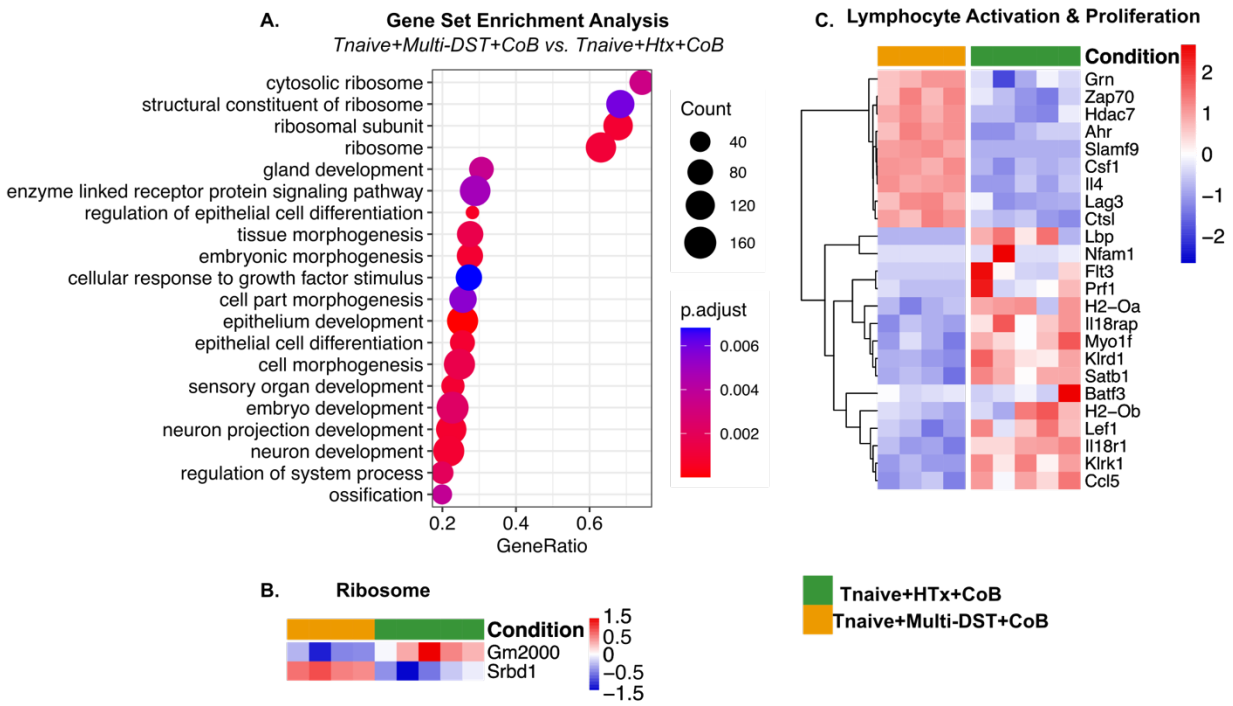

Figure 4.8 Multi-DST+CoB treatment promotes the most robust transcriptional signature of Tnaive hypofunction.
(A) GO (Gene ontology) classifications of differentially expressed genes between Tnaive+Multi-DST+CoB (n=4) and Tnaive+HTx+CoB (n=5) TCR75 T cells, determined using hte gseGO function from the clusterProfiler R package. **(B)** Heatmap of differentially expressed genes found in the gene ontology term 'ribosome'. **(C)** Heatmap of differentially expressed genes found in the gene ontology term 'lymphocyte activation & proliferation'. Significantly differentially expressed threshold is adjusted p val ≤ 0.01, log2 fold change threshold ≥ 1. Colors for heatmaps determined by z score based on the long-normalized counts**.** Diagrams generated by Lexi Cassano.

4.10 Discussion

In this study, we find that alloreactive Tmems become more hypofunctional in terms of cytokine production following repeated exposure to cognate antigen (Figure 4.1); however, they can still damage transplanted hearts, even if less so than unmodified Tmems (Figure 4.2). We transcriptionally interrogated these alloreactive Tmems and identified that while repeated exposure to cognate antigen promoted Tmems dysfunction, Tmems failed to become as dysfunctional as either naïve T cells exposed to Multi-DST+CoB or even Tnaive+HTx+CoB

TCR75 cells (Figures 4.3-4.8). Our findings suggest that the Multi-DST+CoB protocol is insufficient to fully reprogram Tmems into dysfunctional T cells, underscoring the difficulty of promoting T cell dysfunction in Tmem. T cell exhaustion is well published as a terminal differentiation state; however, it remains unclear whether Tmems can differentiate into terminal exhaustion. Our findings are preliminary, and further work is needed to understand the Tmems hypofunctional phenotype. Using our model, further studies will investigate the reversibility of this phenotype as well as the reliance of Tmems hypofunction upon continuous antigen exposure. Regardless, our findings are novel, as we describe the capacity of Tmems to develop some level of hypofunction, when canonical models of T cell dysfunction suggest that exhaustion and memory are distinct fates.

Recent work in $CD8^+$ T cells identified pregnancy-mediated epigenetic imprinting as capable of reprogramming Tmems to durable hypofunction (Pollard et al. in submission). Memory fetus/graft-specific $CD8^+$ T cells were hypofunctional post-partum, with extensive epigenetic and transcriptional alterations distinct from previously characterized exhaustion (Pollard et al. in submission). Interestingly, the memory fetus/graft-specific $CD8^+$ T cells retained some cytokine production capacity post-partum, whereas naïve cells were profoundly dysfunctional (Pollard et al. in submission). It is particularly interesting to consider if our Multi-DST+CoB approach would synergize with the pregnancy-mediated epigenetic imprinting to further promote dysfunction, and whether this could be a viable option to promote future transplant acceptance in mothers without endangering future semi-allogeneic offspring. As B cells are sensitized following semiallogeneic pregnancy[232], more work is needed to understand how the Multi-DST+CoB treatment would impact the sensitized B cells.

To develop a more holistic understanding of the impact of the Multi-DST+CoB protocol

on T cell function, we will perform ATAC sequencing in the future. We hope to identify the key molecular pathways/chromatin states associated with deeper dysfunction, which we can then target in an attempt to either promote more robust tolerance, or use identified surface biomarkers as fingerprints to monitor tolerance over time after transplantation. It has been previously published that the epigenetic landscape associated with exhaustion is fixed even in the face of high transcriptional variability post-antigen clearance [252]. Perhaps this is also the case with Tmem, and even though Multi-DST+CoB can promote transcriptional changes, epigenetically they are "scarred" as memory cells. This hypothesis is in part supported by our PC analysis, where Tmems and Tmem+Multi-DST+CoB cluster more closely than Tmem+Multi-DST+CoB and Tnaive+Multi-DST+CoB (Figure 4.3). ATAC sequencing has previously identified a set of around 6000 open chromatin regions unique to exhaustion [253]. We will use this knowledge to further characterize the differentiation state of the Tmem+Multi-DST+CoB, as well as compare how our model of transplantation tolerance compares epigenetically to exhaustion.

Our transcriptional analysis also identified Satb1 expression as highly variable among our populations of interest, with Satb1 enriched in less dysfunctional populations (Figures 4.5-4.8). This is concurrent with previous findings from our lab, which identify Satb1 as mediating T cell susceptibility to Treg suppression [237]. Satb1 expression was enriched in Tmem+Multi-DST+CoB compared to Tnaive+Multi-DST+CoB, suggesting that susceptibility to Treg suppression further enhances the dysfunctional phenotype *in vivo*. As Tmems can be susceptible to some Treg suppression [254], this may be an appealing pathway to target for more robust Tmems dysfunction.

Another interesting finding was that the aryl hydrocarbon receptor (AhR) was highly enriched in Tnaive+Multi-DST+CoB. The AhR is a ligand-activated transcription factor, and in CD8+ T cells has been associated with exhaustion [255]. In the tumor microenvironment, high

levels of IL-2 stimulate STAT5, which leads to downstream accumulation of tryptophan to 5-hydroxytryptophan (5-HTP) [255]. 5-HTP can then activate the AhR to translocate to the nucleus, which results in expression of inhibitory receptors and limits cytokine production. Whether signaling via IL-2/STAT5 also drives AhR translocation in Tnaive+Multi-DST+CoB is unclear; however, as we do not T-deplete the DST prior to injection, if the donor CD4 T cells are activated during processing, they may represent an exogenous source of IL-2 [256]. Future work will investigate whether the Multi-DST+CoB protocol promotes the most robust $CD4^+$ T cell dysfunction due to AhR related transcriptional changes.

We did not investigate proliferative capacity following our Multi-DST+CoB protocol, and it remains possible that Tmem+Multi-DST+CoB cells retain some functional capacity. Indeed, a recent publication describing serial stimulation events with long periods of rest in between found T cells to take on exhaustion markers, but retain their proliferative capacity [33]. Although this model does not describe true antigen persistence, and therefore does not describe true exhaustion, it is still unknown if Tmems can develop exhaustion, and therefore our Multi-DST+CoB protocol may be generating a comparable phenotype.

Chapter 5: Discussion

5.01 Introduction

In this thesis, we identified two potential threats to the robustness of transplantation tolerance. Memory T cells represent a threat to the induction of tolerance, whereas naïve allospecific T cells that recognize transiently expressed graft antigens are a threat to the maintenance of transplantation tolerance. We found that forcing naïve T cells to encounter persistent antigen results in robust dysfunction and is protective against Lm-mediated rejection. In contrast, memory T cells were more resistant to the development of dysfunction: although they displayed reduced cytokine production following repeated alloantigen stimulation in the presence of CoB, they remained transcriptionally distinct from naïve T cells that develop hypofunction upon transplantation and CoB therapy or upon repeated alloantigen stimulation in the presence of CoB. These findings suggest that promoting dysfunction in a wider array of allospecific T cells is not only possible, but also protective, and that clinical treatments focusing on exposing the immune system to a wider range of alloantigens, and more persistently, may prove beneficial for graft outcomes.

5.02 Working models

Although allospecific T cells that do recognize antigen persistently are profoundly dysfunctional [112], the T cell response to transplanted organs is heterogenous, and there exists a population of T cells that retain functionality due to lack of persistent antigen stimulation. Our results indicate that we can promote more robust tolerance by forcing antigen exposure and enhancing dysfunction in a wider array of allospecific T cells (Figure 5.1). This approach

improved the robustness of tolerance by protecting heart transplanted mice from Lm-mediated rejection.

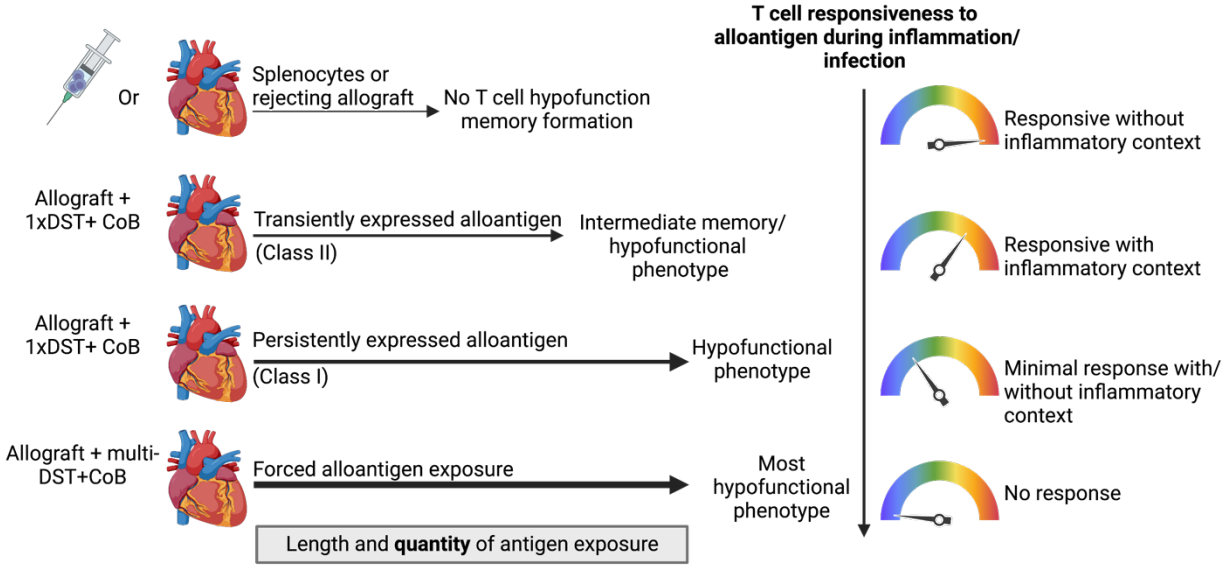

Figure 5.1 Persistent antigen exposure in the context of CoB drives robust T cell dysfunction.
In our model, the T cell dysfunction is driven by cooperation between exposure to persistent antigens and costimulation blockade (CoB). Antigen is cleared quickly following donor splenocyte injection, and heart allografts are rejected quickly in the absence of CoB, resulting in memory T cell formation in both cases. Transient antigens are antigens expressed by the transplant initially but downregulated upon accommodation of the graft in CoB-treated hosts, such as donor derived-MHCII. Persistent antigen can either occur naturally, such as donor MHCI protein or MHCI-derived peptides expressed within the heart allograft, or be therapeutically enforced, such as by using repeated injections of donor splenocytes containing the alloantigen. The longer an antigen is expressed, the more dysfunctional the allospecific T cell becomes in the context of CoB. It remains possible that quantity of antigen available also contributes to more robust dysfunction.

With this work, we describe a unique biological context in which both persistent and non-persistent antigens exist simultaneously within a transplanted organ. In other models of T cell hypofunction, including cancer and chronic LCMV, tumors persistently express antigen (except for antigen-loss variants) and LCMV infection is never cleared. As such, transplantation provides a unique biological model in which T cell responses to persistent and non-persistent antigens are heterogenous. Our findings are also novel, because we identified that T cells specific for non-persistent antigen represent a vulnerability to the transplanted organ, particularly upon antigen

re-expression during inflammatory challenges. The finding that Lm-infection is incapable of reinvigorating tolerant TCR75 T cells, unlike the tumor or LCMV-specific T cells that are not terminally exhausted and can be reinvigorated with checkpoint blockade, highlights persistent antigen as a driver of robust dysfunction.

In this thesis, we utilized a mouse experimental setting where the model antigen OVA can be expressed in a tightly controlled manner dependent upon administration of oral doxycycline: TGO mice (Chapter 3). Future work with this model will investigate more nuanced aspects of the impact of persistent antigen on T cell function. Our results demonstrate that persistent antigen is necessary for robust dysfunction during the induction of tolerance; however, whether persistent antigen is necessary for the maintenance of tolerance is unclear. We will investigate whether periods of antigenic rest during the maintenance phase of tolerance can reinvigorate previously dysfunctional T cells, whether sterile re-expression of antigen after periods of rest is sufficient to drive graft-rejection, and whether there is a temporal threshold of exposure to antigen necessary for irreversible T cell dysfunction. These studies have the potential to inform future clinical antigen treatments, by providing a framework for how much antigen is necessary and for how long.

Our findings have direct implications for patients undergoing transplantectomy, where the transplanted organ is removed either following rejection, or for serious complications such as infections or tumors within the graft. For kidney transplant patients, transplant removal is also commonly performed as patients return to dialysis [257]. There is no global consensus on whether a transplanted organ should be surgically removed following rejection, with rates of nephrectomy varying from 9 to 74 percent, dependent upon the treatment center [258–260]. Our findings suggest that, except in the cases of infection or cancer, leaving the transplanted organ in the host might

be beneficial, as it would provide a persistent source of antigen and, should the patient remain on immunosuppression, suppress allospecific T cells. We have attempted to model this hypothesis in heart transplanted mice; however, our immunosuppressive regimen may not accurately represent human transplantectomy. As such, we are currently developing a more clinically relevant mouse model of transplantectomy, where mice are maintained on a clinical immunosuppressive regimen to prevent transplant rejection, such that we can investigate immune responses following graft removal.

The second threat to transplantation tolerance that we investigated was memory T cells, which are resistant to immunosuppression [221–225], Treg suppression [226], and can prevent the induction of transplantation tolerance [132,261]. Additionally, Tmems are better able to target transplanted organs as they do not require reactivation in secondary lymph nodes [262]. Suppressing Tmem function in transplantation is therefore desirable.

Our findings are novel, not only in using repeated alloantigen stimulation and CoB as a pretreatment for Tmems prior to transplantation, but also providing evidence that Tmem can develop some level of hypofunction. Having found that Multi-DST+CoB promoted more robust tolerance in naïve allospecific T cells than 1xDST+CoB, we hypothesized that pretreating Tmems with this suppressive protocol would promote Tmem dysfunction. Forced antigen exposure prior to transplantation promoted loss of IFNγ and TNF production upon restimulation, demonstrating that Tmems can develop the canonical dysfunctional phenotype. Additionally, Tmems demonstrated an intermediate transcriptional profile in-between classic memory and transplantation tolerance. In this way, providing Tmems with Multi-DST+CoB is sufficient to promote partial dysfunction, but as this dysfunction is still more partial than that seen with naïve T cells, it is unclear if Tmems are progressively developing canonical dysfunction and have not

reached their full potential for hypofunction if they are differentiating toward a distinct state (Figure 5.2).

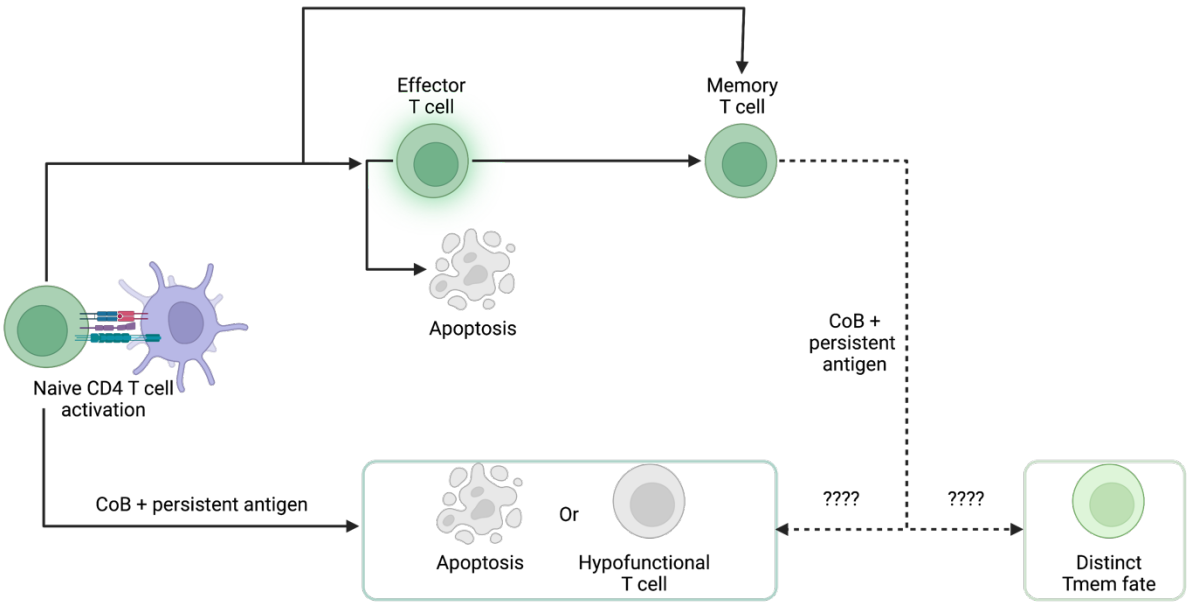

Figure 5.2 Achieving dysfunction of naïve and memory T cells during transplantation tolerance.
Memory T cells are considered a distinct differentiation state from naïve and effector T cells; however, our preliminary data suggest that Tmems exposed to persistent antigen and CoB develop some degree of T cell hypofunction. However, whether this dysfunctional phenotype is comparable to canonical dysfunction of naïve T cells following exposure to CoB or represents a distinct Tmem fate is unclear.

Previous efforts at suppressing Tmems took advantage of memory-specific surface markers, including LFA3, CD27, and OX40. LFA3-Ig, known as alefacept, is a depleting antibody preferentially targeting Tmems [263]. Co-treatment with alefacept and the costimulation blockade cytotoxic T lymphocyte antigen-4 (CTLA-4)-Ig prevented renal allograft rejection in non-human primates [263]; however, kidney transplant patients who received alefacept with combination therapy (tacrolimus, MMF, and corticosteroids) showed no improvement and instead higher incidence of malignancy compared to patients who received the placebo, indicating the drug had no clear benefit in patients [264].

CD27 is a costimulatory marker that binds to CD70 and has also been targeted due to its association with memory. CD27 functions alongside of CD28 to regulate the magnitude of T cell expansion and accumulation upon stimulation; however, unlike CD28, CD27 has no impact on the cell cycle [265]. In transplantation, CD27-deficient recipients showed prolonged graft survival in mice presensitized 40 days but not 10 days prior to transplantation compared to CD27-sufficient mice [266]. Importantly, CD27-deficient recipients showed impaired secondary responses due to a failure to form Tmems rather than failed reactivation of Tmems [266]. These data suggest that co-administering anti-CD27 with our Multi-DST+CoB approach is unlikely to further tolerize memory T cells. However, Multi-DST+CoB is insufficient to induce permanent dysfunction, in naïve mice, i.e. maintained dysfunction after the cessation of treatment, it would be interesting to test if coadministration of anti-CD27 with Multi-DST+CoB could eliminate any partially-functional T cells with an intermediate memory and dysfunctional phenotype, therefore improving tolerance.

OX40, also known as CD134, is only expressed on recently activated T cells. Following ligation with CD134L, expressed on APCs or endothelial cells, downstream signaling of OX40 promotes expression of the anti-apoptotic molecules Bcl-2 and Bcl-x_L [267]. This, in turn, promotes memory T cell formation/survival, as it allows for a larger subset of effector T cells to persist [267]. Furthermore, OX40 promotes Tmem survival after reactivation, as seen in a models of arthritis and allergic asthma [268]. In transplantation, anti-OX40 can synergize with CTLA4-Ig to prolong survival of murine heart and skin allografts; however, it is insufficient on its own [269]. As our Multi-DST+CoB protocol promotes more robust tolerance than CTLA4-Ig, but is still insufficient to completely suppress Tmems, perhaps anti-OX40 and Multi-DST+CoB can synergize suppress presensitized T cells.

Lymphosequestration has been proposed as a way to prevent rejection. In this manner, even if a patient has heterologous immunity, the alloreactive T cells will be incapable of migrating to the transplanted organ and thus unable to mediate rejection. This is important for Tmems in particular, as Tmems are often found in circulation, whereas naïve T cells are more often found in secondary lymphoid organs (SLOs) [22]. Administration of sphingosine 1-phosphate receptor-1 agonist FTY720 restricts circulating T cells to the SLOs and results in prolonged survival of murine heart transplants [270]. Importantly, $CD4^+$ T cells in the SLOs retained the ability to provide T cell help to $CD8^+$ T cells as well as B cells, allowing alloantibody formation, which can subsequently promote graft rejection [270]. Rather than lymphosequestration alone, these data reinforce the notion that concurrent treatments aimed at tolerizing would prevent Tmems from delivering B cell help for alloantibody production remains to be determined.

Future work should focus on elucidating any additional effects associated with Multi-DST+CoB. Providing large doses of exogenous antigen has the potential to alter normal immune processes, such as thymic immigration or priming. Although these potential effects are currently unclear in our Multi-DST+CoB protocol, we can speculate outcomes based on other models of persistent antigen, particularly chronic viral infection. It is interesting to consider if Multi-DST+CoB alters priming by DCs. APCs are relatively short lived, and due to anti-CD154 having a half-life of around 10 days [184], the immunosuppressive effects of Multi-DST+CoB must be maintained by multiple mechanisms. Priming requires TCR signaling to persist long enough to prevent cell death, which, for a naïve T cell, is around 20 hours in vitro [271]. In chronic infection, priming of pathogen-specific $CD8^+$ T cells differs between the onset of infection and during established chronic infection. Early during infection, primed $CD8^+$ T cells differentiate into effector T cells that then develop exhaustion [272]. In contrast, later into chronic infection, primed

effector T cells preferentially differentiate into TCF1^{+} memory-like precursor cells, attributed to adaptations in antigen presentation and reduced signaling via the TCR [272]. This is reminiscent of transplantation, where there are clear differences between the induction and maintenance of tolerance, mainly with regards to the immunosuppression present [272].

In the case of chronic infection, priming was assessed following adoptive transfer of naive virus-specific P14 transgenic T cell clones [272]; however, in transplantation the cells that would be primed during the maintenance phase would be new thymic emigrants. Should these cells develop a more memory-like phenotype, it would prove dangerous to the graft. Thymic emigrants have long been established as a potential risk to transplanted organs, and various efforts have attempted to promote their nonresponsiveness. One approach, for younger patients, is the administration of intrathymic alloantigen. In the absence of additional costimulation blockade, injection of immunogenic donor MHCII into rats resulted in prolonged nonresponsiveness to renal transplants [273,274]. Injected rats demonstrated donor-specific nonresponsiveness and hypofunction in the periphery [273,274]. However, the benefits of the injection were abrogated in rats thymectomized within a week of the intrathymic injection [273,274]. Although it is unclear if antigen from our Multi-DST+CoB regimen is capable of migrating to the thymus, we provide our initial DST injection i.v., suggesting that antigen could travel via the blood to the thymus. By persistently providing antigen during the first week of transplantation, we may be promoting anergy. Indeed, similar to the intrathymic injection, it would perhaps be beneficial to start the Multi-DST+CoB protocol prior to transplantation, to better promote nonresponsiveness [273,274].

With regards to new thymic emigrants, with or without Multi-DST+CoB, providing 1xDST+CoB is sufficient to promote a tolerogenic environment and prevent transplant rejection

in many, but not all, transplant models. This relies in part on linked-suppression, where new antigens that have linked expression to previously tolerized alloantigens also become tolerized[130,131]. This is likely reinforced through the development of allospecific Tregs [110,111]. Adoptive transfer of naïve allospecific T cells during the maintenance phase of tolerance fails to precipitate graft rejection [275]. These cells were controlled by the tolerogenic environment, as depletion of Tregs and blocking PD-1 signaling precipitated rejection [275]. Despite these findings, 1xDST+CoB is insufficient to entirely repress new thymic emigrants. In a mouse model of skin transplantation, when B6 recipients were given full thickness B/c skin grafts and 1xDST+CoB, about 80% of the mice rejected these transplants by 100 days [276]. Adding thymectomy to 1xDST+CoB permitted long term graft acceptance, dependent upon the presence of $CD4^+$ T cells, CTLA4, and IFNγ [277]. In the absence of thymectomy, rejection was due to thymic emigrants and not cross-reactivity, as thymectomized mice retained functional allospecific T cells in the periphery [277].These findings are reminiscent of models of chronic viral infection, where, following thymectomy, mice retain the capacity to mount antiviral $CD8^+$ T cell responses [278]. Whether tolerance following the Multi-DST+CoB protocol lacks T cells with the same stem-like proliferative capacity as $CD8^+$ T cells in chronic exhaustion remains to be investigated [66,73,74].

It is unclear if the Multi-DST+CoB protocol is able to promote a more tolerant environment by altering the composition of thymic emigrants, for example by promoting thymic Treg development, or by better suppressing the normal thymic emigrant pool. In models of full thickness skin grafts, 1xDST+CoB was hypothesized to promote either “split tolerance” or “graft accommodation” [276]. Split-tolerance was defined as tolerogenic suppression of high affinity cells, with lower affinity cells retaining function [279]. However, a second skin graft can override

"tolerance" in this model, which may suggest that the graft is simply being accommodated or ignored. Thus, when the new transplant refreshes the population of donor-derived APCs, they are capable of driving alloresponses [276]. As our Multi-DST+CoB protocol provides a high volume of antigen and continuous exposure to donor-derived APCs, we hypothesize that mice receiving skin grafts tolerized with Multi-DST+CoB will neither experience split tolerance, nor graft accommodation, and will rather be truly tolerant and capable of accepting secondary skin grafts.

We found that, contrary to our initial hypothesis, allospecific T cells specific for persistently expressed graft-derived antigen were so profoundly dysfunctional that they were not reinvigorated even following Lm-infection or injection of plasmids encoding IFNβ and IL-6. Although this dysfunction is somewhat intrinsic due to continuous TCR stimulation, Tregs are also very important for maintaining this phenotype. Preliminary findings from our lab demonstrate that, following Treg depletion using Foxp3-diphtheria toxin receptor (DTR) transgenic hosts of allogeneic heart transplants, class-switched donor specific antibodies (DSA) are generated. This is unique to Treg depletion in transplantation, as the other models capable of precipitating rejection (Lm infection, or plasmids of IL-6 and IFNβ) are insufficient to reinvigorate B cells [138]. These data suggest that allospecific T cells are being reactivated and providing help to B cells. Additionally, when assessed transcriptionally, TCR75 T cells post-Treg depletion were transcriptionally distinct from tolerant TCR75, further suggesting some level of reactivation (unpublished). Currently, investigations into whether the Multi-DST+CoB protocol is capable of promoting robust enough dysfunction to resist Treg depletion-mediated rejection are underway.

Previous work using combination therapy of the glycolysis inhibitor 2-deoxyglucose (2-DG) with the glutamine metabolism inhibitor 6-diazo-5-oxo-l-norleucine (DON) and metformin, a hypoglycemic agent able to promote fatty acid oxidation showed efficacy in prolonging survival of both full thickness skin and heart grafts [280]. The authors targeted these pathways to prevent T cell activation: targeting glycolysis and glutamine metabolism profoundly inhibited T cell proliferation and cytokine production without inhibiting Treg differentiation [280]. The metabolic phenotype of exhaustion has been characterized as reduced T cell glucose uptake, reduced glycolysis, and reduced mitochondrial mass coupled with increased depolarization [82–84]. Unlike exhaustion, the metabolic profile of allospecific T cells in tolerance is not understood, in part due to the cell number limitations of current metabolic assays [87]. However, Tmem metabolism has been well characterized to depend upon fatty acid oxidation, facilitated through mitochondrial remodeling, allowing rapid memory responses [216–218,281]. Similar to how metabolic targeting of naïve T cells promotes graft acceptance [280], we hypothesize that metabolically repressing oxidative phosphorylation will promote more robust Tmem dysfunction. Whether this metabolic approach alone would be sufficient for dysfunction or could synergize with our Multi-DST+CoB approach is unclear, although one potential side effect of this treatment is that Tregs also rely on fatty acid oxidation for energy [87]. Thus, any efforts to target memory cells must be cautious of breaking tolerance by suppressing Tregs.

While not well characterized in transplantation, tissue resident memory T (Trm) cells have the potential to mediate graft damage following development within the host [282]. As memory cells, Trms are primed to respond rapidly to antigen. In a mouse model of kidney transplantation, host-derived $CD8^+$ T cells differentiated into Trms that were later able to drive graft rejection [283]. Importantly, mice in these experiments were not given CoB, therefore the

potential effect of our Multi-DST+CoB protocol on Trm development is unclear, and would differ if used as a preventative or a concurrent treatment. In the mouse kidney model, donor derived Trms were rare [283], although whether that directly applies to $CD4^+$ T cells in heart transplantation cannot be assumed. Additional areas of interest are whether our Multi-DST+CoB protocol can prevent host-derived Trm formation by promoting dysfunction, or whether the antigen from the Multi-DST could penetrate the transplanted tissue and promote donor-derived Trm hypofunction. As we further investigate the mechanisms behind improved outcomes in Multi-DST+CoB-treated mice, understanding the effect of Trms will become important.

Future studies will investigate the effect of Multi-DST+CoB on pre-sensitized B cells. Although naive B cells are well controlled by CoB [170], B cells can be sensitized against graft antigens, such as through pregnancy[232]. Donor-specific antibodies (DSA), an indication of presensitization, can precipitate rejection [227,228] and are therefore used as a bio-marker of immunity prior to and following transplantation. In presensitized mice treated with Multi-DST+CoB, Tmems exhibited reduced function; however, skin grafts were still rejected (not pictured). These data indicate that Multi-DST+CoB is insufficient to contain memory B cell function, and additional clinical strategies to prevent DSA-mediated rejection are necessary.

Current immunosuppressive regimens in patients vary depending on the organ being transplanted. Patients generally receive some combination of steroids, calcineurin inhibitors, mTOR inhibitors, and other immunosuppressive agents[113]. In order to limit drug-associated toxicities, about 40-50% of heart transplant programs practice “induction immunotherapy”, which involves an intense immunosuppressive protocol early post-transplantation, when there is the highest threat of rejection, that will eventually taper off to a lower maintenance dose [284].

Induction therapy is particularly beneficial for patients at risk of acute rejection, including patients with high HLA mismatch, patients with many alloantibodies, and younger patients [285].

In terms of timing, the induction protocol is similar to the methods used to study transplantation in mice: high doses of immunosuppression given early post-transplantation and then weaned. One clear difference is that patients will remain on immunosuppression, whereas mice subjected to a tolerogenic protocol do not; however, with our experimental Multi-DST+CoB protocol, mice are continuously treated, with persistent antigen on the graft acting as immunosuppressive maintenance. Importantly, the mouse model uses anti-CD154 blocking antibody as costimulation blockade. Anti-CD154 is not currently available clinically due to past thrombotic complications in clinical trials [115,116]; however, a modified, Fc silent version of the antibody is currently being tested in clinical trials [117,118]. Therefore, the quality of T cell dysfunction we see in our mice is not currently available in patients.

Some patients will spontaneously develop transplantation tolerance [114]; however, in patients it is rare, with only 5% of kidney and 20% of liver transplant patients achieving operational tolerance [286–289]. Therefore, clinical strategies to develop tolerance are incomplete. One deliberate therapeutic strategy to induce tolerance in the clinic is to generate chimeras, where patients are given donor hematopoietic stem cell transfer (HSCT) concurrently, or sequentially, with the solid organ. This model requires bidirectional tolerance between the donor and the host to prevent both transplant rejection and graft-versus-host disease [290]. A recent cohort of kidney transplant patients with living donors received $CD34^+$ hematopoietic progenitor cells and $CD3^+$ T cells from their donors, and received total lymphoid irradiation (TLI) and antithymocyte globulin (ATG) to promote chimerism [291]. Fully-HLA matched patients developed persistent chimerism and were able to be withdrawn from immunosuppression without rejecting

their transplants for at least two years [291]. In contrast, partially HLA-matched patients had reduced incidence of chimerism and although they were able to limit their immunosuppressive regimens, they required continuous administration of the calcineurin inhibitor tacrolimus to prevent rejection [291]. Whether this is due to the need for continuous suppression of memory T cells, which are known to resist radiation [292], is unclear, although the authors found a correlation between patients who retained a high number of Tmems and poor chimerism [291]. Other approaches to chimerism have been studied in patients with malignancy and with a variety of immunosuppressive and suppression-weaning regimens [293].The benefit of our Multi-DST+CoB approach is that it represents a short term treatment suitable for full- and partially-HLA mismatched patients. Although currently incapable of driving complete Tmem dysfunction, our method avoids the negative side effects associated with patient irradiation.

Mixed chimerism can also be used to develop "delayed" tolerance for patients who do not have living donors. Currently, delayed tolerance has only been studied in non-human primates. The primates initially received a renal transplant and conventional immunosuppression and then, 4 months later, received a tolerogenic protocol with donor bone marrow that was frozen/preserved from the original donor [294]. Delayed tolerance is interesting to consider in the context of memory T cells, as Tmems may develop prior to the tolerogenic protocol, even if the recipients were on a strong conventional immunosuppression. Indeed, when depleting anti-CD8 mAb was added to the delayed tolerance protocol, 85% of recipients developed chimerism with 55% maintaining long-term allograft survival in the absence of immunosuppression [294]. Anti-CD8 is not clinically available, therefore strategies that aim to promote Tmem dysfunction should prove beneficial to the delayed tolerance model.

Aside from chimerism, additional cell-based strategies to promote operational tolerance are in clinical trials. Tregs, Bregs, regulatory macrophages, and DCs have all been investigated in adoptive transfer models, with the sources of these cells varying widely: cells from the donor, from the recipient, or sometimes cells from a third party donor [293]. Mesenchymal stem cells (MSCs) are also an area of investigation, due to their immunomodulatory potential, with a variety of approaches currently in clinical trials [293,295]. As current clinical trials are ongoing, the efficacy of these cell-based approaches remains unclear. Should any cell population prove beneficial at promoting tolerance, we could apply that knowledge to the Multi-DST+CoB approach, and enrich that tolerogenic population in our injections.

Our findings rely on using repeated injections of donor splenocytes as a source of persistent antigen. Clinically, providing alloantigen repeatedly prior to transplantation may prove difficult. Patients with living donors could conceivably receive antigen in the form of leukocyte transfusions prior to transplantation; however, this would not be an option for patients with grafts from deceased donors. In the future, this method could potentially synergize with xenotransplantation as well, in a similar sense to living allodonors, xenodonors could provide patients with leukocyte transfusions prior to transplantation. In addition to difficulty of sourcing antigen, patient compliance may be challenging. Patients would have to self-administer the antigen as an injection, and as our current protocol requires injections every-other day, this is likely to lead to low compliance. Other options would be to increase the time between injections, which would need to be tested, or finding a way to provide antigen orally, which has been shown to promote oral tolerance and prolong transplant survival independent of immunosuppression [133]. However, oral alloantigen is likely to only tolerize indirect alloreactive T cells, following capture and processing of the alloantigen by intestinal or mesenteric lymph node APCs. Implanting slow

antigen-release mini pumps containing HLA antigen would eliminate the need for repeated injections and may be able to tolerize both direct and indirect T cells.

5.03 Conclusion

In summary, we identified a population of allospecific T cells that retain function following costimulation blockade. These T cells persist long-term in graft recipients, and pose a threat to the graft during infection, when their cognate antigen is re-expressed. These T cells represent a large population of functional allospecific T cells. By targeting these cells with repeated antigen exposure, we can reduce their functionality and promote graft survival following infection. This approach can be further applied to memory T cells, which also pose a threat to the graft, to promote an intermediate level of hypofunction. Our findings highlight vulnerabilities of tolerance to T cell-mediated rejection and identify strategies that may improve the robustness and longevity of transplantation tolerance toward the goal of achieving one transplant for life.

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